Regulation of native Kv1.3 channels by cAMP-dependent protein phosphorylation

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Chung, Induk, and Lyanne C. Schlichter. Regulation of native Kv1.3 channels by cAMP-dependent protein phosphorylation. *Am. J. Physiol.* 273 (*Cell Physiol.* 42): C622–C633, 1997.—We present evidence that activity of native Kv1.3 channels in human T lymphocytes can be increased by inhibiting phosphatases [using okadaic acid (OA)] or by activating protein kinase A (PKA). OA increased the maximal conductance (G_{max}) by 40% and shifted the voltage dependence of activation and inactivation, resulting in a significant increase in window current around the normal membrane potential. PKA inhibition [using the PKA inhibitor peptide PKI-(5—24)] decreased G_{max} by 43%, whereas PKA activation [by the Sp diastereomer of adenosine 3′,5′-cyclic monophosphothioate (Sp-cAMPS)] increased G_{max} by 60% and shifted the inactivation curve, producing an increase in the window current. These results are consistent with our previously published work using cell-attached patches but differ from some studies of Kv1.3. Because we previously reported a similar upregulation by protein kinase C (PKC) activation in these cells, we tested whether the PKA and PKC effects were additive. Our results suggest that PKC-dependent phosphorylation acts as a master switch, inasmuch as calphostin C greatly inhibited the current even after Sp-cAMPS, OA, or PKC activation was used to increase protein phosphorylation. Inasmuch as phosphorylation by both kinases (phorbol ester followed by Sp-cAMPS) abrogated the effects of either kinase alone, our results support the view that Kv1.3 is regulated in a complex manner by serine/threonine phosphorylation.

adenosine 3′,5′-cyclic monophosphate; potassium channel phosphorylation; okadaic acid; protein kinase A activators; protein kinase A inhibitor peptide; kinase cross talk

A MEMBER OF THE *Shaker*-related subfamily, Kv1.3 is one of the few voltage-gated K^+ channel genes known to produce a specific current in mammalian cells (for review see Ref. 8). It encodes a voltage-gated, delayed-rectifier K^+ channel that was called the *n* type when it was first characterized in T lymphocytes. This channel is highly expressed in human T cells and certain T cell lines, such as Jurkat, where its biophysical and pharmacological properties have been well characterized (8, 24). Several highly homologous genes were cloned from brain and genomic DNA (MK3 in mouse and RGK5, RCK3, and Kv3 in rat) and subsequently from human lymphocytes (HGK5 and HLK3) (8). Expression of each clone in *Xenopus* oocytes resulted in currents biophysically and pharmacologically very similar to the *n*-type K^+ channel found in T lymphocytes. In the recently standardized nomenclature of voltage-gated K^+ channels, this channel was renamed Kv1.3. The tissue distribution of Kv1.3 is relatively narrow, with transcripts being found in thymus, spleen, brain, lung, and pancreatic islets, but apparently not in heart, kidney, liver, or skeletal muscle (8). In addition to lymphocytes, several other immune cells express currents with the same properties as Kv1.3: macrophages, B cells, and natural killer cells (for review see Refs. 12 and 21) and brain microglia (27).

From its initial discovery in T cells, cell biology studies have implicated Kv1.3 in a variety of immune cell functions: proliferation, cell cytotoxic killing, interleukin 2 (IL-2) secretion, and volume regulation (for review see Refs. 12 and 21). These studies are based on channel blockers, originally using compounds that are membrane permeant and not very selective but later using more potent and selective toxins. Because a consistent finding is that T cell functions are inhibited by any treatment that reduces Kv1.3 current and owing to its restricted tissue distribution, several pharmaceutical companies have been targeting Kv1.3 for new immunosuppression therapies. A principal role for Kv1.3 during T cell activation is in maintaining a necessary and prolonged elevation of intracellular Ca^{2+} that relies on Ca^{2+} entry through channels activated secondarily to store depletion (21). This electrogenic Ca^{2+} influx will tend to depolarize the cell and reduce the driving force, but Kv1.3 channels establish and maintain a negative membrane potential and large electrochemical gradient for Ca^{2+} influx. Consequently, depolarizing T cells with high external K^+ by voltage clamp or by reducing the K^+ conductance with channel blockers reduces Ca^{2+} influx and the rise in internal Ca^{2+} (21).

Posttranslational modulation of Kv1.3 by protein phosphorylation is coming under increasing scrutiny, especially inasmuch as the current amplitude varies in the short term with lymphocyte activation and in the long term with T cell development (21). Within the Kv1.3 sequence (for review see Ref. 8) are several potential sites for phosphorylation by the serine/threonine kinases, protein kinase A (PKA), protein kinase C (PKC), and calmodulin-dependent kinase (CaMKII). Biochemical analysis of Kv1.3 protein isolated from the Jurkat T cell line (7) showed that the native channel is partially phosphorylated in vivo and is an excellent substrate for further phosphorylation by PKA and PKC. Lymphocytes express a number of hormone receptors, the activation of which leads to a rise in adenosine 3′,5′-cyclic monophosphate (cAMP) (16). Several attempts have been made to correlate effects on T cell function and Kv1.3 currents of stimulating these receptors or directly raising cAMP. When cAMP is artificially elevated during T cell activation, it is commonly reported that cell function is inhibited; however, there are several reports that small rises in

cAMP stimulate IL-2 secretion, IL-2 receptor expression, and cell proliferation (for review see Ref. 16).

It was widely anticipated that Kv1.3 current would be reduced by agents that raise cAMP; however, it was reported that the maximal conductance (G_{max}) decreased (9, 26, 30), was unchanged (18, 25), or increased (25). Most studies of Kv1.3 modulation have focused on G_{max} (calculated from currents at nonphysiologically positive voltages) and on the kinetics of inactivation. In contrast, we have emphasized modulation that affects channel activity near the normal resting potential, including changes in the voltage dependence of current activation and inactivation. We previously observed an increase in K^+ channel activity in cell-attached patches from normal human T cells (at the resting potential) in response to several compounds that elevate intracellular cAMP levels in these cells, i.e., 8-bromo-cAMP (8-BrcAMP), prostaglandin E_2 (PGE_2), forskolin, 3-isobutyl-1-methylxanthine, isoproterenol, and histamine (25). However, when we used the whole cell configuration, bath-applied 8-BrcAMP had no effect on G_{max} measured at very positive potentials. At that time we proposed that whole cell recordings do not adequately represent the intact cell, because kinases, phosphatases, and accessory molecules might wash out after rupture of the cell membrane. Other possible reasons for discrepancies in studies of cAMP-dependent regulation of Kv1.3 might be differences in the cell types used (normal cells vs. cell lines) or in cross talk between multiple phosphorylation events. Many ion channels, including Kv1.3, contain potential phosphorylation sites for more than one type of protein kinase, raising the possibility that multiple kinases regulate channels in a coordinated fashion or that one kinase dominates. For Kv1.3 channels in the Jurkat T cell line, effects of PKA have been reported to depend on previous phosphorylation by PKC (26).

We recently reported (10) that PKC activation increases Kv1.3 current in these cells. The phorbol ester 4β-phorbol 12,13-dibutyrate (4β-PDBu) increased G_{max} by 69% and caused a shift (by +9 mV) in the voltage dependence of activation and inactivation, which together produced a 270% increase in tonically active current ("window current"). Conversely, several PKC inhibitors reduced the current in unstimulated cells. Intracellular application of PKC pseudosubstrate or substrate peptides reduced K^+ conductance by 38–43%, and the specific PKC inhibitor calphostin C abolished the current in a dose- and light-dependent manner (half-maximal inhibitory concentration ~250 nM). We concluded that phosphorylation by PKC upregulates Kv1.3 channel activity, and as a result of shifts in voltage dependence, this enhancement is especially prevalent at physiologically relevant membrane potentials.

