J. Membrane Biol. 142, 255–266 (1994)

The Journal of
Membrane
Biology

Regulation of an Inwardly Rectifying K Channel in the T_{84} Epithelial Cell Line by Calcium, Nucleotides and Kinases

J.A. Tabcharani, A. Boucher, J.W.L. Eng, J.W. Hanrahan
Department of Physiology, McGill University, 3655 Drummond Street, Montreal, Quebec H3G 1Y6 Canada

Received: 8 June 1994/Revised: 8 August 1994

Abstract. Agonists that elevate calcium in T_{84} cells stimulate chloride secretion by activating K_{BIC}, an inwardly rectifying K channel in the basolateral membrane. We have studied the regulation of this channel by calcium, nucleotides and phosphorylation using patch clamp and short-circuit current (I_{SC}) techniques. Open probability (P_o) was independent of voltage but declined spontaneously with time after excision. Rundown was slower if patches were excised into a bath solution containing ATP (10 μM–5 mM), ATP (0.1 mM) + protein kinase A (PKA; 180 nM), or isobutylmethylxanthine (IBMX; 1 mM). Analysis of event durations suggested that the channel has at least two open and two closed states, and that rundown under control conditions is mainly due to prolongation of the long closed time. Channel activity was restimulated after rundown by exposure to ATP, the poorly hydrolyzable ATP analogue AMP-PNP, or ADP. Activity was further enhanced when PKA was added in the presence of MgATP, but only if free calcium concentration was elevated (400 nM). Nucleotide stimulation and inward rectification were both observed in nominally Mg-free solutions. cAMP modulation of basolateral potassium conductance *in situ* was confirmed by measuring currents generated by a transepithelial K gradient after permeabilization of the apical membrane using α-toxin. Finally, protein kinase C (PKC) inhibited single K_{BIC} channels when it was added directly to excised patches. These results suggest that nonhydrolytic binding of nucleotides and phosphorylation by PKA and PKC modulate the responsiveness of the inwardly rectifying K channel to Ca-mediated secretagogues.

Correspondence to: J.W. Hanrahan

Key words: K_{BIC} — Inward rectifier — Cystic fibrosis — ATP — cAMP — Protein kinase

Introduction

Carbachol activates basolateral potassium conductance [49] and stimulates chloride secretion [14, 15] by the colonic tumor cell line T_{84}. The main intracellular messenger mediating this response is calcium [23, 48], but other signals have also been implicated and these could interact with the calcium pathway at several levels during stimulus-secretion coupling. For example, agonists that elevate cAMP greatly enhance the secretory response to Ca ionophores. This synergism was originally attributed to cAMP activation of Cl conductance in the apical membrane and K conductance in the basolateral membrane [6]; however, more recent studies suggest that neither T_{84} [8, 33] nor another intestinal cell line HT_{29} [1] have cAMP-activatable K conductances. Carbachol does not increase cAMP levels in T_{84} cells [15] but has been proposed to activate some other pathway because much larger secretory responses are observed with carbachol than with Ca ionophores under conditions that cause a comparable increase in $[Ca]_i$ [13, 48, 49]. Although protein kinase C would seem the most likely candidate since its activity in T_{84} cells is also increased by carbachol [9], phorbol esters that activate PKC are reported to acutely stimulate [24, 25] and inhibit [24, 33] secretion, whereas PKC antagonists have little effect on carbachol-stimulated secretion [25]. Thus, the roles of PKA and PKC in modulating secretory responses to Ca-mobilizing agonists remains unclear.

An inwardly rectifying potassium channel similar to one described previously in dog trachea [47] mediates calcium-activated K conductance in T_{84} cells. It is

present only on the basolateral surface of confluent T_{84} monolayers dissected from porous supports [40] but is found on the apical surface of T_{84} cells before they reach confluence [11, 34, 39, 40]. The channel has ~5-fold selectivity for K over Na, is susceptible to "fast block" by external sodium ions, weakly inhibited by external tetraethylammonium ions (TEA), but relatively insensitive to quinidine, 4-aminopyridine, charybdotoxin, or kaliotoxin, and unaffected by external barium [40]. We refer to this K channel in T_{84} cells as the K_{BIC} channel based on its most distinctive properties (Ba-insensitive, inwardly-rectifying, Ca-activated). Evidence for its role in secretion was obtained by recording channel activity on the basolateral surface of polarized T_{84} monolayers, and by comparing the pharmacologic properties of single K_{BIC} channels with those of the carbachol-stimulated short-circuit current (I_{SC}) [40].

In this paper we examine modulation of the K_{BIC} channel by second messengers using excised patches and T_{84} monolayers in which the apical membrane is permeabilized with *Staphylococcus aureus* α-toxin. The results suggest that activity of this channel depends on the integration of several signals, including intracellular free Ca and ATP concentrations, and phosphorylation by protein kinases A and C. These results have been reported in preliminary form [20, 39].

Materials and Methods

Cell Culture

The T_{84} cell line was obtained from the American Type Culture Collection (Rockville, MD) and studied as described previously [40, 41]. Cells were cultured 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 from Collagen Corp. (Palo Alto, CA) was crosslinked on Millicell® inserts using standard methods [7]. Cells were plated at ~5×10^5/cm^2 on glass coverslips for patch clamp experiments and used after 2–5 days. For Ussing chamber studies, cells were plated on collagen-coated Millicell® inserts and studied after reaching confluence (9–14 days).

Experimental Solutions

Cells were transferred to a patch clamp chamber containing (mM): 150 NaCl, 4 KCl, 2 $MgCl_2$, 5 glucose, 10 TES, pH 7.4. In most experiments this was exchanged with a standard bath solution containing 150 mM KCl and various Ca/EGTA mixtures as indicated. The pipette solution contained (mM): 150 KCl, 2 $CaCl_2$, 10 TES, pH 7.4. Dibutyryl cyclic-AMP (db-cAMP; 0.5 mM), forskolin (10 μM) and 3-isobutyl-1-methylxanthine (IBMX; 10 μM) were added to elevate intracellular levels of cAMP. During Ussing chamber experiments with an apical → basolateral K gradient, apical "high-K, low-Ca" solution contained (mM): 115 Kgluconate, 25 $NaHCO_3$, 1.2 $MgCl_2$, 0.4 KH_2PO_4, 2.4 K_2HPO_4, 60 mannitol and 10 glucose. The basolateral "low-K" solution was identical to the apical solution except Kgluconate was replaced by Nagluconate and 1.2 mM $CaCl_2$ was added. Carbachol, forskolin, IBMX, db-cAMP, adenosine triphosphate and *S. aureus* α-toxin were from Sigma (St. Louis, MO). Preparations of the catalytic subunit of Type II bovine cardiac protein kinase A and rat brain protein kinase C (Type II) were from the laboratory of Dr. M.P. Walsh and have been described previously [38]. The free calcium concentration in patch clamp solutions was calculated using association constants after correction for temperature and ionic strength [4]. In the nominally Ca-free "high-K, low-Ca" solution, total calcium concentration was found to be 26.2 μM using flame atomic absorption spectroscopy. The free Ca concentration in this solution was estimated to be 3.3 μM using a generic association constant for gluconate (a mean constant based on determinations for nine monocarboxylic acids) in conjunction with published association constants for ATP and inorganic salts in the saline [4].

The role of nucleotide hydrolysis in channel activation was examined using the poorly hydrolyzable ATP analogue AMP-PNP. It has been reported that commercially available preparations of AMP-PNP contain up to 0.5% ATP contamination; i.e., much higher than specified on manufacturer's data sheets [5]. We therefore depleted the AMP-PNP and ADP nucleotide stock solutions of trace ATP by incubating them with 50 mM glucose and 86 units of Type C-130 hexokinase from baker's yeast (Sigma) at 30°C for 15 min. Protein was removed by centrifugation at 1,500 × *g* using an Amicon 30 microconcentrator as described previously [5]. In control experiments this same protocol abolished channel responses to 100 μM ATP.

Single Channel Currents

Patch clamp studies were carried out as described previously [19, 40]. Briefly, pipettes were pulled (PP-83, Narishige Instr. Lab., Tokyo) from borosilicate glass (KIMAX) capillaries and had resistances of 4–6 MΩ when filled with 150 mM KCl solution. A chlorided Ag wire was inserted into the pipette and the bath was grounded through an agar bridge having the same composition as the pipette solution. Voltages were corrected for liquid junction potentials arising at the agar bridge electrodes, which 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 using an 8-pole Bessel-type filter (902 LPF, Frequency Devices, Haverhill, MA) during digitization by the computer. The final bandwidth (−3 dB) after recording and playback was 230 Hz, except when event durations were measured, it was 818 Hz. Records were sampled at 1 or 2 kHz, respectively, and analyzed using a laboratory microcomputer system (Indec Systems, Sunnyvale, CA) as described previously [19, 40]. V_p refers to the command voltage applied to the pipette interior, referenced to the bath. Current-voltage (*I/V*) relationships were calculated by a semi-automated procedure in which amplitude histograms were computed for short segments of record. Reversal potentials were estimated by interpolation after fitting a polynomial function to the *I/V* curve. The mean number of channels open during successive segments of the record ($\langle I \rangle / i = NP_o$) was computed from the fraction of time spent at each multiple of the single channel current. To minimize the variability caused by rundown, NP_o was normalized to the values obtained with 1 mM MgATP or 400 nM $[Ca]_i$ when determining the ATP or calcium dependence of the channel, respectively. When P_o was relatively high (>0.1), it was estimated by dividing NP_o by the maximum number of simultaneous openings during long recordings. The experiments in Fig. 1 were performed at 37 ± 1°C, all other patch clamp studies were carried out at room temperature (21 ± 1°C).

