Characterization of PKA isoforms and kinase-dependent activation of chloride secretion in T84 cells


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Singh, A. K., K. Taskén, W. Walker, R. A. Frizzell, S. C. Watkins, R. J. Bridges, and N. A. Bradbury. Characterization of PKA isoforms and kinase-dependent activation of chloride secretion in T84 cells. Am. J. Physiol. 275 (Cell Physiol. 44): C562–C570, 1998.—Chloride exit across the apical membranes of secretory epithelial cells is acutely regulated by the cAMP-mediated second messenger cascade. To better understand the regulation of transepithelial chloride secretion, we have characterized the complement of cAMP-dependent protein kinase (PKA) isoforms present in the human colonic epithelial cell line T84. Our results show that both type I and type II PKA are present in T84 cells. Immunoprecipitation of 8-azido-[32P]cAMP-labeled cell lysates revealed that the major regulatory subunits of PKA were RIα and RIIα. In addition, immunogold electron microscopy showed that RIα labeling was found on membranes of the trans Golgi network and on apical plasma membrane. In contrast, RIα was randomly distributed throughout the cytoplasm, with no discernible membrane association. Northern blot analysis of T84 RNA revealed that Cα was the predominantly expressed catalytic subunit. Short-circuit current measurements were performed in the presence of combinations of site-selective CAMP analog pairs to preferentially activate either PKA type I or PKA type II in intact T84 cell monolayers. Maximal levels of chloride secretion (±100 μA/cm²) were observed for both type I and type II PKA-selective analog pairs. Subsequent addition of forskolin was unable to further increase chloride secretion. Thus activation of either type I or type II PKA is able to maximally stimulate chloride secretion in T84 colonic epithelial cells.

protein kinase A; kinase activation; kinase isoforms; adenosine 3′,5′-cyclic monophosphate analogs

INCREASES IN INTRACELLULAR cAMP have been implicated in the regulation of a variety of cellular functions, including cell proliferation, secretion of macromolecules, membrane turnover (5,6), and both anionic and cationic ion channel activity (16, 24, 35). With the exception of direct activation of ion channels by cAMP, all known cAMP-dependent events in eukaryotic cells are mediated through the actions of the cAMP-dependent protein kinase (PKA). Mammalian PKA is a tetramer composed of two catalytic (C) subunits, which phosphorylate specific serine and threonine residues on target substrates, and two regulatory (R) subunits, which bind cAMP and thus regulate catalytic activity. Two major types of mammalian PKA, type I and type II (PKA I and PKA II), were initially described by their pattern of elution from DEAE-cellulose columns. These kinases were distinguished by the presence of different R subunits, termed RI and RII. Through biochemical studies and gene cloning, three isoforms of the C subunit, Cα, Cβ, and Cγ, and four isoforms of the R subunits, RIα, RIβ, RIIα, and RIIβ, have now been identified. In addition to different biochemical and functional properties, several lines of evidence support specific roles for the different PKA isoforms. RIα, RIIα, and Cα represent the ubiquitous mRNA forms found in most tissues, whereas mRNA for RIIβ seems to be cell and tissue specific in its distribution, and its expression is hormonally regulated in ovarian granulosa cells and testicular Sertoli cells (22, 29). RIβ has so far been detected in several human tissues, with the highest expression in the brain. The highest levels of Cβ mRNA have been observed in the human prostate, intestine, brain, and testes, whereas the message for Cγ has only been detected in human testis. In addition, specific subcellular localization of PKA also supports the notion that specific functions are assigned to each isoform. For example, RI is found throughout the cytoplasm, whereas RII is localized to nuclei, nucleoli, the microtubule organizing center, the Golgi apparatus, and the plasma membrane (17, 27).

