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Store-Operated Calcium Channels

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Parekh, Anant B., and James W. Putney Jr. Store-Operated Calcium Channels. *Physiol Rev* 85: 757-810, 2005; doi:10.1152/physrev.00057.2003.—In electrically nonexcitable cells, Ca^{2+} influx is essential for regulating a host of kinetically distinct processes involving exocytosis, enzyme control, gene regulation, cell growth and proliferation, and apoptosis. The major Ca^{2+} entry pathway in these cells is the store-operated one, in which the emptying of intracellular Ca^{2+} stores activates Ca^{2+} influx (store-operated Ca^{2+} entry, or capacitative Ca^{2+} entry). Several biophysically distinct store-operated currents have been reported, but the best characterized is the Ca^{2+} release-activated Ca^{2+} current, I_{CRAC}. Although it was initially considered to function only in nonexcitable cells, growing evidence now points towards a central role for I_{CRAC}-like currents in excitable cells too. In spite of intense research, the signal that relays the store Ca^{2+} content to CRAC channels in the plasma membrane, as well as the molecular identity of the Ca^{2+} sensor within the stores, remains elusive. Resolution of these issues would be greatly helped by the identification of the CRAC channel gene. In some systems, evidence suggests that store-operated channels might be related to TRP homologs, although no consensus has yet been reached. Better understood are mechanisms that inactivate store-operated entry and hence control the overall duration of Ca^{2+} entry. Recent work has revealed a central role for mitochondria in the regulation of I_{CRAC}, and this is particularly prominent under physiological conditions. I_{CRAC} therefore represents a dynamic interplay between endoplasmic reticulum, mitochondria, and plasma membrane. In this review, we describe the key electrophysiological features of I_{CRAC} and other store-operated Ca^{2+} currents and how they are regulated, and we consider recent advances that have shed insight into the molecular mechanisms involved in this ubiquitous and vital Ca^{2+} entry pathway.

I. INTRODUCTION

An increase in cytoplasmic Ca^{2+} concentration is used as a key signaling messenger in virtually every cell through the phylogenetic tree, where it regulates a broad spectrum of kinetically distinct processes (24, 53). Intracellular Ca^{2+} is often considered a "life-giving" signal because it is essential both for sperm motility and the acrosome reaction and, in the form of periodic Ca^{2+} oscillations, for fertilization of the egg. Ca^{2+} is also vital for sustaining the life of the organism. Distinct spatial and temporal patterns of cytoplasmic Ca^{2+} increases drive processes as diverse as exocytosis, gene transcription, and cell motility. Having given life, Ca^{2+} can also take it away. This dark side of the Ca^{2+} signal can be executed through apoptosis or the more destructive and less specific necrosis.

Eukaryotic cells can increase their cytoplasmic Ca^{2+} concentration in one of two ways: release from intracellular stores or Ca^{2+} influx into the cell. We now have a good understanding of the organelles that function as Ca^{2+} stores and how Ca^{2+} can be released from them into the cytosol (27). Although the importance of the endoplasmic/sarcoplasmic reticulum (ER/SR) has been firmly established, growing evidence indicates that functional compartmentalization exists within the reticulum such that its Ca^{2+}-releasing capabilities are not homogeneously distributed throughout the organelle. Instead, some subcompartments play a disproportionally greater role in Ca^{2+} release (290). Use of genetically targeted Ca^{2+} reporter proteins like aequorin and the cameleons together with detailed immunocytochemical mapping and functional studies have identified contributions from additional organelles like the Golgi apparatus, lysosomes, nuclear envelope (continguous with the ER), and possibly secretory granules (290, 339). In addition, mitochondria play a central role in intracellular Ca^{2+} dynamics under physiological conditions (53).

A relatively small complement of second messengers is thought to release Ca^{2+} from the stores. In addition to the ubiquitous second messengers Ca^{2+} and inositol 1,4,5-trisphosphate ($InsP_3$), roles for cyclic ADP ribose and nicotinic acid adenine dinucleotide phosphate (NAADP) have been described in some cell types (51). Just how a limited number of second messengers can generate the vast array of diverse intracellular Ca^{2+} release patterns consequent to receptor stimulation is unclear, but receptor-specific recruitment of different combinations of second messengers together with mobilization of distinct Ca^{2+} stores is likely to be of major significance.

In spite of its importance, the Ca^{2+} release phase is transient, sometimes fully deactivating within a few tens of seconds. This is in part due to Ca^{2+}- and/or ligand-dependent inactivation of the release channels themselves as well as clearance of Ca^{2+} from the cytosol by resequestration into other organelles (notably mictochondria and ER) as well as extrusion from the cell by Na^+/Ca^{2+} exchangers and Ca^{2+}-ATPases in the plasma membrane. However, many key processes require sustained increases in intracellular Ca^{2+}, and this is accomplished through Ca^{2+} entry into the cell.

The ~10,000-fold concentration gradient for Ca^{2+} across the plasma membrane of resting cells coupled with a hyperpolarized resting membrane potential results in a huge electrochemical driving force in favor of Ca^{2+} influx. Resting cells generally have a low membrane permeability to Ca^{2+}, but even modest increases in permeability result in large Ca^{2+} influx. An increase in membrane permeability to Ca^{2+} can be achieved by opening Ca^{2+}-permeable ion channels in the plasma membrane.

A variety of different Ca^{2+}-permeable channels have been found to coexist in the plasma membrane (134), and

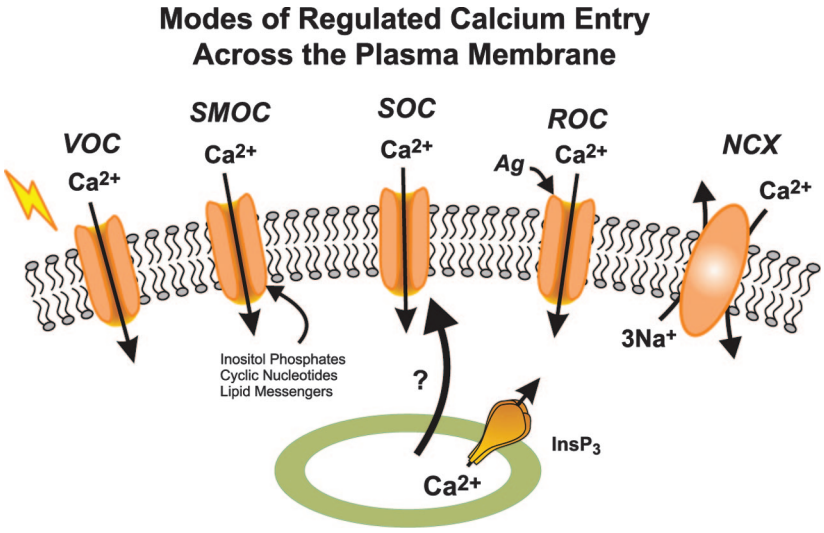

FIG. 1. Modes of regulated Ca^{2+} entry across the plasma membrane. Calcium can enter cells by any of several general classes of channels, including voltage-operated channels (VOC), second messenger-operated channels (SMOC), store-operated channels (SOC), and receptor-operated channels (ROC). VOCs are activated by membrane depolarization, and SMOCs are activated by any of a number of small messenger molecules, the most common being inositol phosphates, cyclic nucleotides, and lipid-derived messengers (diacylglycerol and arachidonic acid and its metabolites). SOCs are activated by depletion of intracellular Ca^{2+} stores, and ROCs are activated by direct binding of a neurotransmitter or hormone agonist (Ag). In addition, under some conditions, Ca^{2+} can enter cells via the Na^{+}-Ca^{2+} exchanger (NCX) operating in reverse mode.

the major ones are depicted in Figure 1. Voltage-gated Ca^{2+} channels are found in excitable cells like nerve and muscle but are largely excluded from nonexcitable cells. Receptor-operated channels, which open rapidly upon binding an external ligand that is usually a neurotransmitter, are also preponderate in excitable cells. Second messenger-operated channels are less widely distributed and are found in some excitable and nonexcitable cells. Store-operated Ca^{2+}-permeable channels on the other hand appear to be widespread, apparently existing in all eukaryotes from yeast (201) to humans (275). Hence, the argument could be advanced that store-operated Ca^{2+} channels represent the primordial Ca^{2+} entry pathway.

II. STORE-OPERATED CALCIUM ENTRY: REFILLING THE STORES AND MORE

The ER is a multifarious organelle carrying out a litany of interdependent processes (26). In addition to its well-documented role as both an agonist-sensitive Ca^{2+} store and sink, protein folding/processing takes place within its lumen. Here, numerous Ca^{2+}-dependent chaperone proteins ensure that newly synthesized proteins are folded correctly and sent off to the appropriate destination. The ER is also involved in vesicle trafficking (114), release of stress signals (165), regulation of cholesterol metabolism (48), and apoptosis (85). Many of these processes require intraluminal Ca^{2+}, and protein misfolding, ER stress responses, and apoptosis can all be induced by depleting the ER of Ca^{2+} for prolonged periods of time. Because of its role as a source of Ca^{2+}, it is clear that ER Ca^{2+} content must fall after stimulation. However, to preserve the functional integrity of the ER, it is vital that the Ca^{2+} content does not fall too low or is maintained at a low level. Replenishment of the ER with Ca^{2+} is therefore a central process to all eukaryotic cells. Because a fall in ER Ca^{2+} content activates store-operated Ca^{2+} channels in the plasma membrane, a major function of this Ca^{2+} entry pathway is believed to be maintenance of ER Ca^{2+} levels that are necessary for proper protein synthesis and folding. However, it is clear that these Ca^{2+} channels have other important roles (see sects. XV and XVI), and it has now been firmly established that store-operated Ca^{2+} entry is central to the physiology of eukaryotic cells. Furthermore, aberrant functioning of store-operated channels has been linked to a growing number of diseases.

III. THE FUNDAMENTAL PROPERTY OF STORE-OPERATED CALCIUM CHANNELS IS ACTIVATION BY STORE DEPLETION

The concept of store-operated Ca^{2+} entry was proposed in 1986 (297). This idea originated from a series of experiments in parotid acinar cells investigating the relationship between Ca^{2+} release from internal stores, Ca^{2+} entry, and store refilling. On the basis of this work, and a few eclectic observations in the literature, it was suggested that the amount of Ca^{2+} in the stores controlled the extent of Ca^{2+} influx in nonexcitable cells, a process originally called capacitative calcium entry. When stores were full, Ca^{2+} influx did not occur but, as the stores emptied, Ca^{2+} entry developed. Subsequently, more straightforward evidence for capacitative or store-operated entry was obtained through the use of thapsigargin, a specific inhibitor of the endoplasmic reticulum Ca^{2+} pump, discussed in more detail in a subsequent section. Direct evidence in support of the basic tenet of this model, namely, that store depletion activates Ca^{2+} influx, was provided by electrophysiological studies which established that the process of emptying the stores activated a Ca^{2+} current in mast cells called Ca^{2+} release-

activated Ca^{2+} current or I_{CRAC} (143). I_{CRAC} is non-voltage activated, inwardly rectifying, and remarkably selective for Ca^{2+} (270, 417). It is found in several cell types mainly of hemapoietic origin. However, as Table 1 indicates, I_{CRAC} is not the only store-operated current, and it is now apparent that store-operated influx encompasses a family of Ca^{2+}-permeable channels, with different properties in different cell types. Nevertheless, I_{CRAC} was the first store-operated Ca^{2+} current to be described and remains a popular model for studying store-operated influx. As a consequence, our understanding of store-operated Ca^{2+} currents is heavily influenced by I_{CRAC}.

Store-operated Ca^{2+} channels can be activated by any procedure that empties the stores (143, 270); it does not seem to matter how the stores are emptied, the net effect is activation of store-operated Ca^{2+} entry. Physiologically, store emptying is evoked by an increase in the levels of $InsP_3$ or other Ca^{2+}-releasing signals followed by Ca^{2+} release from the stores. But there are several other methods for emptying stores. These methods include the following: *1*) elevation of $InsP_3$ in the cytosol [following receptor stimulation or, more simply, dialyzing the cytosol with $InsP_3$ itself or related congeners like the non-metabolizable analog Ins(2,4,5)P_3]; *2*) application of the Ca^{2+} ionophore ionomycin to permeabilize the ER membrane; *3*) dialyzing the cytoplasm with high concentrations of the Ca^{2+} chelators EGTA or BAPTA, which chelate Ca^{2+} that leaks from the stores and hence prevent store refilling; *4*) exposure to the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitors like thapsigargin, cyclopiazonic acid, and di-*tert*-butylhydroquinone which prevent the P-type ATPases from refilling the stores (193, 270); *5*) sensitizing the $InsP_3$ receptors to resting levels of $InsP_3$ with agents like thimerosal (267); and *6*) loading membrane-permeable metal Ca^{2+} chelators like *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylene diamine (TPEN) directly into the stores (135). Through mass action, TPEN lowers free intraluminal Ca^{2+} concentration without changing total store Ca^{2+} such that the store depletion-dependent signal is generated.

These methods of emptying stores are not devoid of potential problems. The key feature of store-operated Ca^{2+} entry is that it is the fall in Ca^{2+} content within the stores and not the subsequent rise in cytoplasmic Ca^{2+} concentration that activates the channels (270). However, ionomycin and SERCA pump blockers generally cause a rise in cytoplasmic Ca^{2+} concentration as a consequence of store depletion, and such a rise in Ca^{2+} could open Ca^{2+}-activated cation channels permeable to Ca^{2+}. One way to avoid such problems is to use agents under conditions where cytoplasmic Ca^{2+} has been strongly buffered with high concentrations of Ca^{2+} chelator such as EGTA or BAPTA. The recent discovery that TRPV6 channels are expressed in many nonexcitable cells has added further complication (72, 379). TRPV6 channels are Ca^{2+} selective but are inactivated at resting levels of Ca^{2+}. Lowering cytoplasmic Ca^{2+} concentration, for example following dialysis with EGTA or BAPTA, removes the Ca^{2+} inhibition and the TRPV6 channels now open (379). Ca^{2+} influx follows, but this is not store-operated. Clearly therefore, one needs to carefully dissect the dependence on lowering intraluminal Ca^{2+} concentration from both the subsequent rise in cytoplasmic Ca^{2+} as well as removal of Ca^{2+}-dependent inhibition of other channels

TABLE 1. *Biophysical properties of store-operated Ca^{2+} channels*

Current	Conductance	Selectivity	Permeability Ratio	Activation	Cell Type	Reference Nos.
I_{CRAC}	0.02 pS; 110 Ca^{2+}	$Ba^{2+}>Ca^{2+}\geq Sr^{2+}$	Ca^{2+}:Na^+; 1,000:1	Receptor agonist	Mast cell	143
				$InsP_3$	RBL-1/-2H3	82, 269
				Thapsigargin	Jurkat T cells	417
				EGTA/BAPTA	Hepatocytes	323
				Ionomycin	Dendritic cells	145
				TPEN	Megakaryocytes	338
					MDCK cells	70
I_{SOC}	11 pS; 10 Ca^{2+}	$Ca^{2+}>Na^+$	Ca^{2+}:Na^+; >10:1	Receptor agonist	Endothelia	369
	1 pS; 100 Ca^{2+}	$Ca^{2+} = Ba^{2+}\gg K^+$	Ca^{2+}:K^+; 1,000:1	Receptor agonist $InsP_3$ Thapsigargin	A431 epidermal cells	405
	2 pS; 160 Ca^{2+}	$Ba^{2+}>Ca^{2+}$	?	BAPTA Thapsigargin	A431 epidermal cells	207
	2.7 pS; 90 Ca^{2+}	$Ca^{2+} = Ba^{2+} = Na^+$	Ca^{2+}:Na^+:K^+; 1:1:1	Thapsigargin BAPTA-AM	Aortic myocytes	364
	2.3 pS; 1.5 Ca^{2+}	$Ca^{2+}>Na^+$	Ca^{2+}:Na^+; 50:1	Cyclopiazonic acid BAPTA-AM Caffeine	Portal vein myocytes	1
	5.4 pS; 20 Ca^{2+}	?	?	Cyclopiazonic acid	Pulmonary artery myocytes	112
	0.7 pS; 90 Ca^{2+}	?	?	Spontaneous	Mesangial cells	213
	43 pS; 1.3 Ca^{2+}	Na^+, $K^+>Ca^{2+}$	Ca^{2+}:Na^+; 1:13	Receptor agonist	Pancreatic acinar cells	178

I_{CRAC}, Ca^{2+} release-activated Ca^{2+} current; I_{SOC}, store-operated Ca^{2+} current; $InsP_3$, inositol 1,4,5-trisphosphate.

following dialysis with exogenous buffer. Hence, store-operated Ca^{2+} entry is best demonstrated using protocols that empty stores under conditions where cytoplasmic Ca^{2+} has been strongly buffered at close to resting levels (~100 nM).

In some cell types, store-operated single-channel currents have been reported in the cell-attached configuration (2, 364, 414). In these experiments, after formation of a cell-attached patch, stores are depleted by either thapsigargin or receptor stimulation, and single-channel events are seen. A potential concern with this approach is that it may be the rise in Ca^{2+} itself or a Ca^{2+} -dependent second messenger but not store depletion that gates the channels. Preincubating the cells with BAPTA-AM would eliminate a Ca^{2+}-dependent current provided the cytosol accumulated enough free BAPTA to prevent a rise in cytoplasmic Ca^{2+} following store emptying. An alternative approach is to carry out whole cell and cell-attached recordings using two pipettes simultaneously, with the whole cell pipette being used to dialyze the cytosol with a high concentration of Ca^{2+} chelator.

Can receptor-evoked Ca^{2+} influx be entirely explained by a store-operated mechanism? In some nonexcitable cells, a solid body of evidence indicates that a variety of Ca^{2+} entry pathways exist. Inositol polyphosphate, cyclic nucleotide, Ca^{2+}, and arachidonic acid-gated Ca^{2+}-permeable channels have all been described and may contribute to agonist-evoked Ca^{2+} influx. In one instance, it has been concluded that agonist-activated entry did not occur by a store-operated mechanism at all, but rather by an entirely distinct mechanism, which the authors termed "ACE" (agonist-activated calcium entry). Reducing expression of either the phospholipase C (PLC)-γ1 or PLC-γ2 isoforms resulted in a pronounced decrease in agonist-evoked Ca^{2+} entry, but Ca^{2+} influx in response to thapsigargin was unaffected. Hence, PLC-γ was not required for the activation of store-operated Ca^{2+} entry, but was essential for the ability of receptor stimulation to evoke Ca^{2+} influx (276). The requirement for phospholipase C-γ was independent of its enzymatic activity because a lipase-deficient mutant was equally effective. On the other hand, it was subsequently suggested that PLC-γ might have a structural role requiring its SH3 domains and which perhaps involved localization of specialized Ca^{2+} stores to the receptors in the plasma membrane (300). In a more recent report, Nishida et al. (258) provided evidence that PLC-γ acts to amplify the agonist-activated PLC signal, necessary for store depletion and activation of store-operated channels (258). It is not clear then whether PLC-γ simply provides additional $InsP_3$ giving adequate release to activate the store-operated channels, or whether PLC-γ provides $InsP_3$ that is localized to regulate specialized stores that in turn are coupled to the entry channels.

As discussed above, the original concept of store regulation of Ca^{2+} entry into cells was termed "capacitative calcium entry," sometimes referred to as CCE. In the current literature, the more descriptive term *store-operated* Ca^{2+} *entry* (SOCE) is now more commonly used. The channels through which Ca^{2+} enters the cells are often called store-operated channels (SOC). These generic terms should be used when the detailed nature of a particular channel or current is not known, and more specific terms such as I_{CRAC} or CRAC channels should be reserved for instances in which the specific electrophysiological properties are those originally described for I_{CRAC}. Finally, there are a number of even more general terms in the literature that imply even more limited knowledge of mechanism, for example, noncapacitative calcium entry (NCCE) or agonist-activated calcium entry (ACE).

Research continues on the relative roles of store-operated and non-store-operated mechanisms in various physiological situations. Discussion of some of these issues, and other aspects of store-operated channels, can be found in the electronically published proceedings of a recent E-conference (104 and references therein).

IV. MEASURING STORE-OPERATED CALCIUM INFLUX

The issue of how best to measure Ca^{2+} influx is a recurring theme in the store-operated channel field. Several different methods have been employed, and these have been discussed in some depth in a previous review (270). The most popular method is to use fluorescent dyes like fura 2, which are easily loaded into cells in a noninvasive manner. In many studies, the cytoplasmic Ca^{2+} concentration is taken as a direct indication of store-operated Ca^{2+} influx. Such an approach is fraught with dangers. First, the membrane potential is not controlled and hence is free to fluctuate. Changes in membrane potential, should they occur, will alter the extent of store-operated influx, through changes in the driving force for Ca^{2+} entry. Second, the cytoplasmic Ca^{2+} signal is not a reliable indicator of Ca^{2+} influx because the former will be determined by the balance between Ca^{2+} influx, cytoplasmic Ca^{2+} buffering, and Ca^{2+} removal. Changes in the activity of SERCA pumps, mitochondrial Ca^{2+} uptake, plasmalemmal Na^+-Ca^{2+} exchange, or Ca^{2+}-ATPases will all alter the size of a cytoplasmic Ca^{2+} signal, if Ca^{2+} influx stays constant. In T lymphocytes, for example, plasmalemmal Ca^{2+}-ATPases increase their activity substantially following Ca^{2+} entry through CRAC channels and so extrude Ca^{2+} more efficiently (19). Inhibiting the pumps would increase cytoplasmic Ca^{2+}, but this would not reflect an increase in Ca^{2+} entry. Third, the nature of the Ca^{2+} influx pathway is not clear. Store-operated Ca^{2+}

entry is not the only Ca^{2+} influx mechanism in nonexcitable cells, and simply measuring cytoplasmic Ca^{2+} levels does not enable one to discriminate between the various possibilities. Even applying thapsigargin in Ca^{2+}-free solution then observing a Ca^{2+} signal on readmission of external Ca^{2+} is not unequivocal evidence for store-operated entry. Thapsigargin-evoked Ca^{2+} release can activate Ca^{2+}-dependent channels permeable to Ca^{2+} and, in some cases, the channels are activated by a Ca^{2+}/calmodulin-dependent protein kinase-mediated phosphorylation that can maintain channel activity for some time after cytoplasmic Ca^{2+} levels have returned to resting levels (41). There are a number of ways to reduce or eliminate these problems when using fluorescent indicators. Membrane potential can be controlled by voltage clamping with patch pipettes in the whole cell configuration. Alternatively, substitution of extracellular Na^+ with K^+ provides essentially a chemical clamp of membrane potential at K^+ equilibrium potential (E_K), which will be close to 0 mV. It is possible to deplete intracellular stores without increasing cytoplasmic Ca^{2+}, by using low concentrations of thapsigargin for prolonged periods (298). Finally, problems of Ca^{2+} buffering, Ca^{2+}-mediated inactivation mechanisms, and Ca^{2+}-activated channels can be reduced somewhat by the use of Ca^{2+} surrogates, such as Ba^{2+}, which will pass through all known store-operated channels, but is not a substrate for Ca^{2+} transporters and does not activate most Ca^{2+}-dependent processes to the extent that Ca^{2+} does. A direct way to eliminate these problems is to use the patch-clamp technique to monitor the store-operated Ca^{2+} current (Fig. 2). Using this method, complications from changes in membrane potential and Ca^{2+} clearance are eliminated. Moreover, by strongly buffering the pipette solution, changes in cytoplasmic Ca^{2+} can be

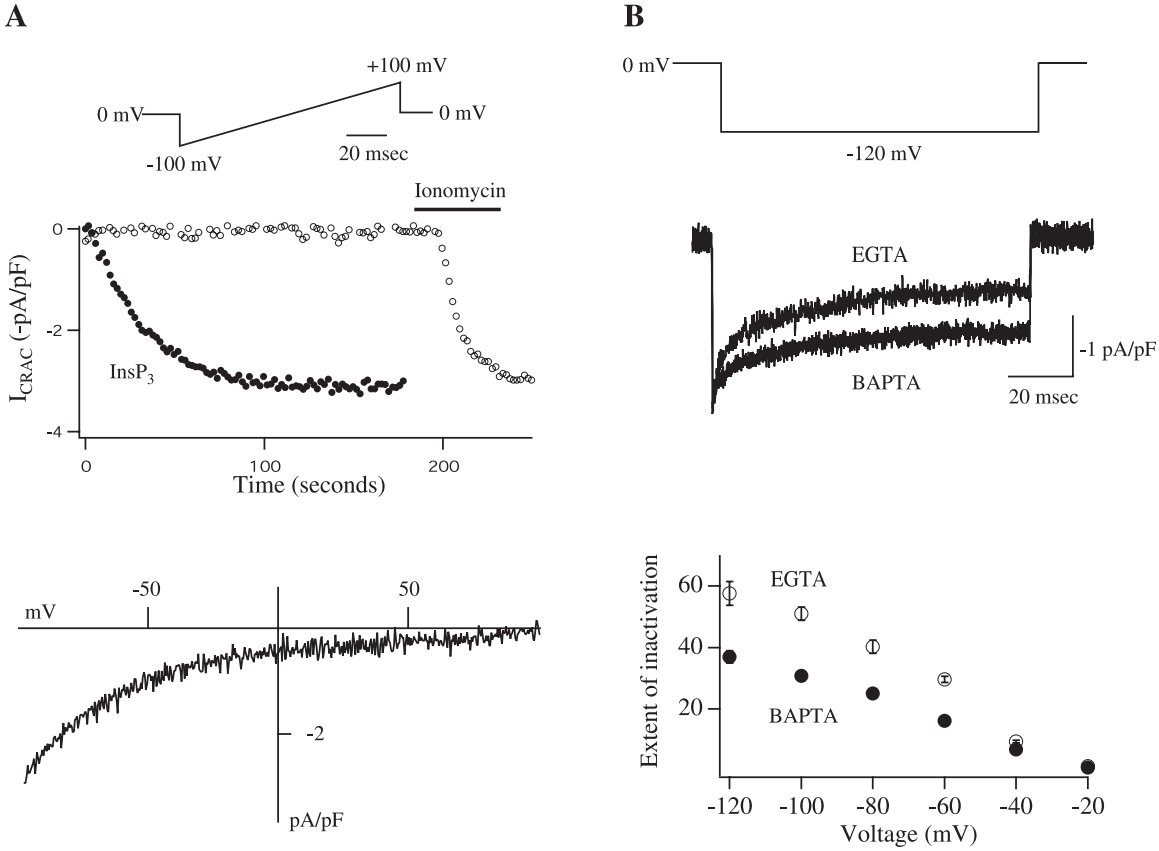

FIG. 2. Fundamental features of the store-operated Ca^{2+} current I_{CRAC}. *A*: time course of development of I_{CRAC} is depicted following store depletion in RBL-1 (rat basophilic leukemia) cells by including inositol 1,4,5-trisphosphate ($InsP_3$; ●) in the patch pipette or by applying ionomycin (○) onto the cell. In both cases, the whole cell patch-clamp technique has been used and Ca^{2+} has been strongly buffered at 120 nM (5 mM free EGTA) in the pipette solution. The voltage-ramp protocol for measuring I_{CRAC} is shown at the *top*. The *bottom panel* in *A* shows the current-voltage relationship for I_{CRAC}, taken when the current has reached steady-state. The current is inwardly rectifying and has a very positive reversal potential (>+70 mV), indicating high selectivity for Ca^{2+}. The current-voltage relationship has the same features irrespective of how stores are emptied. *B*: a general feature of CRAC channels is that they show fast Ca^{2+}-dependent inactivation. Permeating Ca^{2+} feedback locally (within a few nanometers of the pore) to reduce channel activity. Fast inactivation can be revealed by applying hyperpolarizing pulses, as shown in the *top panel*. After depleting store, stepping the voltage from 0 to −120 mV results in an initially large I_{CRAC} (due to the large electrical driving force), but the current then inactivates over a few milliseconds during the hyperpolarizing pulse. The extent of inactivation is ~60% when the slow Ca^{2+} chelator EGTA is included in the pipette, but it is considerably less when the fast Ca^{2+} chelator BAPTA is used instead. The *bottom panel* shows the extent of inactivation at different voltages in the presence of EGTA (○) and BAPTA (●).

suppressed. However, patch clamping is not without its own complications. In the whole cell configuration, which is the most popular way to study store-operated entry at the present juncture since the single-channel conductance of many, but not all, store-operated channels is well beyond the bandwidth of current patch-clamp amplifiers, important but often ill-defined, cytoplasmic factors diffuse into the pipette and are hence lost from the cell. For example, mitochondria are not in a highly energized state due to washout of oxidizable substrates, and therefore, these substrates need to be included in the pipette solution to support I_{CRAC} under physiological conditions of weak intracellular Ca^{2+} buffering (102, 103, 142). Moreover, isolation of I_{CRAC} requires inhibition of other currents, and hence, the ionic composition of the pipette solution is hardly physiological. Some of these problems can be avoided using perforated patch recordings. Finally, in some cell types, the store-operated currents appear to be very small, limiting the usefulness of direct current measurement in such instances. The one advantage of fluorescent indicators in these cases is their greater sensitivity to small Ca^{2+} fluxes compared with direct current measurement. Nevertheless, patch clamping remains the most direct and unambiguous method for studying store-operated influx. It is probably no exaggeration to say that a lot of the discordant results and controversies in the store-operated Ca^{2+} influx field emanate from differences in the techniques used. Ideally, it is probably best to use a combination of experimental approaches.

