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EFFECT OF PHOSPHODIESTERASE INHIBITORS ON BRADYKININ-MEDIATED PROSTAGLANDIN E_2 AND CYCLIC AMP SYNTHESIS IN RENAL PAPILLARY COLLECTING TUBULE CELLS

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Abstract—Isolated rabbit renal papillary collecting tubule cells were used to examine the effects of phosphodiesterase inhibitors on intracellular cyclic AMP and prostaglandin synthesis. Experiments performed on confluent primary tissue cultures demonstrated that bradykinin increases intracellular cyclic AMP by a prostaglandin-dependent mechanism. Phosphodiesterase inhibitors induced a dose-dependent decrease in bradykinin-stimulated prostaglandin synthesis. Fifty percent inhibition occurred with approximately 0.7 mM 3-isobutyl-1-methylxanthine (IBMX). Inhibition was found to be reversible. IBMX did not inhibit bradykinin-induced prostaglandin synthesis as a result of increased intracellular cyclic AMP. The nonmethylxanthine phosphodiesterase inhibitor RO 20-1724 also reduced bradykinin-stimulated prostaglandin synthesis. IBMX inhibited calcium-ionophore-A23187-induced prostaglandin synthesis but did not inhibit arachidonic acid stimulation of prostaglandin synthesis. The data demonstrate that bradykinin increased renal papillary collecting tubule cell cyclic AMP in a prostaglandin-dependent manner. Based on the data presented, phosphodiesterase inhibitors act to decrease arachidonic acid availability for prostaglandin synthesis, independent of changes in cellular cyclic AMP content.

Cyclic nucleotide phosphodiesterase hydrolyzes cyclic AMP and cyclic GMP to 5′-AMP and 5′-GMP respectively. This reaction is inhibited by methylxanthines such as theophylline, caffeine and 3-isobutyl-1-methylxanthine (IBMX). Many of the pharmacological effects of methylxanthines are thought to be mediated by this effect on cyclic nucleotide phosphodiesterase. However, we recently demonstrated that phosphodiesterase inhibitors inhibit bradykinin-induced increases in prostaglandin (PG)E_2 production by slices of rabbit renal inner medulla [1, 2]. Thus, methylxanthines exert effects on two systems, cyclic nucleotides and prostaglandins, which are important local modulators of renal function.

The prostaglandin and cyclic nucleotide systems are known to be interrelated. PGE_2 and PGI_2 are potent agonists for adenylate cyclase in the renal medulla [3]. PGE_2 has been shown to antagonize the antidiuretic action of vasopressin and cyclic AMP in toad bladder [4] and isolated perfused tubule [5]. Work in platelets has shown that agents which increase cyclic AMP diminish agonist-induced increases in prostaglandin production [6]. These observations make it difficult to evaluate the effects of inhibitors that might have significant effects on both the cyclic nucleotide and prostaglandin systems.

Recently, Grenier *et al.* [7, 8] characterized rabbit renal papillary collecting tubule cells grown in tissue culture. These cells were found to be exquisitely sensitive to bradykinin in terms of increasing PGE_2 production. In these cells, in contrast to the renal slice, bradykinin does not directly increase cyclic AMP content. Bradykinin-induced increases in PGE_2 production resulted in increases in collecting tubule cell cyclic AMP content. Because of these characteristics, and because a purified cell preparation avoids some of the difficulties encountered in a multiple cell preparation such as the slice, we utilized the renal papillary collecting tubule cell in primary tissue culture to further investigate the effects of methylxanthines on prostaglandin production and the role of cyclic AMP synthesis on prostaglandin production.

