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Regulation of Cl^- transport in T84 cell clones expressing a mutant regulatory subunit of cAMP-dependent protein kinase

(ion channel/cystic fibrosis/cell transfection/expression vector)

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ABSTRACT **Cl^- channels in the apical membranes of salt-secreting epithelia are activated by both cAMP and Ca^{2+} second-messenger systems, and dysfunctions in their hormonal regulation have been demonstrated in patients with cystic fibrosis. We have transfected the epithelial cell line T84 with an expression vector containing a mutant form of the regulatory subunit of the cAMP-dependent protein kinase. Stable transformants that express this construct have reduced basal cAMP-dependent protein kinase activity and do not increase kinase activity beyond the basal level of control cells in response to cAMP. Forskolin, vasoactive intestinal peptide, and prostaglandin E_2 each stimulate intracellular cAMP accumulation in both mutant and control clones; however, the activation of Cl^- channels in response to elevated cAMP is blocked in mutant clones, indicating direct involvement of the cAMP-dependent protein kinase. In contrast, Ca^{2+} ionophores retain their ability to activate the Cl^- channel in T84 cells expressing the mutant regulatory subunit, suggesting that activation of the channel by means of Ca^{2+} does not require the participation of cAMP-dependent protein kinase activity. These clones will be useful for further studies of the interactions between the cAMP- and Ca^{2+}-dependent regulatory pathways in salt-secreting epithelial cells. They can also be used to identify the mediators of Ca^{2+}-dependent Cl^- channel activation in isolation from interactions with the cAMP second-messenger pathway.**

Many hormones and neurotransmitters influence cellular function by means of second-messenger-mediated phosphorylation of target substrates. The interaction of an extracellular signal with its receptor stimulates the formation of an intracellular second messenger, which, in turn, activates a protein kinase. The kinase then phosphorylates specific cellular proteins and thereby regulates their activity. The cAMP-dependent protein kinase (cA-PK) has been extensively studied and is an important intermediary in the regulation of a variety of cellular functions including metabolism (1), differentiation (2, 3), secretion (4), and the regulation of gene transcription (5, 6).

Recent evidence indicates that some of the actions of neurotransmitters and hormones on ion channels are mediated by cAMP-dependent protein phosphorylation (7). For example, the actions of serotonin on the S-type potassium channel in *Aplysia* sensory neurons (8) and the effects of β-adrenergic stimulation on myocardial Ca^{2+} channel activity (9) are mimicked by the catalytic subunit of cA-PK, indicating that these neurotransmitters modulate channel activity via the cA-PK. Secretory Cl^- channels found in airway and intestinal epithelia and exocrine pancreas are also regulated by a cAMP-dependent mechanism, and a variety of hormones are believed to activate this channel via cA-PK (10). Activation of these channels by cAMP is defective in patients with cystic fibrosis (11, 12). Under normal conditions, Cl^- secretion across epithelial cells is stimulated by hormones that act via the cAMP and Ca^{2+} second-messenger systems. In patients with cystic fibrosis the regulation of airway and sweat gland Cl^- channels by cAMP is defective. In the lung this results in dehydration of the airway surface and an abnormal composition of the secretions that are vital for maintaining the normal function of this tissue.

The T84 cell line is a useful model to study the regulation of Cl^- channels in salt-secreting epithelia. These cells express the regulatory pathways and transport mechanisms for cAMP- and Ca^{2+}-mediated Cl^- secretion and retain the morphology characteristic of columnar epithelial cells (13–15). T84 cells express apical membrane Cl^- channels with the anion selectivity, single-channel conductance, and kinetic properties of the Cl^- channel found in tracheal, sweat gland, and pancreatic epithelial cells (10, 16). In addition, the activation of these channels by drugs and hormones that trigger the cAMP or Ca^{2+} second-messenger systems has been demonstrated (17, 18).