Transepithelial K Currents

The Ussing chambers, voltage clamps, and data acquisition were described previously [40, 41]. Potassium current flowing through the basolateral membrane was measured with high-K gluconate solution bathing the apical side and high-Na gluconate solution bathing the basolateral side (*see above*). These solutions had 25 μM and 1.2 mM total calcium, respectively. α-Toxin (50 U/ml) was added to the luminal side to permeabilize the apical membrane. ATP (5 mM) was also added to maintain the viability of the monolayer. The effects of agonists and blockers on I_{SC} were tested after the current had stabilized under these conditions. At the end of each experiment, the K concentration of the solution bathing the basolateral side was raised to match that on the apical side to confirm that the I_{SC} was dependent on the K gradient. Ussing chamber experiments were carried out at 37 ± 1°C.

Results

Voltage-independent, Inwardly Rectifying K Channel

K_{BIC} is a barium-insensitive, inwardly rectifying K channel in the basolateral membrane of confluent T_{84} monolayers that is activated by carbachol or calcium ionophores [11, 34, 39, 40]. Figure 1*a* identifies this channel in a patch that had been excised into symmetrical 150 mM KCl solution containing 2 mM $CaCl_2$ (37°C). Under these conditions, the mean current-voltage (*I/V*) relationship in Fig. 1*b* yielded a conductance of 21.4 ± 1.3 pS (x ± SE, $n = 6$) near the reversal potential of 0.6 ± 1.1 mV, in agreement with previous results [11, 34, 39, 40]. Replacing 150 mM KCl bath solution with 400 mM KCl shifted the reversal potential from 0.6 mV to −21.0 ± 1.5 mV. This corresponds to a permeability ratio P_{Cl}/P_K = 0.08 ± 0.04 if one assumes that the activity coefficients for potassium ions in the 150 and 400 mM KCl solutions are 0.74 and 0.67, respectively. Moderate (~5-fold) selectivity for potassium over sodium was evident from the reversal potential measured when patches were excised into normal Ringer solution containing 146 mM NaCl and 4 mEq/l KCl (+40.7 ± 2.3 mV; $n = 7$). The effect of membrane voltage was examined over the range −60 to 0 mV with 150 mM KCl and 2 mM $MgCl_2$ in the pipette solution and 146 mM NaCl, 4 mM KCl, 400 nM free Ca and 1 mM IBMX in the bath. Membrane voltage had no effect on P_o under these conditions. Similar results were obtained in cell-attached patches ($n = 2$) and with symmetrical 150 mM KCl solutions over a wider voltage range (±60 mV; $n = 1$).

Channel Rundown in Excised Patches

When patches were excised from unstimulated T_{84} cells into a high-Na bath solution containing 400 nM calcium, activity of the inwardly rectifying K channel at 0 mV increased instantly and then slowly declined during the next 5 min regardless of calcium concentration (Fig. 2*a,b*). Open probability (P_o) also declined spontaneously when patches were exposed to 0.1–5 mM MgATP or to 1 mM isobutylmethylxanthine (IBMX), but the rundown was slower (Fig. 2*c*). Results under each condition are summarized in Fig. 2*d*. The inhibitory effect of MgATP on rundown was not enhanced by PKA, although phosphorylating conditions did increase the mean open and mean closed times (*see below*). Fitting the curves in Fig. 2*d* with single exponentials yielded the following time constants for channel rundown; 47.5 sec under control conditions, 72.3 sec with 0.1 mM ATP alone, and 93.9 sec with 0.1 mM ATP + 180 nM PKA. Channel activity was nearly constant between 1 and 9 min after excision in IBMX-containing solution and was therefore not fitted. Rundown was not slowed by phosphatase inhibitors such as okadaic acid or bromotetramisole [2, 3], nor was it accelerated by the addition of alkaline phosphatase to the bath (*data not shown*).

To further investigate how activity changed during the course of channel rundown, patches were excised

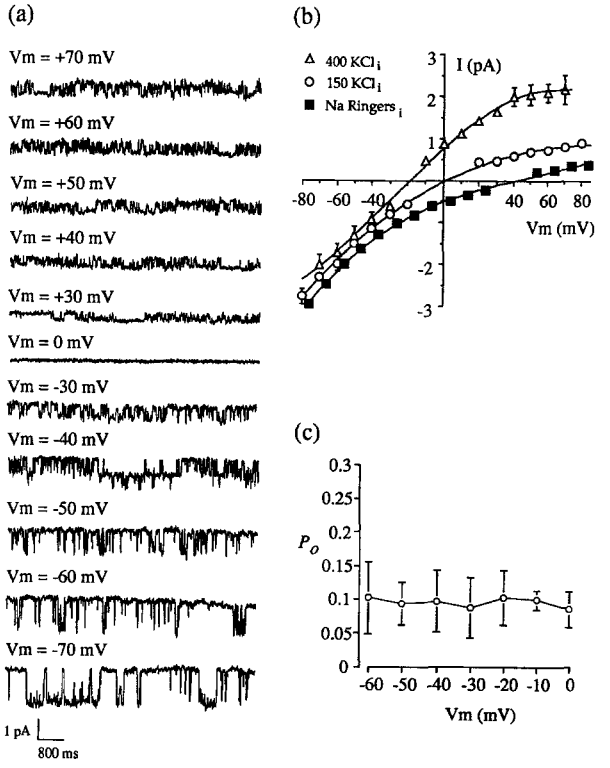

Fig. 1. Identification and voltage insensitivity of the Ba-insensitive, inwardly-rectifying, Ca-activated potassium (K_{BIC}) channel from T_{84} cells. (*a*) Recordings from a patch bathed with 150 mEq/l K on both sides. (*b*) Current-voltage relationship with pipette solution containing 150 mM KCl and either (■) sodium Ringer (146 NaCl, 4 mM KCl), (○) 150 mM KCl or (△) 400 mM KCl in the bath. Means ± SE, $n = 4$–7 patches. (*c*) Open probability (P_o) of the inwardly rectifying K channel in an excised patch bathed on the cytoplasmic side with solution containing 400 nM free calcium and 1 mM IBMX. Means ± SE, $n = 4$ patches.

