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Basolateral K Channel Activated by Carbachol in the Epithelial Cell Line T_{84}

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Abstract. Cholinergic stimulation of chloride secretion involves the activation of a basolateral membrane potassium conductance, which maintains the electrical gradient favoring apical Cl efflux and allows K to recycle at the basolateral membrane. We have used transepithelial short-circuit current (I_{sc}), fluorescence imaging, and patch clamp studies to identify and characterize the K channel that mediates this response in T_{84} cells. Carbachol had little effect on I_{sc} when added alone but produced large, transient currents if added to monolayers prestimulated with cAMP. cAMP also enhanced the subsequent I_{sc} response to calcium ionophores. Carbachol (100 μM) transiently elevated intracellular free calcium ($[Ca^{2+}]_i$) by ~3-fold in confluent cells cultured on glass coverslips with a time course resembling the I_{sc} response of confluent monolayers that had been grown on porous supports. In parallel patch clamp experiments, carbachol activated an inwardly rectifying potassium channel on the basolateral aspect of polarized monolayers which had been dissected from porous culture supports. The same channel was transiently activated on the surface of subconfluent monolayers during stimulation by carbachol. Activation was more prolonged when cells were exposed to calcium ionophores. The conductance of the inward rectifier in cell-attached patches was 55 pS near the resting membrane potential (–54 mV) with pipette solution containing 150 mM KCl (37°C). This rectification persisted when patches were bathed in symmetrical 150 mM KCl solutions. The selectivity sequence was 1 K > 0.88 Rb > 0.18 Na ≫ Cs based on permeability ratios under bi-ionic conditions. The channel exhibited fast block by external sodium ions, was weakly inhibited by external TEA, was relatively insensitive to charybdotoxin, kaliotoxin, 4-aminopyridine and quinidine, and was unaffected by external 10 mM barium. It is referred to as the K_{BIC} channel based on its most distinctive properties (Ba-insensitive, inwardly rectifying, Ca-activated). Like single K_{BIC} channels, the carbachol-stimulated I_{sc} was relatively insensitive to several blockers on the basolateral side and was unaffected by barium. These comparisons between the properties of the macroscopic current and single channels suggest that the K_{BIC} channel mediates basolateral membrane K conductance in T_{84} cell monolayers during stimulation by cholinergic secretagogues.

Key words: T_{84} — Inward rectifier — K_{BIC} — Cystic fibrosis — Chloride transport — Intestinal secretion

Introduction

Transepithelial chloride secretion is stimulated by agents that elevate intracellular cAMP or calcium. While apical membrane Cl channels are the rate-limiting step during secretion and the primary site of regulation, transepithelial transport also depends on the coentry of chloride, sodium and potassium at the basolateral membrane and on sodium extrusion by the basolateral Na/K exchange pump. Passive recycling of potassium ions through basolateral channels maintains a favorable electrical gradient for apical Cl efflux while minimizing K accumulation and thus cell swelling ([41, 42, 52, 53], *reviewed by* [9]).

Exposing the human colonic cell line T_{84} to calcium ionophores or calcium mobilizing agonists stimulates Cl secretion (e.g., [6, 13, 14, 28, 46, 54]). Similar results have been obtained using other intestinal preparations [13, 15]. Results from several laboratories suggest that agonists activate basolateral K conductance in T_{84} cell monolayers primarily by elevating intracellular calcium $[Ca^{2+}]_i$; e.g., carbachol stimulates K current through the basolateral membrane of T_{84} cell monolayers after permeabilization of the apical membrane, and this response is blunted when cells are loaded with a calcium buffer

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[54]. Additional effector mechanisms may be involved [12, 44, 54], but studies with thapsigargin suggest that a rise in intracellular calcium is sufficient to elicit Cl secretion when there is some Cl conductance at the apical membrane [25].

Several calcium-regulated K channels have been described in the basolateral membranes of secretory epithelia. The best known is the "maxi K" or "BK" channel, which is barium and charybdotoxin sensitive and mediates Ca-activated K conductance in many acinar-type gland cells [29, 30, 39, 47]. Another type of K channel has been described on the surface of subconfluent primary cultures of canine tracheal epithelial cells [51]. It was named K_{CLIC} for charybdotoxin-sensitive, low-conductance, inwardly rectifying, calcium-activated K channel.

In this paper we use converging techniques to characterize a carbachol-activated, inwardly rectifying K channel in T_{84} cells which resembles K_{CLIC}. The physiological role of the channel in T_{84} cells is established by recording it on the basolateral membrane of polarized monolayers dissected from porous cell culture supports, and by direct comparison of its single channel properties with those of carbachol-stimulated transport across polarized monolayers—the same approach used previously to identify the cAMP-stimulated chloride channel (CFTR) at the apical membrane of T_{84} cells [46]. The carbachol-activated K channel in T_{84} cells is distinguished by its unusual Ba insensitivity, inward rectification, and calcium activation; it therefore has been named the K_{BIC} channel. Preliminary reports of this work have appeared [20, 45].

Materials and Methods

Cell Culture and Preparation

The T_{84} cell line was obtained from the American Type Culture Collection (Rockville, MD) and studied between passages 35 and 83. Cells were plated at a density of ~500,000/cm^2 on collagen-coated Millicell® inserts and grown to confluence (9–14 days) for Ussing chamber studies. Some patch clamp experiments were performed using monolayers after they had been dissected from the millicells and inverted in a small perfusion chamber to gain access to the basolateral membrane. The cell sheet was sandwiched between two coverslips held together using vacuum grease. The upper coverslip had a small hole to provide access for the patch pipette. Single channel and fluorescence imaging experiments were carried out in parallel studies of cells cultured on glass coverslips. Only cells near the periphery of small areas of monolayer were used for patch recording. Calcium was measured in peripheral cells and in cells near the centers of confluent areas. Cultures were maintained in a 50:50 mixture of DMEM and F-12 media supplemented with 5% FBS, 15 mM HEPES, penicillin (100 U/ml) and streptomycin (100 μg/ml). Media constituents were from GIBCO (Burlington, ONT). Type I collagen was from Collagen Corp. (Palo Alto, CA) and crosslinked into a gel on Millicell® inserts using standard methods [7].

Experimental Solutions

For patch clamp studies, cells were transferred to a recording chamber that initially contained (mM): 146 NaCl, 4 KCl, 2 $MgCl_2$, 5 glucose, 10 TES, pH 7.4. $CaCl_2$ was omitted from the bath solution when recording from excised patches and NaCl and KCl were replaced with 150 mM KCl, RbCl, NaCl or CsCl, or with KCl and Ca/EGTA mixtures. The pipette solution normally contained (mM): 150 KCl, 2 $CaCl_2$, 10 TES, pH 7.4. Calcium imaging was carried out using a perfusate containing (mM): 115 NaCl, 2.4 K_2HPO_4, 0.4 KH_2PO_4, 1.2 $MgCl_2$, 1.2 $CaCl_2$, 25 mM $NaHCO_3$, 10 mM glucose equilibrated with 95% O_2/5% CO_2. Chloride secretion was measured as short-circuit current (I_{sc}) when monolayers were bathed on both sides with (mM): 115 NaCl, 2.4 K_2HPO_4, 0.4 KH_2PO_4, 1.2 $MgCl_2$, 1.2 $CaCl_2$, 25 $NaHCO_3$, 10 mM glucose and equilibrated with 95% O_2/5% CO_2. A 100× stock solution containing 50 mM dibutyryl cyclic-AMP (db-cAMP), 1 mM forskolin and 1 mM 3-isobutyl-1-methylxanthine (IBMX) was used to raise intracellular cAMP in intact cells. Carbachol, forskolin, IBMX, db-cAMP and adenosine triphosphate were from Sigma Chemical (St. Louis, MO). Fura-2-AM and 4-Br-A23187 were from Molecular Probes (Eugene, OR). Two types of charybdotoxin were purchased from different suppliers: native toxin purified from the venom of the scorpion *Leiurus quinquestriatus* was obtained from Latoxan (Rosans, France). Synthetic charybdotoxin was from Peptides Institute (Osaka, Japan). Kaliotoxin was a generous gift from Dr. Marcel Crest, Laboratoire de Neurobiologie, CNRS, Marseille, France.

