J. Membrane Biol. 185, 137–144 (2002)
DOI: 10.1007/s00232-001-0120-3

The Journal of
Membrane
Biology

Cyclic AMP-dependent Cl Secretion Is Regulated by Multiple Phosphodiesterase Subtypes in Human Colonic Epithelial Cells

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Received: 27 April 2001/Revised: 21 September 2001

Abstract. The role of phosphodiesterase (PDE) isoforms in regulation of transepithelial Cl secretion was investigated using cultured monolayers of T_{84} cells grown on membrane filters. Identification of the major PDE isoforms present in these cells was determined using ion exchange chromatography in combination with biochemical assays for cGMP and cAMP hydrolysis. The most abundant PDE isoform in these cells was PDE4 accounting for 70–80% of the total cAMP hydrolysis within the cytosolic and membrane fractions from these cells. The PDE3 isoform was also identified in both cytosolic and membrane fractions accounting for 20% of the total cAMP hydrolysis in the cytosolic fraction and 15–30% of the total cAMP hydrolysis observed in the membrane fraction. A large portion of the total cGMP hydrolysis detected in cytosolic and membrane fractions of T_{84} cells was mediated by PDE5 (50–75%). Treatment of confluent monolayers of T_{84} cells with various PDE inhibitors produced significant increases in short-circuit current (*Isc*). The PDE3-selective inhibitors terqinsin, milrinone and cilostamide produced increases in *Isc* with EC_{50} values of 0.6 nM, 8.0 nM and 0.5 μM respectively. These values were in close agreement with the IC_{50} values for cAMP hydrolysis. The effects of the PDE1-(8-MM-IBMX) and PDE4-(RP-73401) selective inhibitors on *Isc* were significantly less potent than PDE3 inhibitors with EC_{50} values of >7 μM and >50 μM respectively. However, the effects of 8-MM-IBMX and terqinsin on Cl secretion were additive, suggesting that inhibition of PDE1 also increases Cl secretion. The effect of PDE inhibitors on *Isc* were significantly blocked by apical treatment with glibenclamide (an inhibitor of the CFTR Cl channel) and by basolateral bumetanide, an inhibitor of Na-K-2Cl cotransport activity. These results indicate that inhibition of PDE activity in T_{84} cells stimulates transepithelial Cl secretion and that PDE1 and PDE3 are involved in regulating the rate of secretion.

Key words: CFTR — PDE — Bumetanide — Na-K-2Cl cotransport — T_{84} cells

Introduction

Cyclic nucleotide phophodiesterases (PDE) play an important role in cAMP- and cGMP-dependent signaling by controlling the degradation rate of these second messengers (Beavo, 1995; Muller, Engels & Fozard, 1996; Degerman, Belfrage & Manganiello, 1997). PDEs are capable of regulating steady-state levels of cyclic nucleotides under basal conditions and can affect both amplitude and duration of cyclic nucleotide-dependent responses following hormonal stimulation (Beavo, 1995). Perhaps the most dramatic example of amplitude modulation is found in photoreceptors. Photoreceptor PDEs regulate cGMP levels within the cell in response to light intensity (Lagnado and Baylor, 1992). These light-induced oscillations in cGMP levels modulate cGMP-gated cation channels resulting in transduction of light stimulation into depolarization of the outer membrane segment. PDEs also play a major role in regulating responses to prolonged agonist stimulation and are believed to be important mediators of cross talk between Ca^{2+}-dependent and cyclic nucleotide-dependent signaling pathways (Sonnenburg, Seger & Beavo, 1993; Shakur, Pryole & Houslay, 1993; Beavo, Contie Heaslip, 1994). Increases in intracellular $[Ca^{2+}]$ can affect the time course and amplitude of

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cAMP- and cGMP-signaling by increasing calmodulin-dependent (CaM) PDE activity or by increasing Ca^{2+} CaM-dependent phosphorylation of PDEs (Degerman, et al., 1997).

