A forskolin-activated Cl^- current in mouse mandibular duct cells

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Dinudom, A., P. Komwatana, J. A. Young, and D. I. Cook. A forskolin-activated Cl^- current in mouse mandibular duct cells. *Am. J. Physiol.* 268 (*Gastrointest. Liver Physiol.* 31): G806–G812, 1995.—We have previously shown that unstimulated granular duct cells of mouse mandibular gland contain a hyperpolarization-activated Cl^- conductance with characteristics resembling the hyperpolarization-activated volume-sensitive Cl^- channel (ClC-2). We now show that stimulation of these cells with forskolin, but not 1,9-dideoxyforskolin, activates a second whole cell Cl^- conductance with properties resembling the cystic fibrosis transmembrane conductance regulator (CFTR). This conductance has a linear current-voltage relation and is not voltage activated. Its anion permeability sequence is Br^- (1.96) > NO_3^- (1.36) > Cl^- (1) > I^- (0.44), and its conductance sequence is Cl^- (1) > NO_3^- (0.66) > Br^- (0.34) > I^- (0.21). The current carried by this conductance is attenuated 65% by 1 mmol/l diphenylamine-2-carboxylate but is not affected by 0.1 mmol/l 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid or 0.1 mmol/l glibenclamide. The current can be activated by norepinephrine (1 μmol/l), evidently acting via β-adrenergic receptors, since the effect of norepinephrine is inhibited by propranolol (1 μmol/l). We conclude that this adrenergically evoked conductance is due to CFTR, which has previously been shown to be expressed in salivary duct cells, and suggest that it may form part of the mechanism by which β-adrenergic agonists modulate NaCl absorption by salivary ducts.

cystic fibrosis transmembrane conductance regulator; chloride channel blockers; intralobular ducts; mouse mandibular glands

THE PRIMARY SALIVA PRODUCED by the secretory endpieces of the mandibular glands in mouse, rat, and rabbit is modified by electrolyte transport processes in the excurrent duct system, resulting in the formation of a hypotonic final saliva that is poor in NaCl (40). Perfusion studies carried out on the main excretory ducts of the mandibular glands of the rat and rabbit have suggested that ductal NaCl absorption is due to the presence of Cl^- channels (2, 3, 5, 14, 37) and amiloride-sensitive Na^+ channels in the luminal membranes of the duct cells (6, 25, 26, 32, 40). Furthermore, in situ hybridization studies have shown that the cystic fibrosis transmembrane conductance regulator (CFTR) is expressed in salivary ducts (39). Because the anion selectivity sequence of CFTR (1) is similar to that of the Cl^- conductance described in the rabbit mandibular duct (14), we were tempted to speculate that the Cl^- conductance in salivary ducts, similar to that in sweat ducts (31), is attributable to the presence of CFTR. Until now, however, none of these predictions has been tested electrophysiologically.

The intralobular (striated) duct cells of many rodent mandibular glands contain secretion granules in large numbers [hence the name granular ducts (41)], the presence of which enables them to be easily distinguished from the cells of the secretory endpieces. Taking advantage of this property, we have used patch-clamp techniques to investigate the ionic conductances in the granular duct cells of the mandibular glands of male mice. As predicted from the earlier perfusion studies, we have found an amiloride-inhibitable Na^+ conductance (11, 12) and a Cl^- conductance (11). This Cl^- conductance has an anion selectivity sequence (Cl^- = Br^- > I^- > NO_3^-; Ref. 11) similar to that of the Cl^- conductance in the rabbit mandibular main duct (14) and similar to that of CFTR. It is, however, activated by hyperpolarization of the membrane potential, and detailed examination of its properties suggested that it is probably carried by a hyperpolarization-activated volume-sensitive Cl^- channel similar to ClC-2 (28), rather than by CFTR.

In the present study, we show that duct cells contain a second Cl^- conductance that is activated by forskolin and by norepinephrine (acting via β-adrenergic receptors) and has properties similar to those of CFTR.