V. ELECTROPHYSIOLOGICAL PROPERTIES OF STORE-OPERATED CALCIUM CURRENTS

A. I_{CRAC}

1. Current-voltage relationship

Ca^{2+} release-activated Ca^{2+} current is a non-voltage-gated current in that, unlike Ca^{2+} currents through the Cav family, it is not opened by membrane depolarization. Once activated, I_{CRAC} has a characteristic current-voltage relationship (Fig. 2). The current amplitude is large at negative potentials and approaches the zero current level at very positive potentials ($>+60$ mV). In the standard experimental paradigm to monitor I_{CRAC} (voltage ramps spanning -100 to $+100$ mV in 50 ms), the current-voltage relationship reveals a prominent inward rectification at negative voltages (143, 270). Part of this rectification is a consequence of the asymmetric Ca^{2+} concentrations used to measure the current (usually 10 mM outside and a few nanomolar Ca^{2+} inside), which would give rise to some rectification as predicted by the Goldman-Hodgkin-Katz theory. In addition, rectification is accentuated during the ramp protocol by Ca^{2+}-dependent inactivation of the CRAC channels, which leads to a modest steepening of the current-voltage curve at negative potentials. Consistent with this is the finding that, in divalent-free external solution, Na^+ readily permeate through CRAC channels (see sect. v*A5*) and now rectification is less steep (see Fig. 1B in Ref. 17 for superimposition of current-voltage plots with Ca^{2+} and Na^+ as the charge carriers).

The non-voltage-gated behavior and inward rectification are sometimes taken as unequivocal evidence for the presence of I_{CRAC}. However, inward rectification and non-voltage-gated behavior are shared by other Ca^{2+}-selective channels including both TRPV5 and TRPV6, neither of which appear to be store-operated (72, 381). Inward rectification and non-voltage-dependent gating are not unique to I_{CRAC} and therefore should not be considered as diagnostic of the current.

2. Voltage-dependent CRAC conductance

Although CRAC channels are not opened by membrane depolarization, they nevertheless exhibit a slow voltage dependence at least in RBL-1 cells (14, 16). Whole cell CRAC conductance appears to be, directly or indirectly, voltage dependent in that hyperpolarizing holding potentials reduce the size of the current, whereas depolarization increases it, when the current amplitude is measured at -80 mV. Voltage jump relaxation experiments reveal that the voltage-dependent conductance changes develop and reverse slowly, with time constants of several seconds (14). This voltage dependence of the whole cell CRAC conductance can be seen in divalent-free external solution where Na^+ is the charge carrier, although it is less prominent than when Ca^{2+} is the charge carrier. Hence, the slow voltage dependence cannot be wholly explained by Ca^{2+} entry-dependent inactivation of I_{CRAC}. The physiological relevance of voltage-dependent I_{CRAC} remains to be established. Quite large fluctuations in membrane potential have been reported in RBL-1 cells following store depletion (88, 222). Depolarization of the membrane potential would reduce the electrical driving force for Ca^{2+} entry through CRAC channels, but this would be compensated somewhat by the increased macroscopic conductance imparted by the voltage dependence. Hence, different cell-surface receptors may evoke distinct patterns of intracellular Ca^{2+} signals depending on their effects on the membrane potential.

3. Channel selectivity

With Ca^{2+} as the charge carrier, I_{CRAC} approaches zero at very positive voltages ($>+60$ mV), indicative of a high selectivity for Ca^{2+} (270). Indeed, if extracellular Na^+ is replaced by large organic cations like *N*-methyl-D-glucamine ($NMDG^+$), then neither the extent nor apparent reversal potential of I_{CRAC} is altered (144), even in the presence of physiological levels of external Ca^{2+} (1–2

mM; Ref. 90). Moreover, removal of external Ca^{2+} in the continuous presence of external Na^+ and Mg^{2+} abolishes the current completely (90, 143, 417). Unlike current through voltage-operated Ca^{2+} channels, I_{CRAC} does not seem to support any detectable outward currents carried by K^+ or Cs^+, and this renders it difficult to establish a clear reversal potential for the current (270). Hence, the permeability ratio of Ca^{2+} to other ions has been hard to quantify. One way to assess the Ca^{2+} permeability of a Ca^{2+} channel is to relate the amount of Ca^{2+} entering per unit time (integral of the Ca^{2+} current) to the change in the Ca^{2+}-dependent wavelength of fura 2, when this dye is the dominant Ca^{2+} buffer in the cell (252). The assumption is that all incoming Ca^{2+} through the channels is captured by the fura 2. With the use of this approach, it has been concluded that I_{CRAC} in mast and RBL cells is more selective for Ca^{2+} than Cav channels (141). As the latter have a Ca^{2+}:Na^+ permeability ratio of 1,000:1 and Na^+ outnumber Ca^{2+} by more than 70:1 under physiological conditions, CRAC channels are remarkably selective for Ca^{2+}.

Permeability studies of other divalent cations like Ba^{2+} and Sr^{2+} through CRAC channels have been hampered by the fact that CRAC channels seem to require external Ca^{2+} to maintain their maximal activity, a process called calcium-dependent potentiation (60, 420). Because whole cell dialysis with high concentrations of BAPTA fail to affect calcium-dependent potentiation and the nonpermeating cation Ni^{2+} can replace external Ca^{2+} in supporting channel activity, the Ca^{2+} binding site is thought to be extracellular. Ba^{2+} and Sr^{2+} do not support potentiation. Therefore, replacing external Ca^{2+} with either Ba^{2+} or Sr^{2+} results in a decline of channel activity, and steady-state current measurements lead to a significant underestimate of permeability of divalent cations that do not support potentiation. Rapidly replacing Ca^{2+} with Ba^{2+} results in a transiently larger peak Ba^{2+} current (before depotentiation develops), indicating that CRAC channels actually conduct Ba^{2+} better than Ca^{2+}.

4. Anomalous mole fraction

CRAC channels, like Cav channels, are thought to achieve high Ca^{2+} selectivity by high-affinity binding of Ca^{2+} within the channel pore, which prevents Na^+ from permeating (193, 270). When external Ca^{2+} concentration is very low (submicromolar range), large Na^+ currents readily flow through CRAC channels. As Ca^{2+} concentration increases to the micromolar range however, Na^+ permeation is reduced as a Ca^{2+} occupies a high-affinity site within the channel (16, 192). The apparent dissociation constant (K_D) for Ca^{2+} block of the Na^+ current is close to 10 μM in RBL-1 cells (16) and 4 μM in Jurkats (192). As external Ca^{2+} increases further to the millimolar range, mutual repulsion between two Ca^{2+} provides the driving force for selective Ca^{2+} permeation, with apparent K_D of 0.8 mM in RBL-1 cells (90), 2.1 mM in Jurkat T lymphocytes (293), and 3.3 mM in mast cells (144).

Similar behavior is also seen in mixtures of divalent cations Ca^{2+} and Ba^{2+} (141). The conductance of CRAC channels is lower in 10 mM external Ca^{2+} than equimolar Ba^{2+} solutions, but with mixtures of the two ions, the conductance falls to a level less than that seen in either pure Ca^{2+} or Ba^{2+} solutions. Such concentration-dependent permeability ratios are indicative of multi-ion pores and support the idea that CRAC channels select for Ca^{2+} over Na^+ by high-affinity binding of Ca^{2+} to the selectivity filter.

5. Monovalent permeation through CRAC channels: sizing the pore

Like voltage-operated Ca^{2+} channels and TRPV5/6 channels, CRAC channels lose their selectivity in divalent-free external solution (17, 144, 192, 292). Now, Na^+ can readily permeate the channels, resulting in whole cell currents that are five- to eightfold larger than the corresponding Ca^{2+} currents. In divalent-free bath solution and Mg^{2+}/Mg^{2+}-ATP in the pipette, the Na^+ current develops with a similar time course to that of Ca^{2+} following store depletion, and the current is still inwardly rectifying, although to a slightly lesser extent than with Ca^{2+} (16, 17, 144, 292). Unlike the situation with Ca^{2+}, with Na^+ as the permeating species a clear reversal potential can be discerned because of a small but resolvable outward current. Hence, it has become possible to study the selectivity of CRAC channels and hence estimate the minimum pore diameter (17, 292). In RBL cells, ion substitution experiments revealed that the permeability profile for monovalent cations through CRAC channels was $Na^+ = Li^+ > Rb^+$ (0.67) $> Cs^+$ (0.10), and relatively large organic cations like trimethylamine, tetraethylammonium, NMDG, and Tris were essentially impermeant (16). A similarly low P_{Cs}/P_{Na} has been found for CRAC channels in RBL-2H3 (381) and Jurkat T lymphocytes (292). This monovalent permeation profile corresponds to an Eisenmann sequence type X, indicative of a strong-field strength site (17). Such selectivity studies reveal some interesting differences between CRAC and Cav channels (17, 292). Cav1.1–1.4 channels are around six times more permeable to Cs^+ than CRAC channels, and trimethylamine is conducted through Cavs but not CRAC channels. Trimethylamine has a molecular diameter of 0.55 nm, placing the estimated minimum pore size of Cav to be >0.6 nm. The corresponding minimum diameter of the CRAC channel is slightly larger than 0.32 nm (diameter of a Cs^+) but <0.55 nm. CRAC channels, like Cavs, are multi-ion pores, and their selectivity is likely to be achieved by binding of Ca^{2+} to sites (aspartate or glutamate) lining the Ca^{2+}-selective pore. In addition, the relatively small size of the CRAC

channels suggests that molecular sieving may play an auxiliary role in determining channel selectivity and might explain why Cs^+ is relatively impermeable (17).

6. Single CRAC channel conductance

With Ca^{2+} as the charge carrier, single CRAC channel openings have not been seen. Over a wide range of voltages, Hoth and Penner (144) failed to detect any increase in whole cell variance as I_{CRAC} developed in mast cells. They estimated the single-channel conductance to be significantly lower than 1 pS. Stationary noise analysis in Jurkat cells revealed a unitary chord conductance of 24 fS in isotonic Ca^{2+} solution, well beyond the typical bandwidth of a patch-clamp experiment (417). This conductance is almost 1,000-fold smaller than the single-channel conductance of most ion channels. Hoth and Penner (144) observed a small increase in the current variance when Na^+ permeated the CRAC channels. Using this method in lymphocytes, Lepple-Wienhues and Cahalan (192) reported a unitary CRAC conductance of 2 pS. Kerschbaum and Cahalan (166) subsequently found that removal of Mg^{2+} from the pipette solution dramatically increased the size and duration of the monovalent current. Under these conditions of divalent-free solution on both sides of the Jurkat cell membrane, they detected single-channel events of 35–40 pS which developed with a time course that corresponded with passive store depletion and which was inhibited by extracellular divalent and trivalent cations (Ca^{2+}, Mg^{2+}, Ni^{2+}, and Gd^{3+}). The ability to record single CRAC channel activity opened up the possibility for accreting molecular details of the channels as well as directly investigating the activation mechanism. Indeed, recording single-channel events in the absence of external divalents, which was attributed to CRAC channels, has been used to probe both the regulation and gating mechanisms of these channels (42, 92, 323). Furthermore, the single-channel conductance formed one of the central pieces of evidence that the TRPV6 gene encoded the CRAC channel pore (403; see sect. XII*B*). However, subsequent studies by several laboratories have now established that the 35- to 40-pS conductance channels are not CRAC channels (17, 132, 177, 292). Instead, they represent nonselective cation channels that are opened following the removal of intracellular Mg^{2+}/Mg-ATP. These channels, called MagNuM or MIC, are quite widespread, being found in several cell lines including RBL-1, Jurkat, and HEK 293 cells, all of which are popular systems for studying I_{CRAC} (251). MagNuM/MIC is most likely encoded by TRPM7 gene (251, 322). The current through MagNum/MIC channels, unlike CRAC, is not regulated by store depletion, is much more permeable to Cs^+, has a very different current-voltage relationship in both divalent-containing and divalent-free solution dominated, and has a different pharmacological profile (17, 130, 177, 292). A crucial point is that MagNuM/MIC is suppressed by millimolar levels of Mg^{2+}/Mg-ATP in the recording pipette (251). Under these conditions, I_{CRAC} can be studied in relative isolation. In the presence of intracellular Mg^{2+}, fluctuation analysis has revealed that the single-channel CRAC conductance in divalent-free external solution is ~0.2 pS (292). This is an important result for several reasons. First, it establishes a biophysical hallmark of the CRAC channel that can be used to assess the validity of putative CRAC channel genes. Second, it raises intriguing questions concerning channel permeation. Cavs achieve high selectivity by binding Ca^{2+} within the pore. When the pores lack Ca^{2+}, selectivity is compromised, and Na^+, Cs^+, and even large cations like trimethylammonium (TMA^+) permeate with a high throughput rate. Even in the absence of Ca^{2+} and Mg^{2+} however, CRAC channels retain some selectivity by discriminating between monovalent cations, and the throughput rate of Na^+ is relatively low. How this is achieved will require detailed structure-function studies once the channel has been cloned.

B. Non-CRAC Store-Operated Currents

These channels have not been as well studied as CRAC channels. Therefore, their biophysical features are sometimes sketchy. Basic features of these channels are compared with those of CRAC channels in Table 1.

1. Store-operated channels in A431 epidermal cells

Mozhayeva and colleagues (171, 405, 414) have described single store-operated channels from human A431 carcinoma cells. In cell-attached patches, these channels could be activated by stimulation of cell-surface receptors, by thapsigargin or, less frequently, by incubating cells in BAPTA-AM. In inside-out patches, channel activity could be induced by $InsP_3$ applied to the cytoplasmic side. With 105 mM $BaCl_2$ or $CaCl_2$ in the pipette solution, the reversal potential was estimated to be around +65 mV, indicating selectivity for divalent cations. Ba^{2+} and Ca^{2+} were equally permeable but ~1,000 times more so than K^+. The channels were blocked by SK&F-96365 and had a resolvable conductance of ~1 pS, which increased to 6 pS with Na^+ as the charge carrier. Channel mean open time was ~7.7 ms, and the channels were voltage dependent in that open probability increased with hyperpolarizations beyond −40 mV. The channels have been referred to as I_{min} or I_{CRACL} (CRAC-like). The gating of these channels will be discussed in section IX*B*.

Lueckhoff and Clapham (207) have also reported a store-operated channel in A431 cells. These authors used a double patch approach in which one pipette was used to deplete the stores in the whole cell configuration and the second pipette was in the cell-attached mode. Following store depletion with either thapsigargin and high BAPTA

or high BAPTA alone, 2-pS channels were opened in the cell-attached patch in the presence of 160 mM $CaCl_2$, and the conductance increased to 20 pS in the presence of Ba^{2+}. Channel activity was transient, decaying within 4 min. Excision of the patch resulted in rapid rundown of channel activity, and this could not be recovered by $InsP_3$. There are some striking differences between this store-operated channel and I_{min} (171, 405), also reported in A431 cells. The channels differ in single-channel conductance, voltage dependence, and gating by $InsP_3$. The reason for these discrepancies is unclear.

2. Store-operated channels in endothelia

In bovine aortic endothelial cells, application of either the receptor agonists bradykinin or ATP or the SERCA pump blocker, di-*tert*-butylhydroquinone, activated Ca^{2+}-permeable channels in cell-attached patches (369). The Ca^{2+}:Na^+ permeability ratio was estimated to be >10:1, and anomalous mole fraction was seen in mixtures of Na^+ and Ca^{2+}. In the absence of external Ca^{2+}, the single-channel conductance was ~5 pS and fell to 2.5 pS in 1 mM Ca^{2+}. Raising Ca^{2+} to 10 mM increased the conductance to 11 pS. Channel activity was lost quite rapidly on excising the patch to the inside-out configuration but was less resistant to run down in the outside-out mode.

In calf pulmonary artery endothelial cells, a Ca^{2+}-permeable current was described that could be activated by store depletion with $InsP_3$, the SERCA pump blocker di-*tert*-butylhydroquinone or ionomycin (83). The current was small, being only ~20% that of I_{CRAC} in Jurkats at −80 mV. It was inwardly rectifying with a positive reversal potential and was blocked by micromolar concentrations of La^{3+}. Perifusing cells with divalent-free solution increased the size of the current severalfold, and rectification was maintained somewhat. The authors concluded that calf pulmonary artery endothelia expressed a current very similar to I_{CRAC}.

In mouse aortic endothelial cells, Nilius and colleagues (96) have described an inwardly rectifying store-operated Ca^{2+}-permeable current. On switching to divalent-free solution, the current amplitude increased threefold and the channels were quite permeable to Cs^+ as well as Na^+. Another interesting feature of this endothelial current was that Ca^{2+} blocked the monovalent flux with ~20-fold higher affinity than that seen for CRAC channels.

Store-operated currents have been reported in several different types of endothelial cells, and the reader is referred to the excellent discussion of this issue in a recent review (256).

3. Store-operated channels in vascular smooth muscle

In mouse and rabbit aorta, 3-pS store-operated channels have been described (364). In cell-attached patches, the channels were activated by thapsigargin even after the cells had been exposed to BAPTA-AM, suggesting that they were not activated by the thapsigargin-evoked rise in cytoplasmic Ca^{2+} that accompanies store emptying. In 30% of the cells, incubation with BAPTA-AM alone was able to activate the channels. These channels were cation selective but did not discriminate between Na^+, K^+, Cs^+, Ca^{2+}, Ba^{2+}, or Sr^{2+}. In excised patches, the conductance did not change when Ca^{2+} (1 or 10 mM) was added to the Na^+-containing pipette solution, indicating that the channels do not prefer Ca^{2+} over Na^+ when both cations are present. However, with 90 mM Ca^{2+} in the pipette (and no Na^+), a slope conductance of 2.7 pS was found. Hence, these channels are permeable to Ca^{2+}, but it would appear that much of the current is carried by Na^+ (P_{Ca}:P_{Na} = 1). These channels were voltage dependent in that channel activity increased more than threefold at potentials positive to +50 mV. The channels were activated by Ca^{2+} influx factor (363), and this is discussed further in section IX*A*.

In myocytes from rabbit portal vein, single store-operated channels have also been described (1). In cell-attached recordings, the channels could be activated by the SERCA pump blocker cyclopiazonic acid, caffeine, incubating cells with BAPTA-AM, or the calmodulin antagonist W-7. In addition, spontaneous openings of the channels were also observed in the absence of any stimulation. For all cases, the current-voltage relationship was linear over the range −40 to −120 mV, with a slope conductance of 2–3 pS. No single-channel events were observed at positive potentials, so the reversal potential was estimated by linear interpolation to be +20 mV (126 Na^+, 1.5 mM Ca^{2+}). The open lifetime distributions could be fitted by the sum of at least two exponentials, yielding time constants of 5 and 30 ms. Unlike the channels reported in aortic myocytes (364), those in portal vein were affected by external Ca^{2+}. In Ca^{2+}-free solution, the slope conductance increased to 7 pS, and the reversal potential shifted to −4 mV. In isotonic $CaCl_2$, the conductance fell to 1.3 pS, and the reversal potential was estimated to be +80 mV. P_{Ca}:P_{Na} was calculated to be ~50:1.

In proliferating pulmonary artery smooth muscle cells, cyclopiazonic acid activated single channels with a slope conductance of 5.4 pS in the presence of 120 mM Na^+ and 20 mM Ca^{2+} (112). However, the selectivity of these channels was not explored. In human glomerular mesangial cells, which have contractile properties similar to smooth muscle cells, recordings from cell-attached patches revealed spontaneously active channels that were considered to be store-operated (212, 213). These channels had a slope conductance of 0.7 pS in 90 mM $CaCl_2$ and 2.1 pS in 90 mM $BaCl_2$, with estimated reversal potentials of +123 and +63 mV, respectively. Channel activity was not voltage dependent over the range 0 to −80 mV.

It is not always clear whether the cell-attached single-channel events described above are indeed due to store-operated channels as opposed to another Ca^{2+} influx pathway. The best evidence that these channels are store-operated is that channel activity is still seen after cells have been loaded with BAPTA-AM. However, it has not always been shown that sufficient BAPTA has accumulated in the cytosol to suppress the rise in Ca^{2+} following store depletion. Furthermore, for those channels with a P_{Ca}:P_{Na} of 1, most of the current will be carried by Na^+ under physiological conditions. Very large inward currents would be required to elevate Ca^{2+} appreciably. The role of these nonselective channels in muscle, for example, might simply be to provide the depolarization that is necessary for the more Ca^{2+}-selective voltage-gated Ca^{2+} channels to open.

4. Store-operated calcium channels in skeletal muscle

In mouse skeletal muscle, Ca^{2+} leak channels have been observed in resting cells (140). These channels were non-voltage-gated; exhibited a single-channel conductance of between 7 and 14 pS; did not distinguish between Ca^{2+}, Ba^{2+}, and Mn^{2+}; and were inhibited by novel dihydropyridines like AN406 and AN1043 which only weakly affect voltage-operated Ca^{2+} channels. In cell-attached patches, channel open probability was increased by the SERCA pump blocker cyclopiazonic acid. Inhibition of the Ca^{2+} leak channels with AN406 reduced the extent of store refilling following store emptying, indicating that the Ca^{2+} leak channels could contribute to the reloading of the stores. Interestingly, the open probability of these Ca^{2+} leak channels was greater in resting dystrophic muscle cells than in resting normal myocytes. It has been suggested that this higher resting permeability to Ca^{2+} in dystrophic cells results in stimulation of the Ca^{2+}-dependent protease calpain (4). One idea is that calpain might then alter the activity of the Ca^{2+} leak channels resulting in further Ca^{2+} influx. This kind of positive feedback could result in a gradual loss of Ca^{2+} homeostasis leading ultimately to cell death.

5. Neurons and neuroendocrine cells

In bovine adrenal chromaffin cells, store depletion with either thapsigargin or dialysis with 10 mM BAPTA activated a small, inward current at negative potentials (93). The current was nonselective, being carried by both Ca^{2+} and Na^+. Because of the presence of voltage-gated Ca^{2+} channels, the store-operated current could only be measured at potentials more negative than −60 mV. The estimated reversal potential of the current was quite negative (−20 mV), although linear extrapolations do not take into account rectification and hence may underestimate the zero current potential. The small Ca^{2+}-permeable current induced by thapsigargin was able to stimulate exocytosis at negative potentials as well as potentiate secretory responses following activation of voltage-operated Ca^{2+} channels. Although the rate of secretion to thapsigargin was ~30- to 60-fold slower than that elicited by depolarizing pulses which open voltage-operated Ca^{2+} channels, nevertheless the total secretory response to thapsigargin was substantial, amounting to the fusion of 300–400 large dense-core vesicles. In chromaffin cells, movement of vesicles from the reserve pool to the ready-releasable pool is dependent on cytoplasmic Ca^{2+} and protein kinase C (106). Perhaps the main role for store-operated influx in exocytosis is to maintain the size of the ready-releasable pool that would be severely depleted following a train of action potentials.

The existence of store-operated entry in neurons has been much harder to establish. This reflects the complex architecture of neurons rendering it hard to reliably measure small currents that are not located exclusively on the soma, the presence of many ionic conductances which need to be eliminated to dissect out the store-operated pathway, and the fact that neurons coexpress a multitude of Ca^{2+}-permeable channels (voltage operated, ligand gated, second messenger operated). Nevertheless, studies using SERCA pump blockers to deplete stores and fluorescent dyes to monitor Ca^{2+} influx have been interpreted as evidence for store-operated entry (301). A major concern with these sorts of experiments is that the Ca^{2+} influx pathway is not known. It could easily be a second messenger-operated one or, if membrane potential changes, even voltage-operated. The combination of patch-clamp recordings with microfluorimetry in dorsal root ganglion neurons has revealed that depletion of the caffeine-sensitive store evokes Ca^{2+} influx at hyperpolarized potentials (367). Ca^{2+} influx following store emptying was blocked by 2 mM Ni^{2+}, was insensitive to antagonists of voltage-gated Ca^{2+} channels, and was facilitated by hyperpolarizing the membrane potential from −55 to −80 mV, a maneuver that increases Ca^{2+} influx due to the larger driving force. This Ca^{2+} influx pathway was required for refilling the caffeine-sensitive stores. Whether store-operated influx is more widespread in the nervous system and the nature of the underlying channels remain to be determined.

VI. PHARMACOLOGY OF STORE-OPERATED CHANNELS

Progress in the store-operated Ca^{2+} influx field has been severely hindered by the lack of relatively specific inhibitors of the underlying Ca^{2+} channels. Although several agents (econazole, SK&F-96365, 2-aminoethoxydiphenylborane) are known to inhibit store-operated channels, they also can inhibit other channels over similar concentration ranges (95). Hence, they cannot be considered diagnostic of a store-operated mechanism.

Like all Ca^{2+} influx pathways, store-operated channels are inhibited by divalent and trivalent cations, probably via a block by slow permeation. Trivalent cations like La^{3+} and Gd^{3+} are particularly effective, blocking the channels fully in the low micromolar concentration range (144). In experiments employing fluorescent dyes to study Ca^{2+} influx, Gd^{3+} is often used to separate endogenous store-operated channels from recombinant TRPs, since the endogenous pathway is effectively blocked by Gd^{3+} at concentrations that fail to interfere with TRP channel activity (360).

Another pharmacological agent that has become popular for probing store-operated Ca^{2+} entry is 2-aminoethoxydiphenylborane (2-APB). 2-APB is a membrane-permeable inhibitor of $InsP_3$ receptor function, but it exploded onto the store-operated Ca^{2+} entry scene following the report by Ma et al. (211) that it rapidly inhibited thapsigargin-evoked Ca^{2+} influx even when applied after Ca^{2+} entry had developed. This suggested that $InsP_3$ receptors were required for sustaining store-operated influx and was therefore considered compelling evidence in support of the conformational-coupling mechanism for activation of store-operated entry. Although electrophysiological experiments subsequently confirmed that 2-APB inhibited I_{CRAC} activation (13, 291, 381), several observations cast doubt on the conclusion that the effects of the drug arose simply from inhibition of $InsP_3$ receptors. Instead, the most parsimonious explanation was that 2-APB inhibited the store-operated channels directly, most likely on an external site. First, 2-APB was much less effective in inhibiting I_{CRAC} when included in the pipette solution (13, 42), even when the pipette concentration was 20-fold higher than an external concentration which caused full block. However, for hydrophobic drugs that freely cross membranes, the rate of diffusion across the pipette tip constitutes the rate-limiting step. Hence the steady-state concentration of the drug in the cytosol may be significantly lower than the pipette concentration. Nevertheless, 2-APB still blocked I_{CRAC} when applied externally in acidified medium, a condition which presumably protonates 2-APB and thus reduces its membrane permeability (291). Second, following full activation of I_{CRAC}, external application of 2-APB rapidly inhibited the current with a time constant only slightly longer than that seen with the direct channel blocker La^{3+} (13). Third, 2-APB inhibited I_{CRAC} and store-operated entry in the mutant DT40 cell line in which $InsP_3$ receptors are not expressed (45, 210, 291). Hence, $InsP_3$ receptors are not required for 2-APB block of store-operated entry.

