Separate Cl^- conductances activated by cAMP and Ca^{2+} in Cl^- -secreting epithelial cells

(Cl^- secretion/cultured cells/whole-cell patch clamp)

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ABSTRACT We studied the cAMP- and Ca^{2+} -activated secretory Cl^- conductances in the Cl^- -secreting colonic epithelial cell line T84 using the whole-cell patch-clamp technique. Cl^- and K^+ currents were measured under voltage clamp. Forskolin or cAMP increased Cl^- current 2–15 times with no change in K^+ current. The current–voltage relation for cAMP-activated Cl^- current was linear from -100 to $+100$ mV and showed no time-dependent changes in current during voltage pulses. Ca^{2+} ionophores or increased pipette Ca^{2+} increased both Cl^- and K^+ currents 2–30 times. The Ca^{2+} -activated Cl^- current was outwardly rectified, activated during depolarizing voltage pulses, and inactivated during hyperpolarizing voltage pulses. Addition of ionophore after forskolin further increased Cl^- conductance 1.5–5 times, and the current took on the time-dependent characteristics of that stimulated by Ca^{2+} . Thus, cAMP and Ca^{2+} activate Cl^- conductances with different properties, implying that these second messengers activate different Cl^- channels or that they induce different conductive and kinetic states in the same Cl^- channel.

Secretion of salt and water across many epithelia is driven by a secondary active Cl^- transport mechanism in which Cl^- leaves the cell across the apical membranes by diffusion (1). Alterations in apical Cl^- conductance, elicited by hormones and neurotransmitters, is a principal control point determining Cl^- secretion rate (2, 3). Signal-transduction mechanisms involving both cAMP and Ca^{2+} mediate the hormonal control of the secretory Cl^- conductance (4); however, the components of these regulatory pathways have only recently lent themselves to detailed investigation.

The T84 colonic tumor cell line expresses mechanisms for cAMP- and Ca^{2+} -mediated neurohumoral control of NaCl secretion (5). Single-channel patch-clamp studies of T84 cells have identified an agonist-activated Cl^- channel, implying that it underlies the increase in apical Cl^- conductance elicited by secretagogues (6). In this study, we used the whole-cell patch-clamp technique to study the regulated Cl^- conductances of T84 cells. This method allows the examination of macroscopic Cl^- conductance in isolation from other conductances, while retaining a more intact intracellular environment than that offered by excised membrane patches. We identified two secretagogue-induced Cl^- conductances with different properties: One Cl^- conductance is activated by cAMP, and the other is activated by Ca^{2+} .

MATERIALS AND METHODS

Cell Culture. T84 cells were cultured as described (6). Cells were used 1–3 days after plating on collagen-coating coverslips. Individual cells and cells in islands were studied, but the cells in small groups were more frequently responsive to secretagogues than were isolated cells. Voltage-clamp arti-

facts arising from cell-to-cell coupling in islands of cells were insignificant, as evidenced by the small capacitive transients remaining after capacitance compensation.

Whole-Cell Current Recordings. Standard whole-cell tight-seal patch-clamp techniques were used (7, 8). The cells were maintained at 37°C by a constant-temperature microscope stage modified to hold the bath chamber and offer low electrical noise (model 5001, Micro Devices, Jenkintown, PA). At this temperature, the responsiveness of the cells to secretagogues, particularly to cAMP-dependent agonists, was improved.

Increases in Cl^- and K^+ conductances are the major electrical events detected during stimulation of Cl^- secretion (1). Accordingly, bath–pipette ion gradients were chosen so that transmembrane Cl^- and K^+ currents could be monitored independently at clamp voltages equal to the reversal potentials for these ions. The pipette solution was 115 mM $\text{KCl}/25$ mM *N*-methyl-D-glucamine (NMDG) glutamate/0.5 mM EGTA/0.19 mM CaCl_2 (100 nM free Ca^{2+})/2 mM MgCl_2 /2 mM $\text{Na}_2\text{ATP}/0.05$ mM $\text{Na}_3\text{GTP}/5$ mM Hepes, adjusted to pH 7.2 with KOH. The bath solution was 115 mM $\text{NaCl}/40$ mM NMDG glutamate/5 mM potassium glutamate/2 mM MgCl_2 /1 mM CaCl_2 /5 mM Hepes, adjusted to pH 7.2 with NaOH. Bath Na^+ or Cl^- concentrations were reduced by substituting NMDG chloride or sodium glutamate for NaCl . When Na^+ and K^+ -free solutions were used, Na^+ and K^+ were replaced by NMDG $^+$, and Cl^- was reduced by replacing Cl^- by glutamate.