METHODS

Mice were killed by cervical dislocation. The mandibular glands were removed, finely chopped, and incubated in a physiological salt solution containing 50 U/ml collagenase (Worthington type IV, Freehold, NJ) for 40 min with intermittent trituration, until single cells were liberated. These were then washed and suspended in a Na^+-rich solution composed of (in mmol/l) 145 NaCl, 5.5 KCl, 1 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, Na-*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Na-HEPES) (7.5), H-HEPES (7.5), and glucose (10); the pH was adjusted to 7.4 with NaOH. The pipettes were filled with a K^+-rich solution containing (in mmol/l) 80 KCl, 70 K-glutamate, 1 $MgCl_2$, 10 H-HEPES, 5 K_2-ATP, 10 glucose, and 5 ethylene glycol-bis(β-aminoethylether)-*N,N,N*′,*N*′-tetraacetic acid (EGTA); the pH was adjusted to 7.2 with KOH. The Cl^- concentration in the pipette solution was chosen so as to minimize the amiloride-sensitive Na^+ current and the hyperpolarization-activated Cl^- current demonstrable in these cells, since the sizes of these currents depend on the pipette Cl^- concentration (11). For experiments investigating the relative permeability of the Cl^- conductance to various anions, the bath solution used was the same as the standard bath solution, except that 145 mmol/l NaCl was replaced by an equimolar amount of the test salt.

HEPES, EGTA, K_2-ATP, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), forskolin, 1,9-dideoxyforskolin, and norepinephrine were obtained from Sigma (St. Louis, MO), glibenclamide and propranolol from Research Biochemicals (Natick, MA), and diphenylamine-2-carboxylate (DPC) from Aldrich (Milwaukee, WI).

Standard patch-clamp techniques were used (16). Patch-clamp pipettes were pulled from borosilicate microhematocrit tubes (Modulohm, Hevik, Denmark) so as to have resistances of 1–3 MΩ. The reference electrode was a Ag-AgCl pellet connected to the bath by a bridge filled with agar (10 mg/ml) dissolved in the standard NaCl-rich solution. All potential differences were corrected for junction potentials as appropriate (4, 11).

An Axopatch-1D patch-clamp amplifier (Axon Instruments, Foster City, CA) was used to measure whole cell currents. A MacLab-4 data acquisition interface (AD Instruments, Sydney, Australia) attached to a Macintosh IIci computer was used to generate command voltages and sample the whole cell currents.

In some experiments, we used the "slow" whole cell technique (19), in which nystatin (100 μg/ml) is included in the pipette solution. The series resistance achieved with the use of this technique was 28.2 ± 2.6 MΩ (n = 34), which was electronically compensated. The results we obtained did not differ from those we obtained using the more usual "fast" whole cell technique, except that the percentage of cells in which we observed the forskolin-activated current and the magnitude of the current were increased. Thus, when we used the slow (nystatin) whole cell technique, 43 of 48 cells studied had forskolin-activated currents, whereas only 21 of 74 cells studied using the fast whole cell technique responded to forskolin. With the K^+-rich pipette solution and the Na^+-rich bath solution, the mean forskolin-activated current at +80 mV recorded using the slow (nystatin) technique was 1.62 ± 0.19 nA (n = 8), and using the fast whole cell technique it was 0.42 ± 0.02 nA (n = 4). This difference was presumably due to the elimination of washout of intracellular mediators with the nystatin technique. Of the results presented here, data in Figs. 1 and 4 were obtained using the fast whole cell technique, and data in Figs. 2 and 3 were obtained using the slow (nystatin) whole cell technique.

In the anion selectivity experiments, it was not possible to use the slow (nystatin) whole cell technique, because control over the intracellular anion concentration was not adequate, since nystatin has too low a permeability to anions. Consequently, to minimize the effects of washout of the forskolin-activated current, we followed the technique successfully used by Gray and co-workers (15) to determine the anion selectivity of the forskolin-activated Cl^- current in pancreatic duct cells. In this technique the cells are stimulated by exposure to forskolin before formation of the whole cell configuration. Forskolin exposure is then continued throughout the experiment, while the bath solution is changed from the control