A number of different phosphodiesterase isoforms have now been identified in mammalian tissues representing at least eleven unique gene families (Soderling and Beavo, 2000; Hetman, et al., 2000). Many of these families contain multiple genes that are expressed in different tissues as functionally distinct alternative splice variants (Soderling and Beavo, 2000). Classification of these enzymes is based on both primary amino acid-sequence data and on regulatory properties. All PDEs contain a core of highly conserved amino acids that make up the catalytic domain of the enzyme (Charbonneau et al., 1986; Francis et al., 1994; Francis Turko & Corbin, 2000). PDEs within each family exhibit >65% primary sequence homology in contrast to <40% homology between different families (Degerman et al., 1997). Mammalian PDEs have been shown to exist as dimers containing two catalytic subunits. A conserved signature motif (HDX2HX4N) has been identified in the catalytic domain of PDEs, which is absent in enzymes that do not exhibit PDE activity (Beavo, 1995). The mechanism by which these residues contribute to catalysis has not been completely elucidated, but there are some data to suggest that they may be involved in the binding of divalent metal ions.

PDE isoforms have distinct tissue distributions. For example, PDE1, PDE3 and PDE4 represent more than 90% of the total cAMP phosphodiesterase activity present in brain, platelets and renal epithelial cells respectively (Degerman, et al., 1997). Other tissues such as the adrenal cortex possess almost exclusively PDE2. Tissue-specific differences in isoforms also exist within PDE families. In brain and testis for example, multiple subtypes of PDE1 are present that have different specificities for cAMP and cGMP (Muller, et al., 1996). Kidney, liver and airway smooth muscle appear to contain multiple PDE4 subtypes. Identification of PDE4 subtypes in liver and kidney was possible by examining PDE sensitivity to selective inhibitors such as rolipram and Ro 20-1724 (McLaughlin, et al., 1993). In addition to differences in tissue specificity, it appears that PDEs also possess distinct intracellular distributions that are presumably important for determining their regulatory role within the cell. In cardiac muscle cells and hepatocytes, 50–70% of the PDE activity is membrane-associated (Beavo, 1995). In contrast, 80–90% of PDE activity is present in the cytosolic fraction of platelets and smooth muscle cells.

In primary human airway epithelial cells (HAECs) and in A549 cells, PDE4 was shown to be the predominant PDE isoform with lower activities for PDE3, PDE1 and PDE5 (Wright, et al., 1998; Dent et al., 1998). PDE1, PDE3 and PDE4 activities were detected in the cytosolic fraction, but PDE3 activity was not detected within the microsomal fraction of either HAECs or A549 cells (Wright, et al., 1998). These results suggested the presence of cytosolic PDE3 but not membrane-associated PDE3. Inhibition of cytosolic PDE3 activity with ORG-9935 (30 μM) was found to inhibit IL-1β-stimulated granulocyte-macrophage colony-stimulating factor (GM-CSF) secretion. PDE4 inhibition with rolipram also produced a significant inhibition of GM-CSF secretion, indicating a role for both PDE3 and PDE4 in regulation of mediator secretion from airway epithelial cells.

In the present study, we examined the effects of selective inhibitors of PDE activity in the T_{84} human colonic epithelial cell line in an attempt to identify PDEs involved in the regulation of cAMP-dependent Cl secretion. We also examined relative abundance and determined the subcellular distributions of PDE3 and PDE4 isoforms so that enzymatic activities could be correlated with physiological responses to PDE inhibition. The results demonstrate that although PDE4 is present in greatest abundance within T_{84} cells, PDE1 and PDE3 play a role in regulation of Cl secretion. These enzymes were found to be associated with the membrane fraction, making them available for modulating cAMP levels near the membrane.

Materials and Methods

Materials

The PDE inhibitors terqinsin, 8-MM-IBMX and Rolipram were purchased from Biomol (Plymouth Meeting, PA). EHNA, Milrinone the Calcium/Calmodulin-dependent phosphodiesterase (PDE1) and fetal bovine serum (FBS) were purchased from Sigma (St. Louis, MO). DEAE sepharose and 50Q-Macroprep were from Pharmacia, Uppsala Sweden. Dulbecco's Modified Eagle's Medium (DMEM/F12) was from Celox, Penicillin/streptomycin was from BioWhittaker (Walkersford, MD) and Dispase was from CRI. Trypsin/EDTA in HBSS was purchased from Clonetec. T_{84} cells, a human colon carcinoma cell line, were from American Type Culture Collection, MD. The PDE-4 inhibitor RP-73401 (Ashton et al., 1994) and the quinazolinamine PDE5 inhibitor, 6-Chloro-4[(3,4-methylenedioxybenzyl)amino] quinazoline (Takase et al., 1994) were prepared by John Gerster of 3M Pharmaceuticals. Cilostamide was prepared by John Mickelson of 3M Pharmaceuticals (Lugnier et al., 1985).