In the present study whole cell recordings from normal (nontransformed) human T cells were used to determine whether inhibition of protein serine/threonine phosphatases, activation or inhibition of PKA, or sequential activation of PKC and PKA modulates Kv1.3 current in a coordinated fashion. We found that inhibition of serine/threonine protein phosphatases [with micromolar concentrations of okadaic acid (OA)] increases Kv1.3 current and shifts the voltage dependence of activation and inactivation to more positive potentials. A PKA-activating cAMP analog [the Sp diastereomer of adenosine 3′,5′-cyclic monophosphothioate (Sp-cAMPS)] increases G_{max} and shifts the voltage dependence of steady-state inactivation to more positive potentials, resulting in a significantly greater window current. Thus channel activity will increase at voltages around the resting potential, just as we had previously observed using cell-attached patches (25). Because a PKA inhibitor peptide [PKI-(5—24)] decreased G_{max}, this regulation by Sp-cAMPS is probably through PKA. The increase in Kv1.3 current induced by any of the three treatments (PKA or PKC activation or phosphatase inhibition) was completely blocked by calphostin C; thus PKC may provide a "master switch" for the normal functioning of Kv1.3 channels in human T cells. An inhibitory cross talk between PKA and PKC phosphorylation appears to occur, instead of an additive increase in K^+ current, since the currents remained at control levels when first PKC, then PKA, was activated. Thus Kv1.3 appears to be regulated through the interaction of serine/threonine kinases and phosphatases.

MATERIALS AND METHODS

Cells. Human venous blood was collected from healthy donors, and mononuclear cells were separated by centrifugation on Ficoll-Hypaque or Percoll (both from Pharmacia, Dorval, PQ, Canada). After B lymphocytes and monocytes were depleted by adherence to nylon wool (DuPont, Toronto, ON, Canada), the cells were >98% T lymphocytes, as determined by fluorescence-activated cell sorter analysis using anti-CD3 antibody (Serotec, Mississauga, ON, Canada). Cell viability (>95%) was measured by trypan blue exclusion or a live/dead assay (Molecular Probes, Eugene, OR).

Chemicals. ATP (dipotassium salt, K_2ATP) was purchased from Sigma Chemical (St. Louis, MO), calphostin C and Sp-cAMPS from Calbiochem (San Diego, CA), OA and norokadone (norOA) from Research Biochemicals (Natick, MA), and 4β-PDBu from Biomol (Plymouth Meeting, PA). Stock solutions of 4β-PDBu, calphostin C, OA, and norOA were prepared in dimethyl sulfoxide (DMSO), aliquoted, and stored at −20°C. Inasmuch as the highest final DMSO concentration was 0.1%, control solutions contained 0.1% DMSO. Margatoxin (MgTx; a gift of Merck, Sharpe and Dohme Research Laboratories, Rahway, NJ) was prepared as a 1 μM stock solution in distilled water, then aliquoted, lyophilized, and stored at −20°C. A 5 mM stock solution of the PKA inhibitor peptide PKI-(5—24) (Upstate Biotechnology, Lake Placid, NY) was made in 10% acetonitrile, aliquoted, and stored at −20°C. Control pipette solutions contained 0.01% acetonitrile. Freshly thawed aliquots of these drugs were used each day.

Solutions. The bathing medium was NaCl saline consisting of (in mM) 145 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, and 10 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid adjusted to pH 7.4 with NaOH. The standard pipette solution contained (in mM) 95 potassium aspartate, 40 KCl, 10 ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 1 $CaCl_2$, 10 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 2.5 $MgCl_2$, and 2 K_2ATP adjusted to pH 7.4 with KOH. Intracellular free Ca^{2+} (~10 nM) and Mg^{2+} (0.08 mM)

concentrations were held constant in all experiments. Osmolalities of the bath and pipette solutions were 290–295 and 280–285 mosmol/kgH_2O, respectively, as measured with a freezing-point depression osmometer (Advanced Instruments, Needham Heights, MA).

Electrophysiological recordings. An Axopatch 1B patch-clamp amplifier (Axon Instruments, Foster City, CA), pCLAMP software (version 5.5.1, Axon Instruments), and Labmaster hardware were used to control voltage and to acquire and analyze data. All recordings were made at room temperature (22–24°C) in the whole cell patch-clamp configuration. Pipettes were pulled from borosilicate glass (A-M Systems, Everette, WA) to resistances of 5–7 MΩ. The maximum uncompensated series resistance was ~10 MΩ during whole cell recordings, so the voltage error was ~5 mV for a current amplitude of 500 pA. Liquid-liquid junction potentials were compensated using the Axopatch internal circuit; i.e., the potential was zeroed with the pipette in the bath, then remeasured after seal formation and subtracted from the command voltage using the Axopatch internal circuit. Unless otherwise stated, whole cell currents were allowed to stabilize for ~10 min before the K^+ current parameters were measured. Drugs were added to the bath by continuous perfusion or manually by exchanging the entire bath three times with Pasteur pipettes. Where appropriate, values are means ± SE, with the statistical significance of different mean values tested using Student's *t*-test (accepted level $P < 0.05$).

RESULTS

Several salient features of whole cell Kv1.3 currents are relevant to the present results (24). The native Kv1.3 current in T lymphocytes responds to voltage-clamp steps with activation kinetics that are well described by an exponentially rising function raised to the fourth power, so-called Hodgkin-Huxley n^4 kinetics. During a prolonged depolarization the current undergoes C-type inactivation, which exhibits a monoexponential time course. The kinetics and voltage dependence are affected by physical and biochemical factors, including temperature (24) and phosphorylation (Ref. 10 and this study). For example, under the conditions used in the present study, the inactivation time constant is ~325 ms at +30 mV (10). Inactivation is affected by cytoplasmic ions, becoming significantly faster (~200 ms at +30 mV) with 140 mM KF in the pipette (24) than with 40 mM KCl-95 mM potassium aspartate (present study and Ref. 10) or in cell-attached recordings (25). Recovery from inactivation is slow, requiring as long as 30 s to 2 min, depending on the voltage and temperature (24). Because we previously found it necessary to wait 2 min between voltage steps to ensure recovery from inactivation after PKC stimulation (10), we used 2-min intervals in the present study. A systematic study of recovery from inactivation after PKC or PKA activation or inhibition has yet to be undertaken. The voltage dependence of activation and steady-state inactivation is well described by Boltzmann equations; however, the half-maximal voltages depend on numerous factors, including temperature (24) and phosphorylation [PKC (10) and PKA (present study)].

For each set of treatments, activation (g-V) and inactivation (h_∞-V) were plotted as a function of voltage (V), and the data were fitted with Boltzmann equations. The sigmoidal g-V and h_∞-V relations overlap and produce a voltage window that extends from the threshold of the g-V curve to the potential at which the current is completely inactivated. This overlap or window current predicts tonically active channels throughout this voltage range; i.e., a component of the current remains activated even during sustained depolarizations. The magnitude of the window current (pA) was calculated from the area under each actual g-V and h_∞-V curve (pA/mV × mV), using the theoretical fits to these curves and the intersection set over the full voltage range. This value cannot simply be read from the area under the "crossover" of these curves in Figs. 1, 2, and 5, since the summarized h_∞-V and g-V curves were normalized to the G_{max} of each cell. The same result can be obtained by multiplying the area under these curves (a unitless number × mV) by the average maximal K^+ conductance (pA/ mV) given in Fig. 6 and in the text. In addition to this measure of total, tonically activated current, the voltage at which the g-V and h_∞-V curves cross is of interest, since it corresponds with the voltage at which tonic K^+ channel activity is expected to be maximal (i.e., best able to oppose depolarizing currents).