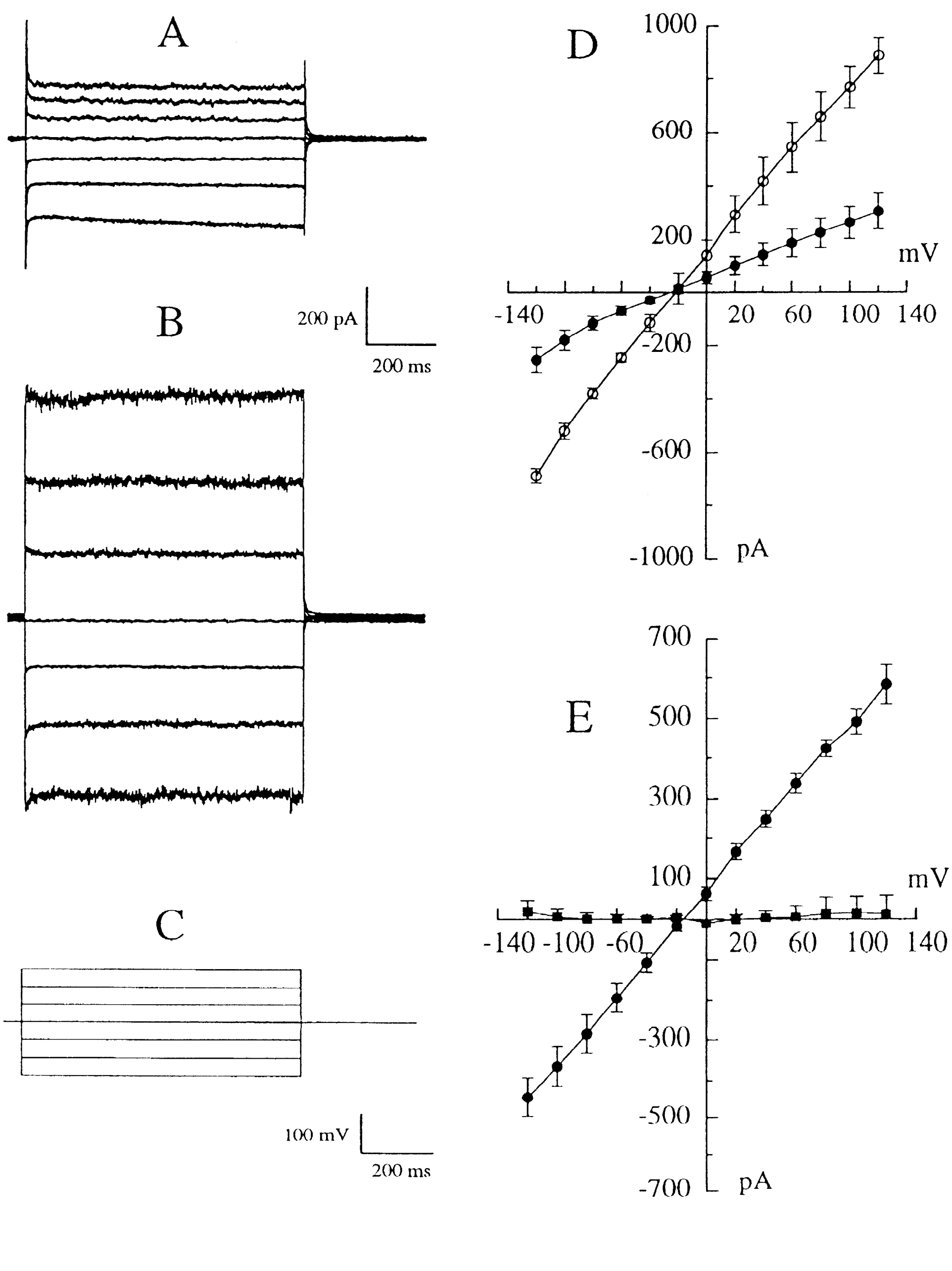

Fig. 1. Whole cell voltage-clamp records of a granular duct cell before (A) and 5 min after (B) treatment with extracellular forskolin (10 μmol/l). C: voltage-clamp protocol. D: mean current-voltage (I-V) relation before (●) and after (○) forskolin application. E: ●, I-V relation of the forskolin-activated current (n = 4), obtained by subtraction of the whole cell I-V data points in D. ■, Mean I-V relation of the current activated by 10 μmol/l dideoxyforskolin (n = 3).

Cl^--rich solution to one in which the Cl^- is replaced by the anion of interest. Finally, DPC (1 mmol/l) was added to the bath to check that the forskolin-activated Cl^- current was the dominant current. In these experiments, K^+ in the pipette solution was replaced by Na^+. This had two effects. First, it eliminated interfering K^+ currents. Second, the high intracellular Na^+ inhibited the amiloride-sensitive Na^+ current that is present in these cells, so as to reduce its interference with the results (12, 27).