Cell Culture for Cl Transport Studies

T_{84} cells were grown in DMEM/F12 supplemented with 3.7 g/l sodium bicarbonate, 10% FBS, 10 μg/ml insulin, 1% nonessential amino acid, 5 μg/ml fungizone, 100 U/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml kanamycin (standard medium). The epithelial cells were then plated onto cell culture dishes and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Culture medium was changed after 24 hours and then every 2–3 days. The epithelial cells were trypsinized and placed on transparent 4.5-cm^2

Table 1. IC_{50} values for the inhibition of cAMP hydrolysis by PDE1, PDE2, PDE3 and PDE4 and cGMP hydrolysis by PDE5

Inhibitor	IC_{50}				
	PDE1 μM	PDE2 μM	PDE3 μM	PDE4 μM	PDE5 μM
8-MM-IBMX	**5 ± 3.0**	37	>400	>400	2 ± 3
EHNA	>400	4.3 ± 2.2	>400	310 ± 250	>400
Cilostamide	221	48	**0.13 ± 0.06**	99	6
Terqinsin	15	1.2	**0.006 ± 0.002**	0.43	0.67
RP-73401	130 ± 100	67 ± 56	50 ± 21	**0.0016 ± 0.0005**	8.9 ± 0.5
Quinazolinamine	230	241	10	340	**0.021 ± 0.08**

Numbers in bold are IC_{50}values of PDE inhibitors tested on Cl secretion in T84 cells..
Data are means ±SEM.

membrane filters (Corning, Corning, NY). The cells became confluent in 4-5 days after seeding on membrane filters.

Measurement of Electrical Parameters

Transepithelial resistance of the cell monolayers was measured using the EVOM epithelial voltohmmeter coupled to Ag/AgCl "chopstick" electrodes (World Precision Instruments, Clearwater FL). After 1 week, the confluent culture inserts (4.5 cm^2) were mounted in Ussing Chambers, bathed on both sides with standard saline solution containing (in mM): 153 Na, 6 K, 143 Cl, 2 Ca, 1 Mg, 20 HCO_3, 0.3 H_2PO_4, 1.3 HPO_4, pH 7.4. The bathing solutions were maintained at 37°C and bubbled with 95% O_2/5% CO_2. Transepithelial potential difference, tissue conductance and short circuit current (*Isc*) were measured with the use of voltage-clamp circuitry from JWT Engineering, Kansas City, KS. The data from the voltage-clamp experiments were digitized, stored and analyzed using Workbench data acquisition software (Kent Scientific, Litchfield, CT) and recorded with a Compaq pentium II microcomputer. All cells were pretreated with indomethacin (10 μM) added to both apical and basolateral solutions at least 10 min before the beginning of the experiment to inhibit basal prostaglandin synthesis and the autocrine effects of cyclooxygenase products on transport properties of the epithelium.

Preparation of Phosphodiesterase from T_{84} Cells

T_{84} cells were grown in 150-mm diameter tissue-culture plates in DMEM/F12 supplemented with 5% FBS and penicillin/streptomycin. Cells were split 1:4 just prior to confluency. Medium was removed from each plate and the cells were dispersed by incubating with 4 ml Dispase and 6 ml 0.025% Trypsin/0.01% EDTA in HBSS, for 15–20 min at 37°C. Cells were collected and washed twice with PBS by centrifugation at 300 × g for 10 min and then plated out in DMEM/F12 medium. Cells were passaged three times to obtain 50 near-confluent plates (approximately 5×10^8 cells total) prior to harvesting. Cells were washed by centrifugation two times in PBS.

T_{84} cells harvested from 50 culture plates were suspended in homogenization buffer (50 mM Tris-HCl, pH 7.4 containing (in mM)10 EDTA, 2 EGTA, 2.5 DTT, 0.5 PMSF, 1 TLCK and 2 μg/ml soybean trypsin inhibitor, 20 μM leupeptin, 20 μM pepstatin A, at 10^7 cells/ml and freeze-thawed for 3 cycles in a dry-ice/ethanol bath. The cells were further homogenized on ice, in a dounce homogenizer and centrifuged 2,000 × g for 30 minutes at 4°C. The supernatant was centrifuged at 100,000 × g for 35 minutes. The cytosolic fraction was saved and the pellet was resuspended in 2 ml of homogenization buffer containing 0.3 M sucrose. The cytosol and pellet fractions were used for the determination of phosphodiesterase activity and for further fractionation using ion exchange chromatography.