Transepithelial Currents

Short-circuit current (I_{sc}) provides a measure of net chloride transport during cAMP and calcium stimulation of T_{84} cell monolayers [6, 14]. These experiments were carried out in temperature-controlled, Ussing-type chambers that had been modified to accept Millicell® inserts. Agar bridges (3 M KCl) were used to measure transepithelial potential and to pass short-circuit current. They were connected through Ag/AgCl electrodes to conventional voltage clamps interfaced to a microcomputer. Voltage pulses (10 mV, 2 sec duration) were applied at 150 sec intervals to monitor resistance, with correction for saline resistance. Currents were digitized, saved to disk, and transferred to a spreadsheet program for calculations and graphics [46]. Ussing chamber studies were carried out at 37 ± 1°C.

Single Channel Recording

Pipettes were prepared using a conventional puller (PP-83, Narishige Instr. Lab., Tokyo) and had resistances of 4–6 MΩ when filled with 150 mM KCl solution and connected to a chlorided Ag wire. The bath solution was grounded through an agar bridge having the same ionic composition as the pipette solution. Liquid junction potentials at the agar bridge were measured against a flowing 3 M KCl electrode. Single channel currents were amplified (Axopatch 1C, Axon Instruments, Foster City, CA), recorded on video cassette tape by a pulse-coded modulation-type recording adapter (DR384, Neurodata Instr., NY) and low-pass filtered during play back using an 8-pole Bessel-type filter (902 LPF, Frequency Devices, Haverhill, MA). The final recording bandwidth was −3 dB at 230 Hz, and records were sampled at 1 kHz and analyzed using a laboratory microcomputer system (Indec Systems, Sunnyvale, CA) as described previously [19]. In excised patches, V_m refers to the membrane potential with respect to the extracellular side of the membrane. In cell-attached patches, $-V_p$ is the applied voltage

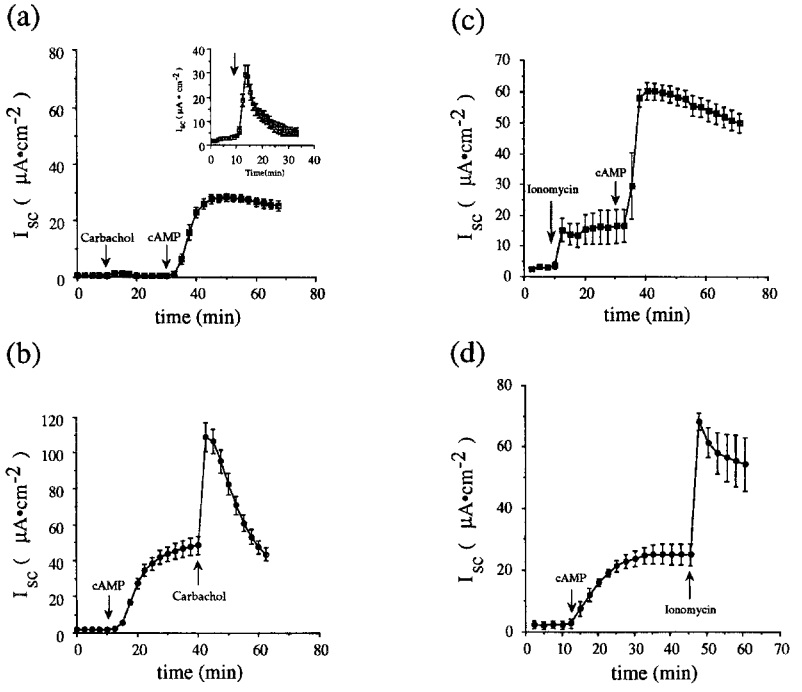

Fig. 1. Effect of secretagogues on short-circuit current (I_{sc}) across T_{84} cell monolayers. (*a*) Carbachol alone had little effect when added to 12 monolayers. Two monolayers that had significant basal I_{sc} before stimulation gave brief responses to carbachol alone. The carbachol transient declined to 50% of the peak value within 5 min (*see* inset). (*b*) Adding carbachol after prestimulation with cAMP mixture (final concentration: 500 μM db-cAMP, 10 μM forskolin, 10 μM IBMX) activated a large I_{sc} that declined to 50% of the peak within 12 min (means ± SE, $n = 5$). (c) Effect of sequentially adding the calcium ionophore ionomycin (2.5 μM) and cAMP mixture on I_{sc} (means ± SE, $n = 5$). Compare the response to cAMP after ionomycin with those in Figs. 1*a* and *b*. (*d*) Effect of sequential addition of cAMP mixture and ionomycin (2.5 μM; mean ± SE, $n = 5$).

referenced to the pipette (i.e., extracellular) solution. Current-voltage (*I/V*) relationships were calculated by a semi-automated procedure in which amplitude histograms were computed for short segments of record and displayed sideways next to the data so that peaks could be verified using cursors. Open events were measured at each potential and entered into an *I/V* curve displayed at the end of the run. Reversal potentials were estimated by interpolation after fitting a polynomial function to the *I/V* curve. Slope conductance was determined by linear regression over the voltage ranges specified in Results. The mean number of channels open ($\langle I \rangle / i$) was computed from the fraction of time spent at each multiple of the single channel current. Single channel experiments were performed at 37 ± 1°C or room temperature (~21°C) as indicated.

Intracellular Calcium ($[Ca^{2+}]_i$) Measurements

Cells on glass coverslips were loaded with Fura-2-acetoxymethyl ester (Fura-2-AM, Molecular Probes) by incubation in MCDB medium containing 12.3 μM Fura-2 AM and ~50 μM Pluronic F-127® for 1 hr at 21°C. Coverslips were mounted in a temperature-controlled (37°C) perfusion chamber on the stage of a Nikon inverted microscope (Diaphot, Nikon, Tokyo, Japan) and illuminated using a 75 W mercury-xenon arc lamp. Excitation wavelengths were selected using a filter wheel (340 or 380 nm; ±5 nm) and directed through a 410 nm dichroic mirror and 40× Nikon UV-fluor oil immersion objective (N.A. 1.3). Emitted light (510–530 nm) was deflected to the eyepieces or an intensified CCD camera (Model 2468, Hamamatsu Photonics, Hamamatsu City, Japan). Twelve frames were averaged for each wavelength (340 or 380 nm) to yield a ratio image every 1.4 sec (Fluor-1; Universal Imaging, West Chester, PA). Autofluorescence by unloaded cells was less than 1.5% of the baseline fluorescence of Fura-loaded cells excited at 340 nm, and less than 3% at 380 nm. Pseudo color ratio images and plots of the average ratios for 20 areas (each area ≈10 μm diameter) were displayed on-line and imported into a spreadsheet program (Lotus 123, Lotus Development, Cambridge, MA) for calculations and graphics.

Calculations and Statistics

Estimates of intracellular free calcium concentrations are given for six experiments under each set of conditions (ten determinations per experiment) using the values obtained for $R_{min} = 0.54 \pm 0.05$, $R_{max} = 2.43 \pm 0.93$ and $\beta = 4.3$, where β is the fluorescence intensity, at 520 nm, of cells in nominally Ca^{2+}-free solution containing 1 mM EGTA during excitation at 380, divided by the intensity measured with high (millimolar) $[Ca^{2+}]$ [27]. Significance was determined at the 95% confidence level using paired Student's *t*-tests.

Results

Effects of cAMP, Carbachol and Calcium Ionophores on I_{sc}

In the first series of experiments, carbachol (100 μM) was added to the basolateral side, followed 20 min later by the cAMP mixture (*see* Materials and Methods). Carbachol alone had little effect on short-circuit current in 12 of the 14 monolayers tested (carbachol peak = 1.25 ± 0.5 μA/cm^2; Fig. 1a), although the same preparations later responded to cAMP mixture (cAMP peak $I_{sc} = 28.0 \pm 1.5$ μA/cm^2). Two monolayers did respond briefly to carbachol alone (inset to Fig. 1*a*) as reported previously [14]. The reason for the variable response to carbachol is uncertain but is probably related to residual apical Cl conductance since monolayers that responded also had some basal current prior to carbachol exposure. All monolayers responded to carbachol after cAMP prestimulation (Fig. 1*b;* [46]).