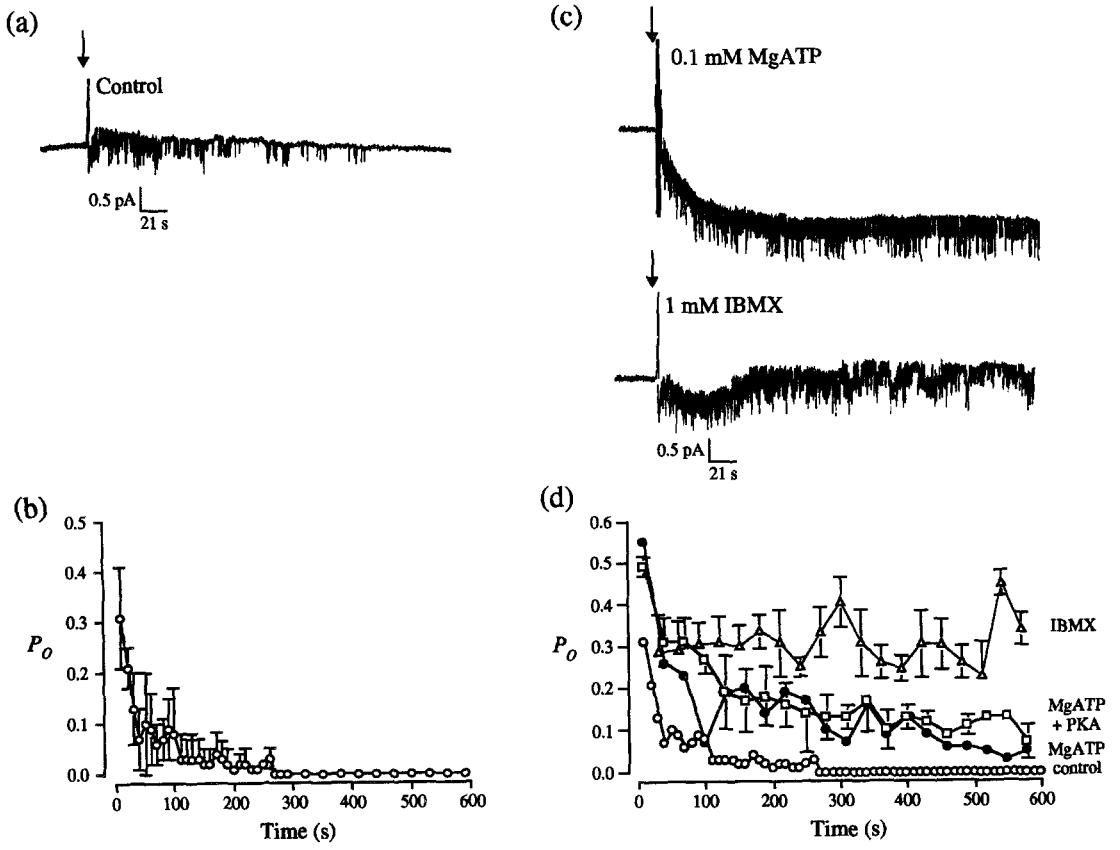

Fig. 2. Spontaneous decline or "rundown" of inwardly rectifying K channel activity after patches are excised into solutions containing 400 nM free calcium (calculated). The pipette solution contained 150 mM KCl and 2 mM $MgCl_2$. The control bath solution was low-calcium Ringer containing 146 mM NaCl and 4 mM KCl and 400 nM free Ca. The membrane potential was 0 mV. (*a*) Representative recording showing rundown of two channels in a patch, excised at the arrow. (*b*) Mean time course of channel rundown when patches were excised into solution containing 400 nM calculated free calcium (mean ± SE, $n = 3$). (*c*) Examples of rundown when patches are excised at the arrows into solutions containing 400 nM calculated free Ca concentration and 0.1 mM ATP or 1 mM isobutylmethyl xanthine (IBMX). (*d*) Mean open probability as a function of time after excising patches into 400 nM free calcium with (●) 1 mM MgATP alone, (□) 1 mM MgATP + protein kinase A (PKA), or (△) 1 mM IBMX.

from unstimulated T_{84} cells into sodium Ringer solution containing 400 nM free calcium. Several of the patches excised under each condition appeared to contain only a single active channel with high P_o during the first few seconds. Long recordings were made of these patches and event durations were determined during three arbitrary time periods; 0–50, 50–100, and 100–150 sec.

Figure 3 shows the events collected during the 100–150 sec interval after excision into a high-Na solution containing 400 nM free Ca with no other agents (control conditions), with 0.1 mM MgATP, with 0.1 mM MgATP + 180 nM PKA, or with 1 mM IBMX. Event histograms were generated for each patch and fitted with two exponentials using a nonlinear, least squares routine. Figure 3 shows the pooled data obtained from three patches under each condition. MgATP and IBMX reduced the overall mean open time by two- to fourfold and decreased the mean closed time more than sevenfold. By contrast, MgATP + PKA increased the mean open time ~9-fold and also increased the closed time. Thus, while MgATP, MgATP + PKA, and IBMX all inhibited rundown, transition rates were increased by MgATP or IBMX and decreased by phosphorylating conditions.

The time constants during rundown are only approximations due to nonstationarity and the small number of events during each interval; nevertheless, they were calculated in an attempt to identify a kinetic state that is responsible for the rundown. The results summarized in Fig. 4 suggest that rundown is complex and that several constants are altered with time after excision. Much of the decline in P_o under control conditions was caused by an increase in the duration of long closures (Fig. 4*d*), with little decrease in mean open time. The increase in the long mean closed time (Fig. 4*d*) was abolished by IBMX or PKA + MgATP and inhibited by MgATP. Exposure to ATP + PKA also increased both mean open times. On the other hand, the open times were much shorter when MgATP or IBMX were present (*compare* control *vs.* MgATP or IBMX in Fig. 3) and this did not change during rundown (Fig. 4).

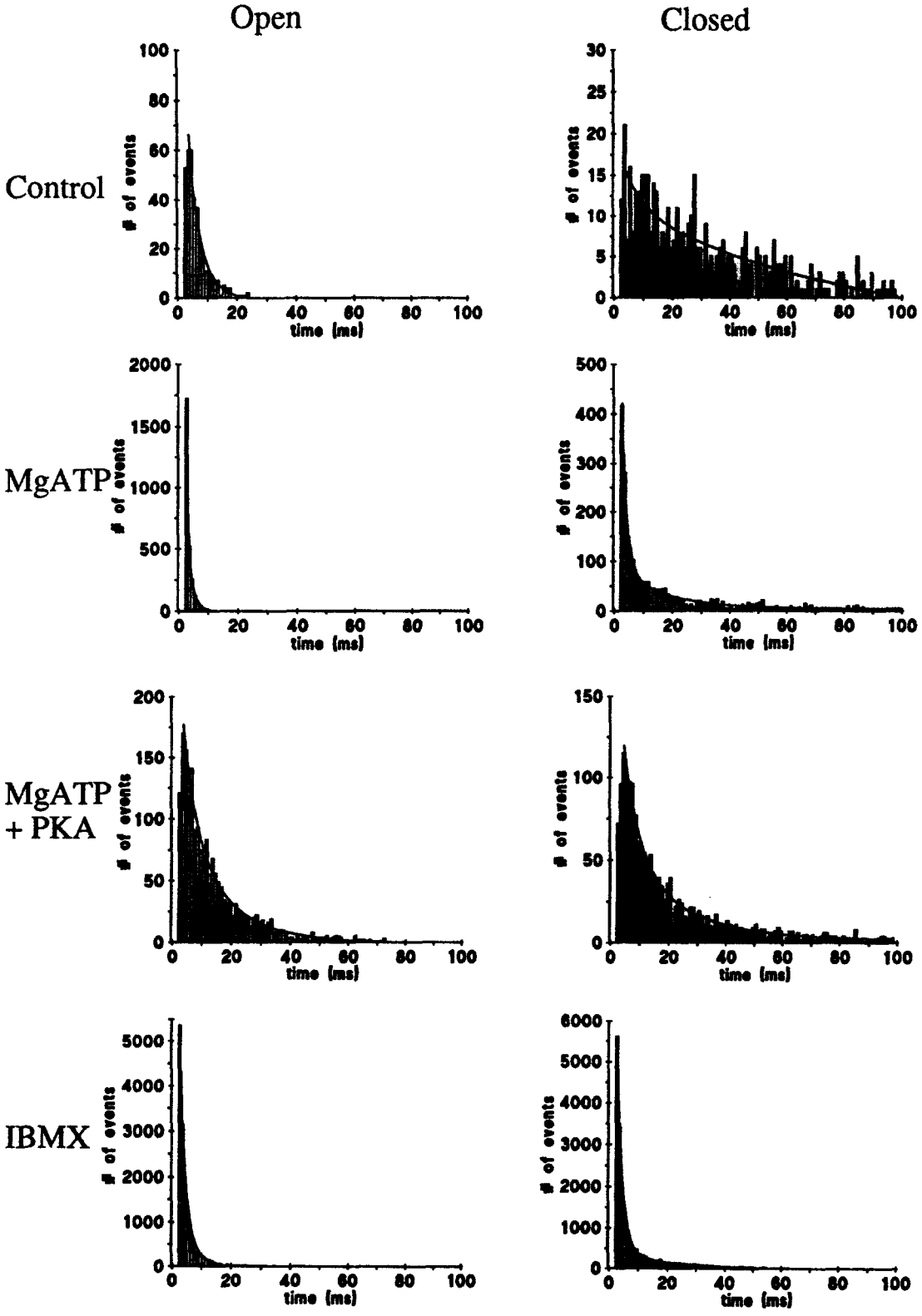

Fig. 3. Histograms of event durations recorded 100–150 sec after patches were excised into solution containing 146 mM NaCl, 4 mM KCl and 400 nM free calcium. Excised patches that apparently contained only a single active channel were analyzed under control conditions or in the presence of 1 mM ATP, 1 mM ATP + 180 nM protein kinase A catalytic subunit (PKA), or 1 mM IBMX. The histograms contain data from three patches under each condition and are well fitted by two exponential components. Note similarity of event durations during exposure to Mg ATP or IBMX.