Preparation of Phosphodiesterase Isoenzymes

The PDE isoenzymes PDE2, PDE3, PDE4 and PDE5 were partially purified by anion-exchange chromatography using a modification of the method of Francis and Corbin, (1988). PDE2 and PDE3 were purified from bovine cardiac muscle. PDE4 was purified from the monocytic cell line, U-937 and PDE5 from bovine lung tissue. Briefly, supernatants from the 100,000 × g centrifugation of the tissue- or cell-homogenate were loaded onto a 44 by 280-mm macroprep-50 anion exchange column previously equilibrated with buffer A (in mM: 25 Tris, pH 7.5, 1 EDTA, 0.2 EGTA, 1 DTT, 2 magnesium acetate, 0.2 PMSF and 1 μM each leupeptin and pepstatin A. The isoenzymes were eluted with a linear gradient of 0–1 M sodium acetate in buffer A. Fractions were assayed for cAMP- and cGMP-phosphodiesterase activity and isoenzyme bands characterized by the inclusion of selective inhibitors. The isoenzyme-selective PDE inhibitors used and their IC_{50} values are shown in Table 1.

Fractionation of PDE isoenzymes in the cytosolic fraction of T_{84} cells was performed as above using a column (11 × 114 mm) of DEAE Sepharose equilibrated with buffer A. The pellet fraction from the high-speed centrifugation of T_{84} cells was solubilized in buffer A containing 20% v/v glycerol and 0.025% luberol prior to loading onto the DEAE Sepharose column prewashed with the same buffer.

Measurement of Phosphodiesterase Activity in Cytosol and Membranes

Aliquots of T_{84} cell cytosol or membranes were diluted into 25 mM Tris buffer, pH 7.5 containing 2 mM $MgCl_2$, 90 mM KCl, 1 mM $CaCl_2$ (buffer C). 75 μl of the enzyme was pre-incubated for 10 min in the presence or absence of inhibitors or modulators at 37°C. Inhibitors were made up in DMSO and added in 2-μl volume. Calmodulin and cGMP were made up in buffer C. 75 μl buffer C containing cyclic nucleotide substrate (cAMP or cGMP containing radioactive tracer) was added to initiate the reaction. The final concentration of cyclic nucleotide was 2 μM, with 0.5–0.6 μCi/reaction of tritiated cyclic-nucleotide tracer. Samples were incubated for 10 min prior to quenching by the addition of 400 μl of 240 mM zinc acetate containing, 0.5 mM cAMP or cGMP, followed by 330 μl of 288 mM Na_2CO_3. The solution was mixed rapidly and centrifuged at 600 × g. The unhydrolized cAMP or cGMP was determined by counting radioactivity in a 200-μl sample of the supernatant mixed with 750 μl of ScintaSafe scintillation cocktail.