Ionomycin (2.5 μM, apical) caused a small, sustained increase in I_{sc} when added alone (Fig. 1*c*), but gave a

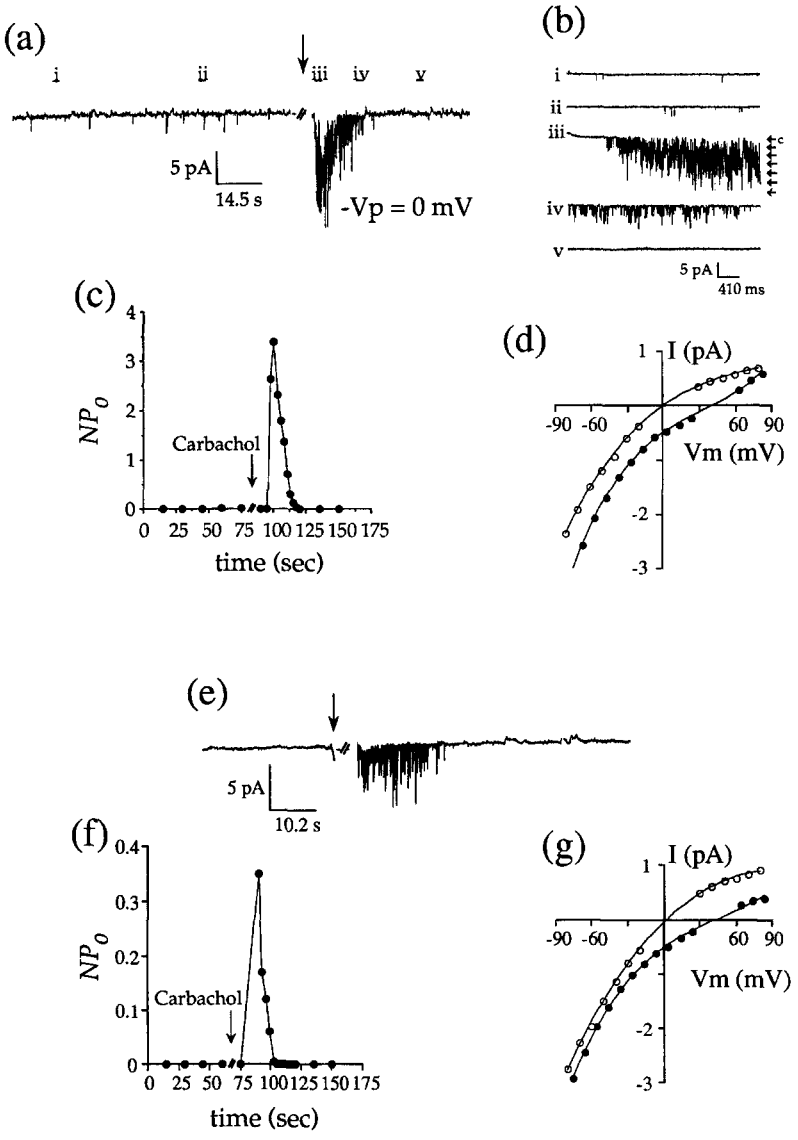

Fig. 2. Activation of inwardly rectifying K channels on the basolateral surface of confluent T_{84} monolayers (*a–d*) and on the surface of unpolarized cells (*e–g*). (*a*) Cell-attached recording on the basolateral surface of T_{84} cell monolayer before, and during, exposure to 100 μM carbachol (37°C). Inward currents were observed at the membrane potential with pipette solution containing 150 mM KCl. Representative of four experiments. (*b*) Expanded traces showing channel activity at the times indicated in (*a*). (*c*) The effect of carbachol on the mean number of channels open, recorded on the basolateral surface of polarized monolayers. (*d*) Mean current-voltage relationship for channels in two excised patches that were bathed externally with 150 mM KCl (pipette solution) and on the cytoplasmic side with Na Ringer (●; 140 mEq/liter Na and 5 mEq/liter K) or (○) 150 mM KCl solution. (*e*) Cell-attached recording on the upper surface of T_{84} cells cultured on glass during stimulation with 100 μM carbachol at 37°C. Representative of three experiments. (*f*) Effect of carbachol on the mean number of channels open, recorded on the surface of unpolarized cells. (*g*) Mean current-voltage relationship for channels bathed symmetrically with 150 mM KCl or with solution containing 145 mM NaCl and 5 mM KCl on the cytoplasmic side (standard errors smaller than symbols, $n = 5$).

much larger response after prestimulation with cAMP mixture (Fig. 1*d*). These data are consistent with the conventional model in which carbachol stimulates Cl secretion through activation of basolateral K conductance, whereas cAMP plays a permissive role by activating apical Cl channels. The carbachol transient is noticeably broader after cAMP pretreatment, suggesting cAMP also prolongs the stimulation of basolateral K conductance. In the next section we identify a K channel in the basolateral membrane of confluent, polarized T_{84} monolayers that mediates the secretory response to carbachol.

Activation of a Channel on the Basolateral Surface of Confluent T_{84} Monolayers and on Unpolarized Cells

Monolayers were cultured to confluence on collagen-coated millicells and dissected for patch clamp studies as described in Materials and Methods. Little spontaneous activity was observed when basolateral membrane patches were recorded in the cell-attached configuration with pipette solution containing 150 KCl (Fig. 2*a,b*). Adding carbachol (100 μM) to the bath caused a rapid increase in channel activity which peaked after 4.0 ± 0.7 sec and then declined spontaneously to the basal level with a variable time course. The mean number of K channels open ($\langle I \rangle / i = NP_o$) increased by approximately 50-fold during carbachol stimulation (Fig. 2*c*). The reversal potential for single channel currents was 44.5 ± 0.35 mV when patches were excised into Na Ringer solution (146 mM Na, 4 mM K), suggesting only moderate (~5-fold) selectivity for potassium over sodium (Fig. 2*d*). When the bath solution was replaced with 150 mM KCl so that patches were bathed symmetrically with high-K solutions, the reversal potential shifted to 0 mV and the slope conductance at the reversal potential was 21.2 ± 3.9 pS. These data suggest that a carbachol-activated, in-

wardly rectifying K channel is present on the basolateral membrane of polarized, confluent T_{84} monolayers.

Since dissecting monolayers from porous supports was tedious and gigaohm seals were difficult to maintain, we examined whether the same carbachol-activated K channel was expressed on the surface of subconfluent cells before they became polarized. Figure 2*e* shows a typical cell-attached recording of channels on the surface of a cell near the edge of a confluent island of cells. The time course for activation and deactivation (Fig. 2*f*) was similar to that for dissected monolayers (Fig. 2*c*) and closely paralleled the carbachol-stimulated Ca transient in the same area of the culture (*see* Fig. 4*a,b*). The conductance in symmetrical high-K solution was 22.9 ± 1.6 pS and the reversal potential with high-sodium solution (146 mEq/liter Na and 4 mEq/liter K) on the cytoplasmic side was 45.8 ± 0.8 mV (Fig. 2*g*), not different from the channels recorded on the basolateral aspect of dissected monolayers (Fig. 2*d*). Channel density was lower on the surface of subconfluent cells compared to the basolateral membrane of polarized monolayers but the channels appeared to be identical with respect to their single channel conductance, P_K/P_{Na} permeability ratio, and gating. We therefore concluded they are the same channel.

Calcium is a second messenger for carbachol and activates K conductance in intestinal crypts (e.g. [50]); we therefore examined whether calcium ionophores stimulate the inwardly rectifying K channel in cell-attached patches. Figure 3*a* shows the effect of adding A23187 (2 μM) to the bath on K channel activity recorded on a cell near the periphery of a confluent area of monolayer. Inward currents were observed at the membrane potential under these conditions because the pipette solution contained 150 mM KCl. Figure 3*b* shows the stimulatory effect of 2 μM A23187 on the mean P_o calculated for five cell-attached patches (mean ± SE, n = 5). Similar results were obtained using ionomycin (1 μM; n = 2). These ionophore responses were more prolonged than carbachol responses, which facilitated estimating the number of active channels in patches.