During whole-cell recording, the membrane potential was clamped alternately to three different test voltages, each for 500-msec duration. Computer-controlled voltage-clamp protocols (pClamp, Axon Instruments, Burlingame, CA) were used to generate current–voltage (I – V) relations when the transmembrane currents were relatively stable by stepping the clamp voltage between -100 and $+100$ mV at 20-mV intervals. Instantaneous I – V relations were constructed from currents recorded 6 msec after a voltage step, thus avoiding capacitive transients.

Pharmacological Agents. 8-(4-Chlorophenylthio)adenosine 3',5'-cyclic monophosphate (cpt-cAMP) and Ca^{2+} ionophore A23187 were obtained from Sigma; forskolin, 1,9-dideoxy-forskolin, and ionomycin were from Calbiochem. Drugs were solubilized in ethanol stock solutions, except for A23187, which was dissolved in dimethyl sulfoxide. Ethanol or dimethyl sulfoxide concentrations never exceeded 0.1% and had no effect on whole-cell currents.

RESULTS

cAMP-Activated Whole-Cell Currents. Whole-cell currents in unstimulated T84 cells were low ($< \pm 100$ pA at ± 60 mV)

Abbreviations: $G_{\text{Cl}}^{\text{cAMP}}$, cAMP-activated Cl^- conductance; $G_{\text{Cl}}^{\text{Ca}}$, Ca^{2+} -activated Cl^- conductance; $G_{\text{Cl}}^{\text{swell}}$, swelling-activated Cl^- b;0 conductance; NMDG, *N*-methyl-D-glucamine; cpt-cAMP, 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate; I – V , current–voltage.

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and typically remained stable under the conditions that prevented cell swelling (see *Methods* and ref. 8). In 22 of 41 cells (54%), forskolin (2–10 μM) produced rapid and sustained increases in cell currents that were entirely reversible with washout of the bath (see Fig. 1A). Prompt onset and reversibility of action (9) were considered essential criteria for identifying agonist-specific effects on cell currents. Of 19 cells that failed to respond rapidly and reversibly to forskolin, 13 showed no change in current, and 6 showed slow, steady increases in current that were not reversed by washing the bath and could be due to leak. The reason for the lack of responsiveness of all cells to forskolin is not apparent but could be related to degree of differentiation because cells growing in islands had a much greater frequency of response than isolated cells.

In responding cells, forskolin stimulated the whole-cell current at -84 mV (E_K) without changing currents at 0 mV (E_{Cl}), suggesting that forskolin increased membrane Cl^- conductance without altering conductances to K^+ or Na^+ (Fig. 1A). Magnitude of the forskolin-induced current varied between 2 and 15 times basal values. Stimulated currents were maintained for at least 30 min, indicating little rundown in the action of forskolin with time. The response to five or six repetitive forskolin challenges also showed minimal rundown. 1,9-Dideoxyforskolin, an analogue that does not activate adenylate cyclase (9), had no effect on whole-cell currents in forskolin-responsive cells (5 μM , $n = 3$). Currents evoked by cpt-cAMP (200–800 μM) were indistinguishable from those activated by forskolin ($n = 14$, see Fig. 1B). Thus, adenylate cyclase activation and increased cAMP mediate forskolin action. The forskolin-stimulated $I-V$ relation is essentially linear over the voltage range -100 to $+100\text{ mV}$ (Fig. 1C). The reversal potential was near 0 mV , as expected for a Cl^- current. There was no time dependence of the currents during hyperpolarizing or depolarizing voltage pulses (Fig. 1D).