Activation of PKA occurs upon binding of four cAMP molecules, two per R subunit monomer, where the binding takes place on two dissimilar sites, designated site B and site A, in a positive cooperative manner. cAMP binding dissociates the holoenzyme, releasing active C subunit and dimers of the R subunit. Various cAMP analogs have been generated that display selectivity for either site A or site B. When two cAMP analogs, each selective for a different binding site, are added in combination to PKA in vitro, the enzyme is activated in a synergistic manner (2, 3, 15). Moreover, because the cAMP analog specificity of site A is different for the two PKA isozymes, the synergistic activation of the PKA I isoyme can be distinguished from the synergistic activation of the PKA II isoyme (2, 15).

Transcellular movement of chloride ions across epithelial cells is brought about by the concerted actions of several basolateral transporters accumulating chloride above its electrochemical equilibrium in the cytoplasm. Chloride then exits the cell through a CAMP-regulated chloride channel located in the apical membrane. The CAMP-regulatable chloride channel in secretory epithelia has been identified, cloned, and expressed. Mutations in the primary sequence of this channel, the cystic fibrosis transmembrane conductance regulator (CFTR), lead to the most common lethal genetic disease of Caucasians, cystic fibrosis. This disease is characterized by impaired transepithelial chloride transport in response to activation of the CAMP-mediated second
messenger cascade (24, 31, 35). Primary sequence analysis of CFTR reveals at least 10 PKA consensus phosphorylation sequences, although several unidentified phosphorylatable sites are also present. Of the consensus phosphorylation sites, four sites (Ser-660, Ser-737, Ser-795, and Ser-813) appear to play a major role in the regulation of CFTR chloride channel activity. Indeed, there is now much compelling evidence to support the view that PKA regulation of chloride secretion has a direct effect on the CFTR chloride channel. Efforts in our laboratory are focused on understanding the molecular and protein-protein interactions surrounding the autoactivation of transepithelial chloride secretion. The aim of our present studies was therefore to determine which PKA isoforms are present in the human colonic epithelial cell line T84 (a well-characterized model cell line of transepithelial chloride secretion). In addition, we have used site-selective cAMP analogs to synergistically activate PKA I and PKA II in intact T84 cells, with the aim of elucidating the role of each isozyme as a modulator of chloride secretion.

MATERIALS AND METHODS

Chemicals. 8-Azido-[32P]cAMP was from ICN Pharmaceuticals (Costa Mesa, CA). cAMP analogs were obtained from Biolot (La Jolla, CA), and fresh solutions were made for each experiment by dissolving analogs in DMSO. Antibodies to PKA R subunits were generated against either purified R subunit or peptides derived from R subunit sequences and have been previously characterized (38, 39, 42). All other chemicals were from Sigma (St. Louis, MO) and were of reagent grade quality.

Cell culture. T84 cells were grown in DMEM-Ham's F-12 (1:1), 14 mM NaHCO3, and 10% fetal bovine serum. The cells were incubated in a humidified atmosphere containing 5% CO2 at 37°C. For measurements of short-circuit currents (Isc), T84 cells were seeded onto Costar Snapwell cell culture inserts (1.0 cm2), and the culture medium was changed every 48 h. Isc measurements were performed on filters after 14–21 days in culture (see Isc measurements).

Northern blot analysis. RNA from T84 cells was extracted using guanidinium isothiocyanate and purified by centrifugation through cesium chloride. RNA samples (20 µg) were fractionated on a 1.5% agarose gel containing 6.7% formaldehyde (vol/vol) in 20 mM sodium phosphate buffer (pH 7.0) and transferred to nylon membranes (Micron Separations, Westhyde, MA). RNA was cross-linked to the filter using a Stratalinker (Stratagene, La Jolla, CA) and probed with 32P-labeled human (h) Cα and hCγ cDNA probes. The PKA C subunit cDNA probes were obtained from a human testis cDNA library. Radioactive labeling of cDNA probes (250 ng) was performed by random primer extension using [α-32P]dATP (3,000 Ci/mmol). Filters were prehybridized in 1× Denhardt's solution, 5× saline sodium citrate (SSC), 50 mM sodium phosphate buffer (pH 6.5), 0.1% SDS, 250 µg/ml salmon sperm DNA, and 50% (vol/vol) formamide at 42°C for 2 h, followed by hybridization at 42°C overnight (10° cpm cDNA probe/ml buffer). Filters were washed four times with 2× SSC-0.1% SDS at room temperature (20 min each), followed by two washes at 65°C (20 min each) with 0.1× SSC-0.1% SDS. After washes, the filter was subjected to autoradiography. Messenger RNA sizes were estimated by comparison to RNA standards.