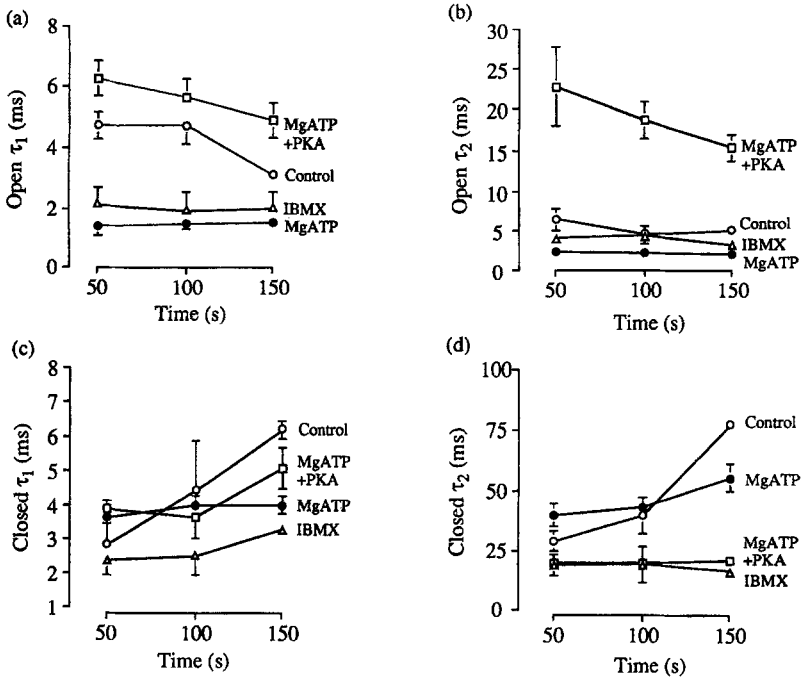

Fig. 4. Time constants obtained by fitting open and closed time histograms for events during three time intervals (0–50, 50–100, and 100–150 sec) after excising patches. (*a, b*) Short and long time constants from open time histograms, respectively. (*c, d*) Short and long time constants from closed time histograms, respectively. Experiments were carried out using patches bathed with solution containing 150 mM KCl, 2 mM $MgCl_2$ and 400 nM free calcium. (○) Control solution, (●) 0.1 mM MgATP alone, (□) 0.1 mM MgATP + protein kinase A (PKA), (△) 1 mM IBMX.

Activation by Nucleotides, Kinases and Calcium

Inwardly rectifying K channels in other cells can be regulated by MgATP, protein kinases, as well as calcium ions [44, 46]; we therefore examined the effects of these modulators on activity of the K_{BIC} channel in excised patches. Figure 5*a* shows the effect of adding 0.1 mM ATP to the cytoplasmic side of a patch containing two channels. The pipette solution contained 150 mM KCl externally and the bath contained 146 mM NaCl, 4 mM KCl, and 400 nM free calcium. In three patches, P_o increased almost tenfold from: 2.4×10^{-3} under control conditions to $22.3 \pm 10.5 \times 10^{-3}$ ($n = 3$) during exposure to 0.1 mM ATP.

Vasoactive intestinal peptide enhances carbachol-activated ^{86}Rb efflux from T_{84} cells [6] but it remains unclear whether this is due to cAMP activation of the carbachol-regulated K conductance or to membrane depolarization induced by activation of Cl channels [21]. We examined direct effects of PKA on the K_{BIC} channel by adding its catalytic subunit to excised patches in the presence of 0.1 mM MgATP. PKA increased P_o more than fourfold (from 22.3 to $90.3 \pm 12.5 \times 10^{-3}$; mean ± SE, $n = 3$) under these conditions (Fig. 5).

Acute stimulation of PKC by phorbol esters can increase [24, 25] or decrease [24, 33] secretion across T_{84} monolayers. On the other hand, downregulation of PKC in T_{84} cells by sustained exposure to phorbol ester inhibits the carbachol-stimulation of ^{86}Rb efflux [24]. To clarify the role of PKC, we examined the effect of adding 3.8 nM PKC II purified from rat brain and the lipid activator 1,2-dioctanoylglycerol (8:0) (DiC_8; 5 μM) to the cytoplasmic side of excised patches in the presence of 400 nM free calcium. PKC was a potent inhibitor of the inwardly rectifying K channel (Fig. 5*c*), reducing P_o by 92 ± 5% ($n = 3$). Inhibition of the K_{BIC} was observed with or without PKA pretreatment. These effects are reminiscent of the actions of PKC on inwardly rectifying K channels in the kidney [45] and on K currents expressed from intestinal mRNA after injection into *Xenopus* oocytes [37]. ADP did not alter channel activity when added to patches already stimulated with 1 mM ATP ($n = 4$ patches).

To examine if stimulation by MgATP requires hydrolysis, we tested the ability of the nonhydrolyzable ATP analogue AMP-PNP to reactivate channels that had rundown in a solution containing 400 nM free calcium. Adding 1 mM MgAMP-PNP to the cytoplasmic side of inside-out patches bathed symmetrically with high-K solutions consistently activated K_{BIC} channels after rundown (Fig. 6*a*). AMP-PNP increased NP_o by 6.6 ± 1.3-fold compared to 21.3 ± 9.0-fold with MgATP (Fig. 6*b*). ADP (1 mM) also activated the K_{BIC} channel in excised patches (Fig. 6*c*). Hexokinase pretreatment did not affect the stimulation by ADP or AMP-PNP, although the same protocol abolished activation by 100 μM MgATP. Thus, it seems likely that nonhydrolytic nucleotide binding is sufficient to activate the K_{BIC} channel.

ATP has a dual effect on ATP-sensitive K channels; it sustains activity when added at low concentrations to excised patches [17, 44], presumably through a phosphorylation mechanism, and inhibits at higher concentrations [10, 18, 29, 30, 36, 46]. We therefore examined the dependence of K_{BIC} channel activity on ATP con-

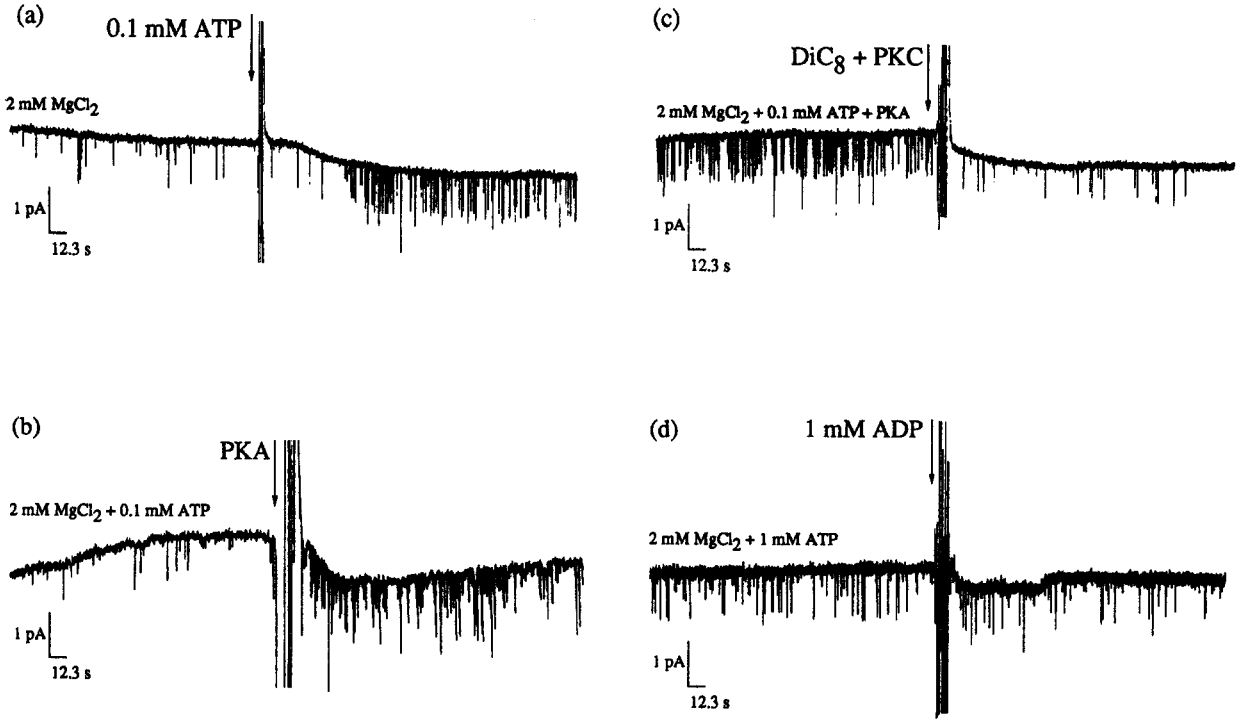

Fig. 5. Effects of nucleotides and kinases on the K_{BIC} channel in excised patches. In each case the pipette solution contained 150 mM KCl and 2 mM $MgCl_2$; the bath solution contained 150 mM KCl, 2 mM $MgCl_2$, and 400 nM free calcium, and the membrane potential was −30 mV. (*a*) Effect of adding 0.1 mM MgATP under these conditions. (*b*) Effect of adding 180 nM protein kinase A to the bath solution containing 2 mM $MgCl_2$ and 0.1 mM ATP. (*c*) Effect of adding the lipid activator DiC_8 and protein kinase C to a patch containing two channels prestimulated by 0.1 mM ATP and 180 nM PKA. (*d*) Lack of inhibition by 1 mM ADP when added in the presence of ATP.