Increases in inward current at -84 mV , with no change in current at 0 mV , could also result from activation of a nonselective cation conductance, which would have a reversal potential near 0 mV with these bath and pipette solutions. Nonselective cation channels are observed in single-channel records from these cells (6). Ion-replacement studies were done to assess this possibility. The forskolin-stimulated component of current was isolated by subtracting the basal current measured before stimulation and is plotted (Fig. 2). In the normal NaCl bath (circles) the forskolin-activated $I-V$ relation is linear; reversal potential is near 0 mV . Reducing bath Cl^- to 6 mM (triangles) decreased outward currents and shifted the reversal potential to $+68\text{ mV}$, a value near the altered E_{Cl} of $+80\text{ mV}$ (Fig. 2A). Replacement of bath Na^+ by NMDG (squares) did not significantly alter the $I-V$ relations (Fig. 2B). These findings indicate that forskolin stimulates a Cl^- -selective current and rule out the possibility that the current monitored at E_K is from cation flow.

Ca^{2+} -Activated Whole-Cell Currents. The Ca^{2+} ionophores, A23187 or ionomycin ($1\text{--}10\text{ }\mu\text{M}$), when added to bath solutions containing 1 mM Ca^{2+} , increased whole-cell currents in 42 of 52 cells (81%). A typical response is shown in Fig. 3. In this experiment, the membrane potential was pulsed to 0 mV to measure outward K^+ current, to -84 mV to measure inward Cl^- current, and to $+40\text{ mV}$ where outward currents from both K^+ and Cl^- are recorded.

In 41 of 42 responding cells, Ca^{2+} ionophore initially increased outward K^+ current at 0 mV , which typically declined within seconds to minutes, even in the continued presence of ionophore and 1 mM Ca^{2+} (Fig. 3). The reason for this transient response of the K^+ current is not known but apparently does not represent a "rundown" phenomenon because a similar response is evoked after washout and readdition of ionophore (see below). Closely following the ionophore-stimulated K^+ current was a marked increase in current at -84 mV . Bath Cl^- and Na^+ substitutions showed that the ionophore-induced inward current was from both Cl^-

FIG. 1. cAMP-activated whole-cell currents. Continuous current recordings during stimulation by forskolin (A) and cpt-cAMP (B). Membrane voltage was held at -20 mV and pulsed to 0 mV and -84 mV . Bath solution changes began at arrows. Pulse protocol for determining the $I-V$ relation (-100 to $+100\text{ mV}$) was run at indicated times. (C) Instantaneous $I-V$ relation before (control) and after forskolin stimulation. (D) Overlay of cell currents recorded during an $I-V$ pulse protocol after forskolin stimulation.

FIG. 2. Cl^- dependence of forskolin-activated whole-cell current (obtained by subtracting currents before and after stimulation). (A) Instantaneous $I-V$ in high- Cl^- (120 mM, NaCl) and low- Cl^- (6 mM, sodium glutamate) baths. (B) Instantaneous $I-V$ in high- Na^+ (115 mM, NaCl) and low Na^+ (0 mM, NMDG chloride) baths.

current and nonselective cation current. The ionophore-induced current at -84 mV occurred in 26 of 42 responding cells (63%). These currents remained elevated with ionophore and 1 mM Ca^{2+} in the bath and declined slowly to control levels when the bath was exchanged for a low Ca^{2+} (100 nM), ionophore-free solution (Fig. 3). Ionophore stimulation of whole-cell currents required 1 mM Ca^{2+} in the bath. Fig. 3 shows that whole-cell currents were not altered by 2.5

FIG. 3. Ca^{2+} ionophore activation of whole-cell currents and dependence on bath Ca^{2+} concentration. Membrane voltage was held at 0 mV and pulsed to +40 mV and -84 mV. Lower trace is a continuation of upper trace.

μM ionomycin in the presence of 1 μM Ca^{2+} but were rapidly stimulated when bath Ca^{2+} concentration was elevated subsequently to 1 mM. Ionophore stimulation was reversed within 3–15 min in low Ca^{2+} media (25 nM–1 μM), and cells could be stimulated several times with no diminution in the current responses.