Photoaffinity labeling of R subunits with 8-azido-[32P]cAMP. Covalent incorporation of the cAMP analog 8-azido-[32P]cAMP was performed by incubating 200 µg protein from a cell lysate in a reaction mixture (final volume 90 µl) containing 50 mM MES (pH 6.2), 10 mM MgCl2, and 1 µM 8-azido-[32P]cAMP, in the presence or absence of 200 µM cAMP. Reactions were carried out for 60 min in the dark at 4°C in the presence or absence of unlabeled cAMP (100 µM). Covalent incorporation was accomplished by exposure of the reactions to ultraviolet light (254 nm), and the samples were irradiated for 10 min at 0°C.

Immunoprecipitations. Samples were adjusted to a volume of 200 µl in buffer containing PBS, 2 mM EDTA, 2.5% Triton X-100, 1 mM benzamidine, 2 µg/ml leupeptin and pepstatin A, and 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF). Staphylococcus aureus cells expressing protein A (Pansorbin cells, Calbiochem, San Diego, CA) were added to the samples, which were incubated by shaking for 20 min at 20°C and thereafter centrifuged (12,000 g for 3 min). Supernatants were transferred to new tubes, and antibody was added. Samples were shaken for 90 min at 4°C, after which S. aureus cells were added, and the incubations continued for a further 60 min. Antigen-antibody complexes bound to protein A were pelleted (12,000 g for 30 s), and pellets were washed twice. Pellets were resuspended in Laemmli sample buffer and resolved by SDS-PAGE using 3.5% stacking gels and 7.5% separating gels.

Cell fractionation. Confluent monolayers of cells were homogenized in a Dounce type homogenizer [10 mM Tris·HCl (pH 7.4) containing 1 mM EDTA, 250 mM sucrose, 1 mM benzamidine, 2 µg/ml leupeptin and pepstatin A, and 0.5 mM AEBSF]. Postnuclear supernatants (5,000 g for 10 min) were centrifuged at 110,000 g for 60 min to yield cytosolic and total membrane fractions. Cytosolic fractions were mixed with Laemmli SDS sample buffer, and the membrane pellets were resuspended in homogenization buffer and recentrifuged (110,000 g for 60 min) before solubilization in sample buffer.

Immunoblot analysis. Samples were resolved by SDS-PAGE and transferred to nitrocellulose. After block of remaining binding sites (Blotto), membranes were incubated overnight at 4°C with the appropriate antibodies. After extensive washing, blots were incubated with horseradish peroxidase-conjugated secondary antibodies, and the signal was visualized by enhanced chemiluminescence. Samples were exposed to X-ray film for equal amounts of time.

Electron microscopy. After fixation in 2% paraformaldehyde-0.01% glutaraldehyde, the cells were scraped from the dish and embedded in 2% gelatin before sucrose infusion. Subsequently, the samples were diced into 1- to 2-mm cubes and mounted on cutting stubs, shock frozen, and stored in liquid nitrogen. Thin sections (70–100 nm) were cut using a Reichert Ultracut S ultramicrotome with an FC4S cryoattachment. Sections were lift-mounted in a small drop of sucrose, mounted on Formvar-coated carbon grids, and washed three times in PBS containing 0.5% BSA and 0.15% glycine (buffer A; pH 7.4). This was followed by a 30-min incubation with purified goat IgG (50 µg/ml) at 25°C and three additional washes with buffer A. All of the preceding steps were designed to ensure minimal nonspecific reaction to the antibodies used. Sections were then incubated for 60 min with primary antibody (either a rabbit polyclonal antibody directed to RIIα or a murine monoclonal antibody directed to RIα), followed by three washes in buffer A and a 60-min incubation in gold-labeled second antibody (1–2 µg/ml). The sections were then washed six times (5 min/wash) in buffer A and washed thoroughly in buffer A (5 changes) and in PBS (3 changes), followed by a
RESULTS