In Figs. 1–4, outward current, defined as positive charge leaving the pipette, is depicted as an upward deflection, and the potential differences are expressed as the potential of the pipette with respect to the bath. All experiments were performed at about 20°C. Results are presented as means ± SE, with the number of cells tested (n) given in parentheses. Statistical significance was assessed using paired or unpaired Student's t-tests at a probability level of $P < 0.05$.

RESULTS

Figure 1 shows representative whole cell current recordings (Fig. 1*A*) and the mean current-voltage (*I-V*) relation (Fig. 1*D*) of granular duct cells studied with the K^+-rich solution in the pipette and the NaCl-rich solution in the bath. The *I-V* relation had a slope conductance of 2.27 ± 0.37 nS (n = 8) and a reversal potential (E_R) of −15.9 ± 6.4 mV (n = 8). (E_R was calculated by averaging the E_R of the individual experiments and hence is close to but does not exactly agree with that of the mean *I-V* relation in Fig. 1*D*). At negative pipette potentials, the whole cell current showed little slow inward current activation (Fig. 1*A*), confirming, as we expected, that the use of a pipette solution containing 70 mmol/l Cl^- suppressed the hyperpolarization-activated Cl^- current (11). After adding 10 μmol/l forskolin to the bath solution, however, we observed a marked increase in the whole cell current, which was not voltage activated (Fig. 1*B*). The *I-V* relation of the cells after forskolin addition (Fig. 1*D*) was close to linear with a slope conductance of 6.54 ± 0.55 nS (n = 4) and E_R of −19.9 ± 4.0 mV (n = 4). Figure 1*E* shows the *I-V* relation of the forskolin-activated current calculated as the difference between the whole cell currents measured before and 2–5 min after forskolin addition. The *I-V* relation was linear with a slope conductance of 4.14 ± 0.34 nS (n = 4) and E_R of −15.9 ± 3.1 mV (n = 4), a value not significantly different from that predicted for a Cl^--selective conductance (−16.1 mV).

Addition to the bath solution of 10 μmol/l 1,9-dideoxyforskolin, a forskolin analogue that does not activate adenylate cyclase (34), caused no changes in the whole cell *I-V* relation (n = 3; Fig. 1*E*).

We also determined the blocker sensitivity of the forskolin-activated current. As shown in Fig. 2, neither DIDS (0.3 mmol/l) nor glibenclamide (0.1 mmol/l) had a significant effect on the magnitude of the forskolin-activated conductance. DPC (1.0 mmol/l), however, significantly reduced ($P < 0.031$, unpaired t-test) the forskolin-activated conductance from 11.89 ± 2.21 nS (n = 4) to 4.20 ± 1.60 nS (n = 4). In these experiments we used the slow (nystatin) whole cell technique to increase the percentage of cells in which we could

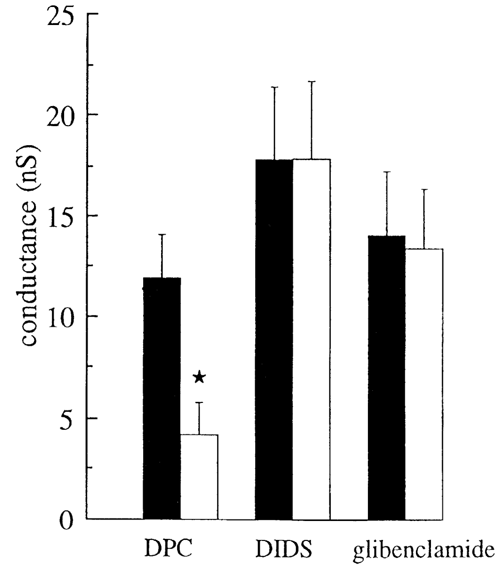

Fig. 2. Effects of addition of diphenylamine-2-carboxylate (DPC; 1.0 mmol/l), DIDS (0.1 mmol/l), and glibenclamide (0.1 mmol/l) on forskolin-activated Cl^- conductance (open bars). Each solid bar summarizes control experiments performed on the same day as inhibitor studies. ★ Significant difference ($P < 0.05$) from control.

demonstrate a forskolin-activated current (see METHODS).