centration. Figure 7*a* shows traces obtained from a patch bathed with 400 nM free calcium and different MgATP concentrations on the cytoplasmic side. Transitions were rarely observed in the absence of ATP but increased progressively as MgATP concentration was elevated to 5 mM. Figure 7*b* shows the relationship between mean number of channels open and MgATP concentration. Significant stimulation was observed when [MgATP] exceeded 100 μM. The potency of 1 mM ATP was similar in nominally Mg-free saline, therefore the active species is probably ATP rather than MgATP. Free Ca was allowed to decline in these experiments as ATP concentration was elevated: compared to control conditions (0 mM ATP), the calculated free calcium concentrations were 99.3% at 0.1 mM ATP, 95.1% at 1 mM ATP, 88.4% at 5 mM ATP and 87.3% at 10 mM ATP. Correcting for this decline in $[Ca^{2+}]_i$ would be expected to increase the steepness of the relationship between NP_o and [MgATP] somewhat but would not explain the decline in NP_o when [MgATP] was raised to 10 mM (*see* Fig. 10). Thus, ATP inhibits the K_{BIC} channel, but at much higher concentrations compared to other ATP-sensitive K channels.

The ability of PKA to activate the K_{BIC} channel was unexpected because the cAMP-stimulated I_{SC} measured across monolayers was blocked by barium whereas the K_{BIC} channel was Ba insensitive. In an attempt to reconcile these observations, we examined whether PKA enhances activity of the inward rectifier only when $[Ca^{2+}]_i$ is elevated. Figure 8 shows a representative recording; the channels were quiescent when exposed to 0.1 mM ATP and 45 nM free calcium (which is within the range of reported free Ca levels in resting T_{84} cells) and did not respond when PKA was also added to the bath until the free calcium concentration was increased to 400 nM, the level observed during carbachol stimulation in our experiments [40]. Considering that 100 nM free Ca usually causes some activation of the channel, the absence of activity with 45 nM free calcium and PKA implies that phosphorylation does not dramatically shift the channel's calcium dependence to lower Ca concentrations. Rather, it appears that calcium is permissive, enabling the K_{BIC} channel to respond to phosphorylation.

To examine cAMP regulation of the inward rectifier *in situ*, K current flowing through the basolateral membrane was studied using T_{84} monolayers that had been exposed to α-toxin on the apical side in the presence of a K gradient, as described in Materials and Methods. Figure 9*a* shows the mean current measured during the permeabilization protocol. At arrow *1*, the apical solution was switched from normal NaCl Ringer to nominally Ca-free, high-K gluconate saline (3.6 mEq/l Cl^-) containing 3.3 μM free calcium. The basolateral solution

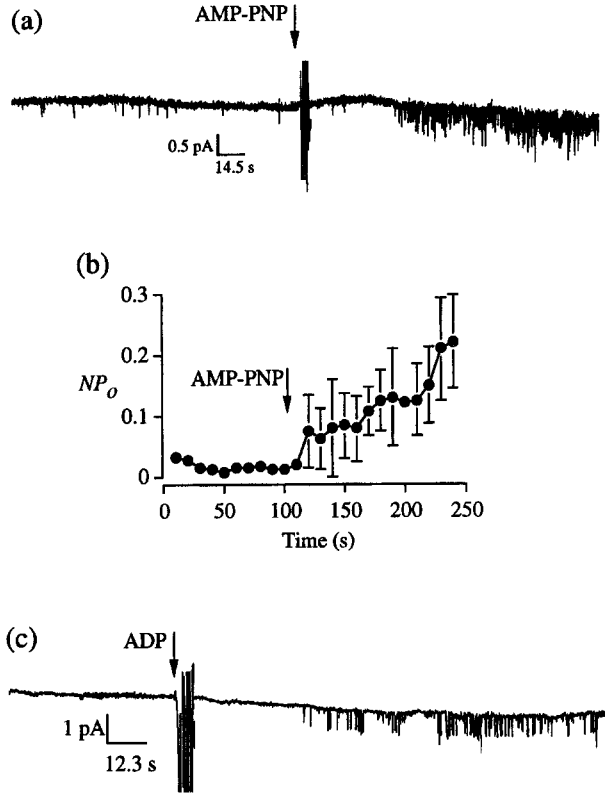

Fig. 6. Effect of the poorly hydrolyzable ATP analogue AMP-PNP or ADP on K_{BIC} channel activity in excised patches. (*a, b*) Representative trace showing activation of K channels by the addition of AMP-PNP at the arrow. Pipette solution: 150 mM KCl, 2 mM $MgCl_2$; bath solution: 146 mM NaCl, 4 mM KCl, 400 nM free calcium. Means ± SE, $n = 4$ patches. (*c*) Activation of channels by 1 mM ADP after they had rundown in 400 nM free calcium solution.

was replaced simultaneously with Nagluconate saline containing 1.8 mM $CaCl_2$. At arrow *2*, α-toxin (50 μg/l) and ATP (5 mM) were added to the apical side. After I_{SC} had stabilized, monolayers were challenged by the addition of carbachol (100 μM) to the basolateral side. Potassium currents generated by carbachol under these conditions were qualitatively similar to $[Ca^{2+}]_i$ and Cl secretory responses which normally occur after addition of carbachol in control saline. I_{SC} probably reflects basolateral K current because it was abolished when the imposed K gradient was eliminated by raising [K] on the basolateral side (*Sym K;* Fig. 9*a*). Figure 9*b* shows the effect of adding cAMP mixture at arrow *3* on I_{SC} after permeabilization of the apical membrane and with micromolar calcium in the apical solution. cAMP caused a two- to threefold stimulation of the I_{SC}, and this K current was relatively insensitive to TEA (10 mEq/l), barium (2 mEq/l)or 4-AP (10 mEq/l). The pharmacological properties of this cAMP-activated current, particularly its insensitivity to barium, are consistent with those of the K_{BIC} channel but clearly different from the K conductance that normally mediates cAMP stimulation, which is strongly inhibited by barium.

Finally, the calcium dependence of the K_{BIC} channel was assessed by determining the mean number of channels open (NP_o) when patches were bathed with different EGTA/$CaCl_2$ mixtures. To reduce the variability caused by rundown, 1 mM IBMX was included in the bath and NP_o was normalized for each patch to the value obtained with 400 nM free Ca (Fig. 10). The $K_{1/2}$ for calcium activation was ~400 nM under these conditions.

In summary, cAMP stimulates single K_{BIC} channels and also increases the macroscopic K conductance that is responsive to carbachol. PKA modulation of the K_{BIC} channel is only observed when calcium is elevated; it would therefore contribute to synergism between cAMP- and Ca-mediated secretagogues [6, 41].

Discussion

Activation of basolateral K channels is thought to enhance transepithelial chloride secretion by increasing the electrical gradient favoring Cl exit through the apical membrane. Basolateral K channels minimize changes in intracellular ion levels and cell volume when K entry on the NaK2Cl cotransporter is increased. The basolateral membrane of T_{84} cells contains a carbachol-stimulated K channel [11, 12, 20, 34, 39, 40]. Its main distinguishing features are barium insensitivity, inward rectification, and calcium activation; therefore, we refer to it as the K_{BIC} channel. Its conductance is similar to that of an inwardly rectifying channel described previously on subconfluent primary cultures of dog tracheal epithelium (K_{CLIC}; [27, 28, 47]); however K_{BIC} is less K selective and more resistant to block by charybdotoxin and barium.