Identification of PKA subunits in human colonic epithelial cells. To resolve which of the C subunits of PKA are expressed in human colonic epithelial cells, mRNA extracted from T84 cells was subject to Northern blot analysis. The cloned cDNAs for hCα, hCB, and hCG were used to identify mRNAs for the various C subunits in T84 colonic epithelial cells. The hCα-specific probe clearly identified a single 2.8-kb mRNA from T84 cells that comigrates with previously studied Cα transcripts (Fig. 1). Using the hCγ probe, no 1.8-kb Cγ mRNA was detected, but, in agreement with earlier studies showing that hCα, hCB, and hCG cDNAs are capable of cross-hybridizing with each of the C subunit mRNAs, the Cγ cDNA hybridized to a single 2.8-kb mRNA, representing human Cα. Interestingly, in addition to the 2.8-kb Cα transcript, a 4.4-kb mRNA corresponding to the size of the Cβ transcript was detected by the hCβ probe. However, detection of the hCβ transcript using a Cγ or Cβ probe required long exposure times to detect (~2 wk; data not shown). Thus, although a small amount of Cβ may be present in T84 cells, it represents a very minor component of the PKA C subunit pool.

To determine which of the PKA R subunits are expressed in colonic epithelial cells, lysates were exposed to 8-azido-[32P]cAMP to photoaffinity label the R subunits. Immunoprecipitations employing subunit-specific antisera revealed that the major PKA R subunit isoforms were RIα and RIα (33 and 58% of total activity, respectively; Fig. 2). Although present in much smaller amounts, RIβ and RIβ (8 and 2% of total activity, respectively) were nonetheless detectable. Photoaffinity labeling of cell extracts in the presence of excess (100 μM) "cold" cAMP revealed that the 8-azido-[32P]cAMP labeling was specific. The incorporation of cAMP analogs. Analogs were added to both the mucosal and serosal solutions. The T84 monolayers had resistances of 500–2,000 Ω/cm². Data were digitized with the aid of a Gould Smartcase recorder at a sample frequency of 10 Hz and saved to a computer hard drive. Changes in Isc were calculated as a difference current between the sustained phases of the response and their respective baseline values.

Protein concentrations. Protein concentrations were determined by the method of Bradford using BSA fraction V as standard.
8-azido-[32P]cAMP was not affected by the presence of 1 mM IBMX or 100 µM adenosine.

Intracellular distribution. Previous studies have documented an unequal distribution and expression of PKA I and PKA II in many cell types. We thus monitored the intracellular distribution of PKA in T84 cells. First, we performed immunoblot analyses of both cytosolic and membrane fractions using isotype-specific regulatory domain antibodies. Immunoblot analysis confirmed that RIα and RIIα were the dominant PKA isoforms. Densitometric analysis of the immunoblots revealed that ~75% of RIα was in the soluble fraction, with ~25% associated with the membrane fraction (Fig. 3). Type RIβ PKA was found only in the cytosol, with no signal detectable in the membrane fraction (Fig. 3). In contrast, >90% of RIIα was found associated with the membrane fraction, and <10% was associated with the soluble fraction (Fig. 3). RIIβ was barely detectable in the membrane fraction and was undetectable in the soluble fraction (Fig. 3). We further investigated the subcellular distribution of PKA isoforms by immunogold electron microscopy, focusing on the dominantly expressed PKA isoforms, namely α types I and II. Results (Fig. 4D) show that RIα was distributed randomly throughout the cytosol and showed no specific association for the intracellular membrane systems. By contrast, cells labeled using RIIα antibodies showed label associated with intracellular membranes, including label in the trans elements of the Golgi (Fig. 4, A and B) and the trans Golgi network (TGN; Fig. 4B), in intracellular spherical vesicles (Fig. 4C), and at the plasma membrane (Fig. 4E).