Phosphatase inhibitor OA increases the K^+ conductance. We examined (Fig. 1) the effect on Kv1.3 current of inhibiting serine/threonine protein phosphatases with the membrane-permeant inhibitor OA. At low concentrations, OA can be used to help separate effects of different protein phosphatases (PP), since inhibition of PP_1 [inhibition constant (K_i) = 0.2–1 nM] is more effective than inhibition of PP_{2A} (K_i = 10–500 nM) or PP_{2B} (K_i = 4 μM) (4). We preincubated intact cells with OA (37°C, 30 min) to avoid the possibility of cytoplasmic disruption and phosphatase loss or inactivation during whole cell recording. An inactive structural analog of OA, norOA, was used as a negative control. Preincubating T cells with 100–500 nM OA had no effect on the current amplitude or voltage dependence (data not shown), whereas 1–3 μM OA (Fig. 1*A*) significantly increased the K^+ conductance (by 40%, $P < 0.05$, Student's *t*-test); G_{max} increased to 6.0 ± 0.4 nS (n = 19) from 4.0 ± 0.2 nS (n = 60) for control cells with no added drugs (summarized in Fig. 6). Incubation with the inactive analog (1 μM norOA) did not affect G_{max} (4.3 ± 0.3 nS, n = 5). Because micromolar OA concentrations were necessary to see an effect, our results suggest that PP_{2B} (K_i = 4 μM) and possibly PP_{2A} (K_i = 10–500 nM) are responsible for regulating Kv1.3.

OA also affected the voltage dependence of Kv1.3 current, as seen from the conductance-voltage (g-V) and inactivation-voltage (h_∞-V) curves in Fig. 1*B*. After treatment with the inactive analog norOA, current activation was half-maximal at −43.3 mV and steady-state inactivation was half-maximal at −64.8 mV. In cells treated with the active analog (OA) at 1–3 μM, these voltages were shifted toward depolarized potentials: half-maximal voltages were −32.5 mV for activation and −61.6 mV for inactivation. After OA treatment, a significantly greater proportion of current was activated by steps to −30 mV, and even though the

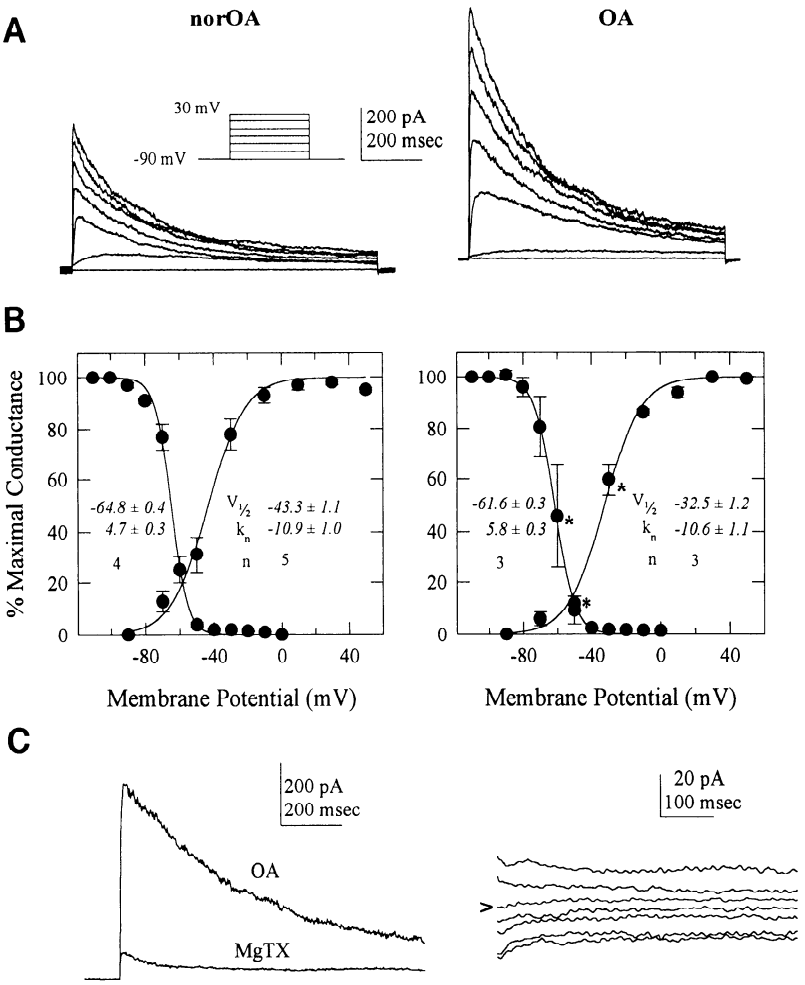

Fig. 1. Phosphatase inhibitor okadaic acid (OA) increases Kv1.3 conductance and causes a shift in activation and inactivation curves to more depolarized potentials. Whole cell K^+ currents were recorded from cells that were preincubated (37°C, 30 min) with 1 μM OA or its inactive analog norokadone (norOA). Bath and pipette contained standard solutions (see MATERIALS AND METHODS). *A*: typical K^+ currents in response to voltage steps between −70 and +30 mV (in 20-mV intervals) applied every 2 min from a holding potential of −90 mV (current at −90 mV is also shown). *B*: voltage dependence of activation and steady-state inactivation. Average values are from cells incubated for 30 min at 37°C in 1–3 μM norOA or OA. At each voltage, K^+ conductance (G_K) was calculated as $I_{peak}/(V_m - E_K)$, where I_{peak} is peak current, V_m is membrane potential, and E_K is Nernst potential for K^+ (−84 mV). For activation, G_K during each voltage step was normalized to maximal value (G_{max}) during a step to +30 mV. For inactivation, peak G_K (at +30 mV) after 3 min at each holding potential was normalized to G_{max} obtained from a holding potential of −100 mV. Activation and inactivation curves were then fitted to Boltzmann equations using nonlinear least-square fits of $g(V) = g_{max}/[1 + \exp[(V - V_{½})/k_n]]$. Adjacent to each curve are values of parameters obtained from curve fits: $V_{½}$ is voltage producing half-maximal G_K, k_n is slope factor indicating steepness of voltage sensitivity, and n is number of cells tested. *Significant differences in normalized G_K ($P < 0.05$, Student's t-test). *C, left*: current enhanced by OA is blocked by Kv1.3 blocking peptide margatoxin (MgTx). K^+ current was evoked by a step to +30 mV from a holding potential of −90 mV before and after 5 nM MgTx was perfused into bath. *C, right*: after OA treatment, current remaining after a long voltage step reverses near E_K. Tail currents were evoked by voltage steps every 10 mV between −60 and −120 mV, each of which followed a 1,150-ms prepulse to +30 mV (holding potential −90 mV). Tail currents are shown beginning 1 ms after each test potential, i.e., after capacitance current has settled. Zero-current (reversal) potential is −80 mV, as indicated by arrowhead.

voltage dependence of inactivation was more variable, there was less inactivation at −60 mV. The increase in G_{max}, together with changes in voltage dependence, produced a 78% increase in the window current (from 880 to 1,565 pA) after OA treatment. Concomitantly, OA caused a +10-mV shift in the voltage at which the window current was maximal (−50 vs. −60 mV) compared with cells treated with the inactive analog norOA.

The outward current did not completely inactivate even after a 1-s depolarizing pulse, particularly after treatment with OA. To test whether the remaining current was Kv1.3 or some other outward current (e.g., Cl^-, K_{Ca}), we determined the reversal potential for the residual current and examined its sensitivity to the Kv1.3-specific toxin MgTx. The residual current after OA treatment reversed at −80 mV (Fig. 1*C, right*), which is very close to the Nernst potential for K^+ in this solution (−84 mV), and the time-dependent current and most of the residual current were blocked by 5 nM MgTx (Fig. 1*C, left*). A similar reversal potential and MgTx block were seen for the residual current in control cells (data not shown) (10). We conclude that the Kv1.3 current was specifically increased by OA.

PKA activation alters G_{max} and the voltage dependence of Kv1.3 current. To activate PKA, we added the hydrolysis-resistant cAMP analog Sp-cAMPS (100 μM) to the pipette solution (Fig. 2) and allowed it to diffuse into cells during whole cell recordings. Sp-cAMPS significantly increased the K^+ currents: G_{max} was 6.4 ± 0.8 nS ($n = 7$), which is a 60% increase compared with control cells (4.0 ± 0.2 nS, $n = 60$, $P < 0.05$). There was no change in the voltage dependence of activation; however, Sp-cAMPS significantly shifted ($P < 0.05$) steady-state inactivation toward more depolarized potentials (by nearly +9 mV). This shift in inactivation, together with the increase in G_{max}, increased the window current by 117% (from 880 to 1,907 pA) and shifted the voltage at which maximal tonic channel activity occurred by ~8 mV.