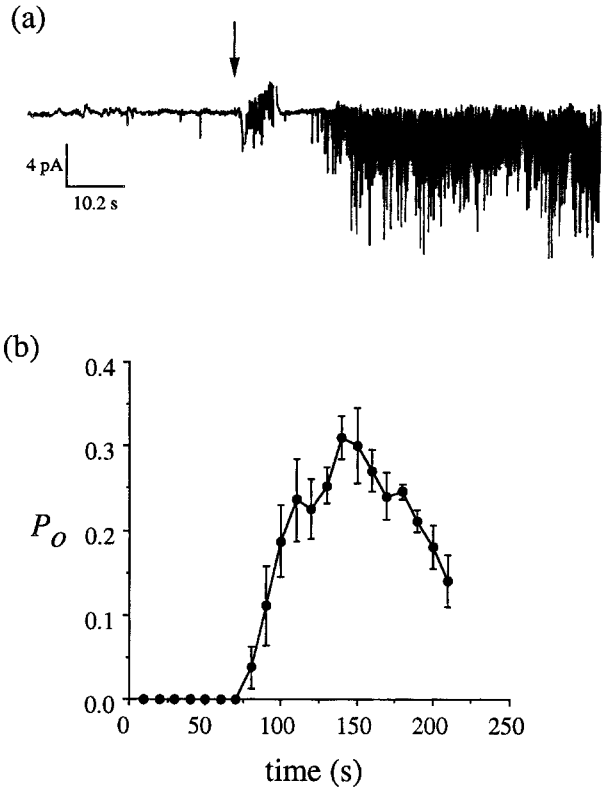

Fig. 3. Activation of K channels by the calcium ionophore A23187. (*a*) Cell-attached recording from a subconfluent T_{84} cell with 150 mM KCl solution in the pipette and normal (high-sodium) Ringer in the bath. (*b*) Mean open probability (P_o) before and during stimulation with A23187 (mean ± SE, n = 5).

$[Ca^{2+}]_i$ During Exposure to Carbachol or Ca Ionophores

For comparison with patch clamp data, the effect of carbachol on $[Ca^{2+}]_i$ was determined using the fluorescent calcium indicator Fura-2 (Fig. 4). Calcium responses were observed in 49 of the 60 areas examined near the periphery of monolayers (six experiments, ten areas/culture). $[Ca^{2+}]_i$ increased to a peak level of 353 ± 33 nM and then declined to near basal levels (149 ± 29 nM) within 4 min, briefer than the I_{sc} response shown in Fig. 1. The declining phase of the intracellular calcium response was well described by a single exponential having a time constant of 1.3 min. $[Ca^{2+}]_i$ reached a similar peak level of 338 ± 30 nM in 38 of the 60 areas assayed near the center of the confluent areas; however, the decline in these cells was slower than in cells near the periphery and had a time course resembling the carbachol-stimulated I_{sc}. Fitting the decline in $[Ca^{2+}]_i$ near the center of confluent areas required two exponentials ($r^2 \gg 0.99$), one having a time constant of 1.3 min (like cells near the edge) and the other having a time constant of 15.9 min. The latter exponential component accounted for about 66% of the decay. By contrast, exposing cells to the calcium ionophore Br-A23187 induced a sustained elevation of $[Ca^{2+}]_i$ (Fig. 5), consistent with the ionophore stimulations of I_{sc} and K channel activity shown above. Pretreating cells with cAMP mixture did not affect the magnitude or time course of the calcium response to carbachol (Fig. 4). It is clear that the synergy between cAMP and carbachol in Fig. 1 is distal to calcium mobilization as reported previously [54], and is not due to phosphorylation of the IP_3 receptor channel [4] or some other mechanism that alters Ca signaling.

Selectivity and Temperature Dependence of the Carbachol-Activated K Channel

Figure 6*a* shows traces of the carbachol-activated K channel in a cell-attached patch with 150 mM KCl in the pipette solution, recorded near the edge of an island of

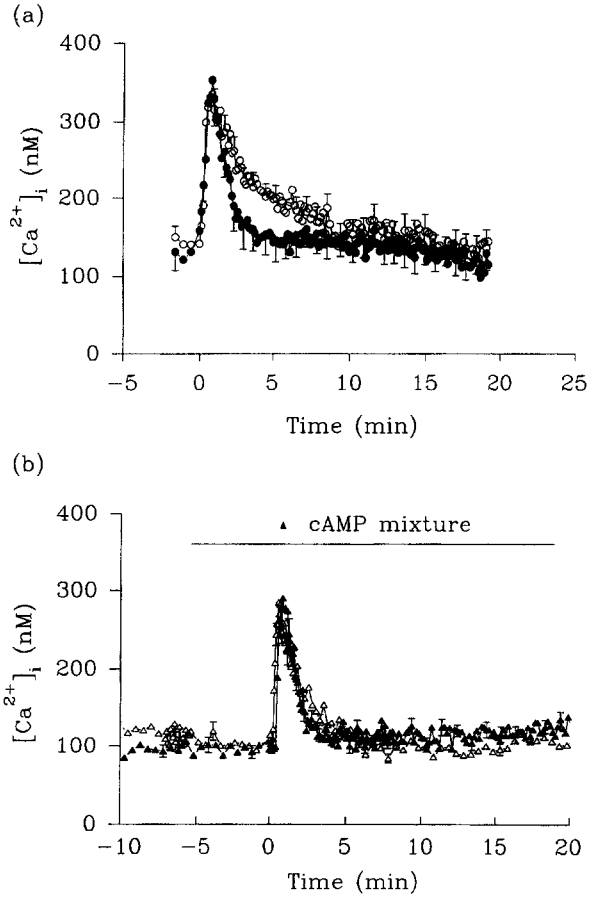

Fig. 4. Effect of carbachol on intracellular free calcium concentration in T_{84} cells estimated by Fura-2 fluorescence. Cells were superfused with HEPES buffered sodium Ringer as in the patch clamp experiments shown in Figs. 2 and 3. Carbachol (100 μM) was added at $t = 0$ min. (*a*) Mean intracellular Ca response observed in cells (●) near the periphery of confluent "islands" or (○) near the center of the confluent areas of monolayer. (*b*) Mean calcium responses obtained in cells near the periphery in the (△) absence or (▲) presence of cAMP mixture (representative of six experiments, ten areas assayed per experiment).

cells. The bath contained control Ringer solution (150 mEq/liter Na, 4 mEq/liter K) at 37°C. The mean current-voltage relationship is shown as filled squares in Fig. 6*b*. The reversal potential under these conditions was $-V_p$ = +71.8 ± 2.1 mV and the slope conductance at the membrane potential in cell-attached patches ($-V_p$ = 0 mV) was 62.6 ± 4.3 pS. Similar slope conductances were obtained on-cell at hyperpolarizing potentials when bath Ringer was replaced with 150 mM KCl solution (open circles, Fig. 6*b*). Conductance at the reversal potential was 32.5 ± 0.9 pS under these conditions. When cell-attached recordings were made using a pipette solution containing 30 mM K (balance sodium) instead of 150 mM K, the reversal potential was +43.5 ± 0.94 mV and the *I/V* curve with 120 mM sodium in the pipette solution was distinctly "S" shaped, yielding a conductance of 34 pS at the membrane potential. Because the S shape was not observed with the KCl pipette solution, the reduced conductance at hyperpolarizing voltages <–40 mV with the Na-containing pipette solution most likely reflects fast block by external sodium. Figure 6*c* shows traces obtained from cell-attached patches at 24 or 37°C with 150 mM KCl in the pipette solution. Single channel conductance determined at the membrane potential ($-V_p$) decreased from 54.6 to 20.5 pS when temperature was lowered from 37 to 24°C (Fig. 6*d*). Conductance was somewhat less temperature sensitive when patches were excised and bathed symmetrically with 150 mM KCl solutions (Q_{10} = 1.4). The reversal potential with 400 mM KCl on the cytoplasmic side and 150 mM KCl in the pipette solution was –21 ± 1.54 mV, yielding a ratio for P_{Cl}/P_K of 0.08 ± 0.04. Slope conductance between +40 and +60 mV was 7.67 ± 0.72 pS with 2 mM $MgCl_2$ bathing the cytoplasmic side (Fig. 7*a*; n = 3) and 7.50 ± 0.57 pS (n = 3) without magnesium (Fig. 7*a*). Thus, inward rectification of this channel is not due to fast magnesium block, unlike other ATP-regulated inward rectifiers such as ROMK1 recently cloned from rat kidney outer medulla [22].