Effect of cAMP analogs on chloride secretion. Previous studies have demonstrated that binding of a cAMP analog selective for either site A or B on the R subunits of PKA stimulates binding of a cAMP analog selective for the other site, and two such site-selective analogs in combination demonstrate synergism in kinase activation. Thus the use of site-selective analog pairs can, to a certain extent, distinguish between effects mediated by PKA I and II. We selected cAMP analog pairs that complement each other in the synergistic activation of either PKA I or PKA II. Figure 5A shows a representative experiment for the activation of PKA II using the site-selective analogs Sp-5,6-DCI-cBIMPS (a PKA II site B-selective analog) and Nε-phenyladenosine-3’,5’-cyclic monophosphate (Nε-Phe-cAMP; a PKA II site A-selective analog). Cells were pretreated with either DMSO (as vehicle control) or Sp-5,6-DCI-cBIMPS at a concentration (400 nM) that results in only marginal chloride secretion (~8 µA/cm²), before exposure to Nε-Phe-cAMP at increasing concentrations. Forskolin (2 µM) was added at the end of the experiment to determine the maximum level of chloride secretion (~112 µA/cm²). Cells pretreated with Sp-5,6-DCI-cBIMPS showed a leftward shift in the Nε-Phe-cAMP dose-response curve (EC50 for Nε-Phe-cAMP alone, 31 µM; EC50 for Nε-Phe-cAMP in the presence of 400 nM Sp-5,6-DCI-cBIMPS, 8.5 µM) consistent with a synergistic activation of PKA II by the two analogs in combination (Fig. 5B). Similar experiments were performed for PKA I activation using Nε-MB-cAMP or Nε-Bzl-cAMP as type I and type II site A priming analogs with increasing concentrations of 8-(6-aminohexylamino)-adenosine-3’,5’-cyclic monophosphate (a PKA I site B-selective analog). Table 1 shows the synergism quotients for a series of PKA I and PKA II isoform-selective cAMP analog pairs. For all analog pairs, the synergism quotients were >1 at low analog concentrations. The extent of synergism was optimal when the sum of the single analog responses was ~30% of the maximum. As the total analog concentration was increased beyond the optimum, the synergism quotient decreased. Because the cAMP analogs used are site selective, and not site specific, high concentrations of a single analog are able to stimulate secretion on their own. Once a maximal level of chloride secretion has been obtained, adding other cAMP analogs or even raising cAMP with forskolin is unable to elicit any increase in chloride secretion.