The K_{BIC} channel is present exclusively on the basolateral surface of confluent T_{84} monolayers when they are cultured on porous supports [40]. Its pharmacological profile is consistent with the carbachol-activated short-circuit current [11, 16, 33, 40, 42], although early tracer studies indicated somewhat higher sensitivity to barium [26, 27]. In the present study, activity of K_{BIC} declined spontaneously when excised into calcium-containing solutions. Many channels run down in excised patches, including ATP-sensitive K channels (e.g., [18, 22, 43]), but rundown has not been reported previously for epithelial inwardly rectifying K channels. Dephosphorylation at PKA sites is probably not responsible for rundown, which was similar during exposure to ATP alone or MgATP + PKA. Moreover, the spontaneous decline in channel activity was not slowed by okadaic acid (10 mM), a Type 1 and 2A protein phosphatase inhibitor or by fluoride (20 mM), a nonspecific inhibitor of phosphatases and other enzymes that catalyze phosphoryl transfer reactions. Channel activity was pro-

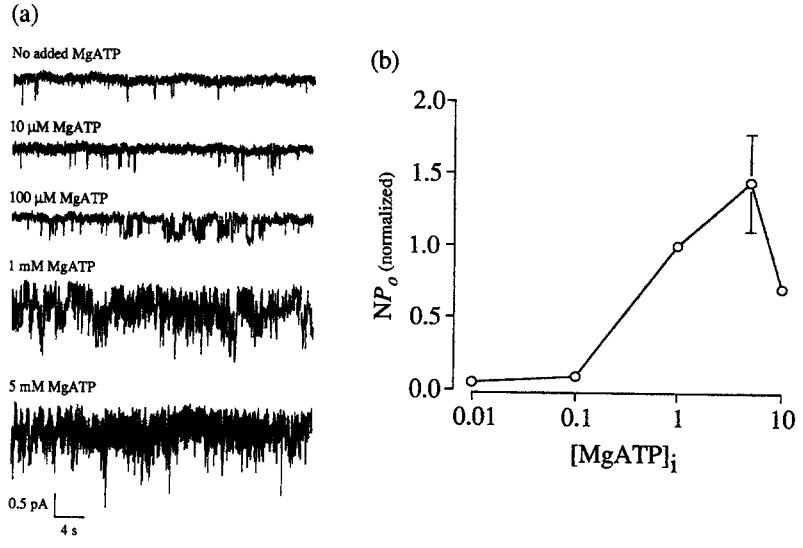

Fig. 7. Effect of increasing MgATP concentrations on activity of the K_{BIC} channel. (*a*) Sequential recordings from a patch bathed with 150 mM KCl pipette solution and with Ringer containing 400 nM free calcium in the bath (V_m = 0 mV). MgATP concentration was increased stepwise from 0 to 5 mM. (*b*) Effect of MgATP concentration on the mean number of channels open (NP_o). NP_o was normalized to the value obtained at 1 mM MgATP.

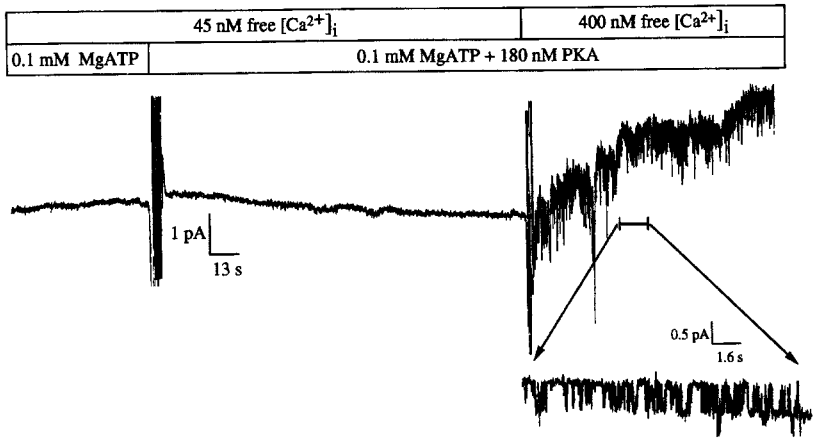

Fig. 8. Activation of the inwardly rectifying K channel by MgATP + PKA requires elevation of calcium. Representative recording showing effects of sequential exposure to MgATP (0.1 mM) and MgATP + PKA (180 nM) with the free calcium concentration buffered at 45 nM and then 400 nM.

longed when patches were excised into solutions containing ATP (1 mM) or IBMX (1 mM). IBMX was recently found to inhibit the rundown of CFTR chloride channel activity and dephosphorylation of CFTR protein [2, 3] but it appears to inhibit rundown of the K_{BIC} channel by a different mechanism that might involve direct interactions with the channel. Exposing active channels to ATP or IBMX reduced event durations and caused a net increase in open probability. PKA + MgATP also slowed the decline in P_o; however, this was associated with an increase in mean open and mean closed times. Taken together, the results suggest that loss of phosphorylation from PKA sites would account for little of the rundown, even though PKA does alter gating.

Activity of the K_{BIC} channel was stimulated by MgATP in the range 0.1–5 mM (Figs. 5*a* and 7) and enhanced further by the addition of PKA. Hydrolysis was apparently not required for nucleotide activation because partial stimulation was obtained using the poorly hydrolyzable ATP analogue AMP-PNP [50] and also by ADP (Fig. 6). The ability of AMP-PNP to mimic ATP has been reported for some ATP-sensitive K channels [10, 36] and not others [44]. Partial activation was not caused by ATP contamination in the present study because pretreating ADP and AMP-PNP stock solutions with hexokinase and glucose did not diminish their potencies, whereas the same protocol abolished activation of the K_{BIC} channel by 100 μM ATP in control experiments. It is uncertain whether ATP would be an important modulator of K_{BIC} activity *in situ*. Cytosolic ATP could fluctuate locally near the basolateral membrane due to, for example, variations in the activity of the basolateral Na/K ATPase pump, and act as a metabolic sensor that inhibits secretion when cellular metabolism is compromised ([32], *reviewed* by [31]). However, the stimulation of K_{BIC} activity by ADP and the lack of effect of ADP on ATP-activated channels implies that this inward rectifier is not regulated by the ATP:ADP ratio *in situ*.

Activation of the K_{BIC} channel by 1–5 mM ATP is reminiscent of ROMK1, a calcium-insensitive inward rectifier cloned from rat kidney outer medulla [22]. The

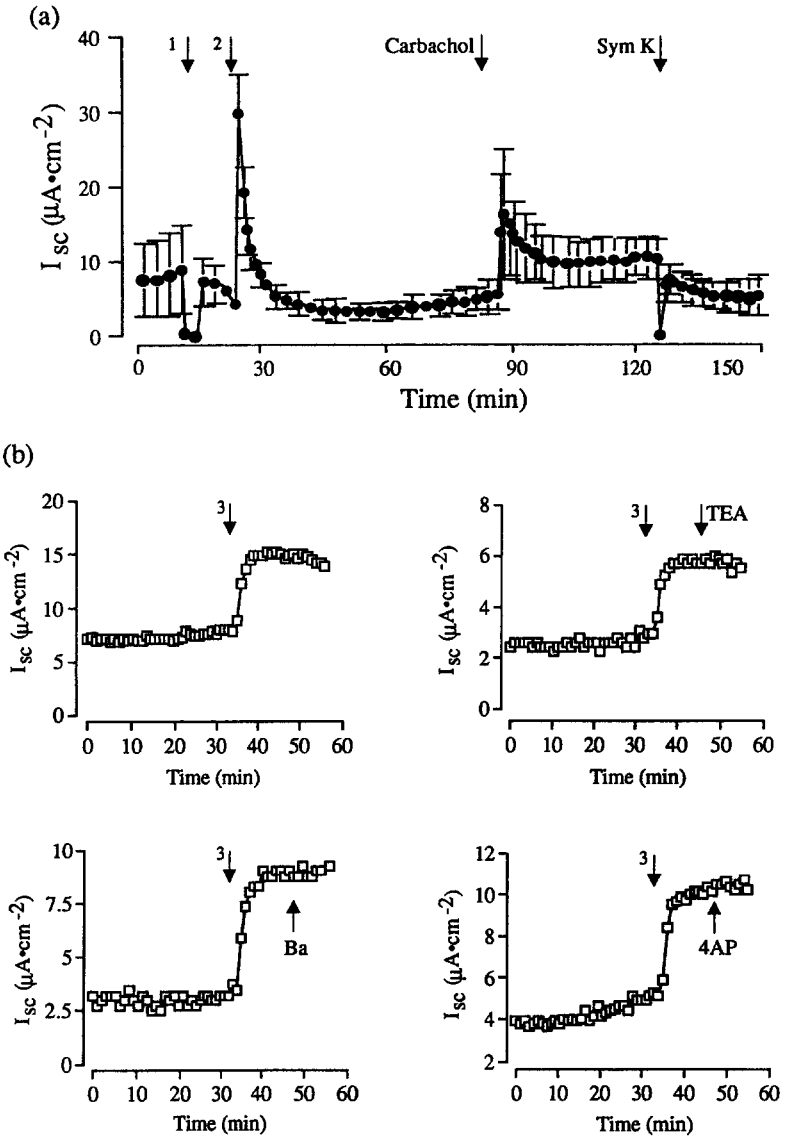

Fig. 9. cAMP stimulation of a carbachol-activated, barium-insensitive K conductance in T_{84} monolayers after permeabilizing the apical membrane. K current was generated by imposing a transepithelial potassium gradient under short-circuit conditions. (*a*) Mean current throughout the permeabilization protocol: (i) monolayers were exposed to sodium gluconate saline on the basolateral side and nominally Ca-free potassium gluconate solution on the apical side at arrow *1*, (ii) α-toxin from *S. aureus* (50 U/ml) and ATP (5 mM) were added to the apical side at arrow *2*, (iii) carbachol (100 μM) was added to the basolateral side at the third arrow, and finally (iv) the K gradient was abolished by replacing serosal sodium gluconate solution containing carbachol with potassium gluconate solution containing carbachol. (*b*) Pharmacology of the potassium-dependent short-circuit current stimulated by cAMP mixture (0.5 mM db-cAMP, 10 μM forskolin and 10 μM IBMX final concentration). Same protocol as in *a* except cAMP mixture was added instead of carbachol. Mean currents were measured with mixture alone (upper left) or after addition of 2 mM Ba, 10 mM tetraethylammonium (TEA), or 10 mM 4-AP; mean of three monolayers under each condition.