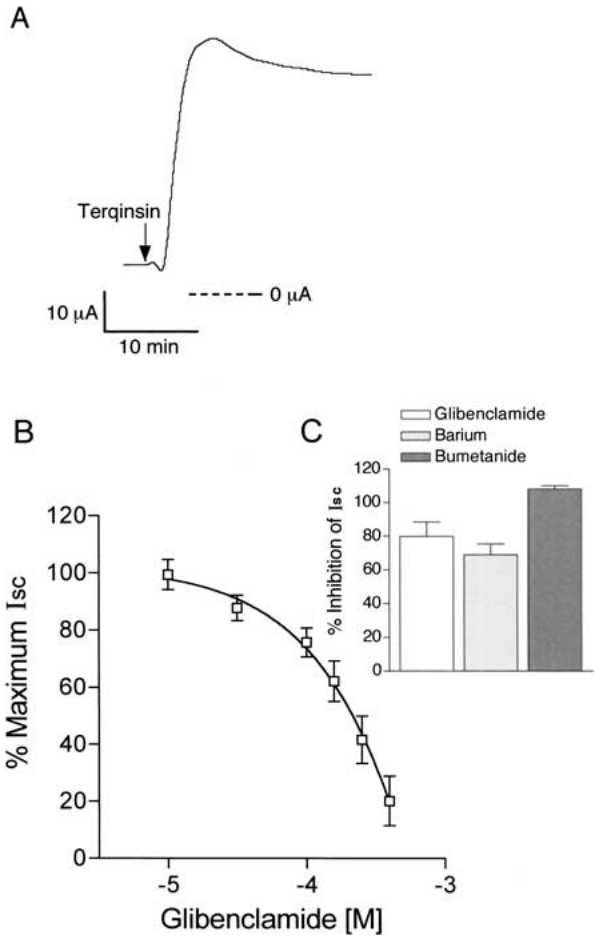


Fig. 1. (*A*) Time course of terqinsin (50 nM) stimulation of *Isc* in cultured monolayers of T_{84} cells ($n = 5$). (*B*) Concentration-response relationship showing glibenclamide inhibition of the PDE inhibitor-activated *Isc* response ($n = 6$). (*C*) Effects of apical glibenclamide (600 μM), basolateral bumetanide (100 μM) and basolateral barium (100 μM) on % inhibition of the terqinsin-stimulated *Isc* ($n = 5$ for each condition).

Statistics

All values are presented as means ± SE. The differences between control and treatment means were analyzed using a *t*-test for paired or unpaired means where appropriate. A value of $P < 0.05$ was considered statistically significant. The IC_{50} values for PDE inhibitors were determined by using linear regression analysis to fit the data in the linear portion of the curves. The concentration of each compound at 50% maximal effect was determined from the equation for the regression line.

Results

Effects of PDE Inhibition on Cl Secretion

The data presented in Fig. 1*A* show the effects of terqinsin on *Isc* of T_{84} cells grown on membrane filters. Addition of 50 nM terqinsin to the basolateral membrane produced a sustained increase in *Isc*. Similar responses were recorded with other PDE3 inhibitors such as milrinone and cilostamide at submicromolar concentrations. 8-MM-IBMX and RP-73401 also produced increases in *Isc* with similar time courses as shown for terqinsin, but significantly higher concentrations were required to elicit an *Isc* response. The effect of terqinsin on *Isc* (Fig. 1*B*) was inhibited in a concentration-dependent manner by apical addition of glibenclamide, an inhibitor of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). Figure 1*C* shows the effects of various inhibitors of anion secretion produced by selective inhibition of PDE3 activity with terqinsin. T_{84} cell monolayers were activated with a maximum concentration of terqinsin (100 nM) and then treated with 600 μM glibenclamide (apical administration), 100 μM barium (basolateral administration) or 100 μM bumetanide (basolateral administration). Treatment with glibenclamide reduced the terqinsin-stimulated *Isc* by 80%. Partial inhibition of Cl secretion was also produced when basolateral K permeability was inhibited with barium. Complete inhibition of the terqinsin-stimulated *Isc* was produced by blocking Cl uptake across the basolateral membrane with the Na-K-2Cl cotransport inhibitor bumetanide.

Identification of the PDE Isoforms Involved in Regulating Cl Secretion

To identify the PDE isoforms involved in regulation of Cl secretion we compared the effects of various PDE3 inhibitors with the PDE4 inhibitor RP-73401 and the PDE1 inhibitor (8-MM-IBMX) and PDE5 inhibitor (Quinazolinamine). Figure 2*A* shows the effects of increasing concentrations of terqinsin on *Isc* with subsequent inhibition of the current response with 100 μM bumetanide. In Fig. 2*B* the concentration-response relationships for terqinsin, milrinone, cilostamide, 8-MM-IBMX and RP-73401 are presented. The EC_{50} values for each of these compounds is reported in Table 2. The rank order of potency indicates that one of the PDE isoforms responsible for regulating Cl secretion in T_{84} cells is PDE3. 8-MM IBMX was found to stimulate *Isc* with a much lower EC_{50} than its IC_{50} for inhibition of PDE3. This EC_{50} value was only slightly greater than the IC_{50} for inhibition of PDEl suggesting that PDE1 may also have some effect on Cl secretion. To test this idea we conducted a set of experiments where monolayers were treated with a maximum stimulatory concentration of terqinsin (100 nM) and then stimulated with 8-MM-IBMX (50 μM). The results (Fig. 3*A* and *B*) show that the effects of terqinsin and 8-MM-IBMX are additive and that addition of these compounds in reverse order produces the same additive response. The results of these experiments indicate that PDE1 is also involved in regulation of anion secretion.