Inactivation was significantly faster in the presence of Sp-cAMPS: the time constant was 285 ± 15 ms ($n = 7$) compared with 325 ± 27 ms for control cells ($n = 60$, $P < 0.05$); nevertheless, the outward current did not completely inactivate, even after a 1-s depolarizing pulse (Fig. 2*C, left*). We confirmed the identity of the noninactivating current as K^+ current, since it reversed at −80 mV (Fig. 2*C, right*), very near the Nernst potential for K^+ (−84 mV). MgTx (5 nM) blocked the time-dependent and residual current (Fig. 2*C, left*), showing that Sp-cAMPS specifically increased activity of Kv1.3 channels.

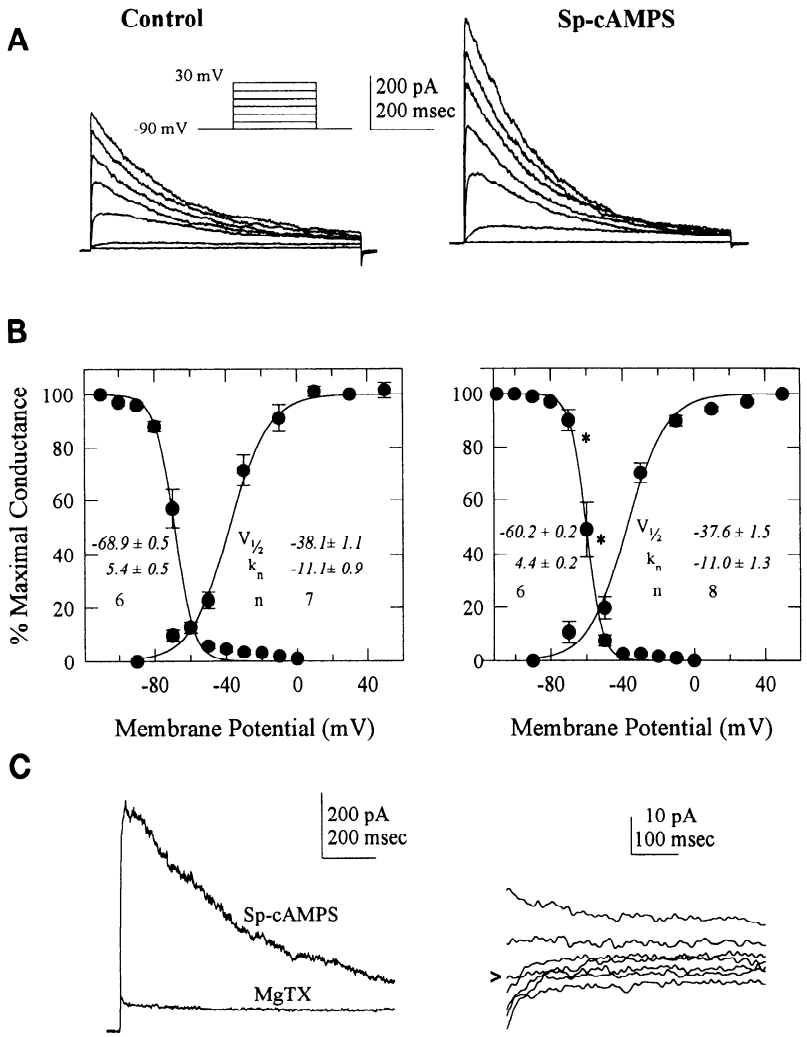

Fig. 2. cAMP analog Sp diastereomer of adenosine 3′,5′-cyclic monophosphothioate (Sp-cAMPS) increases Kv1.3 conductance and causes a shift in inactivation curve to more depolarized potentials. Pipette contained standard solution without (Control) or with 100 μM Sp-cAMPS, and bath contained standard saline. All data were obtained at least 10 min after break into whole cell configuration. (Stability of control currents is shown in Fig. 3*A*.) *A*: K^+ currents were evoked as in Fig. 1*A* by voltage steps between −70 and +30 mV applied every 2 min from a holding potential of −90 mV. *B*: activation and inactivation were determined as a function of voltage as in Fig. 1*B* with and without 100 μM Sp-cAMPS in pipette. *Significantly different ($P < 0.05$). *C*, *left*: whole cell current recorded at +30 mV (holding potential −90 mV) with Sp-cAMPS in pipette before and after 5 nM MgTx was perfused into bath. *C*, *right*: tail currents at end of 1,150-ms pulses to +30 mV (as in Fig. 1*C*) reversed near E_K (arrowhead at −80 mV).

Because Kv1.3 in the Jurkat T cell line is already phosphorylated at serine residues in vivo without stimulation of protein kinases (7), we asked whether inhibition of PKA affects the current without prior PKA stimulation. We used an inhibitor peptide [PKI-(5—24)] that binds to the catalytic subunit of PKA and masks its active site cleft, making it a very specific PKA inhibitor at concentrations <100 μM. Including 5 μM PKA inhibitor peptide in the pipette decreased G_{max} by 43%: to 2.3 ± 0.2 nS (n = 19) from 4.0 ± 0.2 nS (n = 60) in control cells ($P < 0.05$; summarized in Fig. 6). The inhibition of current by the PKA inhibitor peptide (mol wt ~2,000) took a few minutes to develop (Fig. 3*B*), whereas the current was stable for >30 min in control cells (Fig. 3*A*). We have not compared the voltage dependence of activation or inactivation after treatment with PKI-(5—24).

Possible cross talk between PKC and PKA. We previously found (10) that PKC activation produces an increase in G_{max} similar to that described above for Sp-cAMPS and shifts activation and inactivation curves to more depolarized potentials. Thus we asked whether cross talk between the two kinases occurs: Are the PKA and PKC effects additive, or does one dominate? Our results (Fig. 4; summarized in Fig. 6) suggest that PKC-dependent phosphorylation can act as a master switch. First, we increased Kv1.3 currents (and presumably protein phosphorylation levels) by adding the phosphatase inhibitor (OA, 1 μM) or the PKC activator (4β-PDBu, 1 μM) to the bath before recording or by adding the PKA activator (Sp-cAMPS, 100 μM) to the pipette solution. For the individual cells shown in Fig. 4, the current at +30 mV was increased by 51% (OA), 25% (Sp-cAMPS), and 69% (4β-PDBu) compared with two or three control cells from the same cell batches. It was not feasible to allow the PKA peptide inhibitor to diffuse into the cell before PKC activation, because preincubation of intact cells with 4β-PDBu was needed to see an effect on the current (10).

Next we added the PKC inhibitor calphostin C (500 nM) to the bath. Because calphostin C is activated by light (6), it can be added in the dark for control recordings, then illuminated to inhibit PKC. [This compound contains several perylene quinone groups, which can potentially release free radicals when it is activated. Although this is thought to occur only if very high calphostin C concentrations are used (6), as a precaution we added a free radical scavenger (1 mM ascorbic acid) to the bath and pipette solutions (10). Ascorbic acid itself did not affect G_{max}, the current