K_{BIC} channel was activated by ATP at concentrations up to 5 mM ($K_{1/2}$ = ~0.3 mM) and inhibited by 10 mM ATP. This dual regulation of K_{BIC} by ATP qualitatively resembles an inwardly rectifying K channel in rat cortical collecting duct (CCD), which is activated at low ATP concentrations (10–100 μM) and inhibited by high concentrations (100 μM–5 mM; K_i = 0.5 mM [44]). Unlike the K_{BIC} channel, the K channel from rat CCD is sensitive to ADP, which partially relieves ATP inhibition and enables P_o to be high at physiological ATP_i and ADP_i concentrations. Interestingly, ATP and IBMX both stimulated the K_{BIC} channel when added to excised patches and may involve a common mechanism; they had similar effects on the kinetics of single K_{BIC} channels in excised patches (Figs. 3 and 4) and their effects were not additive when they were added sequentially (F. Becq, J. Tabcharani and J. Hanrahan, *in preparation*).

Protein kinases A and C had opposite effects on activity of the inwardly rectifying K channel in T_{84} cells; PKA was a weak stimulator whereas PKC was a potent inhibitor. Whether these kinases phosphorylate the channel itself or some other membrane-associated protein is unknown. PKA was not expected to modulate the K_{BIC} channel because cAMP-stimulated I_{SC} in this preparation is blocked by barium whereas the K_{BIC} channel is not (Fig. 10 in ref. [40], *see also* [16, 33]). Moreover, forskolin does not stimulate K currents when T_{84} cells are studied using the whole-cell patch configuration with $[Ca]_i$ buffered at 100 nM [8]. These apparent discrepancies may be explained by the present finding that PKA only stimulates K_{BIC} activity when cytoplasmic calcium is elevated (400 nM; Fig. 8). This permissive role of calcium represents a novel mechanism for the synergism commonly observed between calcium- and cAMP-mediated secretagogues.

PKC inhibition of the K_{BIC} channel is consistent

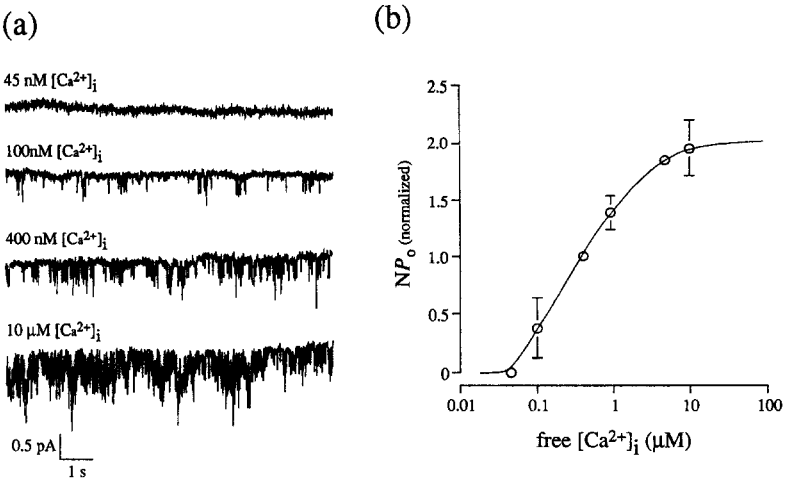

Fig. 10. Effect of free calcium concentration on activity of the inwardly rectifying potassium channel. (*a*) Recordings from a patch bathed with 150 mM KCl pipette solution and Na Ringer in the bath (V_m = 0 mV). The bath was perfused with solutions containing different (calculated) free calcium concentrations. (*b*) Effect of free calcium concentration ($[Ca^{2+}]_i$) on the mean number of channels open (NP_o). IBMX was included in the bath solution to inhibit rundown and NP_o was normalized to the value obtained with 400 nM Ca.

with the reduced activation of basolateral K current by carbachol following acute pretreatment of T_{84} monolayers with phorbol 12-myristate 13-acetate (PMA; [33]), and also the acute inhibitory effect of PMA on carbachol-stimulated ^{86}Rb efflux from T_{84} cells [24]. PKC inhibits inwardly rectifying K currents from intestine expressed in *Xenopus* oocytes [37] and ATP-sensitive inward rectifiers from rat kidney studied using the patch clamp technique [45]. The barium-sensitive channel that mediates basolateral K conductance during cAMP-stimulated Cl secretion is also inhibited by PKC [33, 35].

To study regulation of basolateral K currents *in situ,* the apical membrane was permeabilized using α-toxin (pore diameter 4,000 M_r) and monolayers were exposed to a high-K, low-Cl solution containing 5 mM ATP and 3.3 mM free calcium on the apical side and low-K, low-Cl solution on the basolateral side. Carbachol still activated a small transient current under these conditions, presumably because intracellular calcium levels were lower than 3.3 μM and the IP_3-sensitive stores were full and responsive to receptor activation. More importantly, elevating cAMP caused a sustained increase in transepithelial current that was strictly dependent on the K gradient and insensitive to barium, consistent with the pharmacology of the K_{BIC} channel and with carbachol-activated chloride secretion in normal solutions [40]. Thus, cAMP modulates the carbachol-activated K channel in intact monolayers, and this would contribute to the synergism between cAMP- and Ca-mediated secretagogues.

This work was supported by the Canadian Cystic Fibrosis Foundation and the Medical Research Council of Canada. J.W.H. is a Chercheur-Boursier of the Fonds de la recherche en santé du Québec.

References

1. Bajnath, R.B., Augeron, C, Loboisse, C.L., Bijman, J., De Jonge, H.R., Groot, J.A. 1991. Electrophysiological studies of forskolin-induced changes in ion transport in the human colon carcinoma cell line HT-29 cl.19A: Lack of evidence for a cAMP-activated basolateral K^+ conductance. *J. Membrane Biol.* **122:**239–250
2. Becq, F., Jensen, T.J., Chang, X.-B., Savoia, A., Rommens, J.M., Tsui, L.-C., Buchwald, M., Riordan, J.R., Hanrahan, J.W. 1994. Phosphatase inhibitors activate normal and defective CFTR chloride channels. *Proc. Natl. Acad. Sci. USA* **91:** (*in press*)
3. Becq, F., Jensen, T.J., Riordan, J.R., Hanrahan, J.W. 1994. Phosphodiesterase-independent regulation of CFTR channels by IBMX and theophylline. *Ped. Pulmonol.* **Suppl. 9:**177
4. Brooks, S.P.J., Storey, K.B. 1992. Bound and determined: A computer programme for making buffers of defined ion concentrations. *Anal. Biochem.* **201:**119–126
5. Carson, M.R., Welsh, M.J. 1993. 5′-adenylylimidodiphosphate (AMP-PNP) does not activate CFTR chloride channels in cell-free patches of membrane. *Am. J. Physiol.* **265:**L27–L32
6. Cartwright, C.A., McRoberts, J.A., Mandel, K.G., Dharmsathaphorn, K. 1985. Synergistic action of cyclic adenosine monophosphate- and calcium-mediated chloride secretion in a colonic epithelial cell line. *J. Clin. Invest.* **76:**1837–1842
7. Cereijido, M., Robbins, E.S., Dolan, W.J., Rotunno, C.A., Sabatini, D.D. 1978. Polarized monolayers formed by epithelial cells on a permeable and translucent support. *J. Cell Biol.* **77:**853–880
8. Cliff, W.H., Frizzell, R.A. 1990. Separate Cl^- conductances activated by cAMP and Ca^{2+} in Cl^- secreting epithelial cells. *Proc. Natl. Acad. Sci. USA* **87:**4956–4960
9. Cohn, J.A. 1990. Protein kinase C mediates cholinergically regulated protein phosphorylation in a Cl^--secreting epithelium. *Am. J. Physiol.* **258:**C227–C233
10. Cook, D.L., Hales, C.N. 1984. Intracellular ATP directly blocks K^+ channels in pancreatic B-cells. *Nature* **311:**271–273
11. Devor, D.C., Frizzell, R.A. 1993. Calcium-mediated agonists activate an inwardly rectified K^+ channel in colonic secretory cells. *Am. J. Physiol.* **265:**C1271–C1280
12. Devor, D.C., Frizzell, R.A. 1993. Calcium-mediated agonists activate an inwardly rectified K^+ channel in colonic secretory cells. *FASEB J.* **7:**A350 (*Abstr.*)
13. Dharmsathaphorn, K., Cohn, J., Beuerlein, G. 1989. Multiple calcium-mediated effector mechanisms regulate chloride secretory responses in T84-cells. *Am. J. Physiol.* **256:**C1224–C1230
14. Dharmsathaphorn, K., McRoberts, J.A., Mandel, K.G., Tisdale, L.D., Masui, H. 1984. A human colonic tumor cell line that maintains vectorial electrolyte transport. *Am. J. Physiol.* **246:**G204–G208