Selectivity among cations was studied under bi-ionic conditions using inside-out patches at 37°C. Traces and mean current-voltage relationships obtained with cytoplasmic NaCl, RbCl and CsCl are shown in Figs. 7*b–d*, respectively. Inward currents were easily resolved at 0 mV when the bath solution contained 150 mM sodium or cesium chloride. As shown in Fig. 7*b*, the reversal potential with sodium on the cytoplasmic side (●) was +39.0 ± 1.3 mV, yielding the ratio P_{Na}/P_K = 0.18 ± 0.01 (with P_{Cl}/P_K = 0.08 ± 0.04 as estimated above). Replacing bath KCl with RbCl shifted the reversal potential by only +3.3 ± 1.5 mV, yielding P_{Rb}/P_K = 0.88 ± 0.06 (Fig. 7*c*). Negative currents were observed at potentials up to +40 mV when the bath was replaced with solution containing 150 mM CsCl, beyond which transitions could not be resolved at the largest potentials used (+100 mV; Fig. 7*d*).

In summary, the inwardly rectifying K channel in T_{84} cells resembles some K channels by its high permeability to rubidium and inability to conduct cesium. It has only moderate selectivity against sodium and therefore might carry significant inward Na current during cholinergic stimulation *in situ* if not for voltage-dependent "fast block" by external sodium ions.

Blocker Sensitivities of Carbachol-Stimulated I_{sc} and Single Channel Current

If the inwardly rectifying K channel described above mediates basolateral membrane K conductance during muscarinic stimulation, blockers that are effective from the extracellular side of single channels should also inhibit the carbachol-stimulated I_{sc} in Ussing chamber

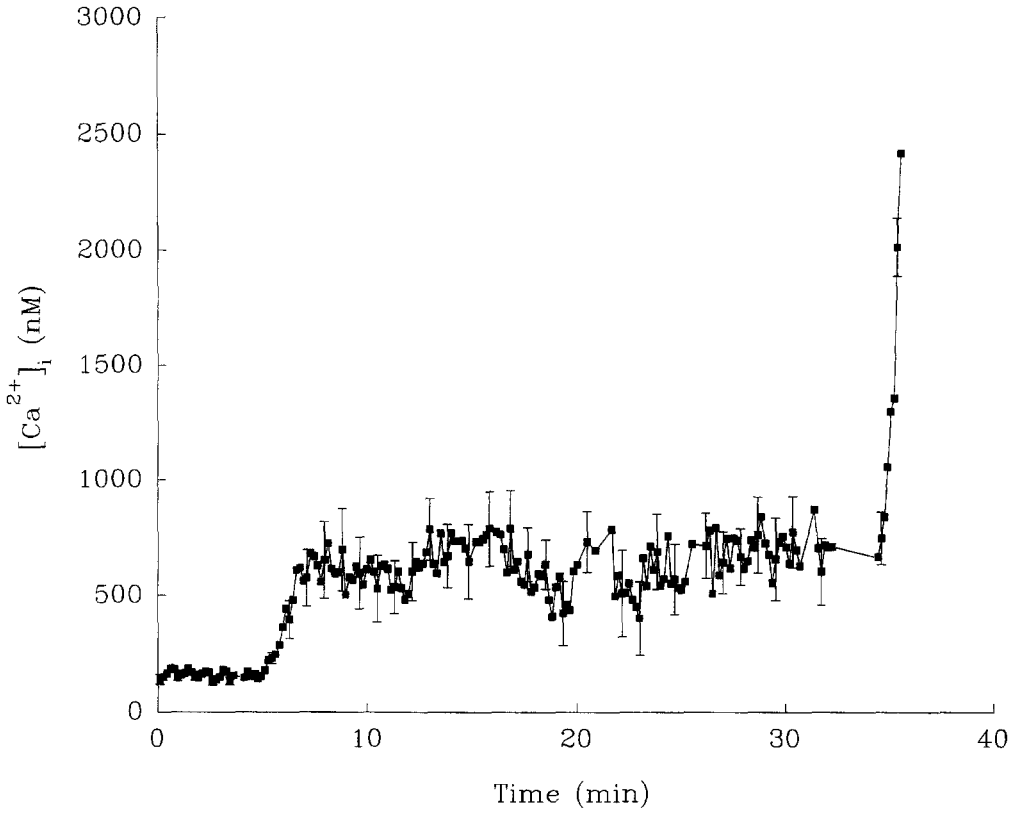

Fig. 5. Effect of Br-A23187 (5 μM) on intracellular free calcium concentration in subconfluent cultures. Ionophore was added at time = 5 min and then increased to 50 μM at t = 35 min.

studies. We compared the potencies of several K channel blockers against single channel and transepithelial currents.

Most potassium channels are sensitive to external TEA and barium although half-maximal block requires millimolar external concentrations in some preparations [21]. Figure 8*a* shows cell-attached recordings under control conditions and with 40 mM TEA in the pipette solution. Single channel currents recorded in the cell-attached configuration with the membrane potential hyperpolarized by –30 mV were reduced only 16% with 10 mM external TEA and 40% with 40 mM TEA. Variation between excised patches in the extent of channel run-down prevented an analysis of blocker effects on open probability; however, P_o was not reduced noticeably by either blocker.

Charybdotoxin has been reported to block the inwardly rectifying K channel in airway cells, with half-maximal inhibition at 10 nM [32]. We examined whether charybdotoxin blocks the carbachol-activated K channel on T_{84} cells. The channel was active when 50 nM, 200 nM or 1 μM charybdotoxin were included in the pipette solution (Fig. 8*b*). Slight inhibition by external charybdotoxin was observed when patches were held at –30 mV with 200 nM charybdotoxin in the pipette solution, but at concentrations normally used to inhibit other Ca-activated K channels (*ca.* 50 nM) charybdotoxin had no effect. Similar results were obtained using charybdotoxin purified from venom or synthesized in vitro. To compare the effectiveness of charged inhibitors at a constant membrane potential, putative blockers were included in the pipette solution containing 150 mM KCl and patches were excised into normal Ringer solution. External TEA (10 mM) reduced the currents slightly under these conditions but quinidine (300 μM), 4-aminopyridine (10 mM) and barium (10 mM) had little effect on the amplitude of single channel currents measured at –40 mV (Fig. 8*c*).

Insensitivity to extracellular blockers under these conditions might be explained if there was competition with potassium, which was at high concentration in the pipette solution. To examine this possibility, blockers were added to sodium Ringer pipette solution, and patches were excised into high-K bath solution to simulate the ionic conditions existing in intact cells. Positive currents recorded at 0 mV under these conditions were not affected significantly by extracellular 10 mM 4-AP or 300 μM quinidine (Fig. 8*d*). Thus, we conclude that the inwardly rectifying K channel in T_{84} cells is not inhibited by these blockers from the extracellular side and that this