Because 2-APB seems to block CRAC channels directly and rapidly, it is becoming a popular tool to probe functional consequences of inhibiting store-operated entry. A caveat here is that the drug is now known to interfere with a variety of transport processes including SERCA pumps (237), K^+ channels (385), MagNuM/MIC channels (130), and mitochondrial Ca^{2+} efflux (291). Furthermore, 2-APB has been found to activate the heat-gated recombinant TRPV1, TRPV2, and TRPV3 channels in HEK 293 cells (61, 146) as well as in native keratinocytes (61). The concentration range over which 2-APB activates TRPV3 is similar to that with which it affects store-operated entry (61). Hence, great care is needed in interpreting results based on the use of 2-APB.

At low concentrations (1–5 μM), 2-APB potentiates I_{CRAC} up to fivefold in Jurkat lymphocytes (291). At higher concentrations, the drug first enhances I_{CRAC} but then the inhibitory effect dominates (291). Low concentrations of 2-APB actually accelerate Ca^{2+}-depedent fast inactivation, whereas higher concentrations reduce it. In RBL-1 cells on the other hand, the potentiation is either much weaker (291) or absent (13, 381). In some systems then, 2-APB seems to potentiate I_{CRAC} even after maximal store depletion. A similar potentiating effect has been reported by the antidiarrheal agent loperamide. This drug increased store-operated influx, measured using fura 2, in a variety of cell types (124), following store emptying with thapsigargin, ionomycin, or receptor-induced elevation of $InsP_3$. Loperamide did not enhance Ca^{2+} signals to sphingosine. Loperamide, like 2-APB, is a promiscuous drug and hence should not be considered a specific tool for modulating store-operated influx. Nevertheless, understanding how these drugs enhance I_{CRAC} could provide new insight into CRAC channel gating.

Another inhibitor of I_{CRAC} is the vanilloid capsaicin, the piquant component of red chili peppers. Capsaicin inhibited I_{CRAC} rapidly and reversibly in Jurkats, with an IC_{50} of 30 μM (91). The block was not voltage-dependent. Capasaicin also inhibited the voltage-dependent K^+ current (Kv1.3) in these cells with a similar concentration dependence. Because capsaicin activates vanilloid type I (VR1) receptors as well as affecting other channels, the block should not be considered specific for I_{CRAC}.

Diethylstilbestrol, a synthetic estrogen agonist, has been found to elicit a rapid and reversible block of store-operated entry in RBL-1 cells, aortic smooth muscle, and platelets (404). Fifty percent inhibition was seen in the 0.1–1 μM concentration range, and the inhibitor was effective only from the extracellular side. Diethylstilbestrol did not affect the nonselective current carried by non-store-operated TRPM7 channels in RBL cells. *Trans*-stilbene, a closely related compound but lacking hydroxyl and ethyl groups, did not inhibit Ca^{2+} influx.

It is important to note that all pharmacological studies on CRAC channels have been carried out in the whole cell configuration, since single-channel recordings are not possible. It is difficult in whole cell recording to identify unequivocally a blocking action as directly on the channels themselves rather than through a local regulatory pathway.

VII. ACTIVATION OF STORE-OPERATED CALCIUM ENTRY: NEED FOR A RETROGRADE SIGNAL

In the initial formulation of capacitative Ca^{2+} influx, incoming Ca^{2+} was considered to pass directly into the stores from the external solution before being released into the cytosol (297). This was thought to occur because the stores were somehow preferentially connected to the external solution. Upon an increase in the levels of $InsP_3$, Ca^{2+} would be released from the stores and so store Ca^{2+} content would fall. Because of this preferential link between stores and the extracellular space, Ca^{2+} would first enter the stores only to pass through into the cytosol via open $InsP_3$ receptors. As $InsP_3$ levels fell, the release channels closed and hence Ca^{2+} no longer entered the cytosol. The build-up of Ca^{2+} within the stores led to inhibition of further Ca^{2+} influx. Activation of store-operated entry would therefore be a simple passive process, without the need for a dedicated activation signal. However, subsequent work did not support the idea that incoming Ca^{2+} first passed into the stores. First, patch-clamp experiments combined with fluorescent recordings revealed that Ca^{2+} influx occurred only after a substantial delay following $InsP_3$-mediated Ca^{2+} release (279). A pronounced dissociation between release and influx is not easily reconcilable with a direct refilling route. Second, following store depletion with the SERCA ATPase inhibitor thapsigargin, Ca^{2+} influx occurred resulting in an elevation of cytoplasmic Ca^{2+} concentration. Thapsigargin releases Ca^{2+} through ill-defined leak channels in the ER membrane. However, the thapsigargin-dependent Ca^{2+} signal was not increased further by elevating $InsP_3$ (350). If Ca^{2+} first entered the stores and then was released into the cytosol, opening of $InsP_3$-gated Ca^{2+} channels in the ER membrane would be expected to increase the rate of Ca^{2+} release from the stores and hence should elevate cytoplasmic Ca^{2+} further. This was not the case. Finally, the rate and extent of store refilling was found to correlate well with the global increase in cytoplasmic Ca^{2+} concentration arising from Ca^{2+} influx, consistent with the notion that incoming Ca^{2+} first entered the cytoplasmic space before being taken up into the stores (155, 246).

The fact that Ca^{2+} entry did not pass directly into the stores had one enormous consquence. Information about the store Ca^{2+} content had to be conveyed to the Ca^{2+} entry channels indirectly, by an intermediate signaling mechanism. This mechanism requires both a Ca^{2+} sensor that detects the fall in intraluminal Ca^{2+} content and then a signal which conveys this information to the plasma membrane. Despite intense investigation and some interesting leads (discussed below), the nature of this retrograde signaling mechanism is far from resolved.

VIII. THE CALCIUM SENSOR

The initial step in the activation of store-operated influx is the detection of a fall in intraluminal Ca^{2+} content. A sensor is required for this, but its identity is unknown. In the conformational-coupling idea (see below), the sensor would be the $InsP_3$ receptor, since it has been reported to change its affinity for cytoplasmic $InsP_3$ as intraluminal Ca^{2+} content is altered (25), although this has been challenged (332). The role of the $InsP_3$ receptor will be discussed in more detail in section IX*B1*.

Calreticulin is a conserved, high-capacity and low-affinity Ca^{2+}-binding protein found in the ER of virtually all eukaryotic cells. It comprises ~1–2% of total ER protein. Because of the large amount of calreticulin in the stores and its low affinity for Ca^{2+}, it is believed that calreticulin is a major intraluminal Ca^{2+}-binding protein. This has led to the suggestion that calreticulin might be an important regulator of store-operated entry, perhaps even as the elusive Ca^{2+} sensor. Stable overexpression of calreticulin in a fibroblast cell line was reported to reduce thapsigargin-evoked Mn^{2+} influx through a mechanism unrelated to its effects on buffering Ca^{2+} within the stores (232). On the other hand, studies employing a calreticulin knock out mouse indicated that store-operated entry was unchanged (65). It has been argued that these stably transfected and knock out cells allowed for alternative, intrinsic homeostatic mechanisms to develop (84). Hence, the observations made do not reflect the role of calreticulin itself but rather that of adaptive processes to compensate for the altered levels of expression. When RBL-1 cells were transiently transfected with calreticulin, it was found that I_{CRAC} developed more slowly when stores were depleted passively (by dialysis with strong buffer) compared with nontransfected cells (84). However, there were no differences in rate of development or extent of I_{CRAC} when stores were emptied rapidly following dialysis with $InsP_3$. The authors concluded that the effects of calreticulin were entirely due to its role as a Ca^{2+} buffer within the stores.

There are several other Ca^{2+} binding proteins associated with the ER including BiP, endoplasmin, PDI, erp72, and calnexin, all of which have low affinity for Ca^{2+} at least in vitro. Moreover, certain SERCA pumps (like SERCA3) are regulated by intraluminal Ca^{2+} (230). Hence, there are still many potential candidates that could function as all or part of the sensor.

IX. ACTIVATION MECHANISM

Perhaps the simplest mechanism for store depletion to activate store-operated Ca^{2+} channels would involve some kind of Ca^{2+}-induced Ca^{2+} entry whereby the Ca^{2+} that is released into the cytosol following store emptying

activates the Ca^{2+} entry channels. Three lines of evidence suggest that this mechanism is unlikely. First, when intracellular Ca^{2+} is tightly clamped at very low levels by dialyzing cells with high concentrations of Ca^{2+} chelator (EGTA or BAPTA), I_{CRAC} can still be activated by $InsP_3$ and thapsigargin (89, 143). Indeed, dialysis with high levels of EGTA or BAPTA alone can deplete the stores and open the store-operated Ca^{2+} channels (270). Second, dialysis of cells with Ca^{2+}-containing pipette solutions up to the micromolar range or flash photolysis of caged Ca^{2+} does not activate I_{CRAC} (90, 227). Third, the membrane-permeable metal chelator TPEN can enter the stores and lower intraluminal Ca^{2+} content without triggering any Ca^{2+} release (135). This reduction in intraluminal Ca^{2+} alone is sufficient for I_{CRAC} to activate. Collectively, these results demonstrate that a simple rise in cytoplasmic Ca^{2+} is not the signal linking store depletion to activation of store-operated influx.

In the past few years, several general models have been proposed for the activation of store-operated influx. However, most studies addressing this issue are plagued with a number of uncertainties. First, Ca^{2+} influx has not always been measured directly but inferred instead from steady-state cytoplasmic Ca^{2+} measurements. Hence, changes in membrane potential as well as alterations in Ca^{2+} removal mechanisms may affect the size of the signal. Second, evidence is generally obtained using pharmacological inhibitors, and many of these drugs have multiple targets. Indeed, as the studies with 2-APB demonstrate, one potential target is the Ca^{2+} channels themselves rather than, or in addition to, the activation mechanism.

A. Diffusible Messenger

The idea that a diffusible messenger might link the process of intracellular store emptying to activation of the plasmalemmal Ca^{2+} channels was first put forward by Putney and colleagues (350). They proposed that, upon store depletion, a messenger was released from the stores into the cytosol where it then diffused to the plasma membrane and opened the Ca^{2+} channels. A variety of mobile messengers have been proposed to fulfil this function (see Table 2). Most interest has focussed on an extract that is thought to contain a factor that is released from the stores and which can trigger Ca^{2+} entry in nonstimulated cells.

1. Ca^{2+} influx factor

Randriamampita and Tsien (303) first reported the existence of a low-molecular-weight factor in an acid-extracted fraction from a Jurkat cell line which was capable of activating Ca^{2+} influx in several different nonexcitable cells (303). This factor, dubbed Ca^{2+} influx factor (CIF), was released from the ER of Jurkat T cells following store depletion with either thapsigargin or an agonist of cell-surface receptors linked to the phosphoinositide pathway. CIF was a phosphorylated, nonproteinacious material that could be broken down by okadaic acid-sensitive protein phosphatases (304). However, subsequent work demonstrated that the acid-extracted fraction from Jurkat T cells evoked Ca^{2+} release and hence that the Ca^{2+} entry phase was simply a consequence of store emptying by $InsP_3$ (32). Crucially, pretreatment with the muscarinic receptor antagonist atropine suppressed the actions of the extract. Hence, this acid-extracted fraction from Jurkat T cells seemed to contain a multitude of factors capable of causing intracellular Ca^{2+} signals, at least in part by promoting Ca^{2+} release from intracellular stores. In an effort to isolate the putative CIF, subsequent work involved a series of purification steps on the acid-extracted fraction from store-depleted Jurkat cells and led to the identification of a fraction which, upon injection into *Xenopus* oocytes, stimulated Ca^{2+} entry [measured

TABLE 2. *Intracellular diffusible messengers that have been reported to activate store-operated Ca^{2+} entry*

Messenger	Cell Type	Measurement	Store-Operated Channel	Reference Nos.
Ca^{2+} influx factor	Aortic smooth mucle;	Single channel	2 pS nonselective	363
	Xenopus oocytes	Fura 2	Unknown	66
Protein kinase C	RINM5f	Fura 2	Unknown	36
	Rat mesangial cells	Single channel	2.1 pS, Ba^{2+} permeable	213
	Portal vein smooth muscle	Single channel	3 pS, Na^+ and Ca^{2+} permeable	2
Tyrosine kinase	Fibroblasts	Fura 2	Unknown	9
5,6-EET	Endothelia	Fura 2	Unknown	324
	Astrocytes	Fura 2	Unknown	396
Lysophospholipid	Aortic smooth muscle	Single channel	2 pS nonselective	337
Lipoxygenase derived	RBL-1 cells	Whole cell current	I_{CRAC}	109
GTP-binding proteins	RBL-1 cells	Whole cell current	I_{CRAC}	34,82
	Lacrimal gland	Fura 2	Unknown	34
Calmodulin	Hepatocytes	Fura 2	Unknown	52
	Skeletal muscle	Fura 2	Unknown	375
Sphingosine-1-phosphate	Neutrophils	Fura 2	Unknown	153

5,6-EET, 5,6-epoxyeicosatrienoic acid.

indirectly through a Ca^{2+}-dependent Cl^- current (167, 354)]. This activity was clearly distinct from that described earlier in that it acted only when applied internally. The active moiety had a molecular mass of 600 Da. However, the issue was confounded by a later study from the same group reporting that CIF, acting at lower concentrations, potentiated Ca^{2+} release from $InsP_3$-sensitive Ca^{2+} stores after all (355). More recent work, however, has arguably ushered in a resurrection of CIF. In *Saccharomyces cerevisiae*, mutation of the Ca^{2+}-ATPase encoded by pmr1 results in depletion of Ca^{2+} from secretory pathway organelles, and this is associated with enhanced Ca^{2+} uptake. Extracts from pmr1 mutant yeast evoked an increase in Ca^{2+} concentration when injected into *Xenopus* oocytes, and this increase was not seen when the extract was applied externally, or when injected into cells in the absence of extracellular Ca^{2+} (66). Extract derived from wild-type yeast was ineffective in promoting Ca^{2+} signals. Hence, extract from store-depleted yeast seemed to contain a factor that could promote Ca^{2+} influx into oocytes, but only via an intracellular action. The action and pattern of isolation of this activity suggested it was the same as that isolated previously from store-depleted Jurkat cells. The effects of the CIF-containing extract were also examined on I_{CRAC}, using whole cell patch-clamp experiments conducted on Jurkat T cells (66). Cells were dialyzed with 10 mM EGTA, a condition which passively empties the stores and thereby activates I_{CRAC} slowly. Including extract from either pmr1 mutant yeast or store-depleted Jurkat T cells significantly accelerated the rate of development of I_{CRAC}.

In addition to accelerating the development of I_{CRAC}, CIF-containing extracts from pmr1 yeast activated a second current in the Jurkat T cells (345). The channels underlying this current had a lower sensitivity to CIF, being activated at CIF concentrations greater than those required to accelerate the development of I_{CRAC}. Moreover, this second current was much larger than I_{CRAC}, exhibited outward rectification, and had a reversal potential around −15 mV. The authors stated that this nonselective current was store-operated (345). The underlying channels had a unitary chord conductance of 24 pS. In excised inside-out patches taken from the plasma membrane of Jurkat T cells, channel activity was very low. Direct application of CIF to the cytoplasmic side of the patch evoked single-channel events, suggesting that CIF directly gated these nonselective channels. These apparently store-operated nonselective channels were not seen in RBL cells, which might argue against a nonspecific or membrane-destabilizing effect of such high concentrations of CIF-containing extract (345).

Although CIF seems to directly activate the 24-pS nonselective channels, its interaction with CRAC channels appears more complex. The ability of low concentrations of CIF to accelerate development of I_{CRAC} in whole cell recordings from RBL-2H3 cells was compromised by including the *Drosophila* protein inactivation-no after potential D (INAD) in the recording pipette (344). Raising the CIF concentration partially rescued I_{CRAC} in the presence of exogenous INAD. INAD is a multivalent PDZ domain-containing scaffold protein that regulates the activity of *Drosophila* TRP/TRPL channels. Affinity-purified INAD that was covalently linked to a Sepharose matrix was found to retain the CIF component of acid-extracted fraction from Jurkat T cells when the latter was passed over the column (344). CIF can therefore bind directly to INAD, at least in vitro. This has led to the suggestion that, at resting Ca^{2+} levels, INAD maintains CRAC channels in the closed state. Upon store depletion however, CIF is generated and subsequently binds to INAD. This causes the complex to dissociate from the CRAC channels, resulting in channel opening (344). In this scenario, CIF would be functioning not as an activating signal in the true sense but rather as a means for removing channel inhibition.

Another study suggested a more direct action of CIF on store-operated influx (363). A 3-pS store-operated nonselective cation channel was reported in smooth muscle cells from thoracic aorta. In inside-out patches, channel activity was low. But direct application of CIF extracted from pmr1 mutant yeast rapidly activated the channels. Furthermore, if excised patches were exposed to permeabilized human platelets in which the stores had been depleted with thapsigargin, then the 3-pS channels were activated (363). Exposure of the patches to either thapsigargin alone (in the absence of platelets) or to permeabilized platelets but in which the stores had not been depleted, failed to activate the channels. Hence, store depletion resulted in the generation of an endogenous factor that was able to diffuse out of the platelets and activate the Ca^{2+}-permeable channels in the smooth muscle membrane. These same Ca^{2+} channels were activated by the acid-extracted fraction containing CIF from store-depleted platelets. Interestingly, subsequent experiments indicated that CIF activated the 3-pS channels indirectly, albeit in a membrane-delimited manner. Smani et al. (336) reported that active CIF extract could activate Ca^{2+}-independent phospholipase A_2 (PLA_2), an enzyme located close to the plasma membrane. This enzyme then cleaved phospholipids to generate lysophospholipid and arachidonic acid, with the former directly opening the store-operated channels. Calmodulin binds tightly to Ca^{2+}-independent PLA_2 under resting conditions, and this maintains the enzyme in an inactive state. Smani et al. (336) found that active CIF could displace Ca^{2+}-independent PLA_2 from calmodulin-Sepharose columns indicating that CIF could displace calmodulin from Ca^{2+}-independent PLA_2 thereby activating the enzyme. Importantly, the Ca^{2+}-independent PLA_2 inhibitor bromoenol lactone (BEL) suppressed activation of store-operated channels

following either store depletion with thapsigargin or exposure to CIF (336, 337). Antisense oligonucleotides directed against Ca^{2+}-independent PLA_2 in aortic smooth muscle reduced the activation of store-operated 3-pS cation channels, supporting the pharmacological results obtained with BEL (337).

These recent studies support a role for CIF in regulating store-operated influx in some systems. However, there are still some unresolved issues.

In Jurkat T lymphocytes and RBL-2H3 cells, CIF accelerates the rate of activation of I_{CRAC} (66). In these experiments, stores have been depleted passively by dialysis with 10 mM EGTA, so I_{CRAC} develops slowly. Inclusion of CIF increases the rate of development of this current. This is a striking result, but it is important to be clear what this experiment actually shows. It shows that CIF can increase the rate at which I_{CRAC} activates, but it does not show that CIF is the elusive signal that links store emptying to opening of the CRAC channels. Passive depletion of stores following dialysis with EGTA is a kinetically complex process reflecting Ca^{2+} leak from the stores, cytoplasmic Ca^{2+} chelation, and SERCA pump-mediated Ca^{2+} reuptake (89). By affecting any one of these processes, CIF would facilitate store depletion and hence accelerate activation of I_{CRAC}. Thapsigargin, like CIF, accelerates the speed of activation of I_{CRAC} following passive depletion of stores with 10 mM EGTA (89). However, thapsigargin is accomplishing this, not by mimicking the activation signal but rather by facilitating the rate of store depletion.

In *Xenopus* oocytes, where CIF has been studied in the most detail, Ca^{2+} influx following store depletion with thapsigargin was completely suppressed by 0.1 mM La^{3+}. However, concentrations of La^{3+} as high as 2 mM failed to reduce Ca^{2+} entry in response to high concentrations of CIF (66). This large difference in La^{3+} sensitivities between Ca^{2+} influx evoked by store depletion compared with CIF is puzzling. One possibility is that CIF and store depletion activate the same population of Ca^{2+} channels, but CIF somehow alters these channels so that they become much less sensitive to La^{3+} block. Because La^{3+} blocks Ca^{2+} channels by binding tightly within the pore, CIF would presumably have to induce quite substantial changes in pore properties to explain the reduced La^{3+} sensitivity. Alternatively, the difference in La^{3+} sensitivities between CIF-induced and store-operated Ca^{2+} influx could point to the existence of two distinct Ca^{2+} entry pathways, the corollary being that CIF does not activate the typical store-operated pathway. Consistent with this latter interpretation is the finding that CIF activates normally quiescent 24-pS outwardly rectifying nonselective cation channels in Jurkat T cells which are apparently store-operated (345). Store depletion in Jurkat T lymphocytes using $InsP_3$, thapsigargin, or receptor stimulation, in either the whole cell or perforated patch configuration, consistently activates only I_{CRAC} (417). Stationary noise analysis reveals that receptor stimulation (the physiological trigger) or exposure to thapsigargin both activate Ca^{2+} channels that have a very low conductance (2 fS). The fact that these diverse methods for store depletion all fail to activate the outwardly rectifying nonselective current in T cells suggests that this latter pathway is specifically recruited by CIF but not apparently by store depletion. Although CIF does not activate a nonselective current in RBL cells, one nevertheless needs to be careful in separating potential actions on store-operated entry from effects on other non-store-operated Ca^{2+} entry pathways. Single-channel recordings (as conducted in vascular smooth muscle) obviate this concern because it can be shown that the store-operated channels are identical to those activated by CIF (363).

Finally, CIF does trigger Ca^{2+} release. Csutora et al. (66) noted that CIF consistently evoked Ca^{2+} release in oocytes, but this was $<5\%$ of the size of the cytoplasmic Ca^{2+} signal that arose following Ca^{2+} entry (66). This is a concern because growing evidence implicates a small subset of the ER that is specifically associated with the activation of store-operated Ca^{2+} entry (see below). A small amount of Ca^{2+} release by CIF could reflect a quite marked reduction in the Ca^{2+} content of this subset of the ER that controls Ca^{2+} influx. Hence, CIF could evoke Ca^{2+} influx indirectly by triggering Ca^{2+} release from a specialized Ca^{2+} store that gates Ca^{2+} influx.

Although recent work on CIF extract is encouraging, it is important to bear in mind that this is not the only diffusible messenger that has been proposed (Table 1). Nevertheless, perhaps a more fundamental question to ask is whether there is any compelling evidence for a diffusible messenger (whatever its identity) at all. Few experiments have been devised to tackle directly this question. The first study to address this was carried out by Parekh et al. (272) who used the technique of patch cramming in *Xenopus* oocytes. In this study, stores were depleted after the formation of a cell-attached patch and the patch current was measured. After excision to the inside-out configuration, the patch current was lost rapidly. However, reinserting (cramming) the patch back into the oocyte resulted in restoration of the current. Because the patch had been reinserted into a region of the oocyte spatially distinct from where it had been excised, it was concluded that the current was gated by a mobile signal (272). Yao and Tsien (400) identified a store-operated Ca^{2+} current in whole oocytes which had similarities to I_{CRAC} (400). However, this whole oocyte current had a very different current-voltage relationship from the patch current reported earlier (272), suggesting that the patch current was not a purely store-operated one. Yao et al. (399) subsequently recorded a current in oocytes following depletion of stores using giant macropatches. Excision of the patch current did not result in loss of activity,

leading these authors to conclude that a diffusible messenger was not central to the gating of the channels (399). As in the earlier study by Parekh et al. (272) however, the current-voltage relationship of the patch current recorded by Yao et al. (399) differed significantly from the whole oocyte store-operated current. Hence, it is not clear whether a diffusible messenger is involved in store-operated entry in oocytes.

In RBL cells, Fasolato et al. (82) reported that the activation mechanism of I_{CRAC} washed out of the cell as a function of whole cell dialysis time. If stores were depleted shortly after the onset of whole cell recording, large I_{CRAC} could be generated. But if stores were dialyzed for a few tens of seconds before store depletion, the size of I_{CRAC} was significantly reduced. The current washed out with a time constant of ~200 s. Correcting for cell size and series resistance (indication of size of connection between the patch pipette and the cytosol), Fasolato et al. (82) calculated that the time constant of loss was compatible with a molecule of ~10 kDa (82). A similar washout of the activation mechanism of I_{CRAC} was reported in megakaryocytes (338). However, subsequent studies found that the activation mechanism of I_{CRAC} in RBL was relatively stable. The ability of store depletion to activate the current was not detectably impaired even if stores were depleted 10–15 min after the onset of whole cell recording (87, 267). Hence, the activation mechanism of I_{CRAC} does not seem to require a small, freely mobile molecule that preexists in the cytosol.

B. Conformational Coupling and Secretion-like Coupling

The conformational coupling model (25, 151), in which $InsP_3$ receptors spanning the ER are proposed to bind to Ca^{2+} channels in the plasma membrane, took its roots from excitation-contraction coupling in skeletal muscle. In skeletal muscle, the dihydropyridine-sensitive Ca^{2+} channel in the plasma membrane is physically linked to ryanodine type 1 Ca^{2+} release channels in the adjacent SR. One should note, though, that such direct physical coupling does not occur in either cardiac or smooth muscle cells. In these latter myocytes, Ca^{2+} channels in the plasma membrane communicate with Ca^{2+} release channels through a local diffusible messenger, namely, Ca^{2+} itself. In the initial version of conformational coupling (151), $InsP_3$ receptors on the stores were proposed to couple to plasmalemmal inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) receptors, which also functioned as Ca^{2+} channels. As evidence accumulated that $InsP_4$ was unlikely to be involved in Ca^{2+} influx, the model was revised by Berridge (25). He suggested that $InsP_3$ receptors on the stores were physically attached to the store-operated Ca^{2+} channels in the plasma membrane. A prediction of this conformational-coupling model is that store-operated Ca^{2+} entry should activate rapidly following store depletion. Protein-protein interactions can occur over a submillisecond time scale, and in skeletal muscle, action potentials invading the t tubules can trigger SR Ca^{2+} release within a few milliseconds (312). However, in most cell types, store-operated Ca^{2+} influx activates slowly. In RBL-1 cells for example, I_{CRAC} activates relatively slowly following rapid store depletion with ionomycin, receptor stimulation, or dialysis with high concentrations of $InsP_3$. However, in Jurkat T cells, flash photolysis of caged $InsP_3$ activated a small current very rapidly (within the duration of the flash artifact of <100 ms), and this was followed by the slower development of a larger I_{CRAC} (227). The rapid component amounted to ~10% the size of the total current, having an average amplitude of just −1.5 pA at −70 mV. It is not clear whether this small but rapidly activating current represents I_{CRAC}, $InsP_3$-gated Ca^{2+}-permeable channels in the plasma membrane, or another conductance. It would be important to see whether rapid increases in $InsP_3$ can activate a proportion of the CRAC channels within a fraction of a second in other systems.