Table 2. Comparison between IC_{50} and EC_{50} values for cAMP or cGMP hydrolysis and Cl secretion in T_{84} cells

Inhibitor	Inhibition of PDE activity (membranes)		Cl secretion	cAMP Hydrolysis (PDE3)
	% Maximum %	IC_{50} [nM]	EC_{50} [nM]	IC_{50} [nM]
Terqinsin	15–20[a]	0.5 ± 0.4	0.6 ± 0.3	0.6 ± 0.2
Milrinone	ND	ND	8.0 ± 0.2	ND
Cilostamide	15–20[a]	295 ± 35	500 ± 100	130 ± 60
8-MM-IBMX	ND	5000 ± 300	> 7000	> 400,000
RP-73401	70–80[a]	1.16 ± 0.6	50,000	50,000 ± 2100
Quinazolinamine	50–75[b]	16 ± 2	ND	10,000

[a] % maximum inhibition of cAMP hydrolysis.
[b] % maximum inhibition of cGMP hydrolysis.

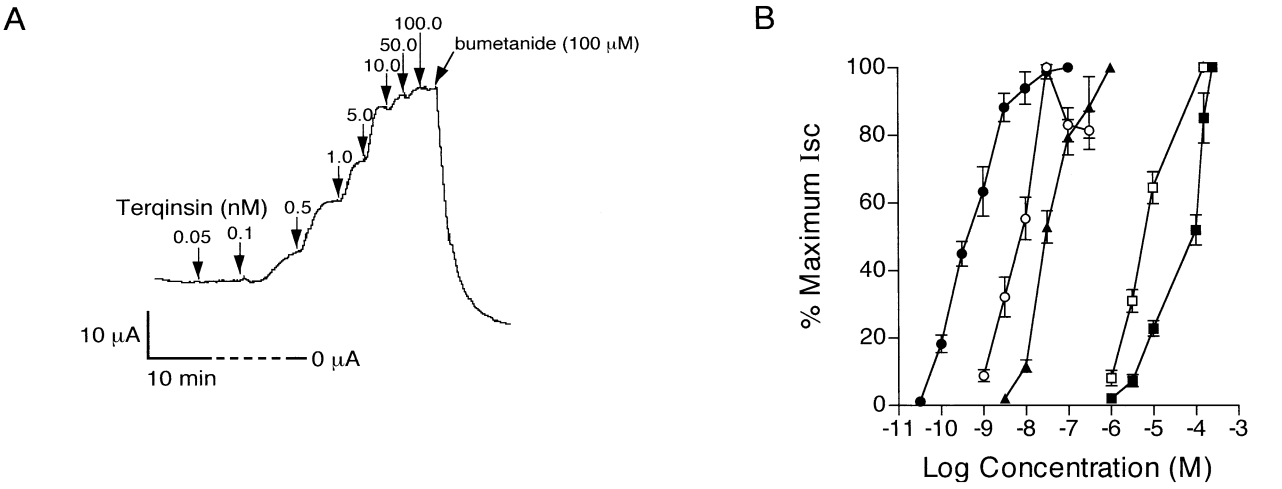


Fig. 2. (*A*) Representative tracing showing the concentration-response time course for *Isc* stimulation by terqinsin and subsequent inhibition by basolateral addition of 100 μM bumetanide. (*B*) Concentration-response relationships for various PDE inhibitors on *Isc* (filled circles: terqinsin; open circles: milrinone; filled triangles: cilostamide; open squares: 8-MM-IBMX; and filled squares: RP-73401.

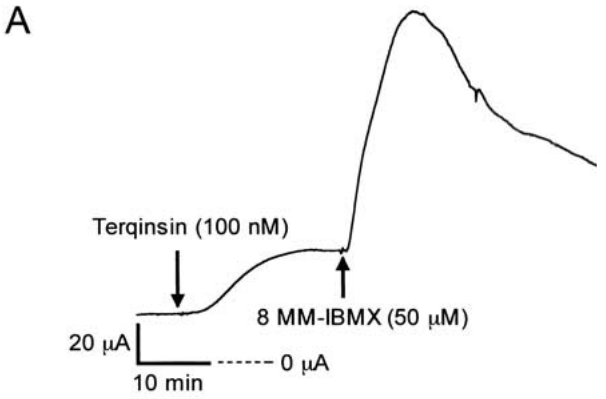
Effects of PDE Inhibitors on Cyclic Nucleotide Hydrolysis in Cytosol and Membrane Fractions

