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ARACHIDONIC ACID METABOLISM IN CULTURED AORTIC ENDOTHELIAL CELLS

EFFECT OF cAMP AND 3-ISOBUTYL-1-METHYLXANTHINE

A. RICHARD WHORTON,* JAMES B. COLLAWN, MALCOLM E. MONTGOMERY, STEPHEN L. YOUNG and RICHARD S. KENT

Divisions of Clinical Pharmacology, Cardiology and Pulmonary Medicine, Duke University Medical Center, Durham, NC 27710, U.S.A.

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Abstract—To investigate the hypothesis that cyclic AMP (cAMP) regulates arachidonic acid metabolism in vascular tissue, we have studied the effects of forskolin (FSK), an activator of adenylate cyclase, and 3-isobutyl-l-methylxanthine (IBMX), a phosphodiesterase inhibitor, on hormone-stimulated prostacyclin (PGI_2) synthesis in porcine aortic endothelial cells grown in culture. In these experiments, bradykinin (1 μg/ml) and A23187 (0.2 μM) potently stimulated PGI_2 biosynthesis (9- and 10-fold respectively). However, prostaglandin synthesis in response to either of these agents was not affected by FSK even though FSK elevated intracellular levels of cAMP 10-fold. IBMX failed to elevate basal cAMP levels when incubated with unstimulated cells. Stimulation of IBMX-treated (0.1 but not 1.0 or 4.0 mM) cells with bradykinin, however, did result in increased cAMP levels, presumably due to PGI_2 formation and subsequent activation of adenylate cyclase. In addition to phosphodiesterase inhibition, IBMX inhibited PGI_2 formation (72% at 1 mM) in a dose-dependent manner so that, at higher doses of IBMX, cAMP levels returned to baseline. Thus, prostacyclin synthesis inhibition by IBMX could not be attributed to elevated cAMP. In other experiments, IBMX (1 mM) was found to directly inhibit arachidonic acid release (32%) and arachidonic acid metabolism (65%) in endothelial cells and to inhibit arachidonic acid conversion to PGE_2 by sheep seminal vesicle microsomes (65%). These data suggest that IBMX directly inhibits both phospholipase and cyclooxygenase activities. These experiments do not support the contention that cAMP regulates these enzymes in cultured aortic endothelial cells.

Prostacyclin (PGI_2), synthesized by the vascular endothelium, has been shown to be a potent inhibitor of platelet aggregation and adhesion and a potent vasodilator [1, 2]. In addition, PGI_2 has been found to exert an inhibitory effect on vascular smooth muscle cell growth [3]. This prostaglandin is formed after release of arachidonate from phospholipids by phospholipases and sequential metabolism by fatty acid cyclooxygenase and prostacyclin synthetase [4, 5]. The rate of formation depends not only upon the activity of the phospholipase but also upon the activity of cyclooxygenase and prostacyclin synthetase [6]. Among compounds which regulate phospholipase activity, cAMP has been suggested to inhibit both phospholipase [7, 8] and cyclooxygenase [9] activities. It has also been suggested that PGI_2, through activation of adenylate cyclase and formation of cAMP, regulates its own biosynthesis [10, 11].

In view of the large number of endogenous compounds and drugs which activate endothelial adenylate cyclase [10, 12], it is obviously important to understand the exact nature of the effect of cAMP upon vascular PGI_2 formation. In many of the reported experiments where beta-agonists or prostaglandins have been suggested to inhibit arachidonate release and metabolism, co-incubation with 3-isobutyl-l-methylxanthine (IBMX) was an absolute requirement [9, 10, 13]. Since IBMX is a potent inhibitor of cyclic nucleotide phosphodiesterases, it has been assumed that cAMP mediated the effects seen.

In the present study, we have further examined the specific role of cAMP in regulation of PGI_2 synthesis in cultured porcine aortic endothelial cells. We chose to use forskolin (FSK), a direct activator of adenylate cyclase [14], to elevate intracellular cAMP levels and have investigated the effects of FSK and IBMX on phospholipase and cyclooxygenase activity. In previous studies we have shown that bradykinin and the ionophore A23187 potently activate phospholipases in endothelial cells releasing arachidonic acid which is then metabolized primarily to PGI_2 [15, 16]. In the present study we used these agonists to study possible cAMP-mediated changes in phospholipase activity. We also examined potential cAMP-mediated effects on cyclooxygenase activity, both in intact cells and in sheep seminal vesicle microsomes using arachidonic acid as substrate. The results of our study suggest that elevations of cAMP do not alter PGI_2 synthesis in endothelial cells, while IBMX directly inhibits both phospholipase and cyclooxygenase. Thus, in experiments where IBMX is used as a phosphodiesterase

* Send correspondence to: A. Richard Whorton, Division of Clinical Pharmacology, Box 3813, Duke University Medical Center, Durham, NC 27710.

inhibitor, interpretation of results may be erroneous unless adequate precautions are taken.