The slow activation of I_{CRAC} in spite of rapid store depletion led Gill and colleagues (277) to propose the secretion-like coupling model. In this version, plasmalemmal store-operated Ca^{2+} channels are still activated by binding to $InsP_3$ receptors, but the complex is not preformed. Instead, upon store depletion, trafficking of peripheral ER to the plasma membrane occurs, and when the two membrane systems are optimally juxtaposed, the coupling reaction takes place. This movement of peripheral ER will be relatively slow and hence provides an explanation for the time course of activation of I_{CRAC}. The secretion-like coupling model predicts that first, store-operated channels should be activated directly by $InsP_3$ receptors and that functional $InsP_3$ receptors are required for the maintenance of store-operated entry, and second, maneuvers that interfere with trafficking of the ER to the plasma membrane should impair store-operated influx. There is evidence to support each of these predictions, but there is also evidence that cannot be easily reconciled with the model. We shall discuss each prediction in turn.

1. Store-operated channels potentially gated by $InsP_3$ receptors

Three lines of evidence have led to the suggestion that store-operated channels can be activated by $InsP_3$ receptors. First, coimmunoprecipitation experiments indicate that $InsP_3$ receptors can bind to certain members of the canonical TRP family (TRPC1 and TRPC3; Refs. 39, 170, 318) which are candidates for store-operated channels. Second, $InsP_3$ receptors seem to activate certain endogenous store-operated channels in excised patches

(405, 414). Third, the membrane-permeable $InsP_3$ receptor antagonist 2-APB inhibits store-operated influx even when applied after Ca^{2+} influx had been activated by thapsigargin (211).

Coimmunoprecipitation experiments have revealed that recombinant TRPC3 channels can bind to $InsP_3$ receptors in HEK-293 cells (39, 170). Similarly, in human platelets (318), TRPC1 can be coimmunoprecipitated with the type II $InsP_3$ receptor. In the platelet study, coimmunoprecipitation only occurred after store depletion, and no binding of TRPC1 channels with $InsP_3$ receptors was evident after stores had been allowed to refill (318). One note of caution is that the specificity of the antibodies has not been extensively characterized and is in fact the matter of some debate (see discussion in sect. XII*B*). Moreover, in HEK293 cells, large complexes can be pulled down that include TRPC3, $InsP_3$ receptors, SERCA2 pumps, caveolin-1, Gα(q/11), PLC-β, and ezrin (202). This suggests that TRPC3 might be part of a large macromolecular complex involving not only $InsP_3$ receptors but also a host of other proteins involved in cellular signaling and transport, and hence, coimmunoprecipitation of $InsP_3$ receptor-TRP proteins may not reflect functional protein-protein interactions but rather coassociation with a common "signalplex." Evidence for functional coupling between Ca^{2+} release and Ca^{2+} entry channels has been proposed from single-channel recordings. Kiselyov et al. (174) reported that the activity of single TRPC3 channels in excised patches ran down with time but could be rescued by applying $InsP_3$ together with microsomes rich in $InsP_3$ receptors. This coupling was mediated by the cytoplasmic NH_2-terminal domain of type I $InsP_3$ receptor (170; see sect. XII*A2* for more detailed discussion). Surprisingly, the coupling was somewhat promiscuous because ryanodine receptors could also activate the channels (173). Whether TRPC3 channel activity requires $InsP_3$ and, more fundamentally perhaps, if the channels are indeed store-operated are controversial issues. Although it seems that TRPC3 channels can be store-operated when expressed at low levels, most studies do not support a requirement for $InsP_3$ in channel gating. Following expression of TRPC3 channels in the $InsP_3{}^{-/-}$ DT40 chicken B lymphocyte cell line, Ba^{2+} influx through TRPC3 channels was similar between control and mutant cells following stimulation of the B cell receptor, which couples to PLC-γ through syn kinase (210). Crucially, no Ca^{2+} release, and therefore presumably no store depletion, occurred following receptor stimulation in the $InsP_3{}^{-/-}$ DT40 cells expressing TRPC3 protein, yet the channels activated in a manner indistinguishable from control cells. In HEK293 cells, whole cell dialysis with $InsP_3$ evoked Ca^{2+} release in TRPC3-expressing cells, but this was not associated with Ca^{2+} influx nor with activation of the TRPC3 conductance. However, subsequent stimulation of muscarinic receptors opened the TRPC3 channels and in a manner that was not prevented by inhibiting $InsP_3$ receptors with heparin (359). In both cell types, TRPC3 could be activated not by store depletion but by diacylglycerol analogs instead.

Store-operated single-channel events have been observed in cultured bovine aortic endothelial cells (369). Channel activity in inside-out patches could be sustained for a couple of minutes by adding $InsP_3$ to the cytoplasmic side of the patch, and this was prevented by heparin. Another store-operated current thought to be gated by $InsP_3$ receptors is the I_{min} of A431 epidermal cells (405, 414). The I_{min} current differs from I_{CRAC} in several biophysical features (see Table 1). I_{min} channels have a conductance of ~1 pS and can be observed in cell-attached patches after stimulation of bradykinin receptors or exposure to thapsigargin. Single-channel activity is lost upon patch excision but can be rescued by application of $InsP_3$. In inside-out patches, channel activity is potentiated by addition of microsomes enriched in $InsP_3$ receptors. Moreover, I_{min} channel activity is potently blocked by the $InsP_3$ receptor antagonist heparin (171).

Because of the very low single CRAC channel conductance, single-channel events are not resolvable, and hence, it has not been possible to see whether $InsP_3$ and $InsP_3$ receptors can directly open CRAC channels. Nevertheless, there is quite a large body of evidence documenting that $InsP_3$ receptors are not required for the activation of I_{CRAC}. First, Sugawara et al. (347) showed that thapsigargin-evoked Ca^{2+} influx was normal in a mutant DT40 B-lymphocyte cell line in which all three isoforms of the $InsP_3$ receptor had been deleted ($InsP_3{}^{-/-}$ DT40; Ref. 347). Subsequent work confirmed that these cells lacked both $InsP_3$ binding sites and $InsP_3$-dependent Ca^{2+} release, yet store-operated influx was intact (45, 210). Recent patch-clamp experiments have demonstrated that the mutant cells express I_{CRAC} at normal levels (291). Hence, I_{CRAC} can activate in the absence of $InsP_3$ receptors.

Furthemore, several studies have found that dialysis with heparin, a competitive antagonist of $InsP_3$ receptors, fails to affect the ability of I_{CRAC} to activate (13, 89, 143). In RBL-1 cells for example, cells were dialyzed with a pipette solution containing $InsP_3$, heparin, strongly buffered Ca^{2+} (~225 nM), and ATP (13), the latter two components being included to expedite store refilling. Following the onset of whole cell recording, $InsP_3$ rapidly diffused into the cell, emptied the stores, and hence activated I_{CRAC}. Because of its much larger size, heparin entered the cells more slowly, but as it accumulated in the cytoplasm, it displaced bound $InsP_3$ from $InsP_3$ receptors. Now, SERCA pumps were able to refill the stores and I_{CRAC} deactivated. Following full deactivation of I_{CRAC}, stores were subsequently depleted by applying ionomycin. I_{CRAC} could be activated once more. Under these conditions, heparin would presumably still be occupying

the $InsP_3$ receptors, yet the current could be evoked again. Hence, I_{CRAC} can be activated even though $InsP_3$ is no longer bound to the $InsP_3$ receptors.

Electrophysiological studies have revealed that, although 2-APB did indeed block the activation of I_{CRAC}, a major target seemed to be the CRAC channels themselves (see sect. VI). Therefore, the 2-APB block of I_{CRAC} can no longer be considered as evidence for the coupling model.

It seems clear therefore that functional $InsP_3$ receptors are not required for the activation of I_{CRAC}. Moreover, the fact that I_{CRAC} can activate in DT40 cells lacking any $InsP_3$ receptor suggests that $InsP_3$ receptors are unlikely to be involved in CRAC channel gating, but this does not mean that the conformational-coupling model should be abandoned. It is conceivable that another protein on the stores links to the Ca^{2+} channels in the plasma membrane. In the DT40 $InsP_3$ receptor triple knockout cells, it has been suggested that ryanodine-sensitive Ca^{2+} release channels substitute for $InsP_3$ receptors in coupling to the CRAC channels (172). Ruthenium red, an inhibitor of ryanodine-sensitive release channels, inhibited I_{CRAC} by ~50% both wild-type and $InsP_3$ receptor triple knockout DT40 cells (172). However, dialysis of RBl-1 cells with ruthenium red, an inhibitor of ryanodine-sensitive channels, together with heparin failed to affect the rate or extent of activation of I_{CRAC} (89). Hence, in these cells at least, functional $InsP_3$ receptors and ryanodine-sensitive Ca^{2+} channels may not be required for the activation of CRAC channels.

2. Movement of the ER

A crucial observation that is often regarded as solid evidence for the secretion-like coupling model is that stabilization of the cytoskeleton, specifically the cortical actin network just below the plasma membrane, inhibits the activation of store-operated entry (277, 319). The ability of $InsP_3$-generating agonists to trigger Ca^{2+} release is unaffected by stabilization of the actin network, suggesting that the formation of this subplasmalemmal barrier does not impede diffusion of molecules, at least of the size of $InsP_3$ (277). It has been suggested that the cortical actin barrier prevents ER from making contact with the plasma membrane, and hence, $InsP_3$ receptors on the stores are unable to activate the store-operated channels. However, similar maneuvers that alter the actin network in other cell types had no effect on store-operated Ca^{2+} entry (13, 309). In RBL-1 cells for example, altering the cytoskeleton induced marked changes in cell morphology and distribution of actin, but neither the rate of development nor extent of I_{CRAC} was affected (13). It is well established that the morphology of the ER is maintained through a tight interaction with microtubules (115). Disruption of the microtubular network causes retraction of the peripheral ER that lies close to the plasma membrane towards the cell center. However, prolonged exposure to nocodazole, which disrupts microtubules, failed to affect the development of I_{CRAC} (13).

An important prediction of the secretion-like coupling model is that it requires intimate association between the ER and plasma membrane. With the use of the method of whole cell ballooning (13), which is thought to separate the plasma membrane from underlying structures through the application of strong positive pressure through the patch pipette, the ability of I_{CRAC} to activate was unaffected if stores were emptied after cell inflation had occurred. Cell ballooning after the activation of I_{CRAC} also had no effect on the current. One might have expected cell ballooning to interfere with activation of I_{CRAC} if a trafficking model were to be operating.

Collectively, the idea that CRAC channels are activated by conformational coupling to $InsP_3$ receptors in the ER membrane seems unlikely at least in the majority of instances.

C. Vesicular Fusion

The vesicular fusion hypothesis suggests that store-operated channels are not in the plasma membrane at rest but are inserted into the membrane upon store depletion via an exocytotic mechanism. Such a mechanism would be analogous to insertion of other channels and transporters following cell-surface stimulation including aquaporin2 water channels in collecting tubules of the kidney by vasopressin (47), the glucose transporter GLUT4 in adipocytes and skeletal muscle by insulin (49), and the H^+-K^+-ATPase in oxyntic cells by gastrin (398). There is good evidence for regulated fusion of TRPC channels (29), although as discussed below, their role in store-operated entry is not well established. Strictly speaking, the fusion model should not be considered an activation mechanism in its own right because it does not address the key question of the activation signal. It is not clear how store emptying might drive vesicle fusion, especially because the fusion needs to be independent of a rise in cytoplasmic Ca^{2+} for the reasons delineated in section III. But a consequence of this is insertion of channels into the plasma membrane.

Early arguments for an exocytotic mechanism in the activation of store-operated Ca^{2+} influx included the findings that the drug primaquine, which impairs vesicle budding and transport, affected the development of I_{CRAC} in megakaryocytes (338) and that disruption of the actin cytoskeleton with cytochalasin D inhibited calcium influx in vascular endothelia (139). However, neither of these tools is specific for targeting secretory mechanisms, and different conclusions have been reached by others. Primaquine was reported to act as a direct Ca^{2+} channel blocker in *Xenopus* oocytes (116). Effects of cytochalasin

D seem cell-type specific in that it fails to affect store-operated entry in several systems (13, 277, 309).

Yao et al. (399) revisited the fusion model in *Xenopus* oocytes. They found that activation of a store-operated current (distinct from I_{CRAC}) could be abolished by overexpression of dominant negative mutants of SNAP-25. Consistent with this was their finding that botulinum neurotoxin A, which cleaves SNAP-25, reduced the store-operated current by 50% with a half-time of 1.1 h. However, botulinum neurotoxins B and E and tetanus toxin were all without effect (399). They also found that the small G protein Rho was a modulator of Ca^{2+} influx because treatment with C3 exoenzyme, which inactivates Rho, enhanced Ca^{2+} influx modestly, whereas overexpression of Rho reduced entry. The effects of the neurotoxins were not mimicked by brefeldin A, indicating that an action on constitutive exocytosis was not involved.

Using giant cell-attached patches to record a Ca^{2+}-permeable current, they also reported that seal formation prior to store depletion prevented activation of the store-operated current in the patch (399). On the other hand, the current was seen if seals were formed after stores had been depleted. Excision of the patch resulted in a slight increase in current, arguing against a central role for a small diffusible messenger in maintaining channel activity. However, there are some puzzling features about the currents measured. The whole oocyte store-operated current, measured in two-electrode voltage clamp, had a linear current-voltage relationship with a positive reversal potential ($>+20$ mV; Refs. 399, 400). The current recorded in cell-attached patches appeared curvilinear with a decreasing conductance as membrane potential hyperpolarized (399). In excised patches, the current was curvilinear, noisy, and unstable at potentials negative to -40 mV and reversed at around $+5$ mV (399). Although a characterization of the patch current was not presented, it would seem that the current measured in the whole oocyte compared with that seen in patches may not represent the same population of channels.

In fura 2-loaded HEK 293 cells, it was also concluded that activation of store-operated entry involved vesicular fusion (3). Intracellular injection of botulinum neurotoxin/A1c reduced Ca^{2+} influx by 90% following readmission of external Ca^{2+} to cells preexposed to cyclopiazonic acid in Ca^{2+}-free solution. In contrast to the oocyte study (399), tetanus toxin also reduced calcium entry in HEK cells (3). In both cases in the HEK cell study however, Ca^{2+} was readmitted long after the toxins had been injected, with 2 h elapsing for botulinum neurotoxin/A1c and 3 h for tetanus toxin. Such long exposure times would not distinguish between a role for vesicular fusion in the direct activation of store-operated influx as opposed to maintaining quiescent channels or other signaling proteins in the plasma membrane via constitutive exocytosis. Consistent with the latter was the finding that exposure to brefeldin A for 4 h reduced Ca^{2+} influx by 80% and to an extent not different from tetanus toxin (3).

As with the other two activation models above, the fusion concept also runs into difficulty.

First, it is often difficult to distinguish between constitutive and regulated exocytosis, especially when cells have been exposed to clostridial neurotoxins for a few hours. In HEK293 cells, brefeldin A impaired store-operated entry with a similar time course and to a similar extent to that seen in the presence of tetanus toxin (3). In hepatocytes, just 2 h of exposure to brefeldin A inhibited store-operated influx by 90% (116). In oocytes however, store-operated entry was unaffected by 20 h of treatment with brefeldin A (399). Hence, in some nonexcitable cells, vesicle trafficking can be relatively fast. In these cases, it is therefore difficult to dissect out a regulated exocytotic event from a constitutive process.

Second, inhibition of exocytosis will normally permit endocytosis to continue, at least to some extent. Hence, the cell surface area will fall, and this must be monitored by taking into account the total plasma membrane surface area. A smaller Ca^{2+} influx signal following exposure to toxins could simply reflect a smaller cell size. Unless electrophysiological measurements are made (to measure membrane capacitance), it is difficult to correct for this.

There is also experimental evidence that does not fit with vesicular fusion as the ubiquitous mechanism for activation of store-operated influx. SNAP-25, the target of botulinum toxin, is not expressed in nonexcitable cells, essentially being restricted to neurons and neuroendocrine cells. Hence, the inhibitory effect of botulinum toxin on store-operated entry described in Reference 399 is unexpected as its substrate apparently should not be present. Indeed, Scott et al. (330) found that SNAP-25 was not expressed in either HEK293 or COS-1 cells and that, in contrast to Reference 3, exposure to botulinum toxin failed to alter store-operated influx in these cells. Instead, these cells expressed the botulinum toxin-insenstive SNARE protein SNAP-23. Expression of a truncated form of SNAP-23 failed to affect store-operated entry, whereas it impaired cycling of transferrin receptors. More dramatically, expression of a mutant NEM-sensitive factor (NSF) construct inhibited membrane trafficking events in general including recycling of transferrin receptors to the plasma membrane, retrograde traffic to the Golgi complex, and intracellular events including fusion of endosomes to lysosomes. In spite of these changes, store-operated influx was unaffected. The authors concluded that the vesicular fusion model was not viable, at least in their systems (330).

A key prediction of the fusion model is that any maneuver that impairs exocytosis should interfere with the ability of I_{CRAC} to activate. Using the capacitance technique to record plasmalemmal vesicular fusion events in single patch-clamped RBL-1 cells, recombinant

α-SNAP mutant was found to suppress regulated exocytosis (12). However, the activation of I_{CRAC} following exposure to thapsigargin was unaffected by a similar exposure to the protein. Furthermore, a variety of toxins (tetanus, botulinum neurotoxins) and pharmacological agents that impair secretory events were all without effect on I_{CRAC}. Hence, I_{CRAC} activation could be dissociated from exocytotic events.

D. Removal of Ca^{2+} Inhibition

The lack of an unequivocal signal that activates store-operated Ca^{2+} influx has led some investigators to question whether a specific activation mechanism indeed exists. Instead, it has been postulated that store-operated channels are maintained in an inactivated state (due to local elevation of subplasmalemmal Ca^{2+} concentration) and store depletion, by increasing activity of SERCA pumps on the stores, removes this inhibition by enhancing Ca^{2+} removal from such sites (18). In this interesting, albeit somewhat speculative, scenario, store-operated channels open, not via an activation signal, but rather by removal of inactivation. This scheme requires *1*) very restricted diffusion of Ca^{2+} from subplasmalemmal regions to bulk cytosol to maintain channel inactivation and *2*) an inability of Ca^{2+} chelators like BAPTA to access these sites (otherwise store-operated channels would be activated rapidly following dialysis with high BAPTA). It is not entirely clear how microdomains of elevated Ca^{2+} exist in the vicinity of inactivated store-operated channels. Presumably, there would have to be a very sizeable and local leakage of Ca^{2+} from the stores. More problematic for this model is that BAPTA can and does reach sites within a few nanometers of the channels because it is able to reduce fast Ca^{2+}-dependent inactivation of CRAC channels, a process that reflects local feedback of permeating Ca^{2+} on the channel itself (88, 144, 418).

X. DO STORE-OPERATED CHANNELS DEACTIVATE BY REVERSAL OF ACTIVATION?

If store depletion activates store-operated entry, it seems likely that store refilling deactivates the Ca^{2+} entry pathway. Indeed, inhibition of SERCA pumps with thapsigargin prevents decay of I_{CRAC} and the extent of store refilling, assessed through the recovery of agonist-evoked Ca^{2+} release into the cytosol.

However, just how store refilling switches off the store-operated channels is unclear. The simplest possibility is that it reflects simple reversal of the activation mechanism. As the stores refill, the activation signal decays. Alternatively, refilling stores may send an inhibitory signal that overcomes activation. In this regard, deactivation of I_{CRAC} would not simply be the reverse of activation. Interestingly, there are several examples in the literature of compounds that inhibit the activation of store-operated Ca^{2+} entry but are much less effective once Ca^{2+} influx has developed. For example, inhibitors directed against phospholipase C (U-73122), lipoxygenase (CDC), calmodulin (calmidazolium), and phosphatidylinositol 1,4,5-trisphosphate kinase all impair store-operated Ca^{2+} influx when applied before store depletion (although store emptying is not compromised) but are much less effective if administered after Ca^{2+} influx has developed (45, 52, 109, 321). It is possible that these compounds all block store-operated Ca^{2+} channels directly and in a state-dependent manner. However, in light of the fact that there are so many such examples, it is worth exploring the concept that the signaling mechanism is complex and that store depletion and refilling might not control store-operated entry via the same pathway.

XI. REGULATION OF STORE-OPERATED CALCIUM ENTRY

Like other ion transport pathways, store-operated Ca^{2+} channels are subjected to inhibitory mechanisms that can be direct or indirect and operate over time scales of milliseconds to minutes.

A. Ca^{2+}-Dependent Inactivation

Many Ca^{2+} channels show the property of Ca^{2+}-dependent inactivation, whereby permeating Ca^{2+} feed back to inhibit further flux through the channels. Our understanding of Ca^{2+}-dependent inactivation of store-operated influx comes largely from studies on CRAC channels as these remain the best characterized. For CRAC channels, Ca^{2+} feedback occurs via three kinetically and spatially distinct mechanisms.

1. Rapid inactivation

This well-characterized process reflects the subplasmalemmal build-up of a microdomain of elevated Ca^{2+} in the vicinity of each open channel (88, 144, 418). It develops with biexponential time constants (10 and 100 ms) and can most easily be seen by applying hyperpolarizing steps to negative voltages (>-60 mV, Fig. 2). With this protocol, I_{CRAC} initially increases (due to the increased electrical driving force) but then declines as rapid inactivation takes effect. The inactivation is much less pronounced when Ca^{2+} is replaced by Ba^{2+} as the charge carrier (418), but Sr^{2+} can substitute for Ca^{2+} somewhat (88). Fast inactivation is reduced, but not abolished, by the fast calcium chelator BAPTA, whereas the slower

EGTA has little impact (88, 144, 418). In divalent-free external solution (where Na^+ is the charge carrier), no inactivation occurs during hyperpolarizing steps, revealing no discernible voltage-dependent component to the inactivation process (16, 192). Over the physiological range of membrane potentials (up to −90 mV), rapid inactivation accounts for no more than a 30% decay in I_{CRAC} amplitude (88, 418). Recovery from inactivation is a biexponential process with time constants in the range of 10–500 ms. Neither the rate nor extent of fast inactivation is affected by receptor stimulation in RBL-1 cells, suggesting that the process might not be under receptor control other than through changes in the membrane potential (88).

2. Store refilling

If emptying of stores activates store-operated entry, then it seems likely that refilling of stores would deactivate Ca^{2+} influx. Early studies using fura 2 to track Ca^{2+} influx showed a good correlation between store refilling and loss of Ca^{2+} entry (155, 246). Recordings of I_{CRAC} have also confirmed that store refilling can switch the current off. Following store depletion in Jurkat T lymphocytes, dialyzing cells with a moderate amount of Ca^{2+} chelator (1.4 mM EGTA) resulted in initial development of I_{CRAC} followed by full deactivation within 100–200 s (419). The decline of I_{CRAC} could be partially reversed by exposing the cells to thapsigargin, indicating that store refilling contributed to the decay. Similarly, in RBL-1 cells, store refilling deactivated I_{CRAC} (13). This was largely prevented when either ATP was omitted from the pipette or cytoplasmic Ca^{2+} was clamped at very low levels (a few nM; Ref. 13), consistent with the requirement for both ATP and Ca^{2+} for SERCA-mediated store refilling.

3. Slow inactivation

Although thapsigargin reduces the decay of I_{CRAC} under conditions where stores can refill, it does not fully suppress it. I_{CRAC} still decays, albeit partially, in the presence of thapsigargin, and this occurs over a timeframe of tens of seconds (13, 263, 419). This inactivation pathway is Ca^{2+} dependent in that it is suppressed by high concentrations of the slow Ca^{2+} chelator EGTA and can be distinguished from rapid inactivation (263, 419). The mechanism underlying this slow inactivation is not clear. In T cells, recovery from slow inactivation is prevented by protein kinase blockers, suggesting an ATP-dependent phosphorylation might be involved in the recovery process (194). In RBl-1 cells on the other hand, kinase and phosphatase blockers did not affect the development of slow inactivation (263). In both RBl-1 cells and T lymphocytes, slow inactivation was much less pronounced if mitochondria were maintained in an energized state during whole cell recording (103, 142). By buffering cytoplasmic Ca^{2+}, mitochondria presumably prevent Ca^{2+} from rising high or long enough to trigger slow inactivation. Ca^{2+}-dependent slow inactivation is thought to underlie agonist-induced intracellular Ca^{2+} oscillations in salivary acinar cells (94) and contribute to the frequency of intracellular Ca^{2+} oscillations in T lymphocytes (194).

Could some of the effects of Ca^{2+} on CRAC channels be mediated by calmodulin? Calmodulin is a very important modulator of voltage-operated L-type calcium channels. By binding to amino acids of the IQ (isoleucine-glutamine) motif in the COOH-terminal tail of the α1c channel subunit, calmodulin can regulate both Ca^{2+}-dependent inactivation and facilitation of the Ca^{2+} channels (415). Calmodulin appears to be stably associated with the IQ motif at resting Ca^{2+} levels, suggesting, as with Ca^{2+}-dependent K^+ channels, it might be tethered to the Ca^{2+} channel (415). As with their voltage-operated counterparts, calmodulin regulation of store-operated entry has been reported. In bovine aortic endothelial cells, dialysis with calmodulin impaired, in a concentration-dependent manner, the ability of thapsigargin to activate a store-operated current (368). The rate of development of the current was slowed almost twofold, its peak amplitude was significantly reduced, and slow inactivation was enhanced by calmodulin. A similar trend was seen in CHO cells (370). Here, the delay between the onset of Ca^{2+} release from the stores and activation of store-operated entry increased 10-fold when cells were dialyzed with calmodulin. Furthermore, calmodulin reduced the overall extent of the store-operated current by 40%.

In *Xenopus* oocytes, store-operated influx has been reported to be potentiated by calmodulin, following stimulation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). Following store emptying, the subsequent rise in cytoplasmic Ca^{2+} concentration activates CaMKII, which then enhances store-operated influx apparently by altering channel gating and not through effects on the activation signal (214). It is not yet clear whether Ca^{2+} influx can also activate CaMKII. If so, this would constitute an intriguing positive feedback mechanism whereby Ca^{2+} influx initially potentiates further Ca^{2+} entry. This would not be self-perpetuating however, because the ensuing high cytoplasmic Ca^{2+} concentration would presumably accelerate both store refilling and Ca^{2+}-dependent inactivation of the entry channels, resulting in a faster decline of the Ca^{2+} current. Activation of CaMKII, at least initially, requires Ca^{2+}-calmodulin. But in some other cell types, calmodulin has been found to inactivate store-operated entry, rather than enhance it (334, 368).

Ca^{2+}-dependent inactivation of TRPC1 channels, which seem to be store-operated in some systems, is mediated by calmodulin (334). Expression of a Ca^{2+}-insensitive calmodulin mutant resulted in an increase in current through TRPC1 channels, and this was associated with a reduction in Ca^{2+}-dependent inactivation of the

current. This effect was not mimicked by overexpressing wild-type calmodulin. Deletion studies revealed that the COOH terminus of TRPC1 (amino acids 664–793) was required for both calmodulin binding and for Ca^{2+}-dependent inactivation.

It is not clear whether calmodulin can regulate I_{CRAC} in a manner akin to that seen for L-type channels or TRPC1. The simple approach of applying inhibitors like calmidazolium is problematic if calmodulin is not free but attached to the channels, where it is much less sensitive to inhibition. Hence, a molecular biological approach may be required to address this.

B. Sphingosine

Application of sphingomyelinase (43, 358), sphingosine (43), or ceramides (223, 358) have all been reported to reduce thapsigargin-evoked Ca^{2+} entry, indicating that signals from the sphingomyelin pathway may regulate store-operated influx. In both RBL-2H3 cells and human peripheral T lymphocytes, sphingosine and related sphingolipids were found to inhibit I_{CRAC} (191, 223).