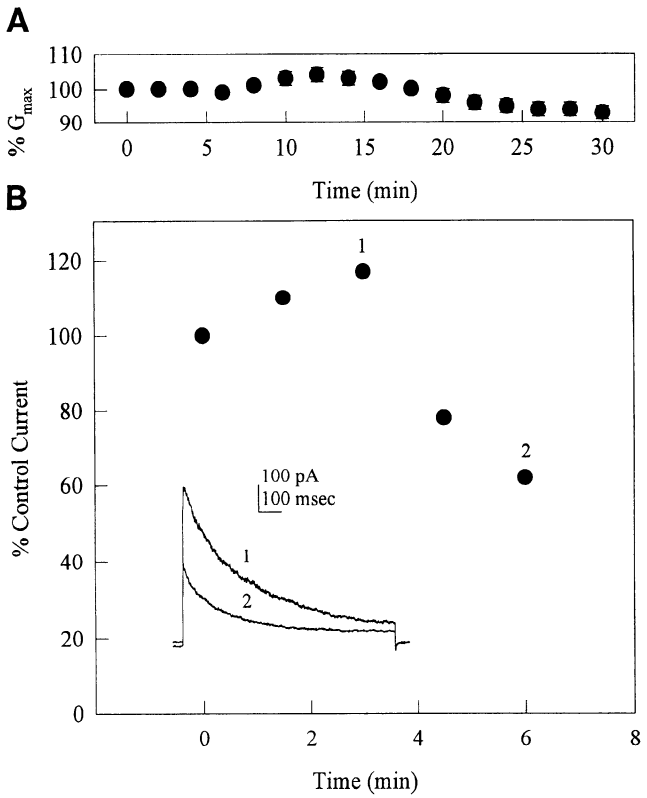

Fig. 3. Kv1.3 conductance is reduced by diffusion of a protein kinase A inhibitor peptide [PKI-(5—24)] from pipette into cell. *A*: no significant change in maximal conductance (G_{max}) in control cells over a 30-min recording period with standard pipette solution (no added ATP, $n = 11$). *B*: G_{max} decreased significantly with time after a whole cell recording was established at 0 min with PKI-(5—24) in pipette (see summary of conductances in Fig. 6). Bath contained standard saline, and pipette contained standard solution + 5 μM PKI-(5—24) (no added ATP). K^+ currents were evoked by steps to +30 mV (holding potential −90 mV) applied every 90 s after whole cell recordings were established at 0 min, and G_{max} is plotted as a function of time. *Inset*: currents at 3 (*trace 1*) and 6 min (*trace 2*) after break into whole cell configuration.

kinetics, or the current's sensitivity to calphostin C.] We found that calphostin C had no effect in the dark. Then, after illumination with the standard 50-W halogen lamp of the inverted microscope, Kv1.3 currents were inhibited by 90–100% ($n = 5$ each for OA and Sp-cAMPS; $n = 15$ for 4β-PDBu) (13). With this calphostin C concentration, inhibition reached a steady state within 2–3 min. If PKA phosphorylates a different site from that phosphorylated by PKC and the two sites behave independently, then a selective PKC inhibitor should not inhibit the portion of current activated by PKA (and possibly by OA). Thus it appears that phosphorylation by PKC may provide a master switch, the closing of which turns off the channel, even when the current is first enhanced by PKA or phosphatase inhibition.

We then asked whether phosphorylation by both kinases (PKA and PKC) would produce additive effects. For all sequential activation experiments, we first confirmed the increase in G_{max} by Sp-cAMPS or 4β-PDBu alone in cells from the same batches (Figs. 4, *B* and *C*, and 5*A*). Then an aliquot of cells was preincubated with 1 μM 4β-PDBu (15–30 min, 37°C) to activate PKC, and whole cell recordings were established with pipettes containing 100 μM Sp-cAMPS (Fig. 5, *B–D*). To our surprise, G_{max} was 4.3 ± 0.3 nS ($n = 5$), a value that was not different ($P > 0.05$) from control cells (4.0 ± 0.2 nS, $n = 60$). If phosphorylation by PKC and PKA additively increased the conductance, one would expect the resulting G_{max} to be >8.0 nS. As before, the time-dependent current was entirely Kv1.3, since it was fully blocked by 5 nM MgTx (Fig. 5*B*). Calphostin C (Fig. 5*C*) also abolished the current.

Moreover, sequential kinase activation did not cause further shifts in voltage dependence; instead, the values were not significantly different from control cells. Half-maximal voltages for control cells (Fig. 2) were −38.1 mV ($n = 7$) for activation and −68.9 mV ($n = 6$) for inactivation compared with −37.6 ($n = 8$) and −60.2 mV ($n = 6$) after Sp-cAMPS alone (Fig. 2) and −38.0 ($n = 9$) and −59.0 mV ($n = 4$) after 4β-PDBu alone (10). However, after sequential PKC and PKA stimulation in the same cells (Fig. 5*D*), half-maximal voltages were

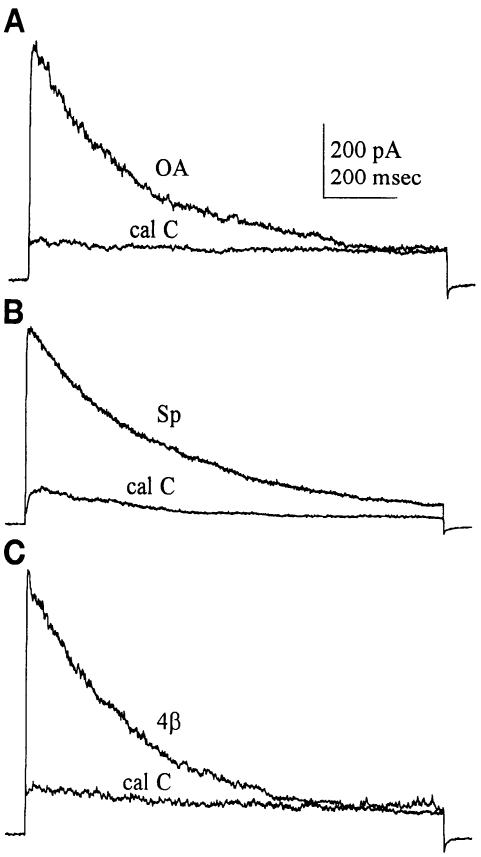

Fig. 4. Light-activated PKC inhibitor calphostin C (cal C) inhibits Kv1.3 currents after their enhancement by 3 treatments. *A*: cells were preincubated with phosphatase inhibitor OA (1 μM, 15–30 min, 37°C). Pipette contained standard solution. *B*: cells were preincubated (15–30 min, 37°C) with 0.1% DMSO, solvent used for OA and 4β-phorbol 12,13-butyrate (4β). Pipette contained standard solution + 100 μM PKA-activating cAMP analog Sp-cAMPS (Sp). *C*: cells were preincubated with protein kinase C-activating phorbol ester 4β-phorbol 12,13-butyrate (1 μM, 15–30 min, 37°C). For each cell, whole cell currents were recorded, 500 nM calphostin C was added to bath for ~10 min in dark, and then calphostin C was activated by illumination using a standard 50-W halogen microscope lamp. All K^+ currents were recorded at +30 mV from a holding potential of −90 mV.

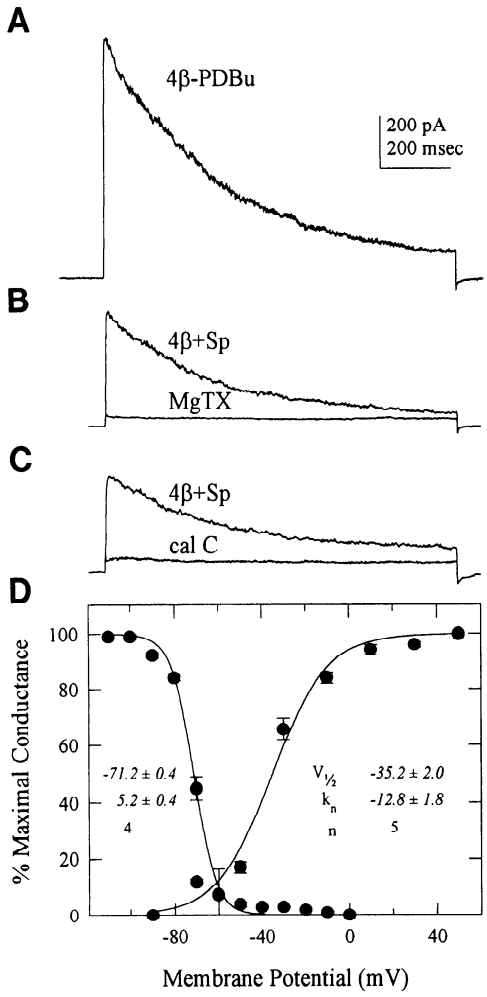

Fig. 5. Sequential activation of protein kinase C and then protein kinase A does not affect Kv1.3 in same manner as activation by each kinase alone. All cells were preincubated with 1 μM 4β-phorbol 12,13-butyrate (4β-PDBu; 37°C, 15–30 min), and then whole cell recordings were established. Traces were recorded at +30 mV from a holding potential of −90 mV. *A*: phorbol ester treatment alone increased current compared with controls (by ~70%, see Fig. 6). *B–D*: after 4β-phorbol 12,13-butyrate preincubation, whole cell recordings were established with 100 μM Sp-cAMPS in pipette, and then ~10 min were allowed for currents to stabilize. Resulting current was blocked by 5 nM MgTx (*B*) or 500 nM calphostin C (*C*). *D*: activation and steady-state inactivation were determined as a function of voltage (as in Fig. 1*B*).