Many of the actions of cAMP are mediated by the cAMP-dependent protein kinases. These serine/threonine kinases exist as inactive tetramers composed of two regulatory and two catalytic subunits. Binding of cAMP to the regulatory subunits results in release of the active catalytic subunits and subsequent phosphorylation of target substrates. Regulatory subunits with mutations in the cAMP-binding domains confer a dominant inhibition of the cA-PK system in cells (19). We have constructed expression vectors containing a cDNA coding for a mutant form of the type I regulatory subunit (RIα) of cA-PK and have found that production of relatively small amounts of this protein blocks the activation of cA-PK in response to hormonal or pharmacological manipulations in NIH 3T3 fibroblasts, Y-1 adrenal, and AtT-20 pituitary cells (20, 21). Similar results have been obtained by other laboratories using these vectors in MA-10 Leydig (22) and UMR-106-01 osteosarcoma (23) cells. We now report that expression of this mutant regulatory subunit in T84 cells blocks the activation of Cl^- channels by drugs and hormones that elevate cAMP but does not block their activation by Ca^{2+} ionophores.

MATERIALS AND METHODS

Cell Culture and DNA Transfection. T84 cells were grown in a 1:1 mixture of Dulbecco's minimal essential medium and Ham's F-12 medium supplemented with 5% newborn calf serum, streptomycin at 0.1 mg/ml, and penicillin at 100 units/ml. Experiments were performed on 80–90% confluent

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Abbreviations: cA-PK, cAMP-dependent protein kinase; RIα, type I regulatory subunit of cA-PK; VIP, vasoactive intestinal peptide; PGE_2, prostaglandin E_2; HPBR, Hepes/phosphate-buffered Ringer's solution.

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cultures 5–7 days after plating. Cells were transfected by electroporation (400 V for 0.5 msec) with 25 μg of linearized DNA in 1 ml of Hepes-buffered sucrose (300 mM sucrose/6.7 mM KCl/142 mM NaCl/10 mM Hepes). After plating for 48 hr, medium containing G418 at 725 μg/ml (GIBCO) was added. G418-resistant colonies were isolated by using glass cloning cylinders and assayed for the presence of mutant RIα mRNA by solution hybridization with a ^{32}P-labeled RNA probe complementary to the 3′-untranslated sequence of human growth hormone (24).

cAMP-dependent Protein Kinase Assay. Soluble protein extracts were prepared from T84 cell cultures by sonicating the cells in a homogenization buffer (10 mM $NaPO_4$, pH 7.0/1 mM EDTA/1 mM dithiothreitol/250 mM sucrose) and centrifuging at 4°C for 15 min to remove the particulate fraction. Protein concentrations were determined by Bradford assay (Bio-Rad), and cA-PK activity was assayed by the method of Roskoski (25) using the synthetic substrate Kemptide. All samples were tested with and without the addition of 5 μM cAMP to the assay, and each sample was assayed in triplicate. Values are expressed as units of kinase activity per mg of protein.

cAMP Assay. T84 cell cultures in 60-mm plates were rinsed with Hepes/phosphate-buffered Ringer's solution (HPBR: 140 mM NaCl/3.3 mM KH_2PO_4/0.83 mM K_2HPO_4/1 mM $CaSO_4$/1 mM $MgSO_4$/10 mM Hepes/10 mM glucose, pH 7.4) and incubated in 2 ml of HPBR with or without hormone for 2 min at 37°C. The buffer was removed, and the plates were treated with 1 ml of ice-cold trichloroacetic acid. The trichloroacetic acid was extracted three times with 5× vol ether, and the samples were lyophilized. Dried samples were reconstituted in 1 ml of 50 mM NaAc, pH 6.2, and cAMP was measured by radioimmunoassay using an assay kit (New England Nuclear). Precipitated proteins in the plate were solubilized in 0.1 M NaOH and measured by using the Bradford assay.