C. cGMP and Protein Kinase G

In some cell types like vascular smooth muscle, it has long been known that nitric oxide (NO) can, via cGMP and then cGMP-dependent protein kinase, stimulate Ca^{2+} reuptake into the stores. Hence, one would expect an increase in cGMP levels to deactivate store-operated Ca^{2+} influx by promoting store refilling. Indeed, in platelets, bovine vascular endothelia and aortic smooth muscle, NO can deactivate Ca^{2+} influx via stimulation of the refilling process (63, 69, 362).

To probe effects of cGMP on store-dependent Ca^{2+} entry independent of store refilling, many investigators have triggered calcium entry by inhibiting SERCA pumps with thapsigargin. Under these conditions, cGMP has been reported to inactivate store-operated Ca^{2+} entry in fura 2-loaded A7r5 vascular smooth muscle cells via stimulation of cGMP-dependent protein kinase (241; see also sect. XI*E*). In HEK-293 cells expressing recombinant TRPC3, cGMP-dependent protein kinase was reported to phosphorylate and inhibit Ca^{2+} influx through TRPC3 channels, the latter apparently functioning in a store-operated mode (184). On the other hand, cGMP did not affect the inactivation of I_{CRAC} in RBL-1 cells (269), nor did membrane-permeable analogs of cGMP alter store-operated calcium entry in *Xenopus* oocytes (281), pancreatic acinar cells (107), or T lymphocytes (30). Regulation of store-operated Ca^{2+} entry by cGMP is likely therefore to be cell type specific.

D. Protein Kinase C

Hydrolysis of phosphatidylinositol 4,5-bisphosphate following receptor activation of PLC generates both $InsP_3$ (the physiological trigger to empty stores) and diacylglycerol. Diacylglycerol can, in the presence of phosphatidylserine and Ca^{2+}, activate protein kinase C. Protein kinase C has disparate effects on store-operated influx, and which probably are cell-type dependent. In populations of thyroid cells and human neutrophils loaded with fura 2, phorbol ester was found to reduce Ca^{2+} influx evoked by thapsigargin or receptor stimulation, suggesting a role for protein kinase C in inhibiting store-operated influx (247, 357). Parekh and Penner (269) found that protein kinase C accelerated inactivation of I_{CRAC} in RBL cells. A similar finding was made in Jurkat T cells (71). Intriguingly, prolonged exposure to the HIV-1 envelope glycoprotein gp160 inhibited the size of I_{CRAC} in Jurkats, and this was prevented by inhibitors of protein kinase C. Western blotting revealed that gp160 altered the distribution of protein kinase C-α and -β1 isoforms only, suggesting that these may be involved in the regulation of I_{CRAC} (71).

In *Xenopus* oocytes, phorbol ester initially increased but then inhibited thapsigargin-evoked Ca^{2+} entry, when the latter was monitored using the endogenous Ca^{2+}-dependent Cl^- current (280). On the other hand, when the store-operated current was measured directly in the oocyte, only the inhibitory effect of protein kinase C stimulation was seen (400).

In some types of smooth muscle, protein kinase C has been reported to open store-operated Ca^{2+} channels rather than inhibit them (Table 2).

E. Arachidonic Acid

The sequential activities of phospholipase C and diacylglycerol lipase liberate arachidonic acid from phosphatidylinositol 4,5-bisphosphate. In addition, the increased cytoplasmic Ca^{2+} resulting from Ca^{2+} release and entry can activate PLA_2 releasing arachidonic acid from membrane phospholipids. Arachidonic acid has numerous effects on Ca^{2+} signaling, and there is growing evidence that arachidonic acid inhibits store-operated entry. In HEK-293 cells, Luo et al. (208) reported that 30 μM arachidonic acid transiently reduced thapsigargin-evoked Ca^{2+} entry. One problem with arachidonic acid is that it is a relatively unstable compound, so researchers generally use high concentrations to see effects. Concentrations of arachidonic acid around 30 μM exceed the critical micelle concentration, so nonspecific effects are possible. In A7r5 cells, stimulation of vasopressin receptors inhibited store-operated entry, an effect mimicked by exogenous arachidonic acid (applied externally at 50 μM; Ref. 242). Using pharmacological tools to dissect out the underlying mech-

anism, Moneer and Taylor and co-workers (241, 242) reported that arachidonic acid stimulated NO synthase III (by an, as yet, unknown mechanism), resulting in generation of NO. NO then stimulated guanylyl cyclase resulting, via cGMP production, in the activation of protein kinase G, which then inhibited store-operated Ca^{2+} entry.

In addition to inhibiting store-operated Ca^{2+} entry, arachidonic acid activates a non-store-operated Ca^{2+} current (233). An intriguing concept is developing which suggests reciprocal regulation between these two Ca^{2+} influx pathways. Arachidonic acid activates non-store-operated entry, via the generation of NO at least in in A7r5 cells, whereas it inhibits store-operated influx over a similar time frame (241, 242). The converse has also been reported, namely, that activation of store-operated influx inhibits arachidonic acid-evoked non-store-operated Ca^{2+} entry, an effect that seems to be mediated by calcineurin (234). On the other hand, Luo et al. (208) suggest the inhibition of arachidonic acid-dependent Ca^{2+} entry by thapsigargin does not reflect store depletion or a rise in cytoplasmic Ca^{2+}. Instead, an unknown step in the cascade that activates store-operated entry inhibits the arachidonic acid-dependent influx pathway. Resolution of how mutual antagonism between the two Ca^{2+} entry pathways is brought about will shed new light into the dynamics of intracellular Ca^{2+} signaling.

Arachidonic acid can also open non-store-operated TRPV4 channels, which are permeable to Ca^{2+} and Mg^{2+}. TRPV4 channels are gated by a variety of diverse stimuli including cell swelling, phorbol ester, and heat. Cell swelling seems to open the channels through a pathway involving stimulation of phospholipase A_2, arachidonic acid release, and then its subsequent metabolism to epoxyeicosatrienoic acid via cytochrome *P*-450 epoxygenase (387). TRPV4 is particularly abundant in some nonexcitable cells like endothelia where it might represent a major route for arachidonic acid-dependent Ca^{2+} influx. However, other stimuli like heat and phorbol ester activate the channels independent of arachidonic acid (383).

F. $InsP_4$

The relationship between the concentration of $InsP_3$ and the amplitude of I_{CRAC} is complex and highly nonlinear (110, 265). The dose-response curve has a Hill coefficient of 12, indicating tremendous cooperativity. In a patch-clamp experiment with $InsP_3$ in the pipette solution, diffusion of $InsP_3$ across the pipette will be rate-limiting, and this could contribute to the nonlinear relationship. The extent of I_{CRAC} activation was still highly nonlinear following cell-surface receptor stimulation, a situation which better represents physiological conditions (265). However, the relationship between the concentration of the nonmetabolizable $InsP_3$ analog Ins(2,4,5)P_3 (265) or adenophostin A (271), a highly potent activator of $InsP_3$ receptors and which is nonmetabolizable, and the extent of I_{CRAC} activation was graded. Hence, the steep nonlinear dependence between $InsP_3$ concentration and I_{CRAC} reflects $InsP_3$ metabolism (265), and it seems that Ins(1,4,5)P_3 5-phosphatase is the important catabolic pathway that underlies this (110). Hence, modifying the activity of this enzyme would be expected to alter the extent of I_{CRAC}. Ins(1,4,5)P_3 phosphatase can be inhibited by $InsP_4$. Dialyzing RBL-1 cells with $InsP_4$ shifted both the relationship between $InsP_3$ concentration and amplitude of I_{CRAC} to the left and reduced the steepness (131), an effect that was mimicked by Ins(1,4,5)P_3 5-phosphatase inhibitors. $InsP_4$ is also an inhibitor of $InsP_3$-dependent Ca^{2+} release (33, 131), so its potentiating effects on I_{CRAC} evoked by low concentrations of $InsP_3$ (by impairing phosphatase activity) may be counterbalanced to some extent by reducing the ability of $InsP_3$ to empty the stores.

XII. CHASING THE STORE-OPERATED CHANNEL GENE(S)

Despite intense investigation, the gene that encodes the CRAC channel and other store-operated channels remains elusive. Research on *Drosophila* phototransduction identified a gene encoding a subunit of a Ca^{2+}-permeable channel that was activated downstream of PLC (121, 245). Because store-operated entry occurs downstream of PLC, speculation arose as to the possible role of this channel in the store-operated Ca^{2+} entry pathway.

In *Drosophila*, absorption of a photon by rhodopsin triggers a cascade of events that results in stimulation of PLC. This is associated with depolarization of the photoreceptor cells, brought about by an inward cationic current that generates the receptor potential (236, 243). The receptor potential has two components: an initial rapid depolarization that is due to a Na^+ current and, if light is maintained, a smaller but sustained depolarization due to influx of both Na^+ and Ca^{2+}. In the *Drosophila* transient receptor potential (TRP) mutant, the response to light is transient and not sustained.

When it was found that the TRP mutant was associated with defective Ca^{2+} influx, that TRP channels were relatively selective for Ca^{2+} and had a predicted topology similar to that of voltage-gated Ca^{2+} channels (with the notable exception that the S4 segments of *Drospophila* TRP were relatively devoid of the voltage-sensing positively charged amino acids), it was suggested that TRP might be a long-sought store-operated channel (121). Expression of *Drosophila* TRP in fura 2-loaded Sf9 insect cells resulted in greater Ca^{2+} influx following exposure to thapsigargin (371). Similarly, in *Xenopus* oocytes, overexpression of *Drosophila* TRP enhanced Ca^{2+} influx-activated Cl^- currents after pretreatment with thapsigargin

(282). However, somewhat ironically, subsequent experiments in *Drosophila* demonstrated that TRP channels were not store-operated after all. Instead, the channels appeared to be activated by downstream lipid products of PLC, either diacylglycerol or polyunsaturated free fatty acids (120, 243). In addition, TRP activity required PIP_2, and depletion of the phospholipid resulted in decay of TRP activity. It was suggested that Ca^{2+} entry through TRP channels both inhibited PLC activity, thereby preventing a reduction in PIP_2, and facilitated PIP_2 recycling (122). In the TRP mutant therefore, PIP_2 levels would rapidly plummet, and this would result in rapid decay of TRP activity.

A number of mammalian homologs of TRP have been found, and the TRP superfamily includes more than 20 related cation channels (125, 244). These TRP channels can be classified into three major subfamilies: TRPC, TRPV, and TRPM. The TRPC subfamily exhibits the greatest sequence homology to *Drosophila* TRP. A review on the properties of TRP channels is beyond the scope of this review, and the reader is referred instead to some recent excellent reviews on this topic (62, 236, 257).

Several groups have examined the possible role of TRP in store-operated Ca^{2+} influx. Essentially, two major types of experiment have been carried out: overexpression of a particular TRP protein in a cell line or interfering with the expression of endogenous TRP in its native environment (antisense oligonucleotides and gene knockout studies). As a result of these approaches, it has been suggested that most of the TRPCs as well as TRPV6 are store-operated. However, the field is bewilderingly confusing, with different laboratories reaching strikingly different conclusions even though the same TRP channels and experimental systems have been employed. Nonetheless, some patterns have developed, and some possible explanations for the discordant findings have emerged that are worthy of discussion.

A. TRPCs

The first mammalian TRPs to be cloned and expressed were TRPC1 and TRPC3 (human TRPC2 was found to be a pseudogene) (409, 411). There are seven related members of the TRPC family, designated TRPC1–7 (the numbering reflects the order of their discoveries). TRPC2 is a pseudogene in humans (but not most other mammalian species). The remaining six appear to fall into two groups based on structural and functional similarities: TRPC1, -4, and -5 and TRPC3, -6, and -7. Zhu et al. (411) expressed TRPC1 and -3 in COS cells and observed that Ca^{2+} entry was slightly increased in response to thapsigargin. Philipp et al. (283) reported that expression of TRPC4 in HEK293 cells resulted in a large inward current activated by store depletion. Zitt et al. (413) expressed TRPC1 in CHO cells and observed nonselective cation currents that were increased by either $InsP_3$ or thapsigargin. Kiselyov et al. (174) demonstrated single channels in TRPC3-expressing HEK293 cells that could be activated by Ca^{2+} store depletion with thapsigargin. These early reports, in highly visible journals, laid a strong case for TRPCs as store-operated channels. However, later reports from other laboratories cast doubt on some of these findings. The original findings with TRPC3 and TRPC1 reported by Zhu et al. (410) were reexamined by the same group; the conclusion was that the previous increased entry likely resulted from constitutive activity of the expressed channels, rather than their regulation by store depletion. Other findings of store-operated behavior of TRPCs could not be reproduced by other laboratories (136, 229, 327, 361, 412). Despite these problems, some intriguing findings deserve consideration. The major findings with respect to the role of specific TRPCs are summarized below.

1. TRPC1

Arguably, among all TRPs, the case for TRPC1 as a store-operated channel (or subunit) is perhaps the strongest. Initial studies by Zhu et al. (411) reported that functional expession of human TRPC1 in COS cells resulted in a modest increase in thapsigargin-evoked Ca^{2+} influx. Zitt et al. (413) carried out whole cell recordings in CHO cells expressing TRPC1 and reported that dialysis with either $InsP_3$ or thapsigargin activated nonselective currents that were permeable to Ca^{2+}, Na^+, and Cs^+. They concluded that TRPC1 encoded a nonselective channel that was gated by store depletion.

Evidence that TRPC1 is intimately involved in store-operated entry in salivary gland cells has also been provided by Ambudkar and colleagues (200). Using RT-PCR, they found that TRPC1 and -3 were expressed in the human submandibular gland cell line, HSG (200). Overexpression of TRPC1 in HSG cells resulted in a twofold increase in the levels of TRPC1 associated with the plasma membrane, detected with an antibody directed against TRPC1. Compared with control cells, cells overexpressing TRPC1 revealed a three- to fivefold increase in the size of the Ca^{2+} signal that occurred upon readmission of external Ca^{2+} following pretreatment with thapsigargin in Ca^{2+}-free solution. Antisense oligonucleotides directed against TRPC1 reduced both the size of the thapsigargin-evoked Ca^{2+} signal and the levels of expression of the protein at the plasma membrane. However, antisense oligonucleotides to TRPC3 had no effect on Ca^{2+} entry induced by thapsigargin. These authors also found that some of the TRPC1 proteins could associate in the same detergent-resistant fraction as caveolin-1, indicating that TRPC1 might be located in the lipid raft domains of caveolae (199). Depletion of plasmalemmal cholesterol

levels in nontransfected cells resulted in loss of thapsigargin-evoked Ca^{2+} influx, leaving release apparently unimpaired. Such loss of Ca^{2+} influx following depletion of cholesterol was also seen in cells overexpressing recombinant TRPC1. An antibody to TRPC1 not only coimmunoprecipitated caveolin-1, and vice versa, but also pulled down the type 3 $InsP_3$ receptor. Not all TRPC1 channels were found in lipid rafts. However, these proteins appeared to be nonfunctional in that no Ca^{2+} influx was observed if cholesterol levels were depleted (presumably disrupting only caveolae). The authors concluded that TRPC1 is part of a signaling complex that is localized to caveolae, and it is this location that is required for the store-operated activity. Expression of truncated TRPC1 proteins in which amino acids 649–673 in the COOH terminal had been deleted were found to actually increase thapsigargin-evoked Ca^{2+} influx. This region of TRPC1 appears to mediate Ca^{2+}-dependent feedback inhibition of TRPC1 channel activity (334).

A crucial question concerning TRPC1 in HSG cells is whether it functions as a regulatory subunit or is directly involved in store-operated entry by contributing to the pore, for example. To address this, TRPC1 was truncated after S5, thereby deleting the putative pore region S6 and the COOH terminus (199). Overexpression of this mutated channel significantly reduced store-operated Ca^{2+} influx as well as single-channel activity by ~70%. Importantly, the mutant channel was able to immunoprecipitate with endogenous TRPC1, indicative of TRPC1 monomers interacting with each other to form multimers. Following site-directed mutagenesis of the seven negatively charged amino acids within the pore region, overexpression of the mutant channel reduced the store-operated Ca^{2+} current. Na^+ permeation in divalent-free solution was largely unaffected, and as a result, the authors argued that the mutant did not inhibit store-operated channel activity per se (199). Collectively, these results provide some support for the notion that TRPC1 forms at least part of the pore of the store-operated channel in HSG cells. However, it is not clear why mutation of specific residues in the channel pore would impart a diminution of Ca^{2+} current but not Na^+ current. If sites imparting Ca^{2+} selectivity were lost, then the channels should have become less selective, and current in the presence of Ca^{2+} would be expected to increase. On the other hand, if the mutations impart a decrease in channel permeability, then one would expect a decrease in current regardless of the charge carrier.

Despite the successful demonstration of store-operated behavior of TRPC1 by Zitt et al. (413) and the Ambudkar lab (200) other investigators have failed to observe store-operated activity of exogenously expressed TRPC1 (198, 335, 342). This may result from the fact that TRPC1, when expressed alone, does not appear to traffic properly to the plasma membrane (138). However, coexpression of TRPC4 or -5 permits proper trafficking of TRPC1. Thus it may be that the few successes with TRPC1 occurred when sufficient quantities of other subunits or associated proteins were available to permit proper trafficking to the plasma membrane.

There are far more reports implicating TRPC1 in store-operated Ca^{2+} entry through knock-down or inhibitor strategies. For example, in a study on the A549 endothelial cell line (46), antisense oligonucleotides directed against TRPC1 reduced thapsigargin-evoked Ca^{2+} signals by 25% and the endogenous I_{SOC} by ~50%. The store-operated pathway in this system seems to be regulated by the protein spectrin (393). Spectrin cross-links F-actin and provides mechanical support for the plasma membrane and internal organelles either by binding to integral membrane proteins directly or by associating with ankyrin and protein 4.1. Disrupting the spectrin-protein 4.1 linkage reduced thapsigargin-evoked Ca^{2+} influx in endothelial cells (393).

Mori et al. (248) found that TRPC1 was expressed in mouse spleen and in a mutant DT40 B cell line, which lacks $InsP_3$ receptors. Importantly, I_{CRAC} is present in both wild-type and $InsP_3$ receptor knock-out cells. Using homologous recombination to disrupt the TRPC1 gene, they reported that the store-operated current was abolished in 80% of the cells, but in the remaining 20% of the cells the current was identical to that seen in wild-type DT40 cells. These authors concluded that TRPC1 was an important component of the store-operated (presumable CRAC) channel and speculated that, in a fraction of the cells in which TRPC1 had been knocked out, a functionally redundant protein was upregulated and subsequently maintained the store-operated current (248). They also found that agonist-evoked Ca^{2+} release was reduced in TRPC1-deficient cells and demonstrated that this was due to a lower sensitivity of the $InsP_3$ receptors to $InsP_3$ (248). This is an important finding and suggests that TRPC1 can modulate $InsP_3$ receptors within the stores. TRPC1 channels could be located within the plasma membrane and bind to, and thereby modulate, $InsP_3$ receptors. Alternatively, some TRPC1 channels could actually be located within the stores themselves and facilitate the Ca^{2+} release process. Growing evidence supports the notion that various TRP channels can be located within the stores where they are capable of functioning as Ca^{2+} release channels themselves (138, 366, 391). A corollary of this is that experiments that affect TRPC1 expression/function could regulate store-operated entry indirectly through effects on store depletion via $InsP_3$ receptors. In addition, biochemical experiments showing coimmunoprecipitation of TRPs with ER proteins such as the $InsP_3$ receptor have been used as evidence in support of conformational coupling for activation of store-operated entry. If, however, some of the TRP channels reside in the ER, these

biochemical findings may not reflect the supposed linkage between the ER and plasma membrane.

In experiments with human platelets, it was reported that depletion of Ca^{2+} stores resulted in association of TRPC1 with intracellular $InsP_3$ receptors (318, 320), based on demonstrated coimmunoprecipitation of the two proteins. These results were taken as supportive of a role for TRPC1 in the store-operated entry in platelets, and also in support of the conformational coupling model whereby store-operated channels communicate with intracellular stores through interaction with $InsP_3$ receptors in the stores. Subsequently, this same group reported that their TRPC1 antibody, which was raised against extracellularly facing amino acids 555–571 of TRPC1, reduced thapsigargin-evoked Ca^{2+} and Mn^{2+} influx in a concentration-dependent manner (318). However, even with high concentrations of the antibody, inhibition was not complete and ~25% of store-operated influx remained. These results have been challenged by another group who report that TRPC1 is located not in the plasma membrane, but in the platelet intracellular stores (127), arguing against a role for TRPC1 in store-operated Ca^{2+} influx in human platelets. The controversy seems to hinge on the specificity of the two different antibodies used, and there is a need to more rigorously clarify which proteins are indeed recognized by the two antibodies. This issue is currently unresolved, and the reader is referred to published correspondence between the relevant authors for more details (127, 325).

Thus, despite some contradictory findings in the literature, there is a considerable body of evidence supporting the idea that TRPC1 can function as a store-operated channel or possibly as a subunit of a store-operated channel. The work by Ambudkar and colleagues (200) clearly suggests a role for TRPC1 in store-operated influx, but the biophysical features of recombinant TRPC1 are markedly different from I_{CRAC}. It is intriguing therefore that the TRPC1 knock-out experiments of Mori et al. (248) in DT40 cells demonstrate that a store-operated current with the phenotype of I_{CRAC} is lost in the majority of cells. Although this result does not necessarily mean that TRPC1 is part of the CRAC channel, it does point towards an important role for TRPC1 in the I_{CRAC} mechanism. This could be a consequence of TRPC1 forming heteromultimers with other CRAC channel subunits, with the presence of TRPC1 imparting an important regulatory aspect but not the pore itself. Alternatively, if a sizable fraction of TRPC1 is indeed located intracellularly, then this raises the possibility that TRPC1 might be involved in an early stage of store-operated Ca^{2+} entry, perhaps by contributing to store depletion by having a structural role, or even by being involved in the activation mechanism itself.

2. TRPC3, TRPC6, and TRPC7

These three TRPCs are considered together because of their similarity of structure, and, as far as we now know, similarity of action. However, by far the majority of investigative work has focussed on TRPC3, the first of the group to be cloned and expressed. Of all the canonical TRPs, the issue of store dependence of TRPC3 is the most polemic. Unlike the case for TRPC1, TRPC3 appears relatively easy to express in cell lines, and most investigators observe a channel that is clearly regulated through PLC activation. The precise nature of this regulation, however, is more controversial, and regulation by store depletion, by interaction with $InsP_3$ receptors, and by PLC-derived diacylglycerols have all been reported.

Initial studies overexpressing TRPC3 in HEK293 cells reported that store-operated Ca^{2+} influx was enhanced (411), although this was later shown to reflect constitutive channel activity (410). Electrophysiological recordings documented that TRPC3 expression in HEK-293 cells resulted in a large nonselective current with an estimated single-channel conductance of 60 pS (149). Although there was some constitutive activity in TRPC3-expressing cells, receptor stimulation substantially increased the size of the current. Zitt et al. (412) expressed TRPC3 in CHO cells and found the channels to be nonselective with a single-channel conductance of 60 pS. The channels were constitutively active, not affected by $InsP_3$- or thapsigargin-evoked store depletion, but were suppressed by strong intracellular Ca^{2+} buffering, suggesting a Ca^{2+} dependence to their activity. Consistent with this were the findings that either dialysis with a pipette solution containing Ca^{2+} buffered at 10 μM or application of ionomycin in the presence of external Ca^{2+} both increased channel activity. However, application of Ca^{2+} directly to inside-out patches containing TRPC3 failed to increase channel activity even in the presence of calmodulin. It was concluded that TRPC3 channels were gated by Ca^{2+}, albeit indirectly, but not by store depletion (412).

Collectively, these early studies favored the idea that TRPC3 proteins represented nonselective cation channels that were activated, in a Ca^{2+}-dependent manner, by receptor stimulation but not store depletion.

This view was challenged by a series of papers from the Muallem lab (170, 174), which advocated that TRPC3 channels were indeed store-operated. Using a TRPC3-expressing HEK 293 cell line, generated by the Birnbaumer laboratory, they found that muscarinic receptor stimulation with carbachol or store depletion with thapsigargin both activated a 66-pS channel in cell-attached patches, but only in cells expressing TRPC3 (170, 174). Interestingly, after exposure to thapsigargin, carbachol increased channel activity further (174). This was interpreted as evidence that both depleted stores and a further signal, associated with receptor activation, ostensibly oc-

cupied $InsP_3$ receptors, were required for maximal channel activation. The ability of thapsigargin to activate TRPC3 channels in the whole cell configuration was suppressed by heparin, indicating that functional $InsP_3$ receptors were required for channel gating even when stores were emptied independently of $InsP_3$ receptor activation. Upon excision of a cell-attached patch containing active TRPC3 channels, channel activity ran down quickly but could be restored by readmission of $InsP_3$ (174). After extensive washing of the patch, $InsP_3$ was no longer able to rescue the current. But activity returned, albeit in 50% of the patches, if the patches were exposed to both $InsP_3$ and either $InsP_3$ receptor-rich cerebellar microsomes or to liposomes containing recombinant type I $InsP_3$ receptors (174).

Using HEK 293 cells, Boulay et al. (39) went on to show that a physical interaction between $InsP_3$ receptors and TRPC3 existed in that both proteins could be coimmunoprecipitated. Because this occurred in resting cells, it would imply that such a complex was already preformed. This is in contrast to the findings of Rosado and Sage (320), discussed in the TRPC1 section above, who reported that coupling of TRPC1 to $InsP_3$ receptors only occurred upon store depletion. Glutathione-*S*-transferase pulldown experiments identified two regions in the $InsP_3$ receptor that interacted with one region in TRPC3. Overexpression of the corresponding peptides affected both agonist and thapsigargin-evoked Ca^{2+} influx; the sequence identified in TRPC3 and one of the $InsP_3$ receptor sequences inhibited entry, while the other $InsP_3$ receptor peptide weakly enhanced Ca^{2+} entry (39). Calmodulin was found to bind to the TRPC3 fragment in a Ca^{2+}-dependent manner, and this prevented both the binding of the $InsP_3$ receptor fragments to TRPC3 protein and the increased channel activity seen when the fragments were applied to excised patches containing TRPC3 (406). It was suggested that Ca^{2+}-calmodulin inhibits TRPC3 channel activity at rest, but this is overcome by occupied $InsP_3$ receptors that activate the channels by displacing calmodulin.

Collectively, the studies of Muallem's laboratory (170, 174) and Birnbaumer's laboratory (39, 351, 406) led to two significant conclusions. First, in contrast to the previous papers on TRPC3, the channels were now considered to be store-operated, and second, the channels were activated by interaction with $InsP_3$ receptors in the ER membrane. This latter point is particularly important because it constitutes direct evidence in support of the conformational-coupling model for activation of store-operated Ca^{2+} entry. Both of these conclusions, however, have been challenged (see, for example, Refs. 359, 412). The issue of involvement of $InsP_3$ receptors in the gating of TRPC3 channels has been discussed in section IX*B*. Here, we focus on whether TRPC3 is store-operated.

As discussed above, initial findings from Zitt et al. (412) and Zhu et al. (410) concluded that TRPC3 was not store-operated. Shortly after the report by Kiselyov et al. (174), Hofmann et al. (136) reported that TRPC3 and TRPC6 channels, expressed in CHO-k1 cells, were not activated by store depletion; rather, these channels were opened by diacylglycerol. Because diacylglycerol would be produced after stimulation of cell-surface receptors that activate PLC, the channels would be activated contemporaneously with an increase in $InsP_3$ (and hence Ca^{2+} release), but it is the DAG produced as a consequence of PIP_2 hydrolysis and not store emptying or occupied $InsP_3$ receptors that opens the channels (136). These results were in good agreement with a study by Kamouchi et al. (163), who expressed TRPC3 in calf pulmonary artery endothelial cells. These latter authors found that expression of TRPC3 resulted in the development of a large nonselective cation current following receptor stimulation, but this was not mimicked by store depletion. Receptor activation of TRPC3 channels could be suppressed by inhibition of PLC with the drug U-73122, consistent with a central role for diacylglycerol in activating TRPC3 channels. A similar conclusion was reached in a series of papers describing studies of TRPC3 stably expressed in HEK293 cells (229, 359, 360). In the latter case, activation of TRPC3 by store depletion could not be detected even in experiments with the same cell line originally used by Kiselyov et al. (174).