−35.2 mV (n = 5) for activation and −71.2 mV (n = 4) for inactivation. Owing to this lack of change of voltage dependence or of G_{max} (Fig. 6), the window current (925 pA) did not differ from that of control cells (880 pA) or from cells treated with the inactive phorbol ester analog 4α-PDBu (950 pA) (10). Finally, the inactivation rate after sequential kinase stimulation (τ_j = 345 ± 32 ms at +30 mV, n = 4) was unchanged from that of control cells (τ_j = 325 ± 10 ms, n = 60). Thus it appears that incubating intact cells with 4β-PDBu before recording, then including Sp-cAMPS in the pipette, abrogates the effects of either kinase alone.

It was difficult with the present experimental system to study the time dependence of the effects of Sp-cAMPS after 4β-PDBu treatment. These experiments are constrained by the small size of T lymphocytes (spherical, 6–7 μm diameter, with a cytoplasmic layer only ~1 μm thick) and the resulting rapid diffusional exchange between the pipette and the cytoplasm (e.g., within seconds for small molecules) (28). With the relatively high concentrations of kinase activators and inhibitors used, effects usually occurred within minutes (Fig. 3*B*). However, the slow recovery of Kv1.3 current from inactivation meant that a single voltage step to a positive potential (to measure G_{max}) could only be delivered every 1.5–2 min. Therefore, only the final, steady-state effects of dual kinase activation are presented.

DISCUSSION

We found that inhibiting multiple phosphatases by preincubating intact cells with high concentrations of OA increased G_{max} by 40% and shifted the activation curve to more positive potentials, which together increased the window current by 78%. Activation of PKA (with Sp-cAMPS in the pipette) increased G_{max} by 60% and caused a shift in voltage dependence to more depolarized potentials, increasing the window current by 117%. (There was no effect on the activation curve.) Inasmuch as diffusion of PKA-inhibitory peptide [PKI-(5—24)] from the pipette reduced G_{max} by 43%, we assume that phosphorylation by PKA is involved. In contrast to the effect of stimulating PKC (10) or PKA alone, sequentially stimulating PKC and then PKA did not change the conductance or voltage dependence from

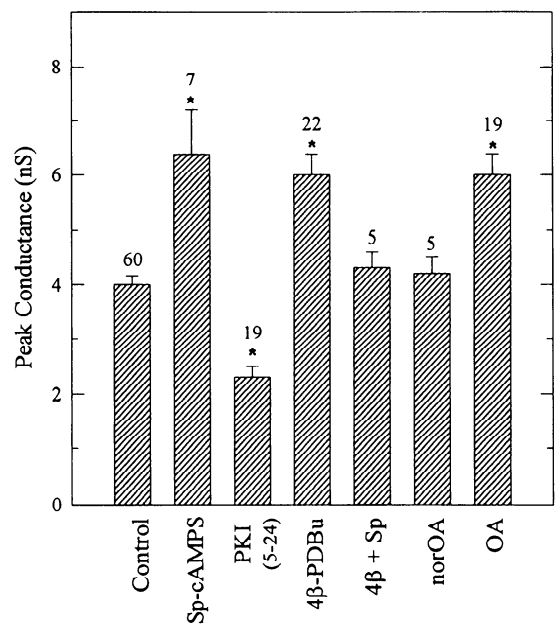

Fig. 6. Summary of average maximal K^+ conductances (G_{max}). Treatments were as follows: control cells with 0.1% DMSO (Control), activation of protein kinase A with 100 μM Sp-cAMPS in pipette, inhibition of protein kinase A by 5 μM PKI-(5—24) in pipette, activation of protein kinase C by preincubation with 1 μM 4β-phorbol 12,13-butyrate alone and 4β-phorbol 12,13-butyrate followed by recording with 100 μM Sp-cAMPS in pipette (4β + Sp), and incubation with 1–3 μM OA or its inactive analog norOA. G_{max} (mean ± SE, with number of cells above each bar) was calculated from maximal current during a step to +30 mV. *Significantly different from control (Student's t-test).

that of control cells. Our results suggest that Kv1.3 is subject to multiple modes of short-term regulation that include cross talk between PKA- and PKC-dependent mechanisms. The fact that high concentrations of OA (1–3 μM) were required suggests that one target phosphatase is PP_{2B} (calcineurin), inasmuch as PP_1 and PP_{2A} are inhibited at nanomolar OA concentrations (4). OA treatment might result in phosphorylation of the same site(s) as PKA, but it might also promote hyperphosphorylation at multiple sites on the channel as well as on other proteins. Because the effects of OA (this study) are similar to those after PKC activation (10), it may be that OA also promotes increased phosphorylation of the same site(s) as PKC.

In principle, an increase in G_{max} could result from any combination of more functional channels, greater probability of opening, or an increase in single channel conductance. Because these increases occurred within minutes (2–5 min for Sp-cAMPS, 15–30 min for OA or 4β-PDBu) (10), it is unlikely that new channels were assembled and inserted into the membrane. In a previous study we observed a similar rapid increase in G_{max} and a shift in activation and inactivation curves simply by raising the recording temperature above room temperature (24). In that study, changes in single channel and whole cell conductance with temperature showed that recruitment of inactive channels contributed to the increase in G_{max}. Such silent channels in the membrane may well be activated by different means, perhaps through phosphorylation at a critical PKA site, leading to an increase in G_{max}. Another possibility is that phosphorylation at the PKA site (by Sp-cAMPS) or PKC sites (by OA) in the S4-S5 loop (see below) increases G_{max} by changing the single channel conductance. Serine at a comparable position in *Shaker* K^+ channels affects the single channel conductance, probably by contributing to the internal vestibule of the channel (14). To discriminate between these two possibilities and an increase in open probability, it would be best, in the future, if single channel recordings were done from excised patches exposed to activated kinase or kinase inhibitors.

Direct phosphorylation of many ion channel proteins does occur, but an important possibility is that effects of manipulating kinases and phosphatases that we and others have described result from changes in phosphorylation of structurally associated proteins, e.g., cytoplasmic β-subunits that are known to regulate some properties (especially inactivation) of voltage-gated K^+ channels (15). Several isoforms of K^+ channel β-subunits (Kvβ) have been cloned, and all have a consensus site for PKA phosphorylation and multiple sites for PKC (for review see Ref. 15). Because Kvβ subunits are ~38-kDa proteins, it is of interest that when Cai and Douglass (7) purified Kv1.3 channel protein from the Jurkat T cell line a ~40-kDa protein coimmunoprecipitated with the channel. PKA phosphorylated the Kv1.3 channel and the coprecipitated protein; thus, on the basis of its size and close association in a channel complex with Kv1.3, this protein may be a regulatory β-subunit, the phosphorylation of which could be involved in modulating the current.