DISCUSSION

There are two major isozymes of PKA, designated type I and type II. In addition to different biochemical properties, several lines of evidence support specific roles for each of the PKA isozymes in cellular responsiveness. For example, activation of PKA I, but not PKA II, in human T lymphocytes inhibits cell replication. Moreover, it is becoming increasingly clear that phosphorylation events are controlled not only by the relative activities of kinases and phosphatases but also by the subcellular localization of these enzymes within the cell. For example, the subcellular targeting of a multifunctional serine/threonine kinase, such as PKA, would enhance the selectivity of PKA by favoring its accessibility to specific substrate proteins. Indeed, there is evidence for subcellular targeting of both PKA I and PKA II. In the present study, we have investigated the levels and subcellular distribution of PKA subunits and the extent to which PKA I or PKA II may mediate the
Fig. 4. Immunogold electron microscopy of PKA R subunits in human colonic epithelial cells reveals distinct subcellular localization of RIIα and RIα. A–C and E show RIIα-labeled cells, and D shows a section labeled with antibodies to RIα. A: at low power, gold label may be seen to be concentrated in region of Golgi apparatus. B: enlargement of boxed area in A; at higher power, this label (arrowheads) appears to be associated with the most trans elements of Golgi cisternae (arrows). C: label is also associated with endosomal compartments (arrowheads). D: in cells labeled with RIα, gold label is randomly distributed throughout the cytoplasm (arrows). No label is seen in region of the Golgi or the trans Golgi network (arrowheads). E: when apical surfaces of cells labeled with RIIα are examined, it can be seen that there is a strong association of marker with plasma membrane. Although most of label is localized to inner face of the membrane (arrows) some appears on the external surface of the cell (arrowheads). This may be due to bound gold particles falling to this side of membrane when section is dehydrated before observations. All bars, 0.1 µm.
FIG. 5. Site-selective cAMP analog pairs indicate activation of chloride secretion by both type I and type II PKA. I_50 short-circuit current: A: site A type II-selective cAMP analog 6-phenyladenosine-3',5'-cyclic monophosphate (6-Phe-cAMP) was added at increasing concentrations in presence (ii) or absence (i) of site B type II-selective cAMP analog 5,6-dichlorobenzimidazole riboside-3',5'-cyclic monophosphorothionate (6-Bzl-cAMP) present; (Fsk; 2 µM) was added at end of experiment to confirm maximal stimulation of chloride secretion. B: data from 3 experiments of A were replotted as dose-response curves and fitted to a Michaelis-Menten function. •, Sp-5,6-DCI-cBIMPS present; ○, Sp-5,6-DCI-cBIMPS absent.

stimulatory effects of increased cAMP on chloride secretion from human epithelial cells.

We initially characterized the isoforms of PKA present in T84 cells by investigating the presence of the subtypes of both the C and R subunits. Human colonic epithelial cells express predominantly the mRNA for the C subunit Cα, although a small amount of Cβ could be detected on longer exposure of the Northern blots. The Cα subunit has been shown previously to represent the C subunit found in most tissues. In contrast, the Cβ subunit has so far been located mainly in the brain. At present we do not know whether the small amount of Cβ identified in T84 cells represents a physiologically important pool of C subunit. Our results from 8-azido-[32P]cAMP labeling and immunoprecipitations revealed that RII (specifically RIIα) constituted most of the remaining R subunit. Although present in much smaller amounts, RIβ and RIIβ were nonetheless detectable, contributing 8% and 2% of the total R subunit population, respectively. We do not know whether the small amount of RIβ and RIIβ present in T84 cells represents a physiologically important pool of R subunits. Two classes of cAMP binding proteins have been reported in bovine and rat liver, one class derived from PKA and a second class characterized by its ability to bind a whole range of adenine analogs (especially adenosine). The absence of an effect of 100 µM adenosine on the photoaffinity labeling from colonic epithelial cells suggests that the adenosine analog binding protein was not a target for the photoaffinity label 8-azido-[32P]cAMP. Similarly, a lack of effect by the phosphodiesterase inhibitor IBMX on 8-azido-[32P]cAMP incorporation indicates that enzymatic hydrolysis of 8-azido-[32P]cAMP or cAMP did not affect the experimental results.

Immunelectron microscopy of T84 cells using antibodies directed against either RIIα or RIIα revealed a striking difference in subcellular localization between the two R subunits. Thus RIIα is randomly distributed throughout the cytosol with no specific membrane association, whereas RIIα is very intimately associated with membranes of the TGN and the apical plasma membrane. In addition, RIIα labeling is also seen on spherical vesicles enclosing an electron transparent space, consistent with early endosomes. These findings are in agreement with previous studies in other polarized epithelia and nonpolarized cells, which show localization of RII to the apical plasma membrane, dithrin-coated pits, early endosomes, and the TGN. It is thus clear that RII is localized to those compartments that are active in endocytosis from the apical plasma membrane and in recycling of cell surface proteins. It is