Some light has been shed on the confusion in the TRPC3 field, and perhaps for other TRPs, by studies of TRPC3 behavior when expressed in the B-cell line DT40. Following overexpression of TRPC3 in DT40 cells, Vazquez et al. (376) found that the rate of Ca^{2+} or Ba^{2+} influx following store depletion was twice as fast as that seen in nontransfected control cells, indicating that TRPC3 channels can be controlled by the Ca^{2+} content of the stores. Importantly, overexpression of TRPC3 channels in another expression system, namely, HEK293 cells, failed to alter the rate of thapsigargin-evoked Ba^{2+} influx when compared with control cells (360). Depending on the expression system then, TRPC3 can function in a store-dependent or store-independent mode. Moreover, TRPC3 exhibits different pharmacological profiles in the different expression systems. In HEK-293 cells transfected with TRPC3, 2-APB reduced Ba^{2+} influx due to receptor activation by ~60%, whereas in DT40 cells, 2-APB was much less effective (360). Perhaps more surprisingly, the receptor-activated TRPC3 channels in HEK293 cells were insensitive to the traditional SOC inhibitor Gd^{3+}, while the store-operated TRPC3 channels in DT40 cells were completely blocked. These findings strongly suggest that the molecular composition of the channels in the two expression systems is not the same. Subsequently, Vazquez et al. (377) found that the difference in mode of regulation and pharmacology probably

resulted in some manner from the level of expression of the protein. Low levels of expression of TRPC3 resulted in enhanced thapsigargin-evoked Ca^{2+} influx, which was inhibited by Gd^{3+} but less so by 2-APB. When the level of expression of TRPC3 was increased, either by increasing the plasmid concentration 10-fold or by using a more efficient avian β-actin promoter, the thapsigargin-activated entry was lost. A reciprocal change of phenotype was seen following receptor stimulation of TRPC3 (377). With the use of DT40 cells lacking $InsP_3$ receptors, the effects of agonist activation independent of store depletion can be examined. With low levels of TRPC3 expression, agonists failed to activate TRPC3, because in this situation store depletion is required. However, in cells expressing higher levels of TRPC3, such that store-operated entry was lost, receptor activation caused a robust activation of TRPC3 channels. Thus the study by Vasquez et al. (377) may provide an explanation for the discordant results in the literature regarding the store dependence/independence of TRPC3 channels (and perhaps other TRP channels as well). This issue is discussed in more detail in a subsequent section.

TRPC3 is widely believed to comprise a nonselective channel, which, more contentiously, may account for nonselective store-operated entry that has been described in some cells. A recent report however has proposed that hTRPC3 is an integral part of the highly Ca^{2+}-selective CRAC channel in T lymphocytes (285). Mutant T cell lines with defective I_{CRAC} and reduced T cell-receptor stimulated Ca^{2+} influx had been generated previously, but the molecular defects of this phenotype had not been elucidated. Philipp et al. (285) found that the TRPC3 gene was defective in all such mutants, but other canonical TRPs were unaffected. Introduction of the complete TRPC3 gene into the mutant cells restored agonist-evoked Ca^{2+} influx. Crucially, whereas I_{CRAC} was considerably diminished in the mutant cell lines, the current was restored fully following expression of TRPC3 (285). Because receptor stimulation and store depletion are believed to activate the same Ca^{2+} entry pathway in T cells, namely, I_{CRAC}, these results indicate that TRPC3 plays a role in store-operated entry possibly by contributing to the make-up of the channel. However, certain issues remain unclear. Only $InsP_3$ was used to deplete the stores (in the presence of 10 mM EGTA). It would be important to know whether thapsigargin or ionomycin ($InsP_3$-independent stimuli) are capable of activating I_{CRAC} in the TRPC3-expressing mutant T cells, under conditions where cytoplasmic Ca^{2+} is strongly buffered at resting levels. Moreover, despite the obvious changes in the TRPC3 gene, Western blotting revealed that the protein levels were not significantly changed.

There are no published reports claiming a store-operated behavior of TRPC6; rather, it more clearly appears to function as a DAG-activated channel (40, 136, 150, 158, 361, 406). Much less work has focussed on TRPC7; Mori's laboratory first cloned TRPC7 and clearly demonstrated that TRPC7 is a DAG-operated, non-store-operated channel (259), whereas another group claimed that it is store-operated (311). In the latter study, it is difficult to evaluate the validity of the conclusion that TRPC7 is store-operated as no information is given on the constitutive activity of TRPC7 (361). In a more recent publication, Lievremont et al. (195) revisited the issue of the regulation of TRPC7. These authors reported that TRPC7, when stably expressed in HEK293 cells, supported Ca^{2+} entry that was either store-operated or activated by DAG depending on the mode of stimulation. Interestingly, no store-operated Ca^{2+} entry was observed when TRPC7 was transiently expressed, providing a possible explanation as to why this activity was not observed in the earlier study from Mori's laboratory (259).

3. TRPC4 and TRPC5

Philipp et al. (283) were the first to clone TRPC4 (bovine), and they reported that the channel was activated by thapsigargin and $InsP_3$ when expressed in HEK 293 cells. Stable expression of TRPC4 in CHO cells also resulted in a store-operated current that could be activated by dialysis with $InsP_3$ in 10 mM EGTA or thapsigargin (386). Surprisingly, the current activated after a sizable delay of 140–330 s following dialysis with $InsP_3$. TRPC4 channels in the CHO expression system were equally permeable to Ba^{2+} and Ca^{2+} but not to Na^+. In the absence of external Mg^{2+}, the channels exhibited anomalous mole fraction effects between Na^+ and Ca^{2+}. Warnat et al. (386) also overexpressed TRPC4 in RBL cells which possess a well-characterized I_{CRAC} and found a modest, but significant, increase in the density of I_{CRAC}. This occurred without an apparent change in either the reversal potential or rectification of the current.

TRPC4 is particularly abundant in the adrenal cortex. The bovine adrenal cortical cell line SBAC expresses TRPC4 and exhibits a small store-operated CRAC-like current that can be activated by $InsP_3$ or thapsigargin (286). Exposing the cells to an antisense oligonucleotide directed against TRPC4 resulted in a 50% reduction in the extent of the store-operated current as well as a 30% fall in endogenous TRPC4 protein expression. The authors concluded that TRPC4 contributes to native CRAC-like channels in adrenal cells (286).

Consistent with a role for TRPC4 in store-operated influx, it was found that overexpression of rat TRPC4 in *Xenopus* oocytes resulted in a twofold potentiation of external Ca^{2+}-dependent Cl^- currents after depletion of intracellular stores with thapsigargin (356).

A major step forward came with the development of a mouse in which the TRPC4 gene had been deleted (96). It was found that the store-operated current seen in nor-

mal endothelial cells was substantially reduced in the knock-out mouse. The endothelial store-operated current was clearly not I_{CRAC} as there were some striking differences in the biophysical features of the two currents. Nevertheless, the results suggested that TRPC4 was important for the function of a native store-operated channel (96). Furthermore, the gene knockout study provided the first real evidence for a physiological role for a TRPC protein, namely, a role for TRPC4 in endothelium-dependent vasorelaxation of blood vessels. The TRPC4 gene knockout impaired the ability of acetylcholine to relax precontracted aortic smooth muscle strips. Muscarinic receptor stimulation of endothelial cells results in Ca^{2+} influx-dependent release of vasorelaxants like NO and prostaglandins, which subsequently vasodilate the adjacent myocytes (96). Hence, TRPC4 might be an important regulator of blood vessel tone.

Collectively therefore, evidence has been accumulating that supports a major role for TRPC4 as a store-operated Ca^{2+}-permeable channel. However, as with the other TRPs, there are conflicting findings in the literature, such that a solid consensus on the role of TRPC4 does not yet exist.

Phylogenetic analysis has established a close structural relationship between TRPC4 and TRPC5. The same laboratory that originally reported that TRPC4 was store-operated subsequently published results, indicating that TRPC5 was similarly activated by store depletion (284). However, in results from a different laboratory, expression of TRPC5 in HEK-293 cells, the same line in which earlier work indicated the channels were store-operated, yielded channels that were activated following receptor stimulation but not store depletion (259). The channels seemed to require intracellular Ca^{2+} for activation because the current could not be detected with a Ca^{2+}-free pipette solution. A similar conclusion was reached by Schaefer et al. (327). They cloned murine TRPC4 and TRPC5 and expressed the channels in the HEK293 system. They found that store depletion following dialysis with $InsP_3$ or application of thapsigargin failed to induce any whole cell currents in cells overexpressing TRPC4 or TRPC5. Similarly, Mn^{2+} quenching of fura 2 following store depletion was unaffected by overxpressing either of the TRP channels. Ca^{2+} influx through the channels was suppressed by an inhibitor of PLC. The channels were nonselective, were equally permeable to Na^+ and Ca^{2+}, and could be activated by guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) in excised inside-out patches. Collectively, the study by Schaefer et al. (327) demonstrates that murine TRPC4 and -5 channels are not store-operated but are instead activated by receptors that stimulate PLC activity.

McKay et al. (229) transiently expressed human TRPC4 channels in HEK 293 cells. They reported an increased basal Ba^{2+} influx into the cells, indicating constitutive channel activity. Whole cell patch-clamp experiments in CHO cells expressing TRPC4 demonstrated the presence of a constitutively active nonselective cation current. Ba^{2+} influx was not enhanced by store depletion with thapsigargin nor by receptor stimulation, suggesting TRPC4 was not store-operated.

A similar conclusion was reached by the Zhu laboratory who produced stable HEK-293 cell lines expressing murine TRPC4 (407). They found that neither agonist- nor thapsigargin-evoked Ca^{2+} influx was affected by the presence of TRPC4. Intriguingly however, they reported that expression of TRPC4 resulted in a three- to fivefold increase in the amplitude of inwardly rectifying K^+ currents. It is not clear as yet whether or not TRPC4 participates in the formation of the inwardly rectifying K^+ channels or how it affects the formation of such channels.

Wu et al. (394) also examined the effects of TRPC4 on store-operated influx in HEK 293 cells, but utilized an antisense oligonucleotide approach. Following stable expression of an antisense construct to TRPC4, Western blotting with a commercially available antibody revealed a reduction in TRPC4 protein to 31% of control. In spite of this reduction in TRPC4 levels, thapsigargin-evoked Ba^{2+} influx was unaffected. Intriguingly, cells stably expressing the antisense construct exhibited a significantly smaller arachidonic acid-dependent Ca^{2+} influx and far fewer Ca^{2+} oscillations to a submaximal dose of carbachol. The authors concluded that TRPC4 was not involved in store-operated entry but helped form the channel that was responsible for arachidonic acid-induced Ca^{2+} entry.

4. TRPC2

Murine TRPC2, a homolog of the human TRPC2 pseudogene, is expressed in very high levels in the testis (374). Transient overexpression of TRPC2 in COS cells resulted in a 35% increase in intracellular Ca^{2+} signals following readmission of external Ca^{2+} to cells exposed either to agonist or thapsigargin (374). The authors suggested that TRPC2 was part of a store-operated channel. However, constitutive activity of TRPC2 was not determined. The particularly high levels of TRPC2 in testes led Jungnickel et al. (159) to examine whether TRPC2 affected sperm-egg interaction. They developed antibodies to TRPC2 and found punctuate labeling of the protein in the anterior head of sperm with lesser labeling at the posterior end. In sperm cells, store depletion with thapsigargin increased Ca^{2+} influx and Mn^{2+} entry (seen with quenching of fura 2). The antibody reduced such cation entry by 73%. Thapsigargin also triggered the release of hydrolytic enzymes necessary for the acrosome reaction, and this was also reduced by the antibody. The antibody also blocked the acrosome reaction triggered by ZP3, a glycoprotein of the egg's extracellular matrix. It was suggested that TRPC2 was an essential component of the ZP3-acti-

vated cation channel in mouse sperm and drove the acrosome reaction.

In rat, TRPC2 RNA was found exclusively in the vomeronasal organ, which detects pheromones (196). The protein was localized to the sensory microvilli, which is presumably the site of pheromone sensory transduction. This region is devoid of Ca^{2+} stores, suggesting that rat TRPC2 is not gated by store depletion. Hofmann et al. (137) cloned two novel murine splice variants, dubbed mTRPC2a and mTRPC2b. They found that mTRPC2b was expressed only in the vomeronasal organ. In contrast to Vannier et al. (374), Hofmann et al. (137) reported that expression of mTRPC2 in HEK-293 cells failed to alter either receptor or thapsigargin-dependent Ca^{2+} influx. This was likely a consequence of the proteins being located in endomembranes rather than the plasma membrane, as revealed by the use of a mTRPC2-GFP fusion protein, leading the authors to question a physiological role for mTRPC2 channels other than in the vomeronasal organ.

B. TRPV6 (CaT1)

TRPV6 (also called CaT1 or ECaC2) is a member of the TRPV subfamily; V denotes vanilloid because the first member of the family that was identified was the vanilloid receptor VR1. TRPV6, and the highly homologous TRPV5 (CaT2 or ECaC1), which share 75.6% amino acid identity, are Ca^{2+}-selective ion channels and serve as a major conduit for apical Ca^{2+} entry in transporting epithelia (intestine and kidney, respectively; Ref. 72). TRPV6 was identified using an expression cloning strategy based on its ability to increase Ca^{2+} influx into *Xenopus* oocytes (278). A detailed characterization of the biophysical properties of recombinant TRPV6 by Nilius and collaborators (72, 379) revealed that the channels were very selective for Ca^{2+} ($P_{Ca}/P_{Na} > 100$), were permeable to Ba^{2+} and Sr^{2+} but not to Mg^{2+}, were inwardly rectifying and non-voltage-gated, became permeable to monovalent cations when external cations were removed, exhibited anomalous mole fraction effects, and exhibited both fast and slow Ca^{2+}-dependent inactivation. All of these features are strikingly characteristic of CRAC channels. However, when expressed in either *Xenopus* oocytes (278) or HEK293 cells (379), channel activity was not enhanced by store depletion with thapsigargin. Hence, TRPV6 was not deemed to be store-operated. Instead, the channels were believed to be constitutively active but could be inactivated by a rise in cytoplasmic Ca^{2+} concentration.

This issue was revisited by Clapham and colleagues (403). They expressed TRPV6 in CHO cells and confirmed the striking similarities between the electrophysiological features of the recombinant channels and those of CRAC. Unlike the preceding studies however, Yue et al. (403) reported that TRPV6 was store-operated. This conclusion was based on the finding that whole cell dialysis with a pipette solution containing $InsP_3$, thapsigargin, and 0.5 mM EGTA transiently activated TRPV6 channels, whereas dialysis with 0.5 mM EGTA alone was ineffective. Crucially, this store dependence was only manifest during the first few hours after transfection with the TRPV6 gene. When recordings were carried out 24 h posttransfection, the channels were no longer store-operated but constitutively active and inhibited by cytoplasmic Ca^{2+}. Dialysis with strong buffer (10 mM EGTA) rapidly activated the channels, probably by removing Ca^{2+}-dependent inactivation, and inclusion of $InsP_3$ in the pipette solution did not alter the kinetics of activation. The authors speculated that a component of the endogenous activation mechanism was rate-limiting and when TRPV6 channels were expressed to such an extent that they overwhelmed this endogenous capacity, then the channels reverted to constitutive mode (403). Put another way, TRPV6 channels would presumably have to become part of an endogenous complex that both suppresses the constitutive activity and confers store dependence. In addition to the store dependence, Yue et al. carried out single-channel recordings on TRPV6 channels in divalent-free external solution and found the single-channel conductance to be very similar to that attributed to CRAC under similar conditions in Jurkat T lymphocytes (~45 pS; Ref. 166). However, it turns out that such measurements supposed to represent CRAC channel conductance are in reality due to TRPM7 channels, which are activated by a fall in intracellular Mg/Mg-ATP (132). A revised estimate of the single-channel conductance of CRAC channels in divalent-free solution is 0.2 pS (292). Hence, the relatively high single-channel conductance of TRPV6 channels indicates that these channels are unlikely to account for the entire CRAC channel pore. There are some other striking differences between the biophysical properties of recombinant TRPV6 channels and those of CRAC in divalent-free solution (17, 381). TRPV6 is almost six times more permeable than CRAC to Cs^+, exhibits a slope of negative conductance negative to −75 mV which is not shown by CRAC channels, and is blocked by Mg^{2+} in a voltage-dependent manner. In addition, TRPV6 and CRAC display different pharmacological properties. Collectively, therefore, although there are some biophysical similarities between TRPV6 and CRAC channels, there are nevertheless several quite marked discrepancies which indicate that TRPV6 probably does not account for the entire CRAC pore. Could TRPV6 be part of the pore? This is possible, but additional components or subunits would have to induce rather extensive changes in the properties of TRPV6 including a change in single-channel conductance, ionic selectivity, pharmacology, and voltage-dependent gating by Mg^{2+}.

Is TRPV6 store-operated? Voets et al. (381) have expressed TRPV6 in HEK 293 cells and reported that the channels were not activated by store depletion following ionomycin application. Instead, the channels were constitutively active and inhibited by a rise in cytoplasmic Ca^{2+} concentration. This pattern was seen irrespective of the posttransfection time. Similarly, overexpression of TRPV6 in RBL-1 cells failed to increase the rate or extent of I_{CRAC} following store depletion in weak Ca^{2+} buffer (37). On the other hand, Schindl et al. (328) reported that TRPV6 interacted with endogenous CRAC channels in RBL cells shortly after transfection and increased the size of the store-operated current. However, this association was no longer manifest 24 h after transfection. This apparent interaction was postulated to create a multimeric channel that was insensitive to 2-APB, in marked contrast to the endogenous CRAC channels.

Vanden Abeele et al. (373) found that TRPV6 was expressed in lymph node carcinoma cells of the prostate cells. Unlike recombinant TRPV6, the channels in the prostate cells were not constitutively active but were gated by store depletion. In addition, they were sensitive to 2-APB. It was reported that knock down experiments with antisense oligonucleotides to TRPV6 resulted in a 50% reduction in the amplitude of the store-operated Ca^{2+} current. On the other hand, antiandrogen treatment upregulated TRPV6 levels by 60% and the store-operated current by 30%. These authors concluded that TRPV6 was part of the endogenous store-operated channel complex.

A similar conclusion was reached by Cui et al. (67) from studies on Jurkat T lymphocytes, one of the model systems for studying I_{CRAC}. These authors found that TRPV6 was expressed in Jurkat cells and that its overexpression resulted in a constitutively active current that had similar biophysical features to endogenous I_{CRAC}. Surprisingly, store depletion only slightly increased the activity of the recombinant channels. However, the key finding was that expression of a pore mutant of TRPV6, dubbed TRIA-CaT1 and which failed to carry any detectable Ca^{2+} current itself, nevertheless suppressed endogenous I_{CRAC} (67). The authors interpreted this as evidence that the mutant interacted with and dominated the endogenous TRPV6 that was responsible, at least partly, for the native store-operated current.

Clearly there are many conflicting reports on whether TRPV6 is store-operated or not. Because similar expression systems have often been used, these differences cannot be simply ascribed to cell-specific posttranslational modifications. In view of the quite striking differences in biophysical properties of recombinant TRPV6 and endogenous CRAC channels, TRPV6 probably is not all or part of the CRAC channel pore. However, it is possible that TRPV6 has a modulatory or auxiliary role in store-operated influx under certain conditions.

C. Summary of TRPs

Although some groups have provided persuasive evidence that certain TRPs may be part of a native store-operated Ca^{2+}-permeable channel, this is by no means widely accepted. How might one explain the startling inconsistencies and contradictions as to whether a particular TRP is store-operated? The discrepancies cannot be simply accounted for by different experimental systems because many groups use the same original source of a particular TRP cDNA and use the same cell type for expression. In one such instance, however, experimental work disclosed the basis for the apparently contradictory results. Two different laboratories expressed TRPC3 in DT40 B lymphocytes. One provided solid evidence that the channels that were formed were store operated (376), and the other provided clear evidence that the channels were not store-operated, rather they were activated in a PLC-dependent manner, probably by diacylglycerol (378). Subsequent work revealed that the variable likely responsible for these distinct behaviors was the level of expression of the protein. At low levels of expression, TRPC3 was store-operated, while at higher levels, it was receptor-activated and not store-operated (377). Similarly, Yue et al. (403) reported that TRPV6 was only store-operated at early times after transfection, which they interpreted as being due to lower levels of expression. But, how might different levels of expression of a particular TRP protein account for the discordant results? An important factor could be a need for the recombinant TRP to form functional complexes with other TRPs or auxillary subunits that might not be present endogenously in sufficient levels to yield the correct stoichiometry. Overexpression of a given TRP might result in such high levels of the recombinant protein being synthesized that it forms mainly homomultimers that are not store-operated. Alternatively, a combination of TRPs in heteromultimers, or with other cellular components may be required to form a store-operated channel and simply overexpressing one component will fail to replicate this. Thus strategies focusing on behavior and regulation as a function of the level of expression might be more informative. What might the factors be with which TRPs need to associate to form store-operated channels? One possibility, already mentioned, is other TRPs, i.e., it might be necessary to assemble a tetrameric channel composed of more than one type of TRP. Among TRPCs, Hofmann et al. (138) reported that only structurally similar TRPCs could come together in functional channels. Thus TRPC4, -5, and -1 can associate, as can TRPC3, -6, and -7. Other combinations apparently did not occur. However, Strubing et al. (343) reported that in situ, other combinations were found, such as TRPC3 or -6 combining with TRPC1 and -4 (343). This raises the possibility that combinations that can be formed in situ are not readily replicated in overexpression experiments.

This is consistent with the idea that high levels of expression preclude the formation of certain, possibly physiological, ion channel complexes.

There are additional complications in interpreting effects of transfected TRP channels, especially when entry is monitored by use of fluorescent indicators under conditions whereby membrane potential is not controlled. For example, TRPC4 increases the size of the inwardly rectifying K^+ current fivefold (407). In many studies examining the effects of TRPs on store-operated influx, Ca^{2+} entry has been measured using fura 2, and the membrane potential has not been controlled. Hence, an increased inwardly rectifying K^+ current would tend to set the membrane potential at hyperpolarized potentials, and this would maintain a very favorable driving force for Ca^{2+} influx. TRPC4 expression could therefore increase the size of store-operated Ca^{2+} influx, but this would be achieved indirectly through effects on the electrical gradient and not because TRPC4 is part of the store-operated complex. Despite the large number of positive reports in the literature, the possibility remains that TRPs do not encode store-operated channels. Instead, they may have an important, albeit supportive role, in store-operated Ca^{2+} entry, by helping form an appropriate signaling complex, for example. TRPCs 1, 4, 5 (but not TRPCs 3, 6, 7) all coimmunoprecipitate with the INAD protein (111), a PDZ-containing protein that helps organize the signalplex in *Drosophila*. Although speculative, certain TRPs may have a similar role in store-operated entry.

XIII. MITOCHONDRIAL REGULATION OF STORE-OPERATED CALCIUM ENTRY

Under physiological conditions of intracellular Ca^{2+} buffering, mitochondria play a central role in the gating of store-operated channels. Whole cell dialysis with $InsP_3$ or stimulation of cell-surface receptors generally fail to activate any detectable I_{CRAC} in weak buffer unless mitochondria are maintained in an energized state (15, 44, 90, 110). By taking up Ca^{2+} that has been released from the stores, mitochondrial buffering results in greater store depletion and hence activation of I_{CRAC}. Depolarization of mitochondria by inhibiting the electron transport chain or suppressing Ca^{2+} uptake through the uniporter prevented I_{CRAC} from developing (102, 103). The size of I_{CRAC} in the presence of energized mitochondria in physiological Ca^{2+} buffer could be increased further by inhibiting the SERCA pumps with thapsigargin (102, 109). This indicates that competition exists between these two major Ca^{2+} removal mechanisms and the extent of this dictates the level of store depletion and hence size of I_{CRAC}. Mitochondrial Ca^{2+} uptake also shifts the relationship between $InsP_3$ concentration and size of I_{CRAC} to the left by around twofold (102). A subthreshold concentration of $InsP_3$ becomes capable of activating the current when mitochondria are energized (102). In this way, mitochondrial Ca^{2+} buffering increases the dynamic range of concentrations over which $InsP_3$ functions as the physiological messenger that activates I_{CRAC}.

In addition to facilitating store depletion and hence activation of I_{CRAC}, mitochondria also sustain Ca^{2+} influx by buffering incoming Ca^{2+} and hence reducing the extent of Ca^{2+}-dependent inactivation of the Ca^{2+} channels. In T lymphocytes and RBL-1 cells, mitochondrial Ca^{2+} uptake maintains I_{CRAC} (103, 142). The rate and extent of Ca^{2+}-dependent but store-independent slow inactivation is reduced twofold by energizing mitochondria, and this is prevented if the mitochondrial Ca^{2+} uptake is inhibited (103, 142). In addition, by competing with SERCA pumps, mitochondrial Ca^{2+} uptake would be expected to slow the rate of store refilling too. This dual role of mitochondria, facilitating activation of I_{CRAC} and then reducing Ca^{2+}-dependent inactivation, results in larger and more sustained Ca^{2+} influx than would be the case if it affected either process alone (103).

Mitochondrial buffering of Ca^{2+} entry through store-operated channels distinct from CRAC has been found in several different nonexcitable cells. In the endothelial cell line ECV304, mitochondria preferentially buffered Ca^{2+} that entered the cell following stimulation with ATP as opposed to Ca^{2+} released from the stores, which probably reflected the fact that three times more mitochondria were located close to the plasma membrane than the ER (189). In another endothelial cell line (EA.hy926), mitochondria located just below the plasma membrane prevented subplasmalemmal Ca^{2+} concentration from rising high enough to open Ca^{2+}-dependent K^+ channels following stimulation with histamine (218). Through maintaining a low Ca^{2+} concentration at these sites, the authors suggest that mitochondria sustain store-operated Ca^{2+} entry by preventing Ca^{2+}-dependent inactivation of the Ca^{2+} entry channels (218). Indeed, mitochondrial depolarization with protonophore or antimycin A substantially reduced the size of the cytoplasmic Ca^{2+} signal following Ca^{2+} readmission to cells challenged with histamine or BHQ. Interestingly, inhibition of mitochondrial Ca^{2+} release by blocking the Na^+/Ca^{2+} exchanger located on the inner mitochondrial membrane also impaired Ca^{2+} influx, suggesting that transmitochondrial flux is essential for maintaining store-operated entry (219).

In pancreatic acinar cells, a population of mitochondria have been found in the basolateral area close to the plasma membrane where they seem to preferentially buffer incoming Ca^{2+} through store-operated channels (273).

Although less well established, recent evidence raises the possibility that mitochondria might have an additional role in supporting store-operated Ca^{2+} channel activity distinct from simple Ca^{2+} buffering (109). One possibility is that mitochondria release factors that sub-

sequently modulate CRAC channel activity (109). Such factors may be related to the release of chemical signals from mitochondria like glutamate (217) and ATP (188) as well as an unidentified messenger generated by mitochondria in skeletal muscle which then diffuses to the plasma membrane to open Na^+ channels (261). Redistribution of mitochondria away from the plasma membrane to the nucleus following overexpression of the mitochondrial fission-promoting protein hfis1, resulted in no change in store-operated Ca^{2+} influx in HeLa cells (97). Subsequent mitochondrial depolarization reduced Ca^{2+} entry by 50%. Hence, mitochondrial regulation of store-operated Ca^{2+} channels does not require local interaction but instead seems to involve a global signal, perhaps a diffusible messenger (97). The involvement of mitochondria in regulating store-operated Ca^{2+} entry is summarized in Figure 3.