To determine the ion selectivity of the forskolin-activated current, we used the fast whole cell technique to ensure adequate control of the cytosolic anion composition. To reduce the effects of washout, we stimulated the cells with forskolin before formation of the whole cell configuration (see METHODS). We first investigated whether the whole cell conductance in the presence of forskolin was dominated by the forskolin-activated Cl^- current. The conductance of the whole cell current in the presence of forskolin was 9.04 ± 0.82 nS (n = 4), and its E_R was −15.05 ± 2.53 mV (n = 4), which was not significantly different from the Nernst potential for Cl^- (−16.1 mV). The addition of 1 mmol/l DPC to the bath solution (which inhibits the forskolin-activated Cl^- current by 65%) reduced the whole cell conductance in the presence of forskolin by 73% to 2.41 ± 0.67 pS (n = 4) without affecting E_R. When we repeated these experiments with the bathing solution diluted by two-thirds with isotonic mannitol, we found that the E_R of the whole cell current was +3.25 ± 3.49 mV (n = 6). This E_R was smaller than that predicted for an anion-selective conductance (+11 mV), which suggested that the whole cell conductance in the presence of forskolin has a small but significant permeability to Na^+ (∼16% of its permeability to Cl^-). The addition of 1 mmol/l DPC to the diluted bathing solution inhibited the whole cell current by 63.4 ± 7.4% (n = 6). The E_R of this DPC-sensitive component of the current, +11.1 ± 3.74 mV (n = 6), was not significantly different from the Nernst potential for Cl^-. Thus the whole cell current in the presence of forskolin has a small contribution from another current (possibly carried by Na^+), which can be eliminated by examining the DPC-sensitive component of the current, and so in the ion selectivity studies that follow we have

Table 1. *Anion selectivity of the whole cell currents in single granular duct cells stimulated with forskolin*

Anion	E_R, mV	Conductance, nS	P_x/P_{Cl}	G_x/G_{Cl}
Cl^-	-15.06 ± 2.53 (4)	9.04 ± 0.82 (4)	1.00	1.00
Br^-	-31.50 ± 4.02 (5)	3.12 ± 0.57 (5)	1.96	0.34
NO_3^-	-22.54 ± 4.28 (4)	5.99 ± 1.19 (4)	1.36	0.66
I^-	4.14 ± 3.29 (5)	1.88 ± 0.36 (5)	0.44	0.21

Values are means ± SE, no. of observations is given in parentheses. Conductance and relative conductance ratios (G_x/G_{Cl}) were measured at positive pipette potentials (0–120 mV). Relative permeabilities (P_x/P_{Cl}) were calculated from the reversal potentials (E_R).

examined the anion selectivity of the DPC-sensitive component of the whole cell current.

The anion selectivity results we obtained using this technique are summarized in Table 1 and in Fig. 3. From the shifts in E_R, we can calculate from the Goldman equation that the permeability sequence relative to Cl^- of the DPC-sensitive component of the forskolin-activated current was Br^- (1.96) > NO_3^- (1.36) > Cl^- (1) > I^- (0.44). The conductance sequence relative to Cl^- was Cl^- (1) > NO_3^- (0.66) > Br^- (0.34) > I^- (0.21). Essentially identical results were obtained when we analyzed the anion selectivity of the entire whole cell current before the addition of DPC (data not shown).

Salivary ducts have been reported to respond to β-adrenergic stimulation with an increased intracellular adenosine 3′,5′-cyclic monophosphate (cAMP) concentration (13) and increased transepithelial Na^+ transport (9, 29, 33), and they respond to α-adrenergic stimulation with an increased intracellular free Ca^{2+} (10) and increased secretion of proteins such as epidermal growth factor (17, 36). We thus used the slow whole cell technique to examine the effects of norepinephrine on the Cl^- current in mouse granular duct cells. As shown in Fig. 4, the addition of 1 μmol/l norepinephrine to the bathing solution evoked a current that was not voltage activated, had a linear *I-V* relation with a slope conductance of 16.92 ± 1.55 nS (n = 13), and had a zero current potential of −11.97 ± 1.88 mV (n = 13), close to the