^{125}I Efflux Assay. Agonist-activated, conductive anion exit from T84 cells was assayed by ^{125}I efflux, as described (26). Briefly, 35-mm cultures of T84 cells were incubated for 30 min at 37°C in HPBR containing ^{125}I at 5 μCi/ml (1 Ci = GBq; DuPont/New England Nuclear). After this loading period, cultures were rapidly washed 4 times with 2 ml of HPBR, after which 1-ml aliquots of isotope-free HPBR were added and removed sequentially at 15-sec time intervals for 4 min. A basal (unstimulated) rate of efflux was established during the first five time intervals. Agonist in HPBR was then added to determine the stimulated rate of efflux in the remaining time intervals. At the end of the experiment, 1 ml of 0.1 M HNO_3 was added to the culture to extract the ^{125}I remaining in the cell layer. ^{125}I content of the samples and cell extract was counted, and isotope in the cell layer at each sample time was calculated from the sample and extract counts. The apparent rate constant (r) for ^{125}I efflux for each sampling period was determined from the equation $r = [\ln(R_1) - \ln(R_2)]/(t_1 - t_2)$, where R_1 and R_2 are the percent of total counts remaining in the cell layer at times t_1 and t_2 (in sec), respectively. This assay monitors conductive anion efflux, as shown by inhibition of forskolin- and ionomycin-stimulated ^{125}I efflux by Cl^- channel blockers and by elevated external K^+ concentration. The absence of significant cation cotransport or anion exchange is inferred from the lack of effect of bumetanide or Cl^--free media on ^{125}I efflux (26).

RESULTS

The expression vector MT-REV(AB)neo is diagrammed in Fig. 1. This construct contains the cDNA for the RIα of cA-PK. Point mutations in both cAMP-binding sites are present, which prevent cAMP binding and activation of the kinase (20). Transcription of the construct is directed by the metallothionein promoter, and a polyadenylylation signal is provided by the 3′ human growth hormone sequence. The metallothionein promoter is responsive to many inducers including Zn^{2+} and Cd^{2+}, and we could demonstrate a 10-fold induction of mutant regulatory subunit after Zn^{2+} treatment of transfected cells. However, basal activity of the metallothionein promoter was sufficient to produce the kinase-deficient phenotype and, therefore, Zn^{2+} induction was not used in any of the experiments described. The neomycin phosphotransferase gene, flanked 5′ and 3′ by simian virus 40 promoter and polyadenylylation sequences, is included to permit selection of stable transformants in G418. An expression vector containing the neomycin phosphotransferase gene alone was used to generate control clones. Kinase activity, Cl^- channel activation, and cAMP generation in response to treatments were examined in two mutant regulatory subunit-expressing clones (R5 and R6) and two control clones (P3 and P4) as well as in wild-type T84 cells.

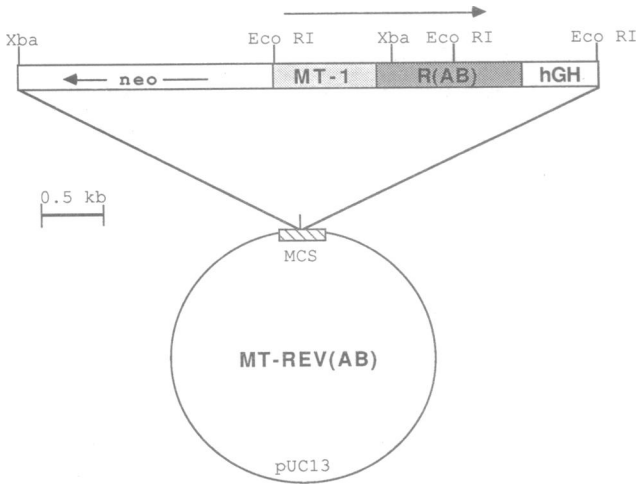

FIG. 1. MT-REV(AB)neo expression vector. MT-REV(AB)neo contains the coding region of the RIα subunit gene of cA-PK with base-pair substitutions that result in 3 amino acid changes: Gly-200 → Glu, Gly-324 → Asp, and Arg-322 → His. The mutant regulatory gene is flanked 5′ by a 700-base-pair (bp) *Kpn* I–*Bam*HI fragment of the mouse metallothionein-1 (MT-1) promoter and 3′ by 630 base pairs (bp) of the human growth hormone (hGH) 3′ untranslated and flanking sequences. The neomycin phosphotransferase gene (neo), flanked 5′ and 3′ by simian virus 40 promoter and polyadenylylation sequences, was included to select stable transformants in G418. The arrows indicate direction of transcription. MCS, multiple cloning site.