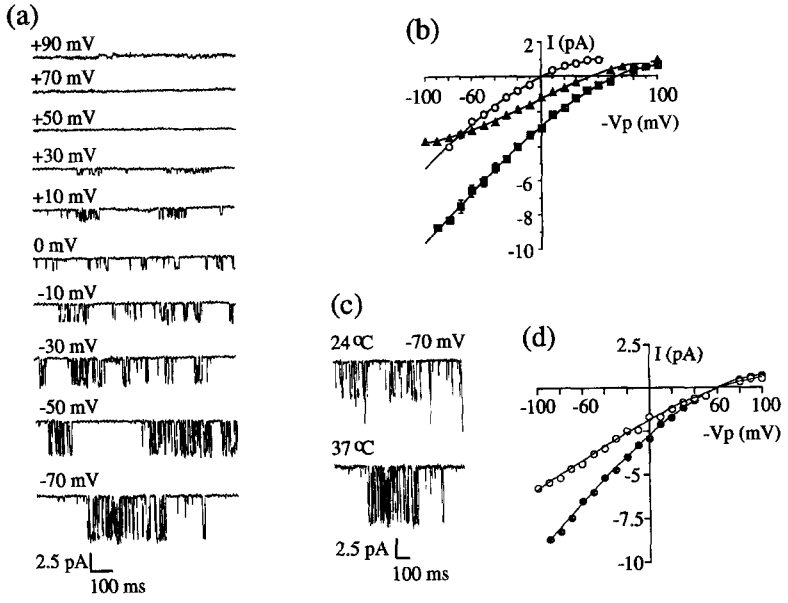

Fig. 6. Temperature dependence and selectivity of the inwardly rectifying channel in cell-attached patches. (*a*) Cell-attached recordings with $-V_p$ between +90 and −70 mV, 150 KCl in the pipette, and sodium Ringer in the bath (37°C). (*b*) Current-voltage relationship on-cell with 150 KCl in the pipette and bath containing (■) sodium Ringer or (○) 150 mM KCl. Also shown (▲): mean *I/V* relationship obtained in the cell-attached configuration with sodium Ringer in the bath and pipette solution containing 30 mEq/liter K and 124 mEq/liter Na. (*c*) Traces obtained at $-V_p$ = −70 mV in the cell-attached configuration with 150 mM KCl in the pipette and normal sodium Ringer in the bath. (*d*) Current-voltage relationships under the conditions described in *c* obtained at (●) 37°C or (○) 24°C.

insensitivity is not explained by voltage or competition with potassium ions.

After characterizing putative blocker effects at the single channel level, we examined whether they inhibit carbachol-stimulated I_{sc} using a sequential protocol similar to the one shown in Fig. 1; (i) monolayers were stimulated with cAMP mixture, (ii) putative blockers were added to the basolateral side, (iii) carbachol (100 μM) was added. I_{sc} during carbachol exposure was normalized to that measured immediately before carbachol addition or, in those experiments that involved barium, immediately before addition of $BaCl_2$ to the basolateral side (Fig. 9).

Carbachol transiently increased I_{sc} by 2.3-fold in the absence of K channel blockers. As in the patch clamp experiments, none of the blockers abolished carbachol stimulation. TEA, quinidine and 4-AP caused some depression of the response, but this probably reflects toxic actions rather than specific K channel block because similar effects were observed after addition of the blockers to the apical side. The I_{sc} response to carbachol was not inhibited by 10 mM barium and was actually enhanced slightly by addition of 2 mM barium to the basolateral side (Fig. 9). This contrasts with the cAMP-stimulated I_{sc}, which was strongly inhibited by extracellular barium at the lowest concentration tested (1 mM), as reported previously [36]. Figure 10 compares the effects of 2.5 mM 4-AP, 2 mM barium (Fig. 10*c*) and 1 μM charybdotoxin (Fig. 10*d*) on cAMP and carbachol-stimulated currents. Barium was the only blocker that distinguished between the K conductances mediating cAMP-stimulated and carbachol-stimulated secretion.

Discussion

The importance of outward potassium current at the basolateral membrane for transepithelial chloride secretion was appreciated in early studies of airway epithelial cells [41, 42, 52]. The hyperpolarizing effect of basolateral K conductance would help maintain a favorable electrical gradient for apical Cl exit, and outward K current would recycle potassium ions that enter with Cl on the basolateral NaK2Cl cotransporter. With only four elements (i.e., apical Cl channels, basolateral K channels, NaK2Cl cotransporters and 3Na/2K exchange pumps), basolateral K channels would be expected to carry 5/6 of the current through the basolateral membrane during electrogenic Cl secretion.

Epithelia contain multiple K conductances which differ in their ion selectivities, blocker sensitivities and regulation [5, 16, 17, 33, 39, 43, 48], *see* [9]. The muscarinic agonist carbachol [3, 23] and calcium ionophores [3, 15] stimulate transepithelial secretion in freshly isolated intestinal tissue [3, 23]. Similar results were obtained in T_{84} cells with carbachol [12, 14, 36, 46] and Ca ionophores [6, 12, 14]. In the present study, carbachol usually had no effect when added alone, although tran-

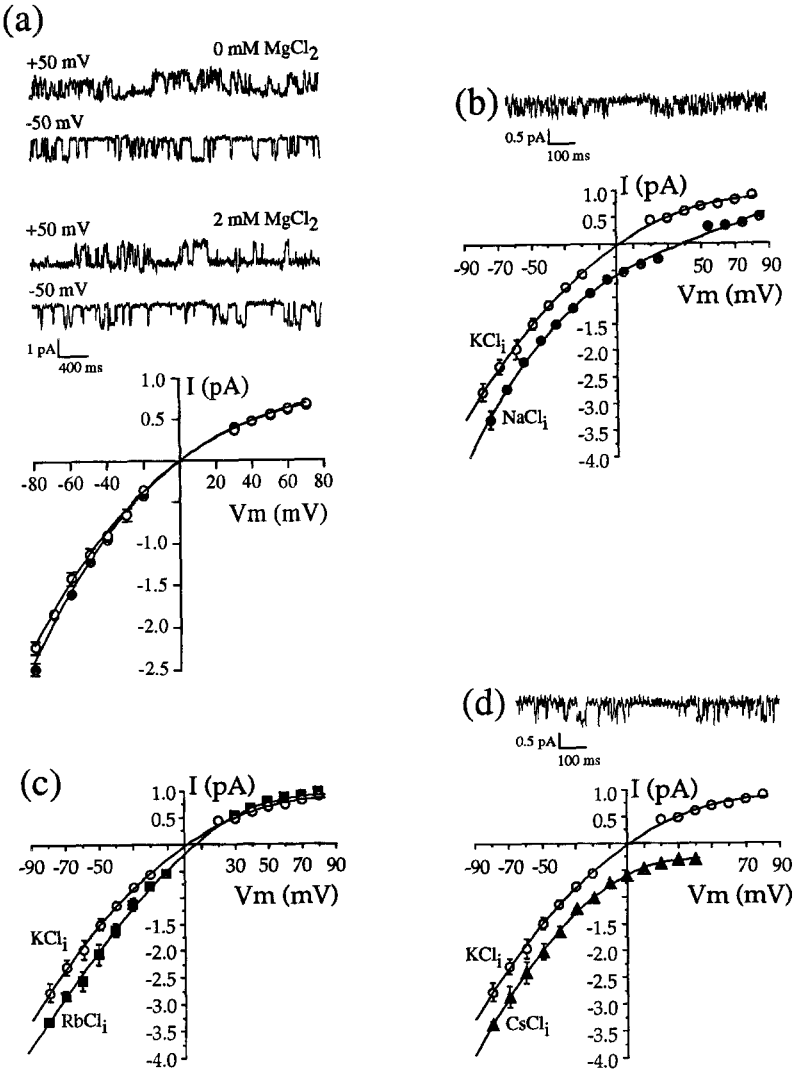

Fig. 7. Magnesium-independent rectification and bi-ionic permeability ratios. Pipette solution contained 150 mM KCl during all experiments. (*a*) Traces obtained at ±50 mV (top) and current-voltage relationships (bottom) in the nominal absence of magnesium or with 2 mM $MgCl_2$ solution bathing the cytoplasmic side. (*b–d*) *I/V* relationships determined with (●) NaCl, (■) RbCl, or (▲) CsCl solutions on the cytoplasmic side. Single channel activity recorded at 0 mV with sodium or cesium bathing the cytoplasmic side is also shown in *b* and *d*, respectively.

sient currents were observed in two monolayers that had basal current prior to carbachol stimulation. Carbachol responses were greatly enhanced in cAMP-stimulated monolayers, consistent with apical Cl conductance serving as a rate-limiting step. Presumably, carbachol activated a K conductance at the basolateral membrane of all the monolayers but generated little transepithelial current until apical Cl conductance was stimulated [14]. We do not know why Cl conductance was partially activated in two of the monolayers, if this accounts for their responsiveness to carbachol alone. Similarly, calcium ionophores caused a modest but sustained stimulation of short-circuit current as in early studies of T_{84} [6] and other secretory cells [1, 3, 15]. There is evidence that the apical membrane of T_{84} cells does not contain significant Ca-activated Cl conductance [2]. Ionophore-stimulated currents are probably carried by the resting Cl conductance of the apical membrane, e.g., by a low level of CFTR channel activity.