Table 1. Synergy quotients for various cAMP analog pairs on chloride secretion

<table>
<thead>
<tr>
<th>cAMP Analog Pair</th>
<th>PKA Isotype Selectively Activated</th>
<th>Synergism Quotient (Chloride Secretion)</th>
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<tbody>
<tr>
<td>N6-6-Bzl-cAMP (60 µM) + 8-AHA-cAMP (630 µM)</td>
<td>I</td>
<td>2.32 ± 0.06</td>
</tr>
<tr>
<td>N6-cAMP (60 µM) + 8-AHA-cAMP (630 µM)</td>
<td>I</td>
<td>4.63 ± 0.36</td>
</tr>
<tr>
<td>N6-6-Bzl-cAMP (60 µM) + 8-CPT-cAMP (30 µM)</td>
<td>II</td>
<td>4.44 ± 0.31</td>
</tr>
<tr>
<td>N6-cAMP (60 µM) + 8-CPT-cAMP (3 µM)</td>
<td>II</td>
<td>2.13 ± 0.26</td>
</tr>
<tr>
<td>Sp-5,6-DCI-cBIMPS (400 nM) + N6-Phe-cAMP (10 µM)</td>
<td>II</td>
<td>2.17 ± 0.10</td>
</tr>
</tbody>
</table>

Values are means ± SE for 6 observations. Synergism quotient is presented as defined in RESULTS. PKA, cAMP-dependent protein kinase; N6-6-Bzl-cAMP, N6-benzoyladenosine-3',5'-cyclic monophosphate; 8-AHA-cAMP, 8-(6-aminoethylamino)adenosine-3',5'-cyclic monophosphate; N6-6-MB-cAMP, N6-monobutyryladenosine-3',5'-cyclic monophosphate; 8-CPT-cAMP, 8-(4-chlorophenylthio)adenosine-3',5'-cyclic monophosphate; 8-Bzl-cAMP, 8-5-benzoyladenosine-3',5'-cyclic monophosphate; 5,6-dichlorobenzimidazole riboside-3',5'-cyclic monophosphorothionate (Sp isomer); N6-Phe-cAMP, N6-phenyladenosine-3',5'-cyclic monophosphate.
interesting to speculate that the subcellular localization of RII, and hence PKA II, may reflect a specific function of the kinase in regulating the subcellular distribution and activity of certain plasma membrane proteins. Support for this view comes from increasing evidence that phosphorylation-dephosphorylation plays a critical role in regulating the activities and surface expression of a wide variety of receptors. Indeed, such subcellular localization of PKA to cation-coated pits and endocytic vesicles may in part explain our observations on the PKA-dependent inhibition of endocytic activity in polarized epithelial cells and the cAMP-dependent trafficking of CFTR chloride channels (30, 41). Accumulating evidence suggests that localization of PKA to specific subcellular compartments can be brought about through the interaction of PKA with specific anchoring proteins (AKAPs) (34, 36, 37). These anchoring proteins serve to tether PKA adjacent to specific substrates, enhancing the specificity of the phosphorylating activity of activated PKA. It will be of interest to determine whether such anchoring proteins are present in T84 cells and, if so, whether they are present in the same subcellular domain as CFTR.