MATERIALS AND METHODS

Theophylline, bradykinin, indomethacin and fraction V bovine serum albumin were obtained from the Sigma Chemical Co. (St. Louis, MO). [N-5,6,8,11,12,14,15-^{3}H]PGE_2 (117 Ci/mmole) and cyclic [G-^{3}H]AMP (31 Ci/mmole) was obtained from the New England Nuclear Corp. (Boston, MA). Dulbecco's minimal essential medium and Alpha minimal essential medium were obtained from K.C. Biological (Lenexa, KS). Fetal calf serum was from the Grand Island Biological Co. (Grand Island, NY). Costar-24 (16 mm) plates and 35 mm plastic dishes were from Costar (Cambridge, MA). 3-Isobutyl-1-methylxanthine (IBMX) was obtained from the Aldrich Chemical Co. (Milwaukee, WI). A23187 and forskolin were obtained from Cal-Biochem (LaJolla, CA). Gelman filter screens (No. 4320)

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were from Gelman Sciences (Ann Arbor, MI). RO20-1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone] was a gift from Hoffmann-LaRoche (Nutley, NJ). Collagenase II was obtained from Worthington Diagnostics, Division of Millipore Corp. (Bedford, MA). First antibody for radioimmunoasssay was obtained from the Regis Chemical Co. (Morton Grove, IL). The source of gloat antisera to rabbit gamma globulins was Antibodies, Inc. (Davis, CA). Normal rabbit serum was obtained from Miles Laboratories (Elkart, IN). PGE_2 was a gift of the Upjohn Co. (Kalamazoo, MI). All other chemicals and reagents were obtained from standard sources.

Renal papillary collecting tubule cells were isolated according to the technique of Grenier *et al.* [7]. Briefly, kidneys from normal male New Zealand white rabbits were removed under Pentothal anesthesia and immediately placed in physiologic saline. Kidneys were halved under aseptic conditions, and papillae were dissected away and minced in Dulbecco's minimal essential medium using a No. 10 scalpel blade. The minced tissue was transferred to a 15-ml tube with 4 ml of Krebs containing 1 mg/ml collagenase II. The tissue was incubated in 5% CO_2 humidified air at 37° for 2.5 hr, passed through a Gelman filter screen, diluted with 2 vol. of distilled sterile water, and allowed to stand for 4 min. This solution was then centrifuged at 600 *g* for 10 min at 4°, and the pellet was resuspended in 10 ml of 10% bovine serum albumin in phosphate-buffered saline and centrifuged. The pellet was then resuspended in Dulbecco's minimal essential medium with 10% fetal calf serum and plated in either Costar-24 wells or 35 mm plastic dishes and incubated in 5% CO_2 humidified air at 37°. The cultures grew to confluence over 6–8 days. Cells were quantified on a Coulter counter using the pronase-cetrimide method of Stewart *et al.* [9].

Experiments were conducted on confluent cultures by aspirating the growth medium, washing with 2–3 ml of phosphate-buffered saline, and replacing with 1 ml of Dulbecco's minimal essential medium containing phosphodiesterase inhibitors as indicated. Indomethacin dissolved in ethanol or IBMX in Delbecco's minimal essential medium was added to the preincubation medium as indicated. After a 30-min preincubation, the appropriate agonist or antagonist was added for the final 30 min incubation. However, forskolin was added after 20 min of preincubation and allowed to preincubate for 10 min prior to the final incubation (see Table 4). All experimental incubations were in 5% CO_2 humidified air at 37°. After the final incubation, the medium was aspirated and assayed for PGE_2 by radioimmunoassay as previously reported [10, 11]. The cells were then either counted or, where appropriate, 300 μl of 85° sodium acetate (0.2 M) at pH 5.0 was added. Acetate-treated cells were scraped with a rubber policeman and assayed directly for cyclic AMP by the protein binding method [12], as modified by Mashiter *et al.* [13].

Results are reported as mean ± standard error of the mean. An unpaired *t*-test was performed with the number noted in the tables.

RESULTS

IBMX induced a dose-dependent inhibition of bradykinin-stimulated PGE_2 production by renal papillary collecting tubule cells (Table 1). Basal PGE_2 production (1.7 ± 0.01 ng $PGE_2/10^5$ cells) was not affected by any dose of IBMX. IBMX also inhibited the effect of the calcium ionophore A23187 to increase PGE_2 production by renal papillary collecting tubule cells. By contrast, there was no effect of IBMX on arachidonic acid-induced PGE_2 production.