I_{sc} declined in the continued presence of carbachol, usually to a level below that observed before carbachol addition. Protein kinase C activity is stimulated by carbachol [8], and PKC can have both stimulatory and inhibitory effects on secretion [18, 26, 36]. Alternatively, it has been suggested that the delayed inhibition may be mediated by IP_4 [24]. In monolayers that responded to carbachol alone, the carbachol transient was briefer than in monolayers that had been prestimulated with cAMP mixture. If carbachol effects are indeed mediated by activation of basolateral K channels, the broader transient observed after cAMP prestimulation suggests that cAMP prolongs their responsiveness to calcium.

Carbachol caused rapid stimulation of inwardly rectifying K channel activity on the basolateral membrane of polarized monolayers that had been dissected from porous supports. By contrast, the K channel was not observed on the apical surface of confluent T_{84} monolayers under these conditions in a previous study [46]. Taken together, these studies demonstrate that the inwardly rectifying K channel activated by calcium-

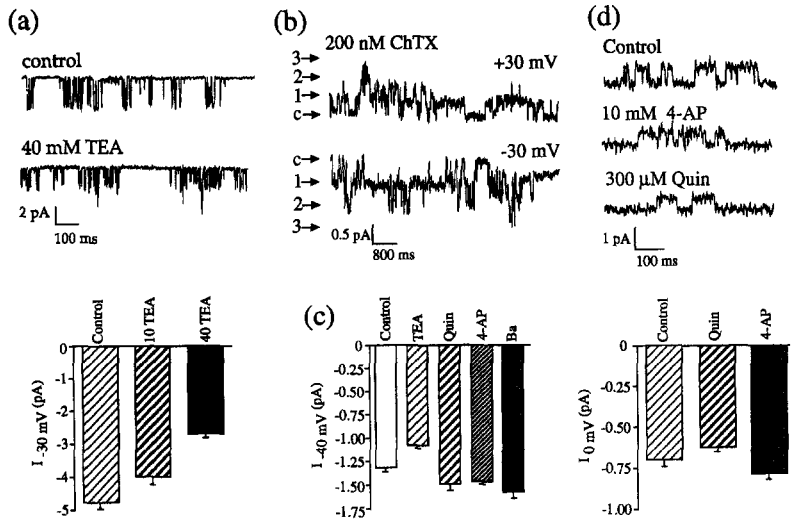

Fig. 8. Effects of common blockers on inwardly rectifying K channel. (*a*) Top: Reduction in single channel current caused by 40 mM TEA in the pipette solution. Bottom: Histogram showing mean current amplitude under control conditions and with 10 or 40 mM TEA in the pipette solution (means ± SE, n = 3–4 patches). (*b*) Transitions recorded at ±30 mV with 200 nM charybdotoxin in the pipette solution. (*c*) Histogram comparing current amplitudes from excised patches with pipette solution containing 150 mM KCl and putative blockers (V_m = –40 mV). (*d*) Traces and histograms obtained with sodium Ringer and putative blockers in the pipette, and with 150 mM KCl and 1 mM IBMX in the bath. Quinidine, 4-AP and barium were all ineffective. Some inhibition was observed with external TEA at high concentrations.

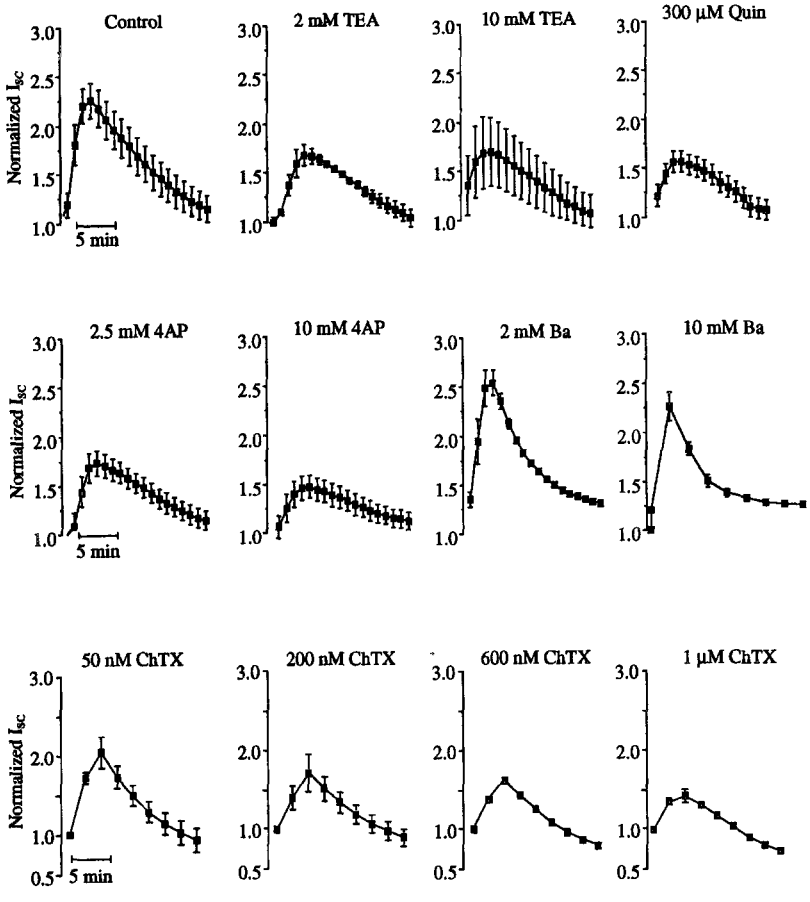

Fig. 9. Carbachol-stimulated I_{sc} transients normalized to the current measured during cAMP stimulation. None of the inhibitors added to the basolateral side of confluent monolayers blocked the carbachol-stimulated I_{sc}. TEA, quinidine, 4-AP and charybdotoxin (ChTX) all caused some inhibition at high concentrations. Barium did not inhibit I_{sc} at any concentration tested.

mobilizing secretagogues is confined to the basolateral membrane as inferred previously based on tracer [6, 14, 28, 34] and short-circuit current studies of monolayers [55]. The presence and identical features of this inwardly rectifying K channel on the basolateral side of polarized monolayers and on unpolarized cells validate the use of subconfluent cultures for studying its single channel properties [10, 45, 51].

Open probability increased from near 0 to about 0.3 during exposure to calcium ionophores and then declined, perhaps due to secondary activation of an inhibitory path such as protein kinase C [18, 26, 36, 44]. In-

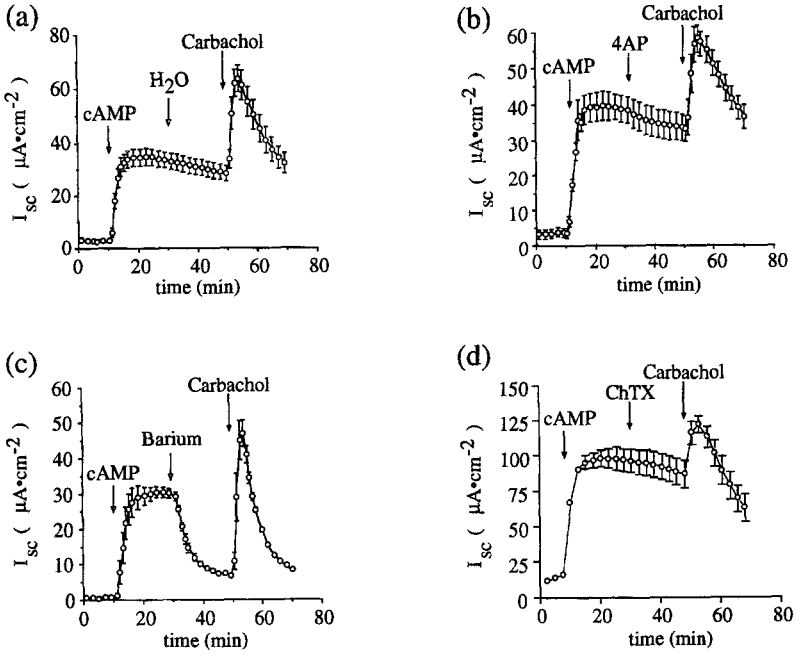

Fig. 10. Comparison of the blocker sensitivities of cAMP- *vs.* carbachol-stimulated short-circuit current across T_{84} cell monolayers. (*a*) Control response to sequential addition of cAMP mixture, vehicle (H_2O), and 100 μM carbachol. (*b–d*) Effects of 4-aminopyridine (2.5 mM), Ba (2 mM), and charybdotoxin (ChTX; 200 nM), respectively, on the cAMP-stimulated current and subsequent response to carbachol.

tracellular free calcium during ionophore stimulation usually declined after reaching an initial peak and then increased. This biphasic response gave the impression that a calcium extrusion mechanism was being activated and then overloaded by the influx.