XIV. QUANTITATIVE RELATIONSHIP BETWEEN STORE DEPLETION AND STORE-OPERATED ENTRY AND EVIDENCE FOR A SPECIALIZED CALCIUM STORE INVOLVED IN REGULATING ENTRY

The parameter that most directly regulates store-operated channels is the content of Ca^{2+} in the ER, or, as discussed below, a specialized component of it. In the simplest situation, the activity of store-operated channels and the depletion of ER stores might be tightly coupled; by this we mean that there exists a roughly linear relationship between the fractional depletion of stores and the fractional activation of store-operated channels. Unfortunately, although it is well-established that store emptying activates these channels, very little is known about the quantitative relationship between the fall in luminal Ca^{2+} content and the extent of store-operated entry. This is in part due to the technical difficulty in measuring store content, or what is likely more relevant, the concentration of Ca^{2+} in stores. As a result, a variety of indirect measures have been used to assess store content and how this correlates with the extent of store-operated Ca^{2+} entry.

In many of the studies discussed below, the store-operated influx does not appear to be tightly coupled to store depletion. The most common situation is that some Ca^{2+} release can occur without ensuing store-operated entry. This dissociation can be explained in one of two ways. One possibility is that a homogeneous Ca^{2+} store exists, but partial depletion of the store does not activate any Ca^{2+} influx. This would mean that a threshold exists below which intraluminal Ca^{2+} content must fall before store-operated entry develops. Alternatively, it might be that at least two functional Ca^{2+} stores exist but only one is linked to store-operated entry. Most authors have interpreted their results in terms of a specialized store that regulates Ca^{2+} entry, and that is a subcompartment of the total $InsP_3$-sensitive store. In fact, in some cases, discussed below, this conclusion seems inescapable.

A number of early studies demonstrated that when populations of cells were analyzed, a roughly linear relationship existed between the magnitude of the release phase of the Ca^{2+} response and the steady-state entry phase (296). This relationship persisted regardless of the means of depletion of the stores (6). This contrasts with entry pathways that are not related to store depletion, and thus may be activated over distinctly different concentration ranges than those associated with release (359). However, this does not ensure that entry and release are necessarily tightly coupled, since the measurements were made on populations of cells, and the behavior of single cells may stochastically vary considerably from the population average. In perhaps the earliest study attempting

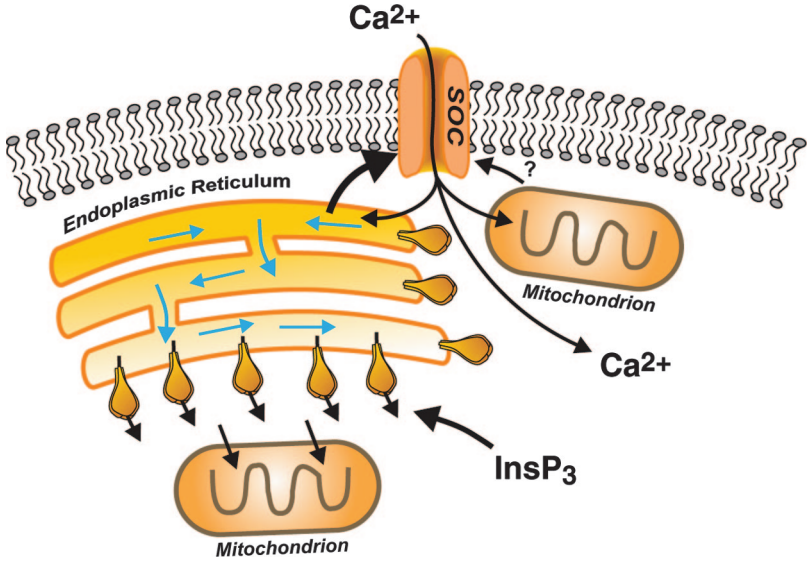

FIG. 3. Regulation of store-operated channels by intracellular Ca^{2+}-buffering organelles. Store-operated channels (SOC) are activated by a signal from a specialized compartment of the endoplasmic reticulum. As depicted in the figure, this compartment may not coincide exactly with the major $InsP_3$-sensitive component of the endoplasmic reticulum. The Ca^{2+} buffering capabilities of the endoplasmic reticulum and mitochondria facilitate SOC activation at least in part by buffering entering Ca^{2+} near the mouth of the channel, and reducing negative-feedback inhibition of the channels. In addition, location of mitochondria in close proximity to $InsP_3$ receptors on the endoplasmic reticulum buffers the release of Ca^{2+} and thereby reduces Ca^{2+} inhibition of release through $InsP_3$ receptor channels. Finally, energized mitochondria appear capable of positively regulating SOC function by a mechanism that is independent of Ca^{2+} buffering.

to deal with this issue quantitatively at the level of single cells, Jacob (155) utilized a clever albeit indirect approach to measuring entry through store-operated channels as a function of the extent of store depletion. He discharged stores from single endothelial cells with histamine, and then blocked the histamine receptors, in the continual absence of external Ca^{2+}. He then assessed divalent cation permeability by the rate of Mn^{2+} entry (which quenches intracellular fura 2). This protocol was one of the first to demonstrate an accelerated cation entry following the depletion of stores that was clearly dissociated from receptor activation, or products of PLC. By varying the duration of the histamine stimulus, or by partially refilling the stores by brief exposures to Ca^{2+}-containing solutions, he subsequently evaluated the rate of Mn^{2+} entry as a function of the degree of store filling. This resulted in a roughly linear relationship, which might suggest that in these cells store emptying was tightly coupled to Ca^{2+} entry.

The majority of subsequent work, however, has revealed some degree of dissociation between global ER Ca^{2+} depletion and the activation of store-operated entry. Perhaps the first study to demonstrate such a dissociation between depletion and influx came from studies on NIH 3T3 cells (310). These authors found that treatment of NIH 3T3 cells with phorbol esters, through activation of protein kinase C, reduced the total Ca^{2+} content of the ER by >50%. However, this depletion of Ca^{2+} did not activate entry, nor did it affect the ability of thapsigargin to activate entry when the remaining stores were emptied. The authors interpreted this result as indicative of distinct stores, a minority of which were involved in store-operated entry regulation, and which were not sensitive to depletion by activators of protein kinase C.

Parekh et al. (265) also found a dissociation between Ca^{2+} release and subsequent activation of I_{CRAC} in RBL-1 cells. Low concentrations of $InsP_3$ released a sizable amount of Ca^{2+} from the stores, but this was not translated into development of I_{CRAC}. Higher concentrations of $InsP_3$, which emptied the stores further, evoked the current. A similar dissociation between Ca^{2+} release and subsequent Ca^{2+} influx was seen using thapsigargin at the single-cell level (265). In spite of clear Ca^{2+} release to thapsigargin, Ca^{2+} influx did not always develop. Collectively, these results suggested a dissociation between the extent of Ca^{2+} release from the total $InsP_3$-sensitive store and subsequent activation of I_{CRAC}. A similar conclusion was reached by Huang and Putney (147), again for RBl-1 cells. These authors compared the development of I_{CRAC} following store depletion with ionomycin and thapsigargin. A low dose of ionomycin activated I_{CRAC} with similar kinetics and to a similar level as that attained with a high dose of thapsigargin. However, as I_{CRAC} started to activate, total store content had been changed only slightly by ionomycin but was substantially reduced by thapsigargin. Hence, the development of I_{CRAC} appeared to require significantly more store emptying by thapsigargin, consistent with dissociation between global Ca^{2+} release and subsequent Ca^{2+} entry. One would expect that the ionophore would have roughly equal access to all Ca^{2+} stores simultaneously. Thapsigargin releases Ca^{2+} by inhibition of SERCA pumps and relies on the passive leak of Ca^{2+} through unknown channels. However, there is evidence that this leak might be associated with protein synthesizing structures (203). This might indicate that the protein-synthesizing ER is less involved in the regulation of store-operated entry. Two other studies, discussed below, support this conclusion.

Recombinant TRPV1 can function as a Ca^{2+} release channel in several cell types (366, 382, 391). Two laboratories have exploited this intracellular location to probe the relationship between store depletion and activation of store-operated entry (366, 391). TRPV1 can function both as an intracellular Ca^{2+} release channel as well as a nonselective cation channel in the plasma membrane. The membrane-permeable agonist capsaicin provides a convenient means for activating intracellular TRPV1 channels. Turner et al. (366) expressed TRPV1 in RBL cells and found that capsaicin released Ca^{2+} from intracellular stores. The capsaicin-sensitive store was contained within the total $InsP_3$-sensitive store because carbachol, acting on muscarinic receptors that increase $InsP_3$ levels, could abolish capsaicin-evoked Ca^{2+} release, whereas capsaicin only partially reduced the size of the Ca^{2+} release signal elicited by carbachol. However, discharge of the capsaicin-sensitive Ca^{2+} store failed to activate I_{CRAC}. Subsequent depletion of the remaining intracellular Ca^{2+} stores with carbachol fully activated the current.

Using a similar approach, Wisnoskey et al. (391) expressed TRPV1 in insect Sf9 and HEK-293 cells and used fura 2 to monitor Ca^{2+} and Ba^{2+} influx. In nontransfected cells, thapsigargin activated a Ba^{2+}-permeable pathway that was blocked by La^{3+} and 2-APB. Store-operated Ba^{2+} influx was unaffected by expression of TRPV1. In TRPV1-expressing cells, capsaicin was able to trigger both Ca^{2+} release and Ba^{2+} entry. However, in marked contrast to the store-operated pathway, capsaicin-evoked Ba^{2+} influx was insensitive to La^{3+} and 2-APB and probably reflected the activity of TRPV1 channels in the plasma membrane with no contribution from endogenous store-operated channels. The key finding was that the capsaicin- and thapsigargin-sensitive stores overlapped considerably (391). Exposure to thapsigargin largely prevented subsequent stimulation of intracellular TRPV1 receptors from eliciting Ca^{2+} release. Similarly, stimulation of these TRPV1 receptors dramatically reduced the size of the thapsigargin-sensitive stores. In spite of this considerable overlap, thapsigargin, but not agonists of the intracellular TRPV1 receptors, was capable of activating store-operated influx. Hence, a sizable amount of Ca^{2+} can be

released from the thapsigargin-sensitive stores, but this is not associated with any store-operated influx (391). Since the TRPV1 expression in both of these studies was driven to excess by a viral promoter, it is possible that the channel has accumulated in a fully functional form at the site of synthesis in the ER. If so, this may be additional evidence for the idea that the component of the ER responsible for signaling to store-operated channels is specialized and distinct from the bulk of the protein-synthesizing ER.

Studies on calf pulmonary artery endothelial cells also point towards a complex relationship between store depletion and subsequent Ca^{2+} influx. Sedova et al. (331) incubated the cells in Ca^{2+}-free solution for different times to passively deplete the stores and then applied Mn^{2+} externally to track Mn^{2+} quenching of fura 2, assuming all Mn^{2+} enters only through the store-operated pathway. Store content was assessed by subsequently stimulating P2Y receptors (which are coupled to $InsP_3$ production) and measuring the ensuing cytoplasmic Ca^{2+} increase. The relationship between the rate of Mn^{2+} quenching and Ca^{2+} store content was sigmoid. Although Mn^{2+} influx was graded with store emptying, this occurred only over a certain range. Crucially, an initial reduction in store content was not associated with detectable Mn^{2+} entry providing evidence for a dissociation between Ca^{2+} release and subsequent Ca^{2+} entry.

Collectively then, studies from a wide variety of cell types and using several different approaches all suggest that a dissociation can exist between the amount of Ca^{2+} released from the total $InsP_3$/thapsigargin-sensitive stores and subsequent store-operated Ca^{2+} entry. Store-operated influx is not always tightly coupled to store depletion. Some Ca^{2+} can be released from the total $InsP_3$-sensitive stores without detectable Ca^{2+} influx occurring.

It is important to be clear that a dissociation between the Ca^{2+} content of the total agonist-sensitive Ca^{2+} store and the extent of I_{CRAC} activation is not incongruous with graded I_{CRAC}. Once the entire Ca^{2+} pool has been emptied sufficiently for I_{CRAC} to activate, then the subsequent relationship can clearly be graded. One example of this comes from RBL cells in which low concentrations of $InsP_3$ can lower the Ca^{2+} content of the total store, but this is not associated with any detectable I_{CRAC} activation (265). However, if the total store is depleted further, then a range of I_{CRAC} amplitudes can be obtained reflecting variable levels of activation (110). As discussed above, in pulmonary artery endothelial cells, the relationship between store depletion and subsequent Ca^{2+} influx was sigmoid (331). Modest levels of store emptying were not coupled with detectable Ca^{2+} influx, but as the stores emptied further, the relationship became more linear. A difficult question is whether this represents a true threshold, below which no entry occurs, or a sigmoid relationship such that very low levels of entry are simply below the level of technical detection, even using sensitive fluorescent dyes.

One study has concluded, however, that store-operated Ca^{2+} influx is tightly coupled to store depletion (135). In this report, the low-affinity Ca^{2+}-sensitive fluorescent dye mag-fura 2 was loaded into the intracellular Ca^{2+} stores, thereby providing an indication of intraluminal Ca^{2+} concentration. Store content was lowered by external application of the multivalent cation chelator TPEN. In its uncomplexed form, TPEN readily crosses cell membranes and would therefore access the intracellular Ca^{2+} stores. Because of its low affinity for Ca^{2+} (K_D 130 μM), TPEN would bind Ca^{2+} in those stores where resting free Ca^{2+} is high (e.g., ER) and rapidly lower the ambient Ca^{2+} concentration. In this way, store content would be lowered, and store-operated influx should activate. By varying the concentration of TPEN that was applied, Hofer et al. (135) constructed the relationship between TPEN concentration and the fall in intraluminal Ca^{2+} concentration. Using different cells, they also constructed the relationship between TPEN concentration and the rate of Ca^{2+} influx, measured using fura 2. Because the spectra of fura 2 and mag-fura 2 are basically identical, it was not possible to measure the rate of Ca^{2+} influx with the cells in which store content was also being measured. They then plotted the rate of Ca^{2+} entry as well as the apparent free Ca^{2+} concentration within the stores against TPEN concentration and found that the two relationships were similar (135). This they interpreted as indicating a graded and tightly coupled relationship between store depletion and subsequent Ca^{2+} influx. As in many studies of this kind, a potential problem is the assumptions that must be made concerning the effects (or lack thereof) of the rather distinct methodologies used to measure release and influx. For example, loading of the ER with a Ca^{2+} buffer (mag-fura 2) could significantly alter the relationship between Ca^{2+} content and signaling. On the other hand, the findings of Hofer et al. (135) may not be that inconsistent with other reports that find more complex relationships. As discussed previously, these dissociations might, at least in some instances, result from heterogeneity in the ER and the fact that a minor component of the ER is involved in signaling to store-operated channels. A reagent like TPEN, however, enters all intracellular compartments due simply to its physical properties, where it rapidly chelates and thus reduces free Ca^{2+}. Thus it would act indiscriminately on all parts of the ER, both the signaling components and the nonsignaling components. Thus the actions of TPEN would be similar to those of the Ca^{2+} ionophore ionomycin, which caused rapid depletion of stores and similarly rapid activation of I_{CRAC} (147).

Unfortunately, despite the circumstantial evidence summarized above, there is as yet no direct evidence that has clearly established the existence of this specialized

store. Based on the indirect evidence, however, we have some ideas as to its features. First, its contribution to the overall size of a Ca^{2+} release transient is small (44, 265, 310). Second, the store that is linked to the activation of I_{CRAC} is probably located close to the plasma membrane. Parekh and Penner (267) showed that the site of $InsP_3$ action for I_{CRAC} activation was likely very close to the site of $InsP_3$ production in the plasma membrane. Third, this store seems to have a lower sensitivity to $InsP_3$ in that higher concentrations of $InsP_3$ are required to activate I_{CRAC} than trigger Ca^{2+} release from the bulk of the $InsP_3$-sensitive store (126, 265). In support of this idea, under certain experimental conditions, the nonmetabolizable high-affinity $InsP_3$ receptor agonist adenophostin A is very effective in activating I_{CRAC}, whereas lower affinity nonmetabolizable $InsP_3$ receptor agonists like L-(α-glycerophospho)-D-*myo*-inositol-4,5-bisphosphate and manno-adenophostin, are not (44, 271). In hepatocytes, adenophostin A activates Ca^{2+} influx at slightly lower concentrations than cause Ca^{2+} release (118). Furthermore, a monoclonal antibody to the $InsP_3$ receptor reduces vasopressin-dependent Ca^{2+} influx but fails to alter Ca^{2+} release in liver cells (118). Hence, the specialized CRAC store appears to differ from the rest of the ER in terms of sensitivity to $InsP_3$, adenophostin A, and an antibody to the $InsP_3$ receptor. It has been suggested that the ability to deplete this CRAC store depends on the affinity of the ligand for the $InsP_3$ receptor and/or the extent of metabolism of the ligand (366). However, metabolism alone is unlikely to be a major factor. The adenophostin A analogs ribophostin and manno-adenophostin, neither of which are metabolizable, have similar affinities for the $InsP_3$ receptor, but only ribophostin activates I_{CRAC} in weak intracellular Ca^{2+} buffer (271). Fourth, this specialized store is refilled by SERCA pumps because blocking SERCA pumps activates I_{CRAC}, and deactivation of I_{CRAC} can be prevented by thapsigargin (13, 419). Finally, because thapsigargin activates I_{CRAC} slowly and after a sizeable delay whereas it triggers Ca^{2+} release much more rapidly, it is thought that the putative CRAC store may be relatively "tight" to Ca^{2+} and hence harder to deplete (147).

Needless to say, none of these properties provides definitive proof of a novel Ca^{2+} store devoted solely to the gating of store-operated channels. Nonetheless, abundant evidence has now established that the ER is not a homogeneous organelle but may be compartmentalized and divided into discrete Ca^{2+} signaling units. The stores are heterogeneous in terms of both Ca^{2+} release channel distribution and SERCA pumps (290). Whether the store coupled to activation of store-operated channels is contained within the ER is unproven, but the clear presence of SERCA pumps would suggest that it is. From a physiological perspective, it seems likely that the organelle that regulates store-operated channels is a part of, or is linked in some way to, the ER because one of the functions of this pathway is believed to be maintenance of ER Ca^{2+} levels that are necessary for proper protein synthesis and folding. This also raises a question about the apparent different sensitivities of the different stores to $InsP_3$. Does this mean that cells can tolerate small losses of stored Ca^{2+} with little impact? In pancreatic acinar cells for example, weak (physiological) cholecystokinin receptor stimulation evokes repetitive Ca^{2+} oscillations that reflect periodic release of Ca^{2+} from internal stores (352). However, a fraction of the released Ca^{2+} is extruded from the cell during each spike. The stores need to refill with Ca^{2+} to sustain the oscillations but, if there were a separate small store with low sensitivity to $InsP_3$ that alone gates CRAC channels, it is difficult to see how this could be accomplished. This problem might be circumvented if the store controlling the channels is a subcompartment of the ER. Because the ER seems to be a largely contiguous organelle (274, 346), at least macroscopically, then a fall in Ca^{2+} content within one region could trigger redistribution of Ca^{2+} within the organelle as a whole. A striking example of such Ca^{2+} "tunneling" within the ER is seen in the pancreatic acinar cell. Here, Ca^{2+} is released from ER in the apical pole, and this store is replenished because Ca^{2+}, entering via store-operated Ca^{2+} channels in the basolateral membrane, is taken up into basolateral ER and then moves rapidly through the lumen of the organelle to the apical pole, where release has lowered the Ca^{2+} concentration (274). A continuous lumen is not incompatible with local heterogeneities within the store, as this could simply reflect asymmetric distribution of Ca^{2+} release channels and Ca^{2+}-ATPases and local intraluminal buffers. In this scheme, $InsP_3$-sensitive Ca^{2+} release channels and Ca^{2+} reuptake sites are spatially separate (in support of this idea, see Ref. 231). $InsP_3$ effectively depletes the region of the ER in immediate vicinity of the $InsP_3$ receptors, but a standing Ca^{2+} gradient is set up whereby Ca^{2+}, taken up by the pumps, tunnels to the release sites and then enters the cytoplasm. Total store content falls but, because of the separation of release and reuptake sites, intraluminal Ca^{2+} content in the region of the reuptake sites falls to a lesser degree. To account for the discrepancy between Ca^{2+} release and Ca^{2+} influx in this scenario, activation of store-operated channels might be determined to a greater extent by the Ca^{2+} content in the vicinity of the uptake sites (90).

XV. PHYSIOLOGICAL FUNCTIONS OF STORE-OPERATED CALCIUM INFLUX: SHORT-TERM RESPONSES

A. General Functions of Store-Operated Ca^{2+} Channels

Store-operated Ca^{2+} influx is the primary albeit not exclusive mode of regulated calcium influx into the cyto-

plasm associated with activation of PLC. When PLC activation results in $InsP_3$-mediated discharge of intracellular Ca^{2+} stores, store-operated calcium entry is required in one of three general roles. First, with transient activation, occurring with cholinergic neurotransmission for example, capacitative calcium entry provides for rapid replenishment of cellular Ca^{2+} stores so that the cell is quickly readied for another stimulus. The store-operated mechanism is uniquely fit to such a role, because it ensures that even when the initial stimulus is removed, as for example by rapid acetylcholinesterase metabolism of acetylcholine, calcium channels will remain open until intracellular stores of Ca^{2+} are completely replenished. Interestingly, in one study in which a non-store-operated Ca^{2+} entry was clearly operating to the exclusion of the store-operated pathway, it was found that upon removal of the agonist, a delayed activation of the store-operated pathway occurred, thereby ensuring refilling of intracellular Ca^{2+} stores (242). Second, store-operated calcium entry provides a means for inducing prolonged, sustained elevation in cytoplasmic Ca^{2+} concentration. Such a sustained elevation may be desirable when tonic responses are needed, for example, in maintenance of smooth muscle tone. This cannot be achieved without a calcium entry mechanism, because intracellular stores contain sufficient Ca^{2+} to provide an elevated cytoplasmic Ca^{2+} concentration for only a finite period, a few minutes at the most. Third, in instances in which sustained cytoplasmic Ca^{2+} signals are associated with repetitive baseline spike oscillations, store-operated Ca^{2+} entry provides a "topping up" of Ca^{2+} stores to maintain the constant amplitude of each intracellular Ca^{2+} spike. In the absence of a coordinated entry of Ca^{2+}, spike height will gradually diminish. In the case of cytoplasmic Ca^{2+} spiking, it is important to coordinate the entry of Ca^{2+} with intracellular release to provide just the amount of Ca^{2+} needed to refill the stores and no more; otherwise, either the baseline Ca^{2+} level will gradually increase, or spike height will gradually decrease. In pancreatic acinar cells, for example, up to 40% of the total mobilizable intracellular Ca^{2+} pool can be pumped out during a single Ca^{2+} spike (352). Compensatory Ca^{2+} entry is therefore required to maintain sufficient Ca^{2+} within the stores so that Ca^{2+} oscillations can be continued. An argument has been made that in some cell types, alternative modes of Ca^{2+} entry may be associated with intracellular Ca^{2+} oscillations (333). However, careful pharmacological characterization of the entry associated with oscillations in hepatocytes demonstrated that it was the store-operated channels that maintained the oscillatory response (117), at least for this cell type. The role of store-operated channels in Ca^{2+} oscillations is discussed in detail in section xv*D*.

The store-operated pathway appears to be the predominant means of regulated influx of Ca^{2+} in "classical" nonexcitable cells, the major types being epithelial cells and blood cells. It is a major component of the signaling pathways that regulate epithelial cell secretion (both discharge of proteins and regulation of transepithelial secretion of salts and water) and carbohydrate metabolism in the liver (both glycogenolysis and gluconeogenesis). In blood cells, various functions are subtended by PLC-dependent rises in intracellular Ca^{2+} concentration, and store-operated Ca^{2+} entry, including secretion (of lysosomal enzymes, for example) and chemotaxis. In some instances, store-operated Ca^{2+} entry channels may be structurally associated with the effectors they regulate (discussed below). Lawrie et al. (189) reported that in an endothelial cell line, mitochondrial calcium uptake responded best to increases in entry of Ca^{2+}, and less well to release of Ca^{2+} from ER or general increases in cytoplasmic Ca^{2+}. This result contrasts with other cell types, such as HeLa cells (314), which have mitochondria in close proximity to ER such that mitochondrial Ca^{2+} may be more efficiently regulated by $InsP_3$-induced Ca^{2+} release. Nonetheless, the major role for mutual regulation of the store-operated Ca^{2+} entry and mitochondrial function is well established, as discussed previously.

A particularly elegant example of how local Ca^{2+} influx can refill the $InsP_3$-sensitive Ca^{2+} store has come from studies on pancreatic acinar cells (240). In these highly polarized cells, store-operated entry is restricted to the basolateral membrane. A sizable portion of the ER is located close to this membrane but also runs through the cell to the zymogen granule-containing apical pole through which it protrudes. Physiological levels of receptor stimulation result in Ca^{2+} oscillations that occur exclusively in the apical pole, where they drive secretion. Stimulation of the acinar cell with an agonist in Ca^{2+}-free solution empties the stores and released Ca^{2+} is extruded out of the cell by the plasma membrane Ca^{2+}-ATPases. However, store content can be maintained somewhat if a small Ca^{2+}-containing pipette is sealed onto the basolateral membrane. This results in focal Ca^{2+} entry, which is rapidly taken up into basolateral ER by SERCA pumps without a detectable increase in cytoplasmic Ca^{2+}. The Ca^{2+} then rapidly tunnels through the ER, a process greatly expedited by the low Ca^{2+} binding ratio with the lumen compared with that in the cytoplasm (239), where it is subsequently released into the apical pole.

Store-operated Ca^{2+} entry may also play a major role in Ca^{2+} signaling in some types of excitable cells as well, including neurons (301). But in such cases signaling involves an "excitation-independent" pathway that is similar to that in nonexcitable cells, i.e., involving receptor activation of PLC and release of Ca^{2+} by $InsP_3$ or other Ca^{2+} releasing messengers. Noteworthy examples of excitable cells which utilize store-operated entry in a significant way are smooth muscle cells and endocrine and neuroendocrine cells (2, 22, 50, 56, 73, 79, 101, 183, 228, 235, 249, 316, 317, 330, 372, 380, 397). More recently,

interest has been increasing in store-operated channels in the central nervous system, and their potential role in fundamental central processes, such as synaptic plasticity (8), or in pathophysiological conditions, such as Alzheimer's disease (133, 168, 295). But it is also clear that in some excitable cells, no such store-operated pathway exists (98, 154, 340). How is store replenishment accomplished in those excitable cells that do not express store-operated Ca^{2+} entry? The answer may be that excitable cells generally do, whereas nonexcitable cells generally do not, express a rapidly turning over Na^+/Ca^{2+} exchange transporter (35, 176). This transporter could provide an energy-efficient route for rapidly adjusting cytoplasmic Ca^{2+} in response to rapid uptake demands of a depleted intracellular Ca^{2+} store. Because the resting turnover of this exchanger is relatively fast, it may be that such a pathway could provide replenishment of intracellular stores in the absence of any store-dependent regulation. In one report (57), acceleration of Na^+/Ca^{2+} exchange activity has been observed in response to depletion of intracellular Ca^{2+} stores by thapsigargin.