Ionophore-stimulated Cl^- currents were studied independent of cation currents by replacing pipette and bath K^+ and Na^+ with NMDG. Under these conditions, the ionophores increased inward and outward Cl^- currents at -60 and $+60$ mV with no change in current at 0 mV (Fig. 4A). Fifteen of 20 cells responded with current increases of 2–30 times. Ionophore-induced Cl^- currents were abolished when bath Ca^{2+} was reduced from 1 mM to 25 nM.

The instantaneous $I-V$ relation was outwardly rectified over the voltage range -100 to $+100$ mV (Fig. 4B, closed circles). Reduction of bath Cl^- from 120 to 17 mM by replacement with glutamate confirmed that the ionophore-activated currents were Cl^- selective (triangles). At low Cl^- ,

FIG. 4. Ca^{2+} ionophore-stimulated whole-cell Cl^- currents. Pipette and bath solutions contained 115 mM NMDG chloride. (A) A23187 stimulation. Membrane voltage held at 0 mV and pulsed to +60 mV and -60 mV. (B) Instantaneous $I-V$ relation before (control) and after stimulation of Cl^- current by 5 μM A23187 in high- Cl^- (120 mM) and low- Cl^- (17 mM) baths. (C) Overlay of Cl^- currents recorded during $I-V$ pulse protocol after A23187 stimulation.

in the bath outward currents were nearly eliminated, and the current reversal potential shifted to +46 mV, close to the E_{Cl} of +52 mV.

The ionophore-stimulated Cl^- currents exhibited both voltage- and time-dependent kinetic components (Fig. 4C). Time-dependent activation of currents occurred at depolarizing clamp potentials $>+40$ mV, and time-dependent inactivation of currents occurred at hyperpolarizing clamp potentials <-40 mV. These time-dependent current components would result in even more pronounced outward rectification were the currents recorded at later times in the voltage pulse.

Extent of outward rectification and time dependence of the ionophore-stimulated Cl^- currents varied from cell to cell and with magnitude of current stimulated. As seen in Fig. 4A, currents measured at ± 60 mV showed pronounced outward rectification during the first minute of ionophore activation; however, as current stimulation reached maximum, the difference in current amplitude at ± 60 mV became progressively smaller. Outward rectification of the instantaneous currents decreased, and the currents recorded at depolarizing and hyperpolarizing voltages also became less time dependent at maximal levels of stimulation.

Elevation of pipette free Ca^{2+} concentration from 100 nM to 500 nM also led to the progressive development of Cl^- current (data not shown). This current showed the same outward rectification and time-dependent kinetic behavior as that stimulated by Ca^{2+} ionophores and supports the notion that the ionophore effects are from increased intracellular Ca^{2+} .

Additive Actions of Ca^{2+} and cAMP on Whole-Cell Cl^- Currents. Experiments were done to determine the additivity of the cAMP- and Ca^{2+} -stimulated Cl^- currents. Fig. 5A

FIG. 5. Additive actions of forskolin and ionomycin on Cl^- currents. (A) Continuous recording. Na^+ was removed from bath to eliminate inward nonselective cation current. (B) Detail of forskolin-stimulated whole-cell current (①) and forskolin plus ionomycin-stimulated whole-cell current (②) obtained at times 1 and 2 during the continuous recording shown in A.

shows that addition of forskolin stimulated whole-cell currents 6 times at -84 mV with no change in current at 0 mV. These currents exhibited no time-dependent components (Fig. 5B1). Subsequent replacement of bath Na^+ with NMDG had no effect on cell currents, indicating that the stimulated current was carried by Cl^- . Addition of ionomycin increased the outward K^+ current recorded at 0 mV and doubled the Cl^- current at -84 mV (see Fig. 5A). In seven cells examined, Cl^- conductance was increased between 1.5- and 5-fold by the ionophore after forskolin activation. After ionophore addition, the Cl^- current showed time-dependent inactivation at hyperpolarizing voltages (Fig. 5B2), as observed with ionophore alone. The time-dependence of current at +40 mV is influenced predominantly by the properties of the ionophore-induced K^+ current. Thus, the combined actions of forskolin and ionophore add the time-dependent, Ca^{2+} -stimulated Cl^- current to the time-independent, cAMP-stimulated Cl^- current.