An inwardly rectifying K channel similar to the one described here has been reported on unpolarized primary cultures of canine tracheal epithelial cells during stimulation by epinephrine or A23187 [51]. The channel in dog trachea, named K_{CLIC} for "charybdotoxin-sensitive, low-conductance, inwardly rectifying, calcium-activated K channel," has similar conductance but is more K selective and appears to have slower kinetics. Preliminary experiments on T_{84} cells at room temperature suggest these differences are not attributable to the higher temperature used in the present study (37°C).

The $[Ca^{2+}]_i$ response to carbachol was shorter in subconfluent cells than the I_{sc} response obtained using confluent monolayers in Ussing-type chambers. A similar temporal difference was noted previously [12] and used as evidence for the existence of additional pathways modulating the action of calcium, although I_{sc} did parallel $[Ca^{2+}]_i$ when they were measured simultaneously in another study [55]. In the present work, the apparent discrepancy in time course between calcium (Fig. 4*b*) and I_{sc} responses disappeared if calcium was measured near the center of confluent areas of monolayers (Fig. 4*a*) and if I_{sc} was measured without cAMP prestimulation (inset to Fig. 1*a*). We had avoided confluent regions during initial imaging experiments so that calcium measured in the peripheral cells could be compared with single channels recorded in those cells.

Additional mechanisms to modulate Ca responses have also been suggested by the greater potency of receptor-mediated agonists when compared to ionophores that give equivalent increases in $[Ca^{2+}]_i$ [38, 54]. On the other hand, recent studies with thapsigargin suggest that elevated $[Ca^{2+}]_i$ is a sufficient signal to cause Cl secretion [25], but do not exclude regulation by kinases and other pathways. The calculated increase in cell calcium ($\Delta[Ca^{2+}]_i$ = 200 nM) is larger than reported previously using cuvette-based fluorescence spectrophotometry (43 nM [14], 100 nM [12]) or microscope-based single-cell spectrofluorimetery (55 nM, [37]; 84 nM [54]), but is comparable to recent studies in which $[Ca^{2+}]_i$ increased from ~100 to >400 nM in the presence of thapsigargin [25]. cAMP did not elevate calcium or alter the calcium response to carbachol. This agrees with previous findings [54] and the observation that vasoactive intestinal peptide (VIP), which elevates intracellular cAMP levels more than 30-fold, does not alter cell calcium [14]. Stimulation of human airway epithelial cells with cAMP mixture also does not raise $[Ca^{2+}]_i$ (R. Harris and J. Hanrahan, *unpublished observation*). These results contrast with those obtained using canine airway primary cultures, where cAMP may regulate the K_{CLIC} channel indirectly by elevating $[Ca^{2+}]_i$ [31].

The conductance of the K channel in T_{84} cells was 18.2 ± 0.4 pS between ± 20 mV when bathed symmetrically with 150 mM KCl solution, similar to that reported for the K_{CLIC} channel from dog airway cells under comparable conditions (19.4 pS; [51]). Inward rectification was an intrinsic property of the carbachol-activated K channel in T_{84} cells and did not require magnesium (Fig. 7). It is unclear whether this rectification would be apparent *in situ;* the membrane potential in colonic crypt

cells probably range between −20 and −60 mV where the *I/V* relationship is relatively linear, and inward currents would be reduced under physiological conditions due to low extracellular [K] and fast block by external sodium ions. The reversal potential of this channel would be −45 mV (assuming $P_{Na}/P_K = 0.178$, $P_{Cl}/P_K = 0.074$ and reasonable values for extracellular and intracellular ion activities (mM); 3.1 K_o, 100 Na_o, 78 Cl_o and 120 K_i, 15 Na_i and 35 Cl_i, respectively). Thus, carbachol activation of this channel would be expected to cause partial depolarization rather than hyperpolarization from the resting potential of −54 mV (*see above*). Nevertheless, the membrane potential would be clamped ~20 mV more negative than the chloride equilibrium potential, and therefore would help maintain the electrical gradient favoring apical Cl efflux. Finally, the high P_{Rb}/P_K permeability ratio would explain the ability of carbachol and A23187 to stimulate large ^{86}Rb effluxes at the basolateral surface of T_{84} monolayers [6, 14, 28].

Single channel and I_{sc} responses to carbachol were relatively insensitive to common K channel blockers. They were unaffected by barium at high concentrations (10 mM), in contrast to the cAMP-stimulated I_{sc}, which was highly sensitive to barium on the basolateral side (Fig. 10*c*). The ability of barium to inhibit most of the cAMP-stimulated current suggests that inwardly rectifying K channels contribute little to basolateral K conductance during cAMP-stimulated secretion. The inwardly rectifying K channel in T_{84} cells described in this study is much less sensitive to barium than was Ca ionophore-stimulated efflux of ^{86}Rb from T_{84} [28] and airway cells [32]. Ionophore-stimulated Rb efflux was sensitive to 5 mM barium in the former study, whereas single inwardly rectifying K channels and the carbachol-stimulated I_{sc} in T_{84} monolayers were insensitive to 10 mM Ba in the present work. It is conceivable that calcium ionophores stimulate flux through other Rb-permeable channels in addition to the inward rectifier, and these contributed to barium-sensitive ^{86}Rb effluxes measured in previous studies.

Activation of additional or alternative K channels could also explain why high concentrations of charybdotoxin [35], quinidine or 4-AP inhibited some of the I_{sc} without significantly affecting single, inwardly rectifying channels. If another K channel is involved in the carbachol response, it is unlikely to be the high-conductance, calcium-activated K channel since these were rarely observed in T_{84} cells. Moreover, kaliotoxin, a peptide which inhibits maxi K channels, had no effect on carbachol-stimulated I_{sc}. High concentrations of external TEA (10–40 mM) did cause small decreases in single channel conductance but had no obvious effects on open probability. Resistance to TEA is not unique to this channel, but it contrasts with some channels such as the maxi K channel, which is inhibited strongly by external TEA ($IC_{50} \leq 1$ mM; e.g., [49]). Quinidine, which blocks Ca-activated K conductances in many cells and swelling-activated K conductance in the basolateral membrane of turtle colon epithelium [17], did not inhibit the inward rectifier, nor did 4-aminopyridine, another commonly used K channel inhibitor [21]. At present, we cannot exclude the possibility that blockers decreased open probability; this effect could have been missed since blockers were present in the pipette throughout the recordings. Regardless of the mechanism of inhibition in intact cells, low sensitivity to external blocker during excised patch clamp experiments was not due to abnormal membrane voltage (Fig. 8*c*) or to competition with high potassium in the pipette solution (Fig. 8*d*).

In conclusion, the regulation and pharmacology of single, inwardly rectifying K channels are both consistent with I_{sc} responses to carbachol, evidence that this channel mediates the carbachol response in T_{84} cells. A channel with similar properties was described in cultured tracheal cells from dog [51] and recently has been reported on the surface of subconfluent T_{84} cells by two other groups [11, 40]. The transient elevation of intracellular Ca evoked by carbachol is consistent with the time course of the I_{sc} response in unstimulated T_{84} monolayers, but the present data suggest that cAMP and other pathways are also likely to modulate the channel's response to calcium.

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