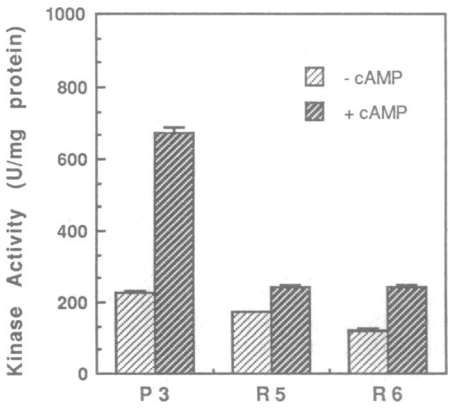

FIG. 2. Expression of mutant regulatory gene inhibits protein kinase activity in transfected T84 cells. Kinase activity with and without 5 μM cAMP in extracts from two mutant regulatory subunit-expressing T84 cell clones, R5 and R6, and in a control clone, P3, transfected with the neomycin phosphotransferase gene only.

Inhibition of cA-PK in T84 Cells Expressing Mutant RIα. The constitutive expression of MT-REV(AB)neo inhibited both basal and cAMP-stimulated cA-PK activity in T84 cells, as shown in Fig. 2. Basal cA-PK activity was reduced in clones that expressed the mutant regulatory subunit (clones R5 and R6) compared with control cells. The addition of 5 μM cAMP to the assay significantly stimulated cA-PK activity in control cell extracts. Although extracts from clones R5 and R6 showed a slight elevation in cA-PK activity in response to cAMP, this increase did not exceed the basal levels seen in the vector-transfected control clone P3.

Blockade of cAMP-Mediated Cl^- Channel Activation. The basal ^{125}I efflux rates in control and mutant regulatory subunit-expressing T84 cells were similar, implying that the reduction in basal cA-PK activity seen in clones R5 and R6 was not associated with reduced basal Cl^- channel activity. Activation of T84 cell Cl^- channels by vasoactive intestinal peptide (VIP) and prostaglandin E_2 (PGE_2) is thought to be mediated via the cAMP second-messenger system (17, 27), and we investigated whether the inhibition of cA-PK activity in T84 clones R5 and R6 was sufficient to block the ability of these hormones to activate the Cl^- channel, as determined by ^{125}I efflux. Representative experiments showing ^{125}I efflux in response to forskolin, VIP, or PGE_2 in control and mutant regulatory subunit-expressing clones are shown in Fig. 3.