DISCUSSION

The stimulatory effects of receptor-mediated agonists on Cl^- -secreting epithelia are transduced by cAMP- and Ca^{2+} -dependent regulatory pathways (4). Cellular models of Cl^- secretion have featured a single apical membrane Cl^- conductance on which the regulatory influences of cAMP and Ca^{2+} converge. We detected two secretagogue-induced Cl^- conductances in the colonic epithelial cell line T84 that have different biophysical properties. The Cl^- conductance activated by forskolin or cAMP analogues, G_{Cl}^{cAMP} , has a linear I - V relation over the range ± 100 mV, and its currents are not time-dependent. In contrast, the Cl^- conductance stimulated by Ca^{2+} ionophores or increased pipette Ca^{2+} concentrations, G_{Cl}^{Ca} , shows an outwardly rectified I - V relation and time-dependent relaxations of Cl^- current at depolarizing and hyperpolarizing voltages. Furthermore, preliminary experiments on the halide permselectivities of the secretagogue-activated Cl^- conductances indicate that G_{Cl}^{cAMP} has the sequence $Br^- > Cl^- > I^-$, whereas G_{Cl}^{Ca} has the sequence $I^- > Br^- > Cl^-$.^{*} These properties, together with the additive effects of cAMP and Ca^{2+} on whole-cell Cl^- currents, suggest that these agonists activate distinct Cl^- conductances.

We had identified another Cl^- conductance in T84 cells that is activated by cell swelling (9). The instantaneous currents carried by G_{Cl}^{swell} are also outwardly rectified, but this current activates at hyperpolarizing voltages and inactivates at depolarizing voltages, behavior kinetically opposite to the G_{Cl}^{Ca} . Thus, three distinct Cl^- conductances are present in T84 cells, each with different conductive and kinetic properties and each of which responds to a different stimulus.

Agonist-Activated Cl^- Currents in Other Cells. Ca^{2+} -activated Cl^- currents, with voltage and time-dependent properties similar to those in T84 cells, are seen in exocrine cells in response to muscarinic receptor activation (10). The single-channel events that underlie this Ca^{2+} -activated Cl^- conductance were not detected in cell-attached recordings (11), and from noise analysis of whole-cell currents, the Ca^{2+} -activated Cl^- channels were thought to be of low conductance (1–2 pS).

Evidence for cAMP-activated whole-cell Cl^- currents in other secretory epithelial cells is sparse. Forskolin-stimulated Cl^- currents with both time-dependent and time-independent components have been seen in canine tracheal cells

^{*}We obtained the following results in experiments where the whole-cell current reversal potential was recorded after bath Cl^- substitution by I^- or Br^- . In two experiments where G_{Cl}^{cAMP} was activated by forskolin, P_I/P_{Cl} was 0.4, 0.4, and P_{Br}/P_{Cl} was 1.3, 1.4. In two experiments where G_{Cl}^{Ca} was activated by ionomycin, P_I/P_{Cl} was 2.2, 2.4, and P_{Br}/P_{Cl} was 1.3, 1.6.

(12). However, these experiments were done without controlling for G_{Cl}^{well} , so that the cAMP-activated currents were not resolved explicitly. cAMP-activated Cl^- currents are also present in nonepithelial cells, including cardiac myocytes (13, 14) and mast cells (15). Like the G_{Cl}^{AMP} in T84, their $I-V$ relations are linear, and the currents are time-independent during voltage pulses. The single-channel events underlying these currents have not been identified.