The data presented in Table 2 show that this inhibition was reversible. In these experiments, a slightly different incubation technique was utilized. After an initial preincubation of 30 min, bradykinin was added where indicated to start incubation No. 1. The medium was removed for analysis, cells were washed to remove IBMX, and the 30-min incubation No. 2 was performed in the absence of IBMX as noted in the table. For incubation No. 1, 1 mM IMBX caused a 60% reduction in the bradykinin-stimulated PGE_2 synthesis from 17.73 ± 1.78 ng $PGE_2/10^5$ cells to 6.56 ± 1.16 ng $PGE_2/10^5$ cells ($P < 0.01$). In incubation No. 2, cells previously treated with IBMX and control cells elicited a similar increase in PGE_2 synthesis following bradykinin addition (8.95 ± 0.63 vs 7.97 ± 0.53 ng $PGE_2/10^5$ cells).

The effects of IBMX on cyclic AMP accumulation and prostaglandin production are fruther analyzed by experiments tabulated in Table 3. In the presence of 2 mM IBMX, there was no measurable increase in either cyclic AMP content or PGE_2 production by bradykinin. However, both arachiodonic acid and PGE_2 increased cyclic AMP content. Arachidonic

Table 1. Dose–response effect of IBMX on bradykinin, A23187, and arachidonic acid stimulated PGE_2 synthesis*

Conc of IBMX (mM)	PGE_2 (ng/10^5 cells) Bradykinin (1 μg/ml)	Arachidonic acid (150 μM)	A23187 (2 μM)
0	12.8 ± 1.3	100 ± 2.0	13.6 ± 0.7
0.2	9.28 ± 0.2	89 ± 18	
0.7	6.59 ± 0.4	107 ± 5	
2.0	3.71 ± 0.5	92 ± 1.5	5.9 ± 0.8

* Cell cultures were incubated for 30 min in the presence of IBMX followed by addition of bradykinin, arachidonic acid, or A23187 for 30 min. Cell counts were 3.37×10^5 cells per culture (N = 3). Values are means ± S.E.M.

Table 2. Reversibility of IBMX inhibition of bradykinin-stimulated PGE_2 synthesis*

Incubation No. 1 Condition	(PGE_2 ng/10^5 cells)		Incubation No. 2 Condition	(PGE_2 ng/10^5 cells)
Medium only	= 3.09 ± 0.18	----------	Medium only	= 0.88 ± 0.03
IBMX	= 0.73 ± 0.05	----------	Bradykinin	= 8.95 ± 0.63
Medium only	= 2.21 ± 0.08	----------	Bradykinin	= 7.97 ± 0.53
Bradykinin	= 17.73 ± 1.78			
Bradykinin + IBMX	= 6.56 ± 1.16†			

* Cell cultures were preincubated for 30 min in medium with or without IBMX followed by a 30-min incubation with conditions indicated in incubation No. 1. For incubation No. 2, the same cells were washed with buffer not containing IBMX and incubated for an additional 30 min in the absence of IBMX with or without bradykinin. IBMX = 1 mM; bradykinin = 1 μg/ml. Cell counts were 3.11×10^5 cells per culture (N = 3). Values are means ± S.E.M.

† $P < 0.01$, bradykinin + IBMX vs bradykinin in incubation No. 1.