Amino acid sequences of Kv1.3 clones from the mouse (MK3), rat (RGK5, RCK3, Kv3), and human (HLK3, HGK5) are very similar, with >98% homology between human and rat clones (for review see Ref. 8). All Kv1.3 clones contain several potential PKA and PKC phosphorylation sites (17), with one (mouse) or two (rat and human) strong PKC sites on the S4-S5 loop and two weaker sites, one on the S2-S3 loop and one near the COOH terminus. There is one strong PKA site at each terminus and one weaker site each on the S4-S5 loop and NH_2 and COOH termini. The previous biochemical analysis of Kv1.3 in the Jurkat T cell line (7) and mutation studies of Kv1.3 and related K^+ channels allow some speculation about which PKA sites may be responsible for the changes in current we observed. In Jurkat cells the Kv1.3 protein is phosphorylated in vivo at serine residues and serves as an excellent substrate for further phosphorylation by PKA and PKC (7), although the identity of these sites (serine and threonine) was not determined. These authors compared the levels of Kv1.3 phosphorylation before and after the cell extracts were treated with alkaline phosphatase or a peptide inhibitor of PKC (to dephosphorylate the channels) and after PKA or PKC was added. Because PKC only phosphorylated Kv1.3 protein after pretreatment with alkaline phosphatase, they concluded that the channel is already highly phosphorylated at the PKC sites in vivo without stimulation (7). Inasmuch as this in vivo phosphorylation is at serine, the PKC sites on the S4-S5 loop are implicated. Phosphoamino acid analysis was only conducted under unstimulated conditions; thus their results do not eliminate any of the potential PKA sites from consideration. Direct addition of phosphatases during patch-clamp recording has shown inconsistent effects on the currents. Intracellular application of alkaline phosphatase during whole cell recordings from Jurkat cells increased G_{max} (26), but the same treatment had no effect when added to excised patches from *Xenopus* oocytes that had been transfected with mouse Kv1.3 (1). Inasmuch as alkaline phosphatase has a very broad substrate specificity, this difference may indicate different starting levels of phosphorylation in the two cell types.

Although we anticipate that more than one site (possibly PKA and PKC sites) becomes phosphorylated when phosphatases are inhibited (e.g., with OA), neither our data nor the literature directly addresses this possibility. One might expect that the increased negative charge of a phosphate group at any of the PKC or PKA sites in the S4-S5 loop (which is near the putative voltage sensor, S4 transmembrane domain) would affect the voltage dependence of activation calculated from the Boltzmann relation. OA produced a positive shift in the activation curve, but Sp-cAMPS did not, suggesting that phosphorylation resulting from PKA and that resulting from phosphatase inhibition are not equivalent. Inasmuch as stimulating PKC (10) shifted the activation curve in the same direction and by the same amount as OA (this study), it is possible that the

same PKC site (on S4-S5) was phosphorylated by the two treatments. It is interesting that OA often causes a preferential increase in phosphorylation at threonine, possibly because of faster turnover than phosphoserine (29).

The few mutation studies of Kv1.3 have focused on kinetics of C-type inactivation, not on G_{max} or the voltage dependence, and results have been inconsistent. In one study, deleting the NH_2-terminal 146 amino acids (which contain 2 of the PKA sites) did not affect the inactivation kinetics (1), whereas in another study, slower inactivation was found when one of the NH_2-terminal sites was mutated from serine to alanine (19). The latter study examined effects on inactivation kinetics of mutating several of the phosphorylation sites, then expressing the channels in *Xenopus* oocytes. Slower inactivation occurred with two of the PKA site mutants: one in the NH_2 terminus and the S4-S5 loop site (19). Much greater slowing occurred when these two sites were mutated together with one of the COOH-terminal sites (a site that had no effect when mutated alone), suggesting cooperative interactions between phosphorylation sites. We observed significantly faster inactivation after Sp-cAMPS (but not OA) treatment. Together, these results suggest that more than one phosphorylation site contributes to the kinetics and voltage dependence of Kv1.3 channels.

Of the several previous studies of cAMP and Kv1.3 currents, most have not examined changes in voltage dependence and none has addressed window current amplitude. Where the G_{max} has been compared, all possible outcomes have been reported: inhibition, no effect, and enhancement. Do the results differ according to cell type (i.e., mouse vs. human, normal vs. cell lines, native channels vs. those expressed in *Xenopus* oocytes), patch-clamp configuration (cell-attached vs. recordings from disrupted cells), recording solutions (e.g., anion species, free Ca^{2+} in the pipette), or the agents used to raise cellular cAMP (cAMP analogs, receptor agonists, phosphodiesterase inhibitors)? We found no definitive correlations but a few points for consideration. The most common response to elevated cAMP is a slow decrease in maximal Kv1.3 conductance, measured during whole cell recordings with frequent voltage steps to quite positive potentials. This is sometimes accompanied by faster current inactivation. Faster inactivation and reduced G_{max} were seen in mouse B cells and a pre-B cell line after cAMP diffusion into the cell (9), whereas in the Jurkat T cell line G_{max} decreased without an apparent change in inactivation kinetics when cells were exposed to 8-BrcAMP or the receptor agonist PGE_2 (26).

Results on normal human T cells have been more variable. Several studies found no effect of elevating cAMP by a variety of means during whole cell recordings: addition of cAMP to the pipette, bath application of permeant cAMP analogs, activation of adenylate cyclase directly or through receptors, or inhibition of cAMP phosphodiesterase (1, 18, 25). One study reported a significant decrease in G_{max}, faster current inactivation, and a shift in the inactivation (but not activation) curve in response to the β-adrenergic agonist isoproterenol (30). Because this decrease was inhibited by propranolol (a β-blocker), attenuated by a PKA inhibitor peptide [PKI-(5—24)], and mimicked by high concentrations of dibutyryl cAMP added to the bath, the authors concluded that PKA activation decreased Kv1.3 current. That study was restricted to a subset of normal human T lymphocytes, the $CD8^+$ cytotoxic T cell population, isolated by positive selection using an anti-CD8 antibody. It is possible that antibody treatment affects T cell responses to isoproterenol. In contrast to these studies using whole cell recordings, we observed an increase in K^+ channel activity around the resting potential during cell-attached recordings from intact T cells that were not subset selected ($CD4^+$ and $CD8^+$ present) (25). Several treatments were used to raise cAMP: the receptor agonists isoproterenol, PGE_2, and histamine, a membrane-permeant cAMP analog (8-BrcAMP), an adenylate cyclase activator (forskolin), and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine. All treatments produced an increase in channel activity for a few minutes, but the duration of this increase was not determined, nor were any possible effects on voltage dependence or kinetics of activation or inactivation.

Although most studies of Kv1.3 regulation have addressed the role of a single protein kinase (PKA or PKC), one study considered interactions between PKA and PKC (26). Using the Jurkat T cell line, Payet and Dupuis (26) first individually activated PKC or PKA and observed effects on G_{max} opposite to those we found (this study and Ref. 10); however, they did not examine the voltage dependence of activation and inactivation or the window current. In that study a slow decrease in whole cell current (over 10–30 min) occurred after bath application of PGE_2 (or 8-BrcAMP), an effect attributed to PKA activation, since it was prevented by intracellular addition of the PKA inhibitor, the Rp diastereomer of adenosine 3′,5′-cyclic monophosphothioate (Rp-cAMPS). A similar decline in current began ~10 min after bath addition of a PKC-activating phorbol ester; thus it was concluded that PKA or PKC activation alone reduces the K^+ conductance. In contrast, after 15–24 h of treatment with phorbol ester (assumed to downregulate PKC) or short-term treatment with PKC inhibitors, 8-BrcAMP had no effect on the current amplitude. The authors proposed that PKA has no effect until PKC has phosphorylated the channel (26). It was assumed that PKC was downregulated; however, prolonged phorbol ester treatment downregulates some (PKC-β) but not all (i.e., not PKC-α) isoforms (20). Thus it is of interest that prolonged treatment with phorbol ester did not appear to change the K^+ conductance in Jurkat cells (26), whereas Deutsch et al. (11) reported an increase in Kv1.3 current in normal human T cells after such prolonged treatment.

The study of the Jurkat T cell line, together with the present study, supports the view that concurrent phosphorylation by PKC and PKA does not have the same effect as phosphorylation by a single kinase. We found that PKA (this study) or PKC activation alone (10)

significantly increased G_{max} and altered the voltage dependence, such that the window current increased significantly. However, sequential activation of PKC (in intact cells) followed by PKA activation (during whole cell recording) negated the effects of activating either kinase alone: neither G_{max} nor the voltage dependence of activation or inactivation was different from control cells. These results suggest that multiple-kinase (site?) phosphorylation can abrogate the effects of each kinase alone.