15. Dharmsathaphorn, K., Pandol, S.J. 1986. Mechanism of chloride secretion induced by carbachol in a colonic epithelial cell line. *J. Clin. Invest.* **77:**348–354
16. Dho, S., Stewart, K., Foskett, J.K. 1992. Purinergic receptor activation of Cl^- secretion in T84 cells. *Am. J. Physiol.* **262:**C67–C74
17. Findlay, I., Dunne, M.J. 1986. ATP maintains ATP-inhibited K^+ channels in an operational state. *Pfluegers Arch.* **407:**238–240
18. Findlay, I., Dunne, M.J., Petersen, O.H. 1985. ATP-sensitive inward rectifier and voltage- and calcium-activated K^+ channels in cultured pancreatic islet cells. *J. Membrane Biol.* **88:**165–172
19. Hanrahan, J.W., Tabcharani, J.A. 1990. Inhibition of an outwardly rectifying anion channel by HEPES and related buffers. *J. Membrane Biol.* **116:**65–77
20. Hanrahan, J.W., Tabcharani, J.A. 1993. Basolateral K channel in T84 cells activated by carbachol. *FASEB J.* **7:**A351 (*Abstr.*)
21. Hanrahan, J.W., Tabcharani, J.A., Chang, X.-B., Riordan, J.R. 1994. A secretory Cl channel from epithelial cells studied in heterologous expression systems. *Adv. Comp. Environ. Physiol.* Vol. 19, pp. 193–220. Springer-Verlag, Berlin
22. Ho, K., Nichols, C.G., Lederer, W.J., Lytton, J., Vassilev, P.M., Kanazirska, M.V., Hebert, S.C. 1993. Cloning and expression of an inwardly rectifying ATP-regulated potassium channel. *Nature* **362:**31–38
23. Kachintorn, U., Vajanaphanich, M., Traynor-Kaplan, A.E., Dharmsathaphorn, K., Barrett, K.E. 1993. Activation by calcium alone of chloride secretion in T_{84} epithelial cells. *Br. J. Pharmacol.* **109:**510–517
24. Kachintorn, U., Vongkovit, P., Vajanaphanich, M., Dinh, S., Barrett, K.E., Dharmsathaphorn, K. 1993. Dual effects of a phorbol ester on calcium-dependent chloride secretion by T84 epithelial cells. *Am. J. Physiol.* **262:**C15–C22
25. Lindeman, R.P., Chase, H.S., Jr. 1992. Protein kinase C does not participate in carbachol's secretory action in T84 cells. *Am. J. Physiol.* **263:**C140–C146
26. Mandel, K.G., McRoberts, J.A., Beuerlein, G., Foster, E.S., Dharmsathaphorn, K. 1986. Ba^{2+} inhibition of VIP- and A23187-stimulated Cl^- secretion by T_{84} cell monolayers. *Am. J. Physiol.* **250:**C486–C494
27. McCann, J.D., Matsuda, J., Garcia, M., Kaczorowski, G., Welsh, M.J. 1990. Basolateral K^+ channels in airway epithelia I. Regulation by Ca^{2+} and block by charybdotoxin. *Am. J. Physiol.* **258:**L334–L342
28. McCann, J.D., Welsh, M.J. 1990. Basolateral K^+ channels in airway epithelia. II. Role in Cl^- secretion and evidence for two types of K^+ channel. *Am. J. Physiol.* **258:**L343–L348
29. Misler, S., Falke, L.C., Gillis, K., McDaniel, M.L. 1986. A metabolite-regulated potassium channel in rat pancreatic B cells. *Proc. Natl. Acad. Sci. USA* **83:**7119–7123
30. Noma, A. 1983. ATP-regulated K^+ channels in cardiac muscle. *Nature* **305:**147–148
31. O'Rourke, B. 1993. Ion channels as sensors of cellular energy. Mechanisms for modulation by magnesium and nucleotides. *Biochem. Pharmacol.* **46:**1103–1112
32. Quinton, P.M., Reddy, M.M. 1992. Control of CFTR chloride conductance by ATP levels through non-hydrolytic binding. *Nature* **360:**79–81
33. Reenstra, W.W. 1993. Inhibition of cAMP- and Ca-dependent Cl^- secretion by phorbol esters: inhibition of basolateral K^+ channels. *Am. J. Physiol.* **264:**C161–C168
34. Roch, B., Baró, I., Hongre, A.S., Escande, D. 1993. Charybdotoxin-sensitive Ca^{2+}-activated K^+ channels in human epithelial cells. *Ped. Pulmonol.* **Suppl. 9:**227
35. Shen, B.-Q., Barthelson, R.A., Skach, W., Gruenert, D.C., Sigal, E., Mrsny, R.J., Widdicombe, J.H. 1993. Mechanism of inhibition of cAMP-dependent epithelial chloride secretion by phorbol esters. *J. Biol. Chem.* **268:**19070–19075
36. Spruce, A.E., Standen, N.B., Stanfield, P.R. 1985. Voltage-dependent ATP-sensitive potassium channels of skeletal muscle membrane. *Nature* **316:**736–738
37. Sullivan, S.K., Swamy, K., Greenspan, N.R., Field, M. 1990. Epithelial K channel expressed in *Xenopus* oocytes is inactivated by protein kinase C. *Proc. Natl. Acad. Sci. USA* **87:**4553–4556
38. Tabcharani, J.A., Chang, X.-B., Riordan, J.R., Hanrahan, J.W. 1991. Phosphorylation-regulated Cl^- channel in CHO cells stably expressing the cystic fibrosis gene. *Nature* **352:**628–631
39. Tabcharani, J.A., Hanrahan, J.W. 1991. Single chloride and potassium channels in the epithelial cell line T_{84}. *Biophys. J.* **59:**6*a* (*Abstr.*)
40. Tabcharani, J.A., Harris, R.A., Boucher, A., Eng, J.W.L., Hanrahan, J.W. 1994. Basolateral K channel activated by carbachol in the epithelial cell line T_{84}. *J. Membrane Biol.* **142:**(*in press*)
41. Tabcharani, J.A., Low, W., Elie, D., Hanrahan, J.W. 1990. Low-conductance chloride channel activated by cAMP in the epithelial cell line T_{84}. *FEBS Lett.* **270:**157–164
42. Trapnell, B.C., Chu, C.-S., Paakko, P.K., Banks, T.C., Yoshimura, K., Ferrans, V.J., Chernick, M.S., Crystal, R.G. 1991. Expression of the cystic fibrosis transmembrane conductance regulator gene in the respiratory tract of normal individuals and individuals with cystic fibrosis. *Proc. Natl. Acad. Sci. USA* **88:**6565–6569
43. Trube, G., Hescheler, J. 1984. Inward-rectifying channels in isolated patches of the heart cell membrane: ATP-dependence and comparison with cell-attached patches. *Pfluegers Arch.* **401:**178–184
44. Wang, W., Giebisch, G. 1991. Dual effect of adenosine triphosphate on the apical small conductance K^+ channel of the rat cortical collecting duct. *J. Gen. Physiol.* **98:**35–61
45. Wang, W., Giebisch, G. 1991. Dual modulation of renal ATP-sensitive K^+ channel by protein kinases A and C. *Proc. Natl. Acad. Sci. USA* **88:**9722–9725
46. Wang, W., Schwab, A., Giebisch, G. 1990. Regulation of small-conductance K^+ channel in apical membrane of rat cortical collecting tubule. *Am. J. Physiol.* **259:**F494–F502
47. Welsh, M.J., McCann, J.D. 1985. Intracellular calcium regulates basolateral potassium channels in a chloride-secreting epithelium. *Proc. Natl. Acad. Sci. USA* **82:**8823–8826
48. Wong, S.M.E., Lindeman, R.P. Parangi, S., Chase, H.S., Jr. 1989. Role of calcium in mediating action of carbachol in T84 cells. *Am. J. Physiol.* **257:**C976–C985
49. Wong, S.M.E., Tesfaye, A., DeBell, M.C., Chase, H.S., Jr. 1990. Carbachol increases basolateral K^+ conductance in T84 cells. *J. Gen. Physiol.* **96:**1271–1285
50. Yount, R.G., Ojala, D., Babcock, D. 1971. Interaction of P-N-P and P-C-P analogs of adenosine triphosphate with heavy meromyosin, myosin and actomyosin. *Biochemistry* **10:**2490–2496