Isoenzyme-selective PDE inhibitors were used to determine the isoenzyme profile present in the 100,000 × g supernatant (cytosol) and pellet (membranes) from T_{84} cells. With the exception of 8-MM-IBMX (Table 1), which inhibits PDE1 and PDE5 equally, the other inhibitors used had adequate selectivity to use as tools for the identification of each isoenzyme in the T_{84} cell preparations. The contribution of each isoenzyme to the total cAMP and cGMP phosphodiesterase activity was determined by varying the concentration of each inhibitor over the concentration range where specific inhibition occurs. Using cAMP as a substrate, the PDE4-selective inhibitor RP-73401 produced 70 to 80% inhibition of phosphodiesterase activity in the cytosolic preparations.

The concentration for half-maximal inhibition determined for RP-73401 was 1.16 ± 0.59 nM and 2.18 ± 0.30 nM for the cytosolic and membrane preparations, respectively. The PDE3 inhibitors terqinsin and cilostamide inhibited 20% of the cytosolic and approximately 25% of the membrane cAMP-phosphodiesterase activity (Fig. 4). The concentrations of terqinsin and cilostamide that gave half-maximal inhibition were 0.6 ± 0.4 nM and 295 ± 35 nM, respectively. The response to the PDE2 inhibitor EHNA was variable, with a maximum inhibition of PDE2 activity of 20%. Inhibition of cGMP phosphodiesterase activity by the PDE5-selective inhibitor quinazolinamine, indicated that PDE5 accounts for 50–75% of the cGMP PDE activity in cytosolic and membrane preparations. The concentration for half-maximal inhibition by quinazolinamine was 16 ± 2 nM.

Discussion

The results of this investigation showed that 70–80% of the cAMP PDE activity present in both membrane and cytosolic fractions from T_{84} cells is associated



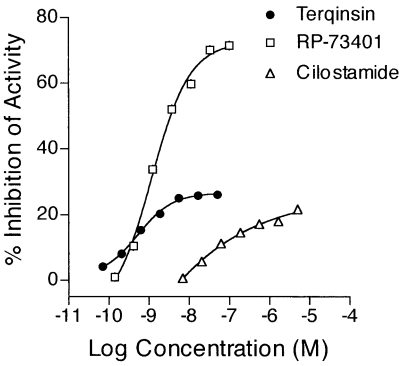


Fig. 4. Concentration-response relationships for PDE inhibitors on cAMP hydrolysis (membrane fraction); terqinsin, filled circles; RP-73401, open squares; cilostamide, open triangles.

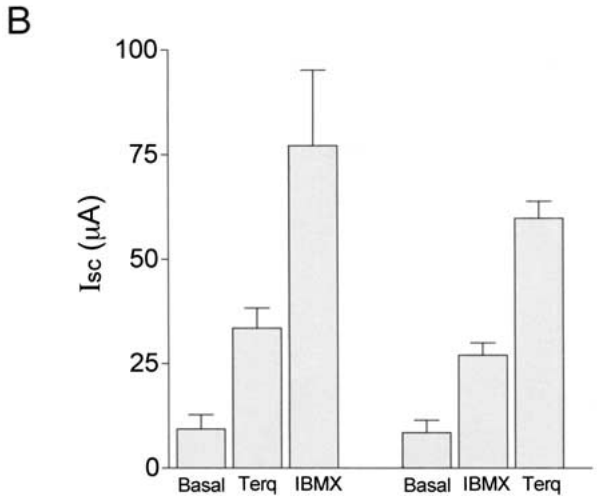


Fig. 3. (*A*) Representative tracing showing the additive effects of 50 μM 8-MM-IBMX on *Isc* after administration of a maximum stimulating concentration of terqinsin (100 nM). (*B*) Bar graph showing effects of terqinsin and 8-MM-IBMX on *Isc*. Note that additive responses were not dependent on the order of addition of terqinsin or 8-MM-IBMX.