Single-Channel Basis of Cl^- Secretion. The single-channel basis of the secretagogue-stimulated apical Cl^- conductance in epithelial cells is currently believed to be an outwardly rectified 30- to 50-pS Cl^- channel (16). This channel, which we refer to as the outward rectifier, is present in apical membrane patches from T84 cells (6). The inference that this channel is responsible for Cl^- secretion has emerged from studies showing that it becomes active in cell-attached patches in the presence of secretagogues (16). However, its distinctive biophysical properties have been characterized mostly in excised, inside-out membrane patches, often after its activation by depolarizing voltages. The relation of this channel to the two secretagogue-induced whole-cell Cl^- conductances of T84 cells reported here is not entirely clear.

An outwardly rectified $I-V$ relation is the most distinctive feature of the 30- to 50-pS Cl^- channel. The conductance properties of the underlying single-channel events should be most accurately reflected by the instantaneous $I-V$ relation of the whole-cell current because time-dependent changes in channel kinetics at different voltages are minimal at the onset of the voltage pulse. In this respect, the properties of the G_{Cl}^a are most consistent with those of the outward rectifier. Both the single-channel and whole-cell currents show outward rectification. Also, the open probability (P_o) of the outward rectifier increases with depolarization (6), and the time dependence of G_{Cl}^a is consistent with this P_o-V relation. Activity of the outward rectifier in excised patches is not reversibly altered by changes in bath Ca^{2+} (16), but relevant Ca^{2+} -dependent regulatory proteins may be lost after patch excision. Additional whole-cell studies should define the elements of this pathway and identify the missing component(s).

The whole-cell Cl^- currents activated by cAMP are difficult to reconcile with the properties of the outward rectifier. Both the instantaneous and steady-state whole-cell $I-V$ relations of G_{Cl}^{AMP} are linear, and the cAMP-activated current is time independent (see Fig. 1 C and D). Thus, the conductance and kinetic properties of the outward rectifier cannot account for the cAMP-activated whole-cell current.

Two hypotheses can reconcile these disparate findings. (i) Stimulation of Cl^- conductance by cAMP could result from activation of a Cl^- channel other than the outward rectifier. This possibility raises the question of why such channels are not regularly seen in isolated membrane patches. The relevant channels may have small unitary conductances (<5 pS) that make them difficult to resolve in single-channel records, particularly if the higher conductance channel is present. Activity of a 4- to 6-pS Cl^- channel, with a linear $I-V$ and no voltage-dependence of its kinetic behavior, was stimulated by secretin in pancreatic duct cells (17). Larger conductance channels compatible with the properties of G_{Cl}^{AMP} have been observed in secretory epithelia (16, 18, 19). In human tracheal cells (16, 19), a 20-pS Cl^- channel with a linear $I-V$ relation was activated by secretory agonists, and in *Necturus* enterocytes, stimulation by forskolin in cell-attached patches or by protein kinase A in excised patches activates a 25-pS channel with a linear $I-V$ relation (20). The possibility that another channel is responsible for G_{Cl}^{AMP} has important implications for cystic fibrosis, in which a defect in cAMP-mediated regulation of the outward rectifier has been implicated as the

mechanism for Cl^- impermeability of secretory epithelial cells.

(ii) If the outward rectifier is activated by cAMP, perhaps its behavior is different in the whole-cell and single-channel measurements. Certain features of this channel clearly depend on measurement conditions. For instance, depolarization-induced activation occurs in excised patches but not in whole-cell (this study) or cell-attached recordings (21). Thus, depolarizing voltages could evoke outward rectification by favoring a conductance state of the channel that does not occur in the intact cell. Certainly, the most parsimonious explanation for the discrepancies between the single-channel and whole-cell Cl^- currents activated by cAMP is a measurement-dependent change in channel properties. That is, the channel may exist in different conductive and kinetic states, and as discussed above, the state routinely seen in the excised patch corresponds to the whole-cell current activated by Ca^{2+} . Activation of the channel by cAMP in the intact cell could induce another state that is not routinely encountered in single-channel studies. Accordingly, the different properties of the whole-cell currents activated by cAMP and Ca^{2+} would be explained by a regulator-dependent change in channel properties (states). However, if only one of these states is preferred in the excised patch, then it is important to show that the details of channel regulation (e.g., by purified proteins) mimic those that occur in the intact cell.

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