To investigate whether one or both of PKA I and PKA II participate in mediating agonist-induced chloride secretion, we employed site-selective cAMP analogs on T84 human colonocytes to stimulate transepithelial chloride secretion. An accurate reflection of the relative roles of PKA I and PKA II in stimulating chloride secretion can be obtained with the use of site-selective analog pairs. The use of site-selective cAMP analog pairs to determine kinase isozyme activation relies on the principle of synergism between the cAMP binding sites of the R subunit. Although the R subunits of PKA I and II are homologous proteins (exhibiting the strongest homology in the cAMP binding sites), PKA I and II exhibit kinetic differences in cAMP analog specificities for binding. This difference in binding affinity, together with the positive cooperative binding interactions between the intrasubunit binding sites, can be exploited to differentiate between the isozymes in vitro and in intact cells. For example, site-selective analog pairs have proven useful in the elucidation of PKA isozyme regulation of processes including inhibition of T-lymphocyte proliferation and adipocyte phosphorylase activation. Our results (Table 1) show synergism in activation of chloride secretion from both type I and type II cAMP analog pairs, suggesting that activation of either PKA I or PKA II can lead to increased chloride secretion. It should be noted, however, that the similarities of the various cAMP analogs for sites A and B on each kinase isozyme are normally expressed relative to the binding of cAMP at those sites. Thus the binding of various cAMP analogs is selective but not necessarily specific. At high concentrations, all analogs will bind to both sites, but they bind selectively to one or the other site at relatively low concentrations. For this reason, optimal synergism is demonstrated at low levels of cAMP analog stimulation. Indeed, optimum synergism between isozyme-selective analog pairs was observed when the sum of responses for the single analogs was between 25 and 30% of the maximum. These results are consistent with observations of PKA-mediated cell responses in several other tissues stimulated with pairs of site-selective cAMP analogs. For example, PKA I-directed analog pairs show maximum synergism in activating phosphorylase in both neutrophils and hepatocytes when the sum of the responses for the single analogs is ~20% of the maximum. Similar observations were also obtained for PKA II-mediated lipolysis in intact adipocytes. Because the mechanism of synergism of intact cell responses using site-selective analog pairs occurs via the exclusive activation of PKA, our observations are consistent with the hypothesis that activation of either PKA I or PKA II can stimulate increased transepithelial chloride secretion.

It is unlikely that any synergism is due to analog-stimulated elevation of endogenous cAMP. Rather, cAMP analogs have been shown to lower endogenous cAMP levels through an activation of phosphodiesterase(s) via protein-kinase-mediated short-term feedback. Another potential concern is the use of 8-(4-chlorophenylthio)adenosine-3',5'-cyclic monophosphate, which in addition to activating PKA II can also result in the activation of type II cGMP-dependent protein kinase (PKG II). Indeed, there have been reports that application of PKG II to excised membrane patches from natively expressing and CFTR-transfected cells can cause the activation of CFTR-mediated chloride transport (18, 26). It is also worth noting that the heat-stable enterotoxin from Escherichia coli (Sta) has been shown to activate guanylyl cyclase in T84 cells, with subsequent increase in chloride secretion. However, subsequent studies have shown that T84 cells do not express PKG II and that cGMP-mediated increases in chloride secretion are due to cross-activation of PKA.

We have demonstrated the presence of both PKA I and PKA II isozymes in human colonic epithelial cells; PKA I accounted for approximately one-third of the total PKA activity, and PKA II accounted for the remaining two-thirds. Although both PKA isozymes are present, they show clear spatial separation within the cell. Thus PKA I is soluble and randomly distributed throughout the cytosol. In contrast, PKA II is predominantly membrane associated. Use of synergistic pairs of site-selective cAMP analogs demonstrated that activation of either kinase isozyme was capable of activating a chloride secretory current. However, the precise physiological role of these isozymes in this process remains to be elucidated. For example, it is not known whether different PKA isozymes preferentially activate different membrane microdomains. Thus although activation of apical membrane CFTR chloride channels (via phosphorylation) can lead to chloride secretion, it is also known that activation of basolateral potassium channels alone can produce chloride secretion by membrane hyperpolarization that increases the driving force for apical chloride exit. It is therefore possible that stimulation of chloride secretion by isozyme-selective cAMP analog pairs may result from activation of different proteins or different pools of the same protein. Thus
future experiments will need to be designed to elucidate the interactions between PKA I and PKA II and activation of apical and/or basolateral components of the secretory machinery. Finally, the results presented make use of exogenous permeable cAMP analogs, a technique which is likely to cause a uniform distribution of these analogs throughout the cell. Because it has been shown that hormonal agonists can lead to increases in cAMP in specific subcellular compartments (1, 21), it is likely that the normal regulators of secretion (e.g., β-agonists, vasoactive intestinal peptide) could lead to a localized accumulation of cAMP, resulting in specific isotype activation.

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