B. Regulated Exocytosis

In many nonexcitable cells, an increase in cytoplasmic Ca^{2+} is an important factor that regulates secretion. In mast cells, Ca^{2+} influx accelerates degranulation (81). In RBL cells, Ca^{2+} entry through CRAC channels drives exocytosis (7). Studies on mutant T cells in which I_{CRAC} was absent revealed a good correlation between the loss of I_{CRAC} and a defective secretory response (80). Contact of a cytotoxic T lymphocyte cell with an appropriate cellular partner results in the activation of Ca^{2+} influx which then triggers the exocytosis of lytic granules containing perforin and granzyme, resulting in the "lethal hit." Contact of AJY cells (a cytotoxic T cell line) with target AY cells resulted in the activation of a Ca^{2+} influx pathway in the AJY cells that exhibited a very similar divalent cation selectivity, measured using fura 2, with CRAC channels in Jurkat cells (416). Ca^{2+} entry through store-operated channels therefore appeared to drive lytic granule fusion. In excitable cells a role for store-operated entry in driving secretion has also been observed. In bovine adrenal chromaffin cells for example, store-operated Ca^{2+} entry was able to evoke robust exocytotic events under conditions where voltage-operated Ca^{2+} channels were largely inactive (93).

C. Regulation of Enzymatic Activity

Ca^{2+} entry through store-operated Ca^{2+} channels can alter the activities of enzymes like adenylyl cyclase and NO synthase. Activation of store-operated Ca^{2+} influx by thapsigargin in C6–2B glioma cells inhibits the type VI adenylyl cyclase isoform but stimulates the type I form (64). This stimulation was specific for store-operated Ca^{2+} influx because changes in enzymatic activity were not apparent following Ca^{2+} release or following nonspecific entry of Ca^{2+} due to a Ca^{2+} ionophore. Given that cAMP can regulate a plethora of intracellular processes, changes in Ca^{2+} entry could have far-reaching consequences on a multitude of cell functions.

Endothelial NO synthase is stimulated by a rise in cytoplasmic Ca^{2+} arising from store-operated influx. This results in increased NO production, which subsequently acts both as an intra- and intercellular messenger (197).

In RBL-1 cells, Ca^{2+} entry through CRAC channels stimulates arachidonic acid production, whereas Ca^{2+} release from the stores is ineffective even though the latter evokes a robust intracellular Ca^{2+} signal (55). The arachidonic acid released by Ca^{2+} entering through CRAC channels is used to synthesize the potent paracrine proinflammatory signal leukotriene C_4 (LTC_4), which is secreted from the cells. Hence, Ca^{2+}-dependent release of arachidonic acid following activation of CRAC channels might be one way whereby a brief pulse of Ca^{2+} influx induces longer lasting downstream effects. Mitochondrial depolarization, which impairs CRAC channel activity, also suppressed arachidonic acid and LTC_4 secretion (55). Hence, through regulation of store-operated influx, mitochondria can influence downstream Ca^{2+}-dependent processes.

PLC-δ is activated by cytoplasmic Ca^{2+} in the micromolar range, and a recent study raises the possibility of a novel positive feedback mechanism that prolongs store-operated entry (169). Expressing the PLC-δ1 isoform in PC-12 cells (which do not normally express the enzyme) resulted in larger bradykinin-evoked Ca^{2+} signals and more pronounced $InsP_3$ production, but only in the presence of external Ca^{2+}. In Ca^{2+}-free solution, overexpressing PLC-δ1 had no effect on either agonist-evoked $InsP_3$ production or the size of the subsequent Ca^{2+} release signal. Agonist-evoked Ca^{2+} influx was larger in the PLC-δ1 expressing cells (169). Although PC-12 cells express many Ca^{2+} entry pathways including voltage-operated Ca^{2+} channels, this nevertheless raises the intriguing possibility that store-operated Ca^{2+} influx, initially induced by store depletion following stimulation of bradykinin receptors that link to PLC-β, stimulates PLC-δ1 resulting in the generation of more $InsP_3$. This would maintain the stores in a relatively empty state and hence prolong the duration of store-operated entry.

In T cells, the plasma membrane Ca^{2+}-ATPase (PMCA) is the primary Ca^{2+} extrusion mechanism and is therefore central to preventing Ca^{2+} overload. PMCA activity is increased severalfold by Ca^{2+} entering through CRAC channels (19), and this modulation reverses slowly (time constant of ~4 min). PMCA activity could be altered effectively by manipulating the amplitude and spatial ex-

tent of Ca^{2+} microdomains near CRAC channels, suggesting a close functional coupling between the Ca^{2+} entry and efflux pathways (20). Modulation of Ca^{2+} extrusion by Ca^{2+} entering though CRAC channels is likely to help determine the time course and size of intracellular Ca^{2+} signals.

It has been reported that store-operated Ca^{2+} influx is also required for remodelling of the plasma membrane. An increase in intracellular Ca^{2+} concentration is thought to promote transmembrane externalization of the phospholipid phosphatidylserine to the outer leaflet of the plasma membrane (181, 221). Such translocation is thought to be a hallmark of cells undergoing apoptosis (discussed below) but also is required for the assembly of enzyme complexes of the blood coagulation cascade on the surface of stimulated platelets. In the very rare inherited disorder called Scott syndrome, phosphatidylserine is not externalized. Intriguingly, in B lymphoblasts taken from a Scott syndrome patient, store-operated Ca^{2+} influx is reduced, whereas Ca^{2+} release from the stores is normal. Moreover, La^{3+} and SKF-96365 inhibit both thapsigargin-evoked Ca^{2+} influx and the degree of phosphatidylserine externalization (221). Ca^{2+} entry via store-operated channels might therefore regulate the distribution of phospholipids in the plasma membrane.

D. Ca^{2+} Oscillations

Repetitive oscillations in intracellular Ca^{2+} provide an important digital mode of Ca^{2+} signaling regulating a variety of physiological processes such as secretion and gene regulation (353). In a variety of cell types, store-operated Ca^{2+} influx has been found to modulate the pattern of intracellular Ca^{2+} oscillations. In *Xenopus* oocyes, Ca^{2+} influx was found to accelerate the speed at which the Ca^{2+} wave propagated through the cytoplasm (108) and modified the pattern of intracellular Ca^{2+} oscillations (266). In T lymphocytes, Ca^{2+} oscillations depend on Ca^{2+} influx, and it is thought that they arise from cyclical interactions between Ca^{2+} release from the stores on the one hand and both CRAC channels and Ca^{2+}-dependent K^+ channels in the plasma membrane on the other (75). In rat hepatocytes, inhibitors of CRAC channels (Gd^{3+}, SKF-96365, and 2-APB) all suppressed Ca^{2+} oscillations (117). Although these agents are not selective for CRAC channels and can block other Ca^{2+} entry pathways, inhibiting or activating Ca^{2+}-permeable nonselective cation channels were without effect on the oscillations. It was suggested that Ca^{2+} entry through CRAC channels was required for supporting Ca^{2+} oscillations. Similarly, store-operated entry is thought to be required to maintain intracellular Ca^{2+} oscillations evoked by stimulation of metabotropic glutamate receptors in type I rat cortical astrocytes (287).

In some other cell types however, Ca^{2+} oscillations are thought to depend critically on another Ca^{2+} influx pathway that is not store-operated. Ca^{2+} entry through arachidonic acid-gated channels maintains oscillations in avian nasal gland cells and HEK-293 cells expressing recombinant muscarinic receptors, and this does not seem to involve store depletion (333).

E. Muscle Contraction

In certain types of smooth muscle, a component of the agonist-evoked contraction is relatively resistant to inhibitors of voltage-gated Ca^{2+} channels, leading to the idea that this component might be store-operated (182). Indeed, inhibition of SERCA pumps increases myoplasmic Ca^{2+} concentration and can evoke contraction in some smooth muscle preparations (228). The fact that thapsigargin can cause contraction is not unequivocal evidence for store-operated entry. Thapsigargin-induced Ca^{2+} release from the stores can activate Ca^{2+}-dependent Cl^- channels in the myocyte plasma membrane, resulting in depolarization and subsequent opening of voltage-gated Ca^{2+} channels (182). Moreover, typically used voltage-operated Ca^{2+} channel blockers like nitrendipine inhibit the channels in a complex manner, by binding with high affinity to the inactivated state (21). Hence, the insensitivity of a thapsigargin-evoked Ca^{2+} signal to dihydropyridine-based Ca^{2+} channel blocker may well depend on the extent of depolarization and does not necessarily eliminate a contribution from voltage-operated channels. On the other hand, Ca^{2+}-permeable nonselective store-operated currents have been reported for some smooth muscle cells (see sect. v*B3*), and it seems likely that Ca^{2+} entering through this route can contribute, directly or indirectly via the ensuing depolarization, to muscle contraction.

Although it is highly controversial as to whether TRPC4 is store-operated or not (see above), deletion of the TRPC4 gene in mice results in an approximately twofold reduction in the ability of acetylcholine to elicit vasorelaxation in precontracted aortic rings (96). Acetylcholine activates muscarinic receptors in the vascular endothelial cell plasma membrane, and this triggers store-operated Ca^{2+} influx. The subsequent rise in cytoplasmic Ca^{2+} is required for the synthesis and release of vasoactive agents like NO and prostaglandins which cause the underlying myocytes to relax.

F. Sperm Chemotaxis and the Acrosome Reaction

In the ascidians *Ciona intestinalis* and *Ciona savignyi*, the sulfated steroid sperm-activating and sperm-attracting factor is released from the egg and induces both sperm activation and then chemotactic behavior of the

activated sperm towards the egg (402). Both processes require Ca^{2+} influx, but in ascidian sperm, chemotaxis was unaffected by voltage-operated Ca^{2+} channel blockers. Instead, a role for store-operated influx was proposed since SKF-96365, Ni^{2+}, and 2-APB all impaired chemotaxis. Surprisingly, thapsigargin, in spite of activating store-operated Ca^{2+} influx, actually inhibited ascidian chemotaxis. Hence, both activation and inhibition of store-operated influx seems to impair chemotaxis. The authors suggest that the discrepant results may arise if the open/closed regulation of store-operated channels regulate chemotaxis and thapsigargin inhibits chemotaxis by clamping store-operated channels in the open state (402).

The acrosome reaction, which involves the exocytosis of acrosomal vesicles from the sperm into the zona pellucida, is an essential step for fertilization. It appears to be triggered by the binding of sperm to ZP3, a glycoprotein component of the zona pellucida, which initiates a sequence of events culminating in the activation of Ca^{2+} influx into the sperm which drives the acrosome reaction (262). Thapsigargin activates a very similar influx pathway (as defined by Mn^{2+} quenching and pharmacological sensitivity) that can trigger the acrosome reaction. These results suggest that ZP3 activates store-operated Ca^{2+} influx, and this is sufficient for the exocytotic event. Ca^{2+} imaging experiments have revealed that the store-operated Ca^{2+} channels are clustered in the postacrosomal region of the sperm near the connective piece (99). Store-operated Ca^{2+} influx is apparently reduced in PLC-$\delta4^{-/-}$ sperm, although the underlying mechanism is unclear (99). The acrosome reaction not only releases proteases and hyaluronidase which are required for the sperm to penetrate through the zona pellucida but also exposes proteins on the sperm surface that bind to components of the zona pellucida like ZP2. This is thought to maintain tight binding of the sperm to the zona pellucida as it progresses through to reach the egg. Upon fusion, the novel sperm-derived PLC-ζ isoform enters the egg where it induces the Ca^{2+} oscillations that drive fertilization (326). Hence, store-operated entry in the sperm not only seems to be a key orchestrator of the fertilization process but also, via stimulation of the acrosome reaction, ultimately induces Ca^{2+} signals in an adjacent cell (the egg) via the release of a mobile signal (PLC-ζ).

XVI. PHYSIOLOGICAL FUNCTIONS OF STORE-OPERATED CALCIUM ENTRY: LONG-TERM RESPONSES

Throughout the life span of organisms, cells make decisions about growth, division, function, and death. The importance of calcium in fertilization and in mitogenesis was pointed out over 50 years ago by Heilbrunn (129). Following mitosis, a cell must either commit to reenter the cell cycle or exit into G_o, a quiescent state in which a differentiated function is maintained (28). In many but not all cell types, such cells may at a later time, with an appropriate stimulus, reenter the cell cycle to further divide. Or, in circumstances in which a particular cell type's function is no longer needed, as in the case of the thymus, systemic signals may instruct cells to undergo a complex process of self digestion and packaging, termed apoptosis (329). Mutations in key genes that control mitogenesis can lead to inappropriate cell division or cancers; these genes are designated protooncogenes, and the transforming forms of the genes are oncogenes. It is noteworthy although perhaps not unexpected that the vast majority of protooncogenes code for proteins involved in signal transduction pathways. One protooncogene, *Bcl-2*, acts as a suppressor of apoptosis, such that if it is expressed in excess cancerous growth can result (5, 238).

A. Gene Transcription

Calcium signaling is believed to play an important role in the regulation of cell growth and differentiation (23, 105, 206). Thus it would not be surprising if elevations in intracellular Ca^{2+} concentration through store-operated channels were able to activate the expression of specific genes. This has been most clearly demonstrated for T lymphocytes, wherein Ca^{2+} entry through CRAC channels has been shown to play a significant role in gene regulation (76). As in many cell types, readmission of external Ca^{2+} to T lymphocytes exposed to thapsigargin in Ca^{2+}-free solution results in Ca^{2+} influx through CRAC channels. The elevated cytoplasmic Ca^{2+} binds to calmodulin, and the Ca^{2+}-calmodulin complex activates the phosphatase calcineurin. Calcineurin then enters the nucleus and dephosphorylates nuclear transcription factors like NFAT, NFκB, and OctA. This results in increased transcription and translation of the interleukin-2 gene and its receptor (76). Hence, Ca^{2+} entry through CRAC channels can regulate gene expression. Interestingly, different patterns of Ca^{2+} oscillation (by varying the time over which external Ca^{2+} is present in thapsigargin-treated cells) differentially regulates the activities of the different transcription factors, suggesting that periodic oscillations in CRAC channel activity might translate into different Ca^{2+}-dependent effects (76). In Jurkat T lymphocytes, the pyrazole derivative YM-58483 was found to inhibit thapsigargin-evoked Ca^{2+} influx as well as subsequent interleukin-2 production and NF-AT-dependent promoter activity but failed to affect the activity of the promoter AP-1, consistent with the idea that store-operated Ca^{2+} influx can selectively activate certain transcription factors (152).

B. Cell Cycle

It is well established that Ca^{2+} is a key regulator of the cell cycle (206). Ion channels are also important regulators of the cell cycle, in part through their effects on the membrane potential and hence driving force for Ca^{2+} influx (255). In proliferating pulmonary artery smooth muscle cells, cyclopiazonic acid-induced Ca^{2+} release was twofold larger than in growth-arrested cells, but the subsequent Ca^{2+} influx had increased fivefold (112). Proliferating cells also exhibited increased levels of TRPC1 mRNA. Exposing proliferating pulmonary artery myocytes to an antisense oligonucleotide directed against TRPC1 mRNA resulted in a decrease in cyclopiazonic acid-induced Ca^{2+} influx and a 50% reduction in the rate of cell proliferation (349).

In interphase HeLa cells, Ca^{2+} release from the stores by stimulation with either histamine or thapsigargin evoked Ca^{2+} influx (294). However, in mitosis, similar levels of Ca^{2+} release were not associated with any detectable Ca^{2+} influx, indicating that the coupling between store depletion and Ca^{2+} influx had been severed. The mechanism underlying this uncoupling is unclear but, given the dramatic subcellular changes that occur during mitosis, it is likely that several processes are involved.

Xenopus oocytes, like mammalian oocytes, are arrested at the G_2-M transition. They must undergo meiotic maturation to be competent for fertilization. Maturation to form the egg can be induced by the steroid hormone progesterone. Following store depletion, store-operated influx was found to be absent in eggs but not in oocytes (215, 216). This loss of store-operated entry occurred rapidly at the germinal vesicle breakdown stage of meiosis. The mechanism seems to involve maturation promoting factor (216), a complex of a serine/threonine kinase (p34cdc2) subunit and a regulatory cyclin B subunit, which is necessary and sufficient for germinal vesicle breakdown and entry into meiosis. However, once store-operated channels were activated, increasing maturation promoting factor had no inhibitory effect. Hence, it was concluded that maturation promoting factor inhibited the coupling mechanism linking store depletion to activation of store-operated channels. Because Ca^{2+} influx can fertilize *Xenopus* eggs, inactivation of store-operated entry at the germinal vesicle breakdown stage might be a safety mechanism to ensure egg activation does not occur in the absence of fertilization.

An interesting switch between store-operated and voltage-gated Ca^{2+} channels has been reported for retinal neuroepithelial cells (348). As the cells progress through the cell cycle, store-operated Ca^{2+} influx is dominant but declines both in M-phase cells and in the subsequent differentiated retinal ganglion cells. On the other hand, voltage-operated Ca^{2+} channels become prominent as the cells leave the cycle. Presumably such changes occur at the level of channel expression, and it would be interesting to know how this reciprocal relationship is brought about.

C. Apoptosis

There is considerable evidence for a role of Ca^{2+} signaling in apoptosis. Glucocorticoid-induced apoptosis of lymphoid cells is a widely studied model of cell death. In a study of glucocorticoid-induced apoptosis, Kaiser and Edelman (160) identified a Ca^{2+} influx that they concluded was associated with glucocorticoid-induced lymphoid cell death. These investigators subsequently found that the action of glucocorticoids to induce apoptosis could be mimicked by Ca^{2+} ionophores (161), a result since confirmed many times (54, 226, 395). Similarly, thapsigargin also triggers a full apoptotic response (31, 100, 157, 164, 250, 365). Investigators have shown that chelation of Ca^{2+} by intracellular chelators, extracellular EGTA, or overexpression of calbindin inhibits apoptosis due to glucocorticoids and other agents (78, 225, 226, 315, 341). Calcium channel blockers also prevent apoptosis in regressing prostate (220). The functional activity of Ca^{2+} is often mediated through binding to calmodulin (10, 175, 206), and calmodulin antagonists have been reported to disrupt apoptosis in a variety of systems (77, 226). The oncogene *Bcl-2*, the product of which is known to inhibit apoptosis in several model systems, has also been suggested to regulate intracellular Ca^{2+} compartmentalization (11, 185). Lam and co-workers (185, 186) demonstrated that dexamethasone elevated cytoplasmic Ca^{2+} concentration and reduced stored Ca^{2+} in a lymphocytic cell line and concluded that glucocorticoids may act in a thapsigargin-like manner. Together these data suggest a central role for calcium in apoptosis in response to glucocorticoids and other agents.

Bian et al. (31) examined the role of Ca^{2+} signaling in apoptosis induced in S49 cells (a lymphocytic line) by the Ca^{2+}-ATPase inhibitors thapsigargin and cyclopiazonic acid and by the synthetic glucocorticoid dexamethasone. These investigators also investigated the effects of overexpression of the antiapoptotic oncogene Bcl-2. In support of the idea that depletion of stored Ca^{2+} may signal apoptosis, Bian et al. (31) found that removal of extracellular Ca^{2+} augmented, rather than inhibited, apoptosis due to thapsigargin (31). Overexpression of the apoptosis suppressor Bcl-2 inhibited apoptosis due to thapsigargin but did not affect thapsigargin-induced Ca^{2+} signaling. Dexamethasone induced apoptosis, diminished the size of the ER Ca^{2+} pool, and caused a small elevation of intracellular Ca^{2+}, results similar to those originally reported by Lam et al. (392). However, this elevation was not due to Ca^{2+} influx because the increase was similar in the presence or absence of Ca^{2+} in the medium. Furthermore,

in contrast to the results with thapsigargin, apoptosis due to dexamethasone was unchanged in a Ca^{2+}-free medium. These findings indicate that changes in Ca^{2+} handling appear to play a lesser role than previously thought in the actions of Bcl-2 and glucocorticoids. They also indicate that depletion of intracellular stores can provide a potent stimulus for apoptosis, independent of the activation of Ca^{2+} entry.

Other studies confound this issue, however. For example, in neurons, Ca^{2+} can have a protective effect against apoptosis, and in this case, thapsigargin-activated entry actually protects against apoptosis (187). Likewise, studies on the effects of the antiapoptotic oncogene Bcl-2 are sometimes contradictory. He et al. (128) found that Bcl-2 protected against thapsigargin-induced apoptosis by diminishing the extent of ER Ca^{2+} depletion and concluded that store depletion is a key signal for apoptosis. Similar findings were reported by Wei et al. (388). However, Williams et al. (390) found that overexpression of Bcl-2 actually enhanced store-operated entry, and this was associated with protection against apoptosis by the calcium channel blocker SKF-96395 (390).

Much of the confusion over the role of calcium stores and store-operated channels likely derives from the fact that multiple factors can lead to apoptosis, and these vary in significance depending on cell type. Thus, while it is clear that a common means for inducing apoptosis involves sustained elevation of intracellular Ca^{2+} concentration (74, 254, 313), it is equally clear that depletion of ER Ca^{2+} can also lead to apoptosis, likely through activation of a stress response (31, 128, 302, 306). However, it seems unlikely that this latter effect is related to the signaling pathway involved in store-operated channels, as it seems to require extensive, pathological reduction in ER stores. On the other hand, it is very likely that the sustained elevation of intracellular Ca^{2+} that more commonly signals apoptosis would involve store-operated Ca^{2+} entry because of the general requirement for prolonged signaling.

XVII. PATHOPHYSIOLOGY

There are to date few documented disease states that can be specifically attributed to a failure or malfunction of capacitative calcium entry. However, there is evidence that chemical toxicity and other environmental factors may act through this pathway. The potential for disease associated with the properties of capacitative calcium entry is obvious given the central role this calcium influx pathway plays in so many physiological systems. Rao et al. (305) documented that in certain patients with congenital platelet dysfunction, platelets showed diminished intracellular Ca^{2+} signaling, including a decrease in Ca^{2+} entry. There are also documented cases of immunodeficiencies apparently derived from impaired store-operated entry, as well as evidence for a role of store-operated entry in acute pancreatitis. There is growing evidence for a role for store-operated entry in the toxic effects of environmental chemicals that affect Ca^{2+} homeostasis.

A. Severe Combined Immunodeficiency

In two studies on human patients, severe combined immunodeficiency has been linked to a loss of store-operated Ca^{2+} entry in T lymphocytes (86, 275). I_{CRAC} was completely absent from the T cells of a patient suffering from a primary immunodeficiency, and this was associated with defective cell proliferation after stimulation with TCR-CD3 complex, due to a failure of expression of Ca^{2+}-dependent genes. Proliferation could be restored by the addition of ionomycin to the medium, indicating that this immunodeficiency was due to defective Ca^{2+} signaling and not downstream events. The number of cases of idiopathic immunodeficiency is large, and it is not known to what extent dysfunctions in store-operated channels or signaling mechanisms may be involved.

B. Acute Pancreatitis

A key step in acute pancreatitis is the premature intracellular activation of the protease trypsin, which is stored within the zymogen granules of the pancreatic acinar cell. Raraty et al. (307) have found that high concentrations of the secretagogue cholecystokinin evoked a sustained increase in intracellular Ca^{2+} concentration via store-operated Ca^{2+} channels. Sustained store-operated entry prematurely activated trypsin resulting in digestion of the granules. Trypsin was then liberated into the cytoplasm, with disastrous consequences. Removal of external Ca^{2+} suppressed the destruction of the granules, as did buffering intracellular Ca^{2+} rises with BAPTA. Hence, it is the rise in Ca^{2+} itself that drives trypsin activation, with the Ca^{2+} entering via store-operated Ca^{2+} channels (264, 307).

C. Alzheimer's Disease

One particularly significant example of a pathological state that seems to involve alterations in Ca^{2+} metabolism is Alzheimer's disease, discussed in detail in a recent review (224). A major cause of the familial form of Alzheimer's disease is a missense mutation in one of three genes coding for amyloid precursor protein, presenilin-1, or presenilin-2. These mutations lead to the production of an aberrant peptide, Aβ, thought to play a significant role in the pathophysiology of the disease. Yoo et al. (401) reported that gene mutations associated with Alzheimer's

disease led to diminished capacitative Ca^{2+} entry and to increased release of store Ca^{2+} by agonists. They speculate that the combination of diminished capacitative calcium entry together with augmented release of stores may lead to reduced Ca^{2+} storage, known to act as an activator of apoptosis (31). Leissring et al. (190) also observed a diminished capacitative Ca^{2+} entry and augmented release, but observed increased rather than reduced Ca^{2+} storage. These findings suggest that the reduced capacitative calcium entry and augmented release both result from the increased storage of Ca^{2+}; that is, increased Ca^{2+} stores result in increased release, yet greater levels of Ca^{2+} remain in the stores resulting in diminished store-operated entry. Regardless of the mechanism involved, it seems clear that one cellular consequence of Alzheimer's disease-associated mutations is diminished capacitative calcium entry, which in some way could contribute to the associated neuropathology (299).

D. Toxicology

It is generally agreed among cellular toxicologists that prolonged elevation of cytoplasmic Ca^{2+} can be toxic to cells (58, 123, 148, 162, 253, 260, 288, 289, 308). As discussed above, at more moderate levels, Ca^{2+} can serve as an inducer of cell proliferation or of apoptosis. It is likely, therefore, that agents which augment capacitative Ca^{2+} entry, whether directly or indirectly through increasing release, could have untoward effects on cellular physiology. The prototypical example of agents in this class is thapsigargin, which is capable of killing cells in vitro by apoptosis (31, 100, 157, 164, 365, 408), or acting as a tumor promoter in vivo (119, 205).

An example of a Ca^{2+}-mobilizing environmental toxin is tributyltin, an important component of marine paints which has been shown to accumulate in coastal waters and estuaries and in marine organisms (cf. Ref. 59). Chow et al. (59) found that in lymphocytes, tributyltin was capable of mobilizing the same intracellular Ca^{2+} stores as the SERCA pump blocker di-*tert*-butylhydroquinone (*t*BHQ), resulting in activation of Ca^{2+} entry (59). This action may underlie the known action of tributyltin as an immunotoxin. Chow et al. (59) point out that this may be an example of a toxicological mechanism involving capacitative Ca^{2+} entry.

A more widely investigated class of environmental toxins is the polycyclic aromatic hydrocarbons (PAH). These potentially toxic substances are generated by the burning of fossil fuels and are also present in cigarette smoke or in charcoal-cooked food. Chemicals of this class are potent carcinogens and are generally immunosuppressive; it has been suggested that this immunosuppressive action contributes to carcinogenicity (68). Kreiger et al. (179) reported that PAHs caused a persistent elevation in intracellular Ca^{2+} concentration in HPB-ALL T-cells and that this response was well correlated with the abilities of the PAHs to act as immunosuppressives. In a subsequent study (180), these same investigators showed that PAHs were inhibitors of SERCA calcium pumps, indicating a thapsigargin-like action. The PAHs are known to be complete carcinogens, not requiring the action of a separate promoter; thus it is possible that this thapsigargin-like action of PAHs contributes to their carcinogenic action by providing a tumor-promoting action.

Finally, a controversial area of environmental toxicology is the possible pathological effects of weak electromagnetic field (EMF; Refs. 113, 384), such as those likely to be generated by power lines, video display terminals, electric blankets, or by personal transmitting and receiving equipment (mobile telephones, for example). Because one of the suggested health effects of such fields is an increase in the incidence of leukemias (204, 389), much of the research on biological effects has focussed on lymphocytes or lymphocytic cell lines. There is a considerable body of published work of cellular effects of weak EMF on lymphocytes, and Walleczek (384) has proposed that changes in cellular Ca^{2+} metabolism may underlie these responses. However, more recent work has cast doubt on the original epidemiological findings (156), as well as the various cell biological results, including effects on Ca^{2+} signaling (38).

XVIII. CONCLUSIONS

Store-operated channels play a fundamental role in both the immediate and long-term regulation of cells. It follows that unexpected perturbations in this process will have the potential for pathological outcomes, while planned pharmacological manipulations may find clinical utility in certain disease states. We may look forward in the future to increasing information on the role of store-operated calcium channels in disease as well as in the therapy of disease.

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