Table 3. Effects of agonists on cyclic AMP content and PGE_2 synthesis in renal papillary collecting tubule cells in the presence of 2 mM IBMX*

Conditions	Cyclic AMP content (pmoles cyclic AMP/10^5 cells)	PGE_2 synthesis (ng PGE_2/10^5 cells)
Absence of IBMX	0.06 ± 0.03†	0.42 ± 0.03
Medium only	0.49 ± 0.03	0.34 ± 0.04
Bradykinin	0.47 ± 0.08	0.38 ± 0.05
Arachidonic acid	4.1 ± 0.3†	15.7 ± 4.03†
Arachidonic acid + indomethacin	0.6 ± 0.1	2.6 ± 0.06
PGE_2	5.1 ± 0.2†	

* Cell cultures (35 mm dishes) were incubated 60 min in 2 mM IBMX. Agonists and antogonists were added after 30 min of incubation. Bradykinin = 1 μg/ml; arachidonic acid = 150 μM; indomethacin = 28 μM; $PGE_2 = 10^{-6}$ M. Cell counts were 15×10^5 cells per dish (N = 3). Values are means ± S.E.M.

† $P < 0.01$, vs medium only.

Table 4. Effects of bradykinin and forskolin on renal papillary collecting tubule cell cyclic AMP content and PGE_2 synthesis*

Conditions	Cyclic AMP content (pmoles cyclic AMP/10^5 cells)	PGE_2 synthesis (ng PGE_2/10^5 cells)
Medium only	0.16 ± 0.09	2.14 ± 0.16
Bradykinin	0.69 ± 0.10†	10.49 ± 0.46†
Forskolin	27.80 ± 1.3	1.72 ± 0.30
Bradykinin + forskolin	46.50 ± 0.1‡	7.92 ± 0.95‡
Bradykinin + forskolin + indomethacin	32.3 ± 2.2§	0.46 ± 0.05§

* Cell cultures were incubated for 60 min in 0.1 mM IBMX. Bradykinin (1 μg/ml) and indomethacin (28 μM) were added after 30 min of incubation. Forskolin (100 μM) was added after 20 min of incubation. Cell counts were 3.21×10^5 cells per culture (N = 3). Values are means ± S.E.M.

† $P < 0.01$, medium only vs bradykinin.

‡ $P < 0.01$, forskolin vs bradykinin + forskolin.

§ $P < 0.01$, bradykinin + forskolin vs bradykinin + forskolin + indomethacin.

acid mediated increases in both cyclic AMP and PGE_2 were inhibited by indomethacin.

A concentration of 0.1 mM IMBX was used in Table 4 to allow bradykinin stimulation of PGE_2 synthesis. At this concentration of IBMX, minimal inhibition of bradykinin-induced PGE_2 production would be expected (Table 1). Using these conditions, bradykinin elicited a 5-fold increase in PGE_2 synthesis and a 4-fold increase in cyclic AMP content. To further examine the effects of cyclic AMP on bradykinin stimulation of PGE_2 synthesis, forskolin, a diterpene stimulator of the catalytic unit of adenylate cyclase which does not interfere with the hormone receptor [14, 15], was added. Forskolin (100 μM) had no effect on PGE_2 production but did markedly increase cyclic AMP content 150-fold. With forskolin, bradykinin caused a doubling of cyclic AMP content but did not alter bradykinin-induced PGE_2 production. Indomethacin prevented bradykinin stimulation of PGE_2 synthesis and the observed

Table 5. Effects of methylxanthine and nonmethylxanthine phosphodiesterase inhibitors on bradykinin-stimulated PGE_2 synthesis*

Conditions	PGE_2 synthesis (ng $PGE_2/10^5$ cells)
Medium only	1.13 ± 0.09†
Bradykinin	11.9 ± 0.5
Bradykinin + IBMX	1.78 ± 0.47†
Bradykinin + RO20-1724	4.08 ± 0.31†
Bradykinin + theophylline (2 mM)	9.84 ± 1.42
Bradykinin + theophylline (10 mM)	3.63 ± 0.37†

* Cell cultures were incubated for 60 min with or without phosphodiesterase inhibitors. Bradykinin was added after 30 min of incubation. Bradykinin = 1 μg/ml; IBMX = 2 mM; RO20-1724 = 1 mM; theophylline as noted. Cell counts were 6.81×10^5 cells per culture (N = 8). Values are means ± S.E.M.