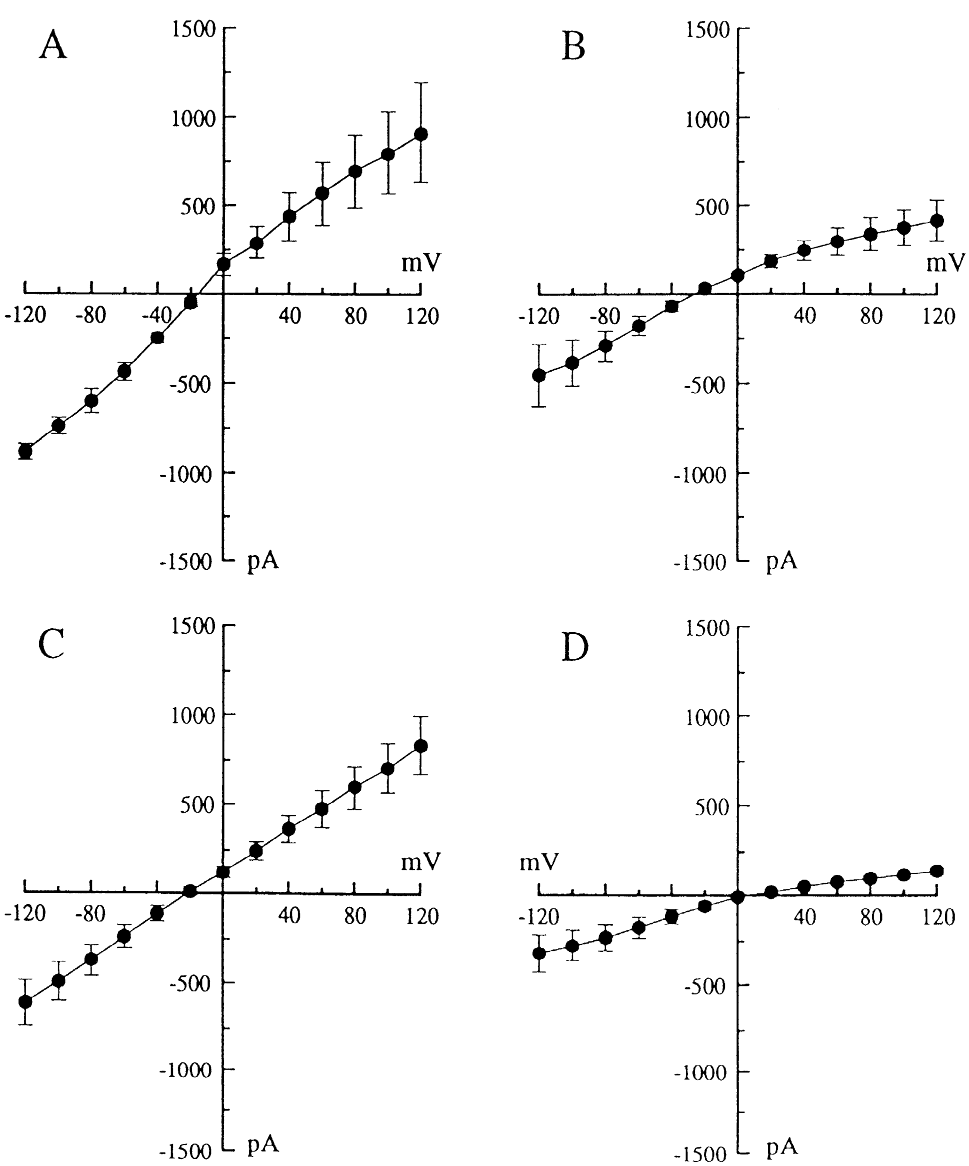

Fig. 3. Mean *I-V* relations of the DPC-sensitive component of the forskolin-activated current in granular duct cells obtained with bath solutions containing Cl^- (n = 4; *A*), Br^- (n = 4; *B*), NO_3^- (n = 4; *C*), or I^- (n = 5; *D*).