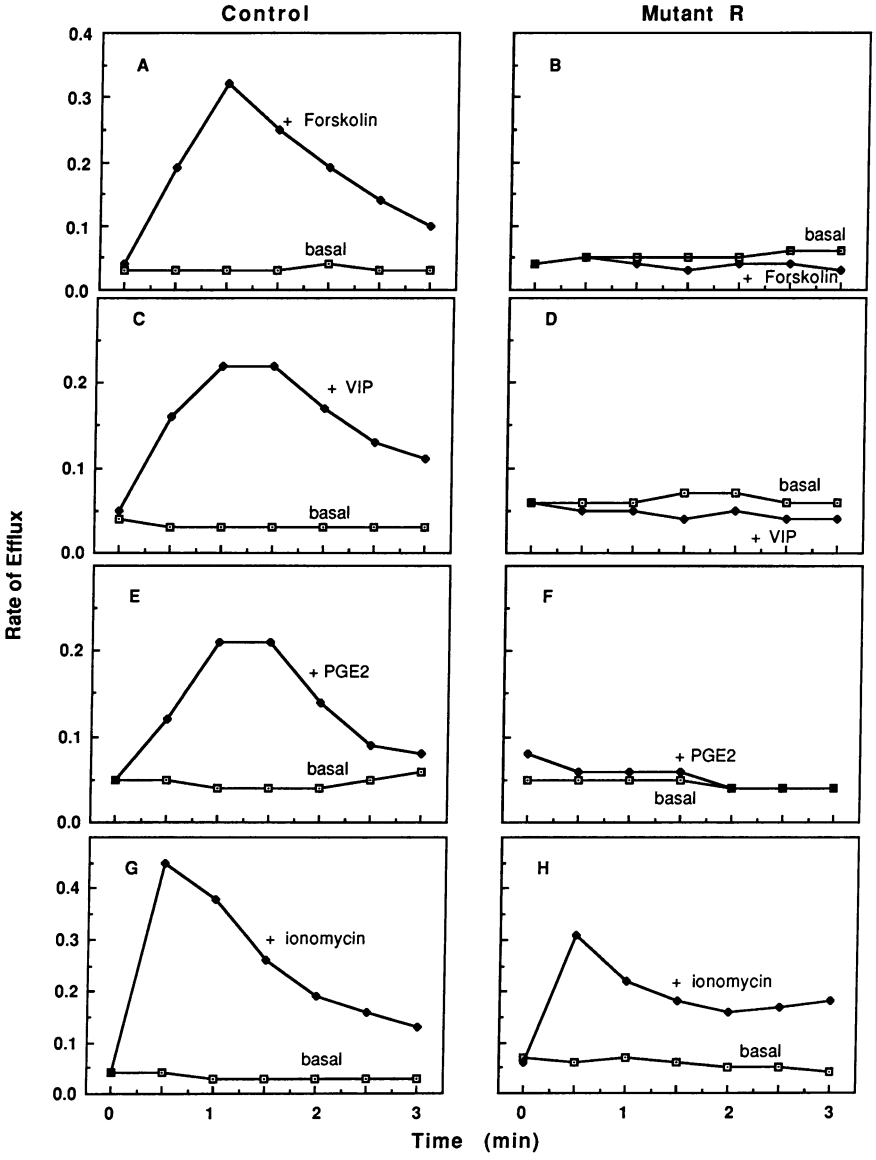

FIG. 3. Expression of mutant regulatory (R) gene inhibits ^{125}I efflux in response to agents that stimulate cAMP production but does not inhibit ^{125}I efflux in response to Ca^{2+} ionophore. ^{125}I efflux in control or mutant regulatory subunit-expressing T84 cell clones in response to 10 μM forskolin (*A* and *B*), 1 μM VIP (*C* and *D*), and 2 μM PGE_2 (*E* and *F*). (*G* and *H*) ^{125}I efflux in control and mutant regulatory subunit-expressing T84 cell clones in response to 2 μM ionomycin compared with basal efflux rates. No significant differences were seen in the efflux rate constants of the two vector-transfected control clones (P3 and P4). Similarly, the two mutant regulatory subunit-expressing clones (R5 and R6) did not show significant differences in their efflux rate constants. Mean efflux rate constants are the averages of independent experiments of control (P3 and P4) vs. mutant regulatory subunit (R5 and R6) clones. *P* values were determined by two-tailed *t* tests. The mean basal efflux rate constant was 0.04 ± 0.005 in control clones and 0.06 ± 0.01 in mutant regulatory subunit clones. The mean efflux rate constants in response to drug or hormone treatment were as follows: 0.27 ± 0.05 (control; $n = 4$ experiments) vs. 0.07 ± 0.02 (mutant regulatory subunit; $n = 6$ experiments) in response to forskolin, $P < 0.01$; 0.21 ± 0.01 (control; $n = 6$ experiments) vs. 0.07 ± 0.01 (mutant regulatory subunit; $n = 6$ experiments) in response to VIP, $P < 0.01$; 0.22 ± 0.03 (control, $n = 10$ experiments) vs. 0.06 ± 0.01 (mutant regulatory subunit, $n = 8$ experiments) in response to PGE_2, $P < 0.01$; 0.23 ± 0.09 (control, $n = 16$ experiments) vs. 0.15 ± 0.07 (mutant regulatory subunit, $n = 18$ experiments) in response to ionomycin, $P > 0.05$; 0.125 ± 0.005 (control, $n = 6$ experiments) vs. 0.14 ± 0.05 (mutant regulatory subunit, $n = 12$ experiments) in response to A23187, $P > 0.05$.

Vector-transfected control clones (P3 and P4) gave similar magnitude and time course of ^{125}I efflux in response to forskolin and PGE_2 as has been observed (26) in wild-type T84 cells. Although treatment with forskolin, VIP, or PGE_2 increased ^{125}I efflux in control T84 clones (Fig. 3 *A*, *C*, and *E*), clones R5 and R6 did not respond to cAMP-mediated agonists (Fig. 3 *B*, *D*, and *F*), showing that expression of the mutant regulatory subunit blocks the ability of forskolin, VIP, and PGE_2 to activate the Cl^- channel.