with PDE4. PDE5 appears to be the predominant PDE responsible for cGMP hydrolytic activity. PDE3 accounts for a relatively minor fraction of the total cAMP hydrolytic activity in both membrane and cytosol fractions, however, PDE3 is responsible for regulation of cAMP-dependent Cl secretion in these cells. Evidence in support of this conclusion is based on the effects of selective PDE inhibitors on transepithelial Cl secretion as determined by measurements of *Isc*. Treatment of T_{84} cells with the PDE3 inhibitors terqinsin, milrinone and cilostamide all produced increases in *Isc* with EC_{50} values that closely corresponded to the IC_{50} values of terqinsin and cilostamide on PDE3-mediated cAMP hydrolysis. In contrast, the effects of the PDE4-selective inhibitor RP-73401 produced inhibition of Cl secretion at concentrations that were several orders of magnitude higher than terqinsin or cilostamide. This concentration range is consistent with inhibition of PDE3 activity by this PDE4-selective inhibitor. These results are similar to those from a previous study showing that selective PDE3 inhibition with milrinone and amrinone produces activation of CFTR in Calu-3 and 16HBE cells, both derived from airway epithelium (Kelley, Al-Nakkash & Drumm, 1995). These investigators observed no effect on CFTR activation with either rolipram (a PDE4 inhibitor) or IBMX (a PDE1 and PDE5 inhibitor). In contrast, results of experiments using 8-MM-IBMX presented in this study suggest that PDE1 is also capable of regulating Cl secretion in T_{84} cells.

Previous studies with T_{84} cells have shown that Cl secretion can be stimulated by agonists that increase intracellular cAMP (Dharmsathaphorn et al. 1984; 1985) and that CFTR mediates Cl efflux across the apical membrane in response to cAMP (Cliff and Frizzell, 1990; Anderson et al., 1992). Thus, CFTR presumably mediates Cl efflux in response to PDE3 or PDEI inhibition in T_{84} cells. This conclusion is supported by the effect of glibenclamide, a known inhibitor of CFTR activity (Sheppard & Robinson, 1997) reported in Fig. 1. It is worth noting, however, that glibenclamide can block other types of Cl channels and so we cannot rule out the possibility that Cl channels other than CFTR may also be involved. Inhibition of the PDE inhibitor-activated current by basolateral bumetanide, an inhibitor of Na-K-2Cl cotransport activity and partial inhibition by barium, an inhibitor of basolateral K channels, are characteristic of cAMP-activated Cl secretion in T_{84} cells (Mandel, Dharmsathaphorn & McRoberts, 1986; Mandel, et al., 1986).

Previous work with canine ventricular myocytes showed that β-adrenergic agonists produced activation of calcium channels by increasing cAMP near the plasma membrane of these cells (Hohl and Li, 1991). A similar observation was reported for single frog

cardiac myocytes (Jurevicius & Fischmeister, 1996). In the experiments with frog myocytes, exposure of half of the cell to β-adrenergic agonists produced activation of Ca^{2+} channels in a region of the cell in contact with the β-agonist. Pretreatment with IBMX greatly reduced compartmentalization of the adrenergic response. These results suggested that PDEs play a role in limiting the actions of cAMP to local regulation of calcium channels by degrading cAMP that diffuses away from its site of synthesis at the membrane. It seems less likely that PDEs in epithelial cells are involved in compartmentalization in the same way as suggested for cardiac myocytes. In T_{84} cells, receptors for neurohormones such as VIP are localized to the basolateral membrane. However, cAMP/protein kinase A (PKA) effector proteins such as CFTR are located in the apical membrane (Mandel, et al., 1986 a,b). Thus, local cAMP synthesis and PKA activation events could not account for phosphorylation of distant substrates, such as apical transport proteins, unless diffusion away from the basolateral membrane was allowed to occur. It seems reasonable to suggest that specific PDE isoforms may be colocalized with PKA phosphorylation substrates such as CFTR. This type of colocalization could explain the specificity of specific PDE isoforms on CFTR activity under conditions where a relatively minor fraction of total PDE activity is attributable to PDE3.

In conclusion, these findings demonstrate that Cl secretion in T_{84} cells is selectively regulated by at least two PDE isoforms. The specific PDE3 subtype (PDE3A or PDE3B) involved in this regulation is presently unknown. The absence of any effect of PDE inhibitors such as RP-73401 at concentrations that block PDE4 activity suggests compartmentalization of the cAMP response to agonist stimulation and perhaps colocalization of PDE isoforms with cAMP-regulated transport pathways involved in Cl secretion.

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