There are several possible biological reasons for differences in PKC and PKA regulation of the same channel in different cell types. Of primary consideration is the presence of multiple phosphorylation sites that could affect channel behavior, including sites on the channel itself as well as on accessory molecules such as β-subunits, anchoring proteins, and the cytoskeleton. A key feature of signal transduction by protein kinase pathways is that the same kinase may achieve substrate specificity by kinase translocation to new sites and by compartmentation and anchoring at specific sites (23). Thus it would not be surprising if the same channel is affected in complex ways that culminate in cell-type-specific regulation. With few exceptions, comparisons have not been made of types (e.g., PKA isoforms), expression levels, and localization of kinases, phosphatases, and accessory molecules in normal lymphocytes, transformed lymphocyte cell lines, or exogenous channel expression systems (e.g., *Xenopus* oocytes). An exception is PKC, where differences are known to exist in expression of specific isoforms between the Jurkat T cell line and normal T cells. Of the 10 isoforms of PKC described so far, 9 have been found in T cells and T cell lines (for review see Ref. 13). Normal T cells express higher levels of PKC-β than PKC-α, and phorbol ester treatment increases PKC-β by two- to threefold with no effect on PKC-α or PKC-γ (3). In contrast, Jurkat T cells express more PKC-α than PKC-β (22), and since prolonged phorbol ester treatment may not downregulate PKC-α (20), it is unclear whether the experiments on Jurkat cells described above (26) successfully removed the relevant isoform. Even the responses to mitogen activation may differ between cell types; when Jurkat cells are activated by concanavalin A, PKC-α is translocated to a greater extent and for a longer duration than PKC-β (20). Finally, differences may exist in localization and substrate specificity for these PKC isoforms that affect their ability to phosphorylate specific channels, accessory molecules, and cytoskeletal proteins (23). Such differences could well culminate in cell-type-specific regulation of ion channels.

There are also some methodological differences between studies of Kv1.3 regulation. *1*) Whole cell recordings, especially those using receptor agonists as stimuli, may not adequately preserve the concentrations and localization of enzymes in the signaling pathway, inasmuch as endogenous kinases and phosphatases located in the cytosol will be diluted. In many cell types, inactive PKC isoforms are mainly cytosolic, but up to 30% of the PKC in resting T cells is membrane associated (22). If this localization differs between normal T cells and other cells in which Kv1.3 regulation is studied, PKC availability in whole cell recordings will differ. In T cells, approximately equal amounts of the phosphatases PP_1, PP_{2A}, and PP_{2B} are present in the cytoplasm, cell membrane, and microsomal membranes (2); thus cytoplasmic dilution is a concern. Evidence that phosphatases may wash out of T cells is that OA had no effect if added to the bath more than ~10 min after whole cell recording began (unpublished observations). *2*) Interactions between channels and accessory molecules may differ between intact cells (cell attached) and whole cell recordings, e.g., if anchoring proteins position kinases and phosphatases near the channel (23). Some isoforms of PKA are anchored through their regulatory domain to the membrane (23). Other accessory molecules (e.g., β-subunits) interact with the channels and the cytoskeleton (15): interactions that could be disrupted during whole cell recordings. *3*) It is well known that the intracellular anion species can affect Kv1.3 inactivation kinetics and current rundown (18; see discussion in Ref. 10). In one study spontaneous current rundown (by 30–50%) occurred within minutes of establishment of whole cell recordings with 130 mM Cl^- or aspartate in the pipette, but the rate of rundown was dramatically slowed when 40 mM KF was added to 90 mM potassium aspartate (18). When we observed different effects of cAMP elevation in intact cells compared with whole cell recordings using 140 mM KF solution in the pipette, we speculated that KF prevented further phosphorylation of the channel (25). It is well known that aluminum can be leached from patch-pipette glass and form complexes with F^-, e.g., AlF_4^-, which mimics phosphate and has many effects on signal transduction pathways (5). Because we were concerned that KF might affect protein phosphorylation, we used a pipette solution with a more physiologically relevant anion content (40 mM KCl, 95 mM K aspartate). Under these conditions, the control K^+ currents were stable for >30 min (whether or not ATP was added to the pipette solution) and PKA activation increased G_{max}. It is notable that all studies reporting a decrease in Kv1.3 current with elevated cAMP used high intracellular Cl^- (≥140 mM) or F^- (≥70 mM) concentrations (9, 26, 30). *4*) Different chemicals and protocols have been used to elevate intracellular cAMP levels. Most often membrane-permeant cAMP analogs have been added to the bath (9, 18, 26, 30). These compounds produce a transient elevation of intracellular cAMP but leave cleaved products (bromine, butyrate) that might have undesirable side effects. To produce a sustained activation of PKA, we added to the pipette solution a hydrolysis-resistant cAMP analog (Sp-cAMPS) that is not cleaved. If receptor stimulation is used to elevate cAMP, there are more steps in the signal transduction cascade at which cross talk could occur and more enzymes, the activity of which might be affected by cell disruption during whole cell recordings. To reduce washout, some authors treated intact cells before establishing recordings, as we did with OA (present study) and PKC activators (10) and

Krause et al. (18) did with PKA activators. However, most authors added drugs and receptor agonists after whole cell recordings were begun (9, 26, 30). *5*) Even the voltage-clamp protocol could have contributed to the decrease in whole cell conductance (G_{max}) observed by some authors after cAMP elevation. Several of the studies (1, 18, 26, 30) applied large voltage steps every 30–60 s for many minutes. Kv1.3 channels undergo C-type inactivation, which is accentuated by voltage steps that are applied too frequently to allow full recovery from inactivation (21, 24). We found that at least 60 s are required to recover from inactivation between depolarizing steps under control conditions, and up to 2 min are required after PKA [or PKC (10)] activation, particularly when large depolarizing steps are used. Thus protocols that apply large and frequent voltage-clamp steps will cause cumulative inactivation and a decrease in G_{max} that may have little physiological relevance to a nonexcitable cell. A more detailed study of the kinetics and voltage dependence of entry into, and recovery from, inactivation after manipulation of the phosphorylation state is needed.

The present results have several implications for T cell function. T cells are electrically nonexcitable; thus they experience relatively small changes in membrane potential within a normal range of about −50 to −70 mV. The relevance of changes in channel activity that occur only at nonphysiological, positive voltages (e.g., where G_{max} is measured) is questionable. An important result of our work is that large changes in channel activity occur in the normal membrane potential range. This is entirely consistent with our previous study of cAMP elevation in intact human T cells (25), wherein single channel activity increased in nonclamped cells (i.e., at their natural potential). From studies reporting a decrease in Kv1.3 current in response to cAMP elevation, the link between channel activity and T cell function (21) was apparently conserved, inasmuch as the majority of studies show inhibition of cell function by cAMP (16). Most treatments that decrease Kv1.3 (including a wide variety of channel blockers) decrease cell function, most notably proliferation and cell cytotoxic killing (21). However, our studies appear to break that link, in that cAMP upregulated Kv1.3 activity. We favor the interpretation that cAMP elevation inhibits T cell function through means other than Kv1.3 activity. One signal transduction step that is clearly involved is the cAMP-dependent downregulation of IL-2 gene transcription, an essential step in T cell activation (16). Another possibility is that the outcome depends on when and by how much cAMP is elevated; small elevations have been reported to enhance lymphocyte function (16). In this case, upregulation of Kv1.3 by phosphorylation would be expected to help maintain a large negative membrane potential to facilitate the Ca^{2+} entry that is necessary for T cell activation.

We are grateful to G. Sakellaropoulos for expert technical assistance and to Dr. P. S. Pennefather for helpful comments on the manuscript.

This study was supported by a Medical Research Council of Canada Grant (to L. Schlichter) and an Ontario Graduate Scholarship (to I. Chung).

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Received 14 January 1997; accepted in final form 9 April 1997.

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