Mutant regulatory subunit clones did, however, retain normally elevated intracellular cAMP levels in response to these agents, as shown in Fig. 4. The level of cAMP production that we observed in response to VIP and PGE_2 agrees with that observed (18, 27) in wild-type T84 cells. These data demonstrate that expression of the mutant regulatory subunit does not interfere with the ability of these agents to bind to their receptors and stimulate adenylate cyclase-catalyzed production of cAMP. Thus, the residual kinase activity present in clones expressing the mutant regulatory subunit is not sufficient to stimulate ^{125}I efflux in response to elevated intracellular cAMP.

Response of Mutant T84 Cells to Ca^{2+} Ionophores. Increases in T84 cell ^{36}Cl and ^{125}I effluxes have been observed in response to treatment with Ca^{2+} ionophores (18, 26). Inhibition of cA-PK in mutant regulatory T84 cell clones did not block ^{125}I efflux in response to 2 μM ionomycin, as shown in Fig. 3*H*. We also tested the response of mutant regulatory clones to a second Ca^{2+} ionophore, A23187, with similar results. There was no statistically significant difference in the response of mutant regulatory T84 cell clones vs. control clones to either Ca^{2+} ionophore. These data indicate that the inhibition of cA-PK activity in T84 cells does not block the ability of the Cl^- channel to respond to Ca^{2+}.

DISCUSSION

The phosphorylation of ion channels by cA-PK has been shown to modify channel activity. For example, cAMP or the purified catalytic subunit of cA-PK closes the S-type K^+channel in *Aplysia* sensory neurons, an action important in producing behavioral sensitization (8, 28). In those experiments, whether the substrate for catalytic subunit is the channel itself or some intermediate protein is unknown; however, the cA-PK-mediated phosphorylation of Ca^{2+}

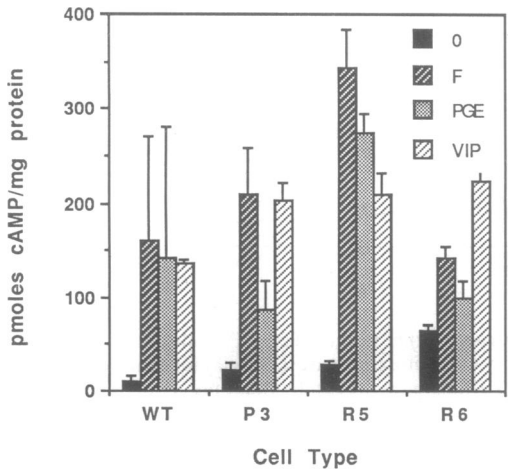

FIG. 4. cAMP generation in control and mutant regulatory subunit-expressing T84 cell clones. Levels of cAMP in pmol/mg of total protein are shown after 2-min treatment with either buffer alone (0), 10 μM forskolin (F), 2 μM PGE_2, or 1 μM VIP. The response is compared in wild-type T84 cells (WT), a control clone expressing neomycin phosphotransferase (P3), or mutant regulatory subunit expressors (R5 and R6).

channels purified from skeletal muscle has been demonstrated (29, 30), and the rate of $^{45}Ca^{2+}$ uptake into vesicles containing reconstituted Ca^{2+} channels is proportional to the extent of channel phosphorylation (31).

Although these studies show that plasma membrane ion channels are substrates for phosphorylation by cA-PK, a direct action of cAMP and cGMP on ion channels has also been documented. cGMP, in the absence of nucleoside triphosphates, increases the conductance of a cation-selective channel in photoreceptor outer segment (32). In addition, both cAMP and cGMP have been shown to activate an increase in conductance in olfactory receptor cilia in the absence of kinase activity (33). The dual regulation of a cation channel in renal inner-medullary collecting duct cells by both a kinase-independent and a kinase-dependent action of cGMP has recently been reported (34). These experiments indicate that the cAMP and cGMP second messengers do not necessarily exert their effect on ion channels by means of a kinase-dependent phosphorylation event.