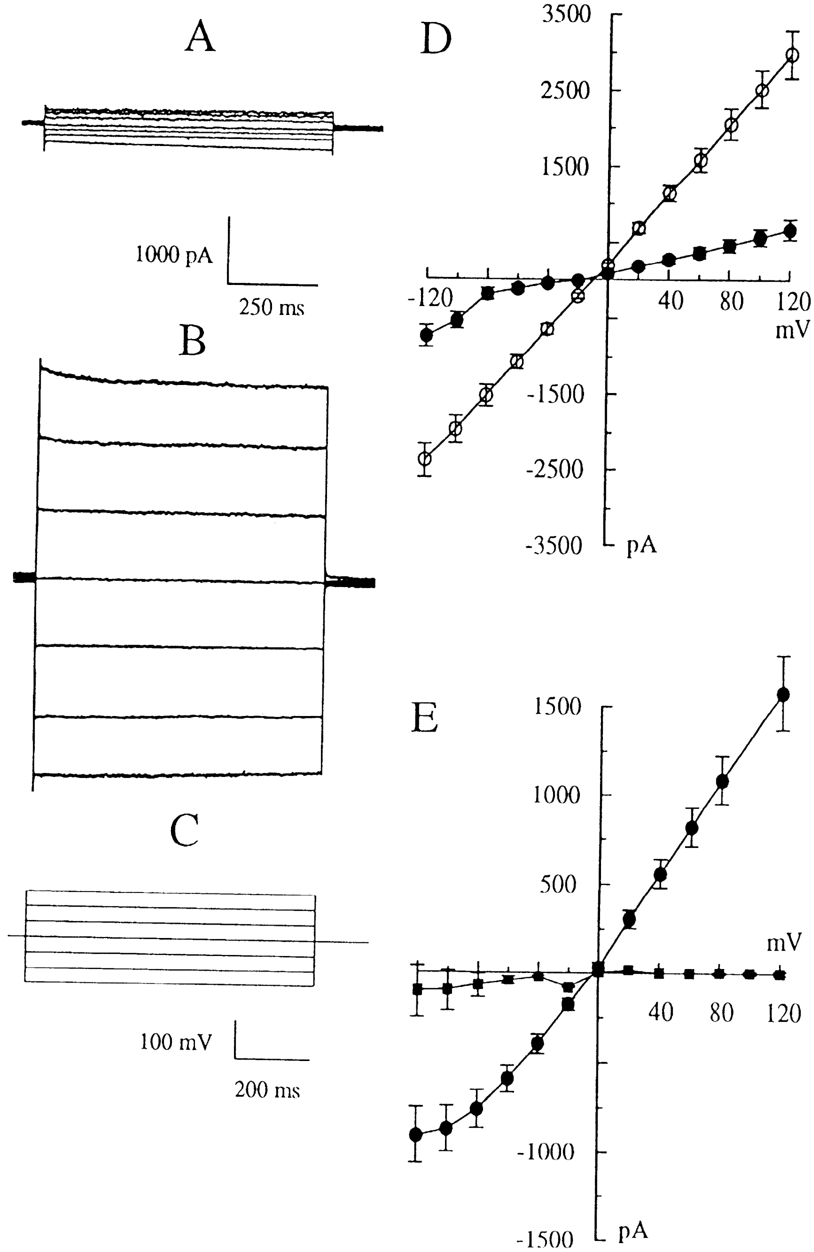

Fig. 4. Representative whole cell recordings before (*A*) and 5 min after (*B*) addition of 1 μmol/l norepinephrine to the bathing solution. *C*: voltage-clamp protocol. *D*: mean *I-V* relation of the norepinephrine-activated whole cell current before (●, $n = 4$) and after (○, $n = 4$) addition of 1 μmol/l norepinephrine. *E*: mean *I-V* relation of norepinephrine-activated current calculated by subtracting the current after norepinephrine addition from the current before norepinephrine addition (●, $n = 13$). Filled squares in *E* show mean *I-V* relation of current activated by 1 μmol/l norepinephrine in presence of 1 μmol/l propranolol ($n = 4$).

Nernst potential for Cl^- (−16.1 mV). Dilution of the bath solution by two-thirds with isotonic mannitol shifted the E_R of the norepinephrine-activated current to +18.86 ± 1.89 mV ($n = 3$). This shift is that expected for a current that is selective for Cl^- (+27 mV). DPC (1 mmol/l) inhibited the norepinephrine-activated current by 50.63 ± 6.58% ($n = 5$). The E_R of the DPC-sensitive component of the current was −10.23 ± 2.30 mV ($n = 5$), which was close to the Cl^- Nernst potential (−16 mV). The norepinephrine-activated current was not inhibited by DIDS (100 μmol/l). The properties of the norepinephrine-activated current are thus indistinguishable from those of the forskolin-activated current. The greater magnitude of norepinephrine-activated current is probably due to the use of the slow whole cell technique in these experiments. Prior addition of the β-adrenergic antagonist propranolol (1 μmol/l) to the bathing solution totally prevented the effect of norepinephrine ($n = 4$; Fig. 4*E*).

DISCUSSION

cAMP-activated Cl^- currents have been described previously in many epithelial cells, including pancreatic ducts (15), sweat ducts (31), epididymis (20), and cultured respiratory epithelia (30). These Cl^- currents are believed to be carried by CFTR. In the mandibular glands of rodents, at least in the rat, CFTR is expressed in the gland ductal system (39), although, until now, no Cl^- conductance attributable to CFTR has been described in the duct cells.