† $P < 0.01$ vs bradykinin.

cyclic AMP content was no different than with forskolin alone.

The effects of methylxanthine and a nonmethylxanthine phosphodiesterase inhibitor are examined in Table 5. The methylxanthines, IBMX and theophylline, inhibit bradykinin-induced increases in PGE_2 synthesis. RO20-1724, a nonmethylxanthine phosphodiesterase inhibitor, also inhibited bradykinin-induced increases in PGE_2 production.

DISCUSSION

These data show that IBMX caused a reversible dose-dependent inhibition of bradykinin-stimulated PGE_2 release from isolated renal papillary collecting tubule cells in primary tissue culture. These results also confirm previous observations made by Grenier *et al.* [7, 8] that bradykinin appears to increase intracellular cyclic AMP content in renal papillary collecting tubule cells by a prostaglandin-dependent mechanism. However, bradykinin appears to increase cyclic AMP content in rabbit renal medullary slices by a mechanism independent of prostaglandin release [1, 2]; therefore, there are cells, other than renal papillary collecting tubule cells, within the renal medulla which respond to bradykinin directly by increasing cyclic AMP content.

The data presented in this report show that phosphodiesterase inhibitors act to inhibit bradykinin-induced prostaglandin synthesis by a mechanism which does not involve changes in cyclic AMP metabolism. The results with forskolin show that greatly increasing cellular cyclic AMP content did not affect bradykinin-induced increases in PGE_2 production. Furthermore, in experiments not shown, incubation of renal papillary collecting tubule cells with 3 mM dibutyryl cyclic AMP or 2 mM cyclic AMP did not diminish bradykinin-mediated PGE_2 release.

This effect is not unique to IBMX in that the methylxanthine, theophylline, as well as the structurally dissimilar imidizolidinone derivative, RO20-1724, exhibited the same effect, although theophyline was 5-fold less active than IBMX. This suggests that phosphodiesterase inhibitors have common effects not only on cyclic nucleotide metabolism but also on bradykinin-mediated prostaglandin release. This study also points out the difficulty in interpreting results from previous reports and ongoing studies in which investigators are assessing both agonist stimulation of prostaglandin or thromboxane synthesis and cyclic AMP in the presence of phosphodiesterase inhibitors.

Experiments in platelets [16] and other tissues [17, 18] have suggested a sequence for agonist-mediated prostaglandin release. Initially there is agonist interaction with a receptor on the cell surface. This interaction results in activation of certain lipases and is associated with an increase in cytosolic Ca^{2+}. This Ca^{2+} is a result of release from intracellular stores and transmembrane Ca^{2+} flux. It is a point of controversy as to which lipases are activated. One theory proposes that there is activation of phospholipase C. This lipase activation would act on polyphosphoinositides and initiate phosphatidylinositol "turnover" resulting in activation of diacylglycerolipase and/or phospholipase A_2 to yield arachidonic acid from cellular phospholipids. These phospholipids are constituents of cell membranes, and certain classes of phospholipids (such as phosphatidylinositol, phosphatidylcholine and phosphatidylethanolamine) are thought to contribute arachidonic acid for metabolism by prostaglandin H synthase to prostaglandin products. In an attempt to localize the site of inhibition, we performed a dose-response experiment with bradykinin, arachidonic acid, and the calcium ionophore A23187 (Table 1). Our data demonstrate that IBMX inhibited both bradykinin and calcium ionophore mediated prostaglandin release, but did not affect arachidonic acid mediated synthesis of prostaglandin. This would suggest that IBMX does not interfere with the prostaglandin H synthase enzyme, but rather interferes with steps in the process of making arachidonic acid available for prostaglandin synthesis. Phospholipase C is a phosphodiesterase, cleaving the phosphodiester linkage of myoinositol at the third carbon of glycerol. We suggest that inhibition of phospholipase C is the most likely site of inhibition by phosphodiesterase inhibitors. Further studies in this regard are required.

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