The use of genetic constructs that specifically inhibit kinase activity will be useful in the analysis of the kinase-dependent and independent actions of cyclic nucleotides on ion channel function. cAMP-mediated activation of Cl^- channels has been reported in T84 cells (16, 35), tracheal epithelia (11, 12, 36), pancreatic duct (37), and *Necturus* enterocytes (38). Our data indicate that cA-PK mediates forskolin, VIP, and PGE_2 activation of T84 Cl^- channels. Patch-clamp analysis of tracheal epithelia has shown that these Cl^- channels open in response to purified catalytic subunit (39, 40), suggesting that the target for the kinase is the channel itself or some closely associated protein. The recently cloned cystic fibrosis gene encodes a 1480-amino acid polypeptide with 10 consensus sequences for cA-PK phosphorylation and may represent either an ion channel or a protein important in the regulation of ion channel activity (41). Transcripts for this gene are abundant in T84 cells (42) and may represent the target for cA-PK phosphorylation in response to hormones that activate the Cl^- channel via the cAMP second-messenger system.

The specific inhibition of the cA-PK is also useful in identifying the pathway that activates a particular substrate when a hormone or neurotransmitter stimulates more than one second-messenger system. For example, VIP stimulates cAMP production and β-endorphin secretion in AtT-20 cells. AtT-20 cell clones that express the mutant regulatory subunit retain the ability to secrete β-endorphin in response to VIP despite a severe inhibition of cA-PK activity, suggesting that an alternate second-messenger system is mediating this response (21). This result constrasts with our results in T84 cells, which indicate that cA-PK does, indeed, mediate the effects of VIP on Cl^- channel activity.

We have found that the Ca^{2+} ionophores A23187 and ionomycin retain the ability to stimulate ^{125}I efflux in the absence of a functional cA-PK system. Thus, the Ca^{2+}- and cAMP-dependent pathways function in parallel. If these pathways converge in their regulation of apical membrane Cl^- channels they must do so distal to cA-PK. Increases in cell Ca^{2+} activate T84 cell Cl^- conductance (35), and our data indicate that this action on the channel does not require participation of the cA-PK. Indirect effects of Ca^{2+} on Cl^- efflux have also been proposed. For example, stimulation of intestinal Cl^- secretion by Ca^{2+} ionophores has been suggested to be mediated by an increase in prostaglandin-mediated cAMP production (43). Because the inhibition of cA-PK activity in T84 cells blocks ^{125}I efflux in response to PGE_2 but not in response to ionomycin or A23187, PGE_2 cannot be a necessary intermediate for Ca^{2+} activation of the Cl^- channel. Others have suggested that the effects of Ca^{2+} on Cl^- efflux are the result of a cooperative interaction between Ca^{2+}-activated K^+ channels and cAMP-mediated

activation of Cl^- channels (18). These experiments have demonstrated synergistic effects of the cAMP and Ca^{2+}-mediated pathways in T84 cells. Our data show that although these two pathways may interact cooperatively, Ca^{2+}-mediated Cl^- efflux does not depend on the cA-PK-regulated pathway.

Attempts to identify the mediators of the Ca^{2+}-dependent regulation of secretory Cl^- channels can benefit from the use of these mutant regulatory-expressing T84 cells. Several studies suggest complex interactions between the cAMP- and Ca^{2+}-second-messenger systems, both at the level of transport mechanisms and between the regulators themselves. The selective lesioning of the cA-PK system in these cells will be useful in dissecting out the contributions due to synergism between the two systems and to potential cAMP-dependent intermediates of the Ca^{2+} pathway.

A variety of isoforms of both the regulatory and catalytic subunits of cA-PK have been characterized (44–47), and this diversity may be important in the targeting of cA-PK phosphorylation to specific substrates. We have found that T84 cells express several different cA-PK subunit isoforms (K.V.R. and G.S.M., unpublished observation). Use of expression vectors that code for either wild-type or modified subunits may help elucidate the specific pathways used by various hormones to activate Cl^- channels and can provide information on the interactions of various second-messenger systems in regulating secretory Cl^- channel activity.

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