In this study we report the presence of a forskolin-activated Cl^- conductance in mouse granular duct cells. This Cl^- conductance is not activated by 1,9-dideoxyforskolin but is activated by norepinephrine acting via β-adrenergic receptors. It thus seems likely that the conductance is activated by cAMP resulting from increased adenylate cyclase activity. Like CFTR, the conductance is not voltage activated (1), and its sensitivity to blockers is similar to that described for CFTR in other tissues, being blocked by DPC and unaffected by DIDS (1). Unlike CFTR in airway epithelia, which has been reported to be sensitive to blockers of ATP-sensitive K^+ channels such as glibenclamide (35), the forskolin-activated Cl^- conductance seen in salivary duct cells is not blocked by glibenclamide. In this respect, it resembles both the cAMP-activated Cl^- conductance in T84 cells, which has been found to be only very weakly sensitive to glibenclamide (18), and the apical Cl^- conductance in *Necturus* gallbladder (8). The anion selectivity of the forskolin-activated current in mouse granular duct cells is similar to the anion selectivity of cAMP-activated Cl^- currents reported in other tissues (1, 15), in which CFTR has been found to be more permeable to extracellular Br^- and Cl^- than to extracellular I^-. Interestingly, CFTR has been found to be more permeable to intracellular I^- than to intracellular Cl^- (38). We have no data on whether this is also true for the forskolin-activated Cl^- current in mouse granular duct cells.

The properties of the forskolin-activated Cl^- current distinguish it clearly from other anion conductances reported in salivary epithelia. Its lack of voltage activation, insensitivity to DIDS, and anion selectivity sequence distinguish it from the Ca^{2+}-activated Cl^- conductance in salivary endpiece cells (21), which is activated by depolarization, blocked by DIDS, and has the selectivity sequence $I^- > Cl^- > Br^-$. It can be distinguished from the outwardly rectifying Cl^- channel that has been reported in the HSD human submandibular gland duct cell line (22), because that channel is blocked by DIDS and does not select among I^-, Br^-, or Cl^-. Finally, it can be distinguished from the ClC-2-like conductance observed in unstimulated mouse mandibular granular ducts, despite the similarity in their anion selectivities, because the ClC-2-like conductance is strongly hyperpolarization activated and is not blocked by DPC (28).

In many of the cells in which CFTR has been described, it has been located in the apical membrane, although in sweat ducts it is found in both the apical and the basolateral membranes (31). As yet we do not have data to determine whether the β-adrenergically activated Cl^- conductance described here is in the apical membrane, the basolateral membrane, or in both.

Studies on the effects of β-adrenergic stimulation on electrolyte transport by salivary ducts indicate that low concentrations of β-adrenergic agonists stimulate NaCl absorption in rat and rabbit mandibular salivary ducts (9, 23, 24, 29, 33) and increase cAMP in duct cells (13). It is thus surprising that the only study on the effects of forskolin on ductal transport in isolated perfused rabbit mandibular glands (7) showed that forskolin reduces the ductal NaCl transport evoked by acetylcholine. The explanation for this is unclear.

Because the Cl^- current described here, which is activated by forskolin and β-adrenergic agonists, is not active in resting cells, it may be involved in the regulation of ductal transport by β-adrenergic agonists. There are two possible mechanisms by which the forskolin-activated Cl^- conductance could influence NaCl absorption. In salivary ducts, a component of NaCl absorption, the magnitude of which is species dependent, is attributable to electrogenic influx of Na^+ across the apical membrane through amiloride-sensitive Na^+ channels (40). This electrogenic Na^+ movement is balanced by electrogenic flow of Cl^- through Cl^- channels in the apical and basolateral membranes (2, 5, 14, 37), thus providing a mechanism by which increasing the Cl^- conductance could increase the rate of Na^+ transport. A second mechanism for linking NaCl transport to the Cl^- conductance is provided by the dependence of the Na^+ conductance of granular duct cells on cytosolic Cl^- (11). Increasing the Cl^- conductance could inhibit or activate the ductal transport rate, depending on whether cytosolic Cl^- is below or above electrochemical equilibrium. The potentially opposing effects of these two mechanisms may explain the reported differences in the actions of β-adrenergic agonists and of forskolin on ductal function.

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