MATERIALS AND METHODS

Materials. Bradykinin triacetate, 3-isobutyl-1-methylxanthine and arachidonic acid were purchased from the Sigma Chemical Co. (St. Louis, MO), ionophore A23187 (free acid) and forskolin from Calbiochem (La Jolla, CA), solvents (HPLC grade) from Fisher, and culture media and buffers from the Grand Island Biological Co. (Grand Island, NY). All compounds were dissolved in incubation buffer immediately prior to an experiment. A23187 was dissolved in ethanol (2 mg/ml) and diluted with incubation buffer before use (final ethanol concentration was less than 30 ppm). Forskolin was dissolved in dimethyl sulfoxide (10 mg/ml) and diluted with buffer before use (final dimethyl sulfoxide concentration was less than 20 ppm). Neither the ethanol nor dimethyl sulfoxide vehicles had any demonstrable effect on the cells. Arachidonic acid was converted to its Na^+ salt with Na_2CO_3 and dissolved in buffer immediately before using. [5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (100 Ci/mmole) was purchased from the New England Nuclear Corp. (Boston, MA). Radioimmunoassays for cAMP were done using commercially available reagents from either New England Nuclear or Becton Dickinson (Orangeburg, NY). Both assays gave equivalent results.

Cell culture. Endothelial cells were collected from porcine aortae and cultured as described by Jaffe *et al.* [17]. Briefly, freshly collected aortae were cleaned, filled with 0.1 to 0.2% collagenase (*Clostridium perfringens,* Sigma) in medium 199, sealed with clamps, and incubated at 37° for 15–20 min. Freed cells were collected, washed by centrifugation at 4°, and resuspended in medium 199 containing Hanks' salts, 10% fetal calf serum, and 1% antibiotic/antimycotic mixture (GIBCO). Cells were then plated (4×10^5/flask) in 25 cm^2 polystyrene flasks (Costar, Cambridge, MA) and monitored until about 50% of the cell clumps adhered. Nonadhering cells were poured off, and the primary isolates were incubated in medium 199 plus 10% fetal calf serum at 37°. The medium was changed every 3 days, and the fetal calf serum was maintained at 10% as the cells grew to confluence. Porcine endothelial cells in culture were polygonal in shape and demonstrated contact inhibition. Further identification was as previously published [16]. Primary and subsequent cultures were treated with 0.1% EDTA at 37° and split 1:3 for subculture. In general, confluent cultures in the first or second passage were used, although cells have been studied after the fifth pass with no apparent difference in response. Immediately prior to an experiment, culture medium was poured off and the monolayer washed once with Hanks' balanced salts, pH 7.4. To study the effects of FSK on cAMP accumulation or on PGI_2 production, FSK or FSK plus bradykinin was added and the incubation was continued for 10 min. The medium was then collected and used for PGI_2 analysis. The cells were used for protein and cAMP determination as described below. To study the effects of IBMX on arachidonic acid metabolism by endothelial cells, IBMX was added 5 min prior to addition of arachidonate (5 μM sodium salt). The incubation was continued for an additional 2 min after arachidonate addition, and the buffer was collected and analyzed for PGI_2 production. To study the effects of IBMX on cAMP and PGI_2 production in control and bradykinin-stimulated endothelial cells, cells were preincubated with IBMX 5 min before addition of buffer or agonist. All incubations were carried out at 37° in room air. To study fatty acid release from cells, fatty acid free albumin (Sigma) was added to the incubation buffers (0.5%) to trap released fatty acids. Under these conditions, no changes in cell morphology or viability (tryphan blue exclusion) were noted [15, 16].

Prostacyclin assay. Prostacyclin, PGI_2, released by cells after incubation was measured as its stable hydrolysis end-product, 6-keto-$PGF_{1\alpha}$, and quantitated by gas chromatography-mass spectrometry employing selected ion monitoring. After addition of a deuterated prostaglandin analog internal standard (3,3,4,4-[^{2}H]-6-keto-prostaglandin $F_{1\alpha}$, Upjohn), the samples were acidified to pH 3 with formic acid and extracted twice with 2 ml ethyl acetate. Before analysis by mass spectrometry, prostaglandins were methylated (diazomethane), silylated [(*N,O*)-bis (trimethylsilyl) trifluoroacetamide] and methoxylated (methoxamine–HCl) as previously described [18] (typical recoveries at this point ranged from 50 to 70%). Selected ion monitoring was done using a quadrapole gas chromatograph-mass spectrometer (Hewlett–Packard 5992-B) equipped with a 4 ft glass column packed with 3% OV-1 on 80/100 mesh Chromosorb WHP and operated isothermally at 230° with helium as the carrier gas (22 cc/min). Ion pairs monitored were *(m/z)* 598 vs 602 for 6-keto-$PGF_{1\alpha}$.

cAMP assay. In experiments where cAMP levels were determined, cells were incubated as described. The reaction was stopped with ice-cold buffer containing 1 mM IBMX. This buffer was poured off and the cells were scraped from the flask and homogenized in ice-cold distilled water using a Brinkmann polytron homogenizer. Aliquots of the homogenate (0.7 ml) were then added to 0.7 ml of 12% trichloroacetic acid (4°), and the remainder was saved for protein determination using acidic Coomassie Blue (Bio-Rad, Richmond, CA) [19]. The trichloroacetic acid extract was centrifuged at 2000 *g* for 15 min at 4° to pellet denatured proteins. The supernatant fraction containing cAMP was collected and extracted four times, each time with 3 ml of water-saturated diethyl ether. The aqueous layer was evaporated to dryness and reconstituted in 0.05 ml of sodium acetate buffer, pH 6.2. All samples were assayed in duplicate at two dilutions using commercially available cAMP radioimmunoassay reagents by procedures essentially according to Steiner *et al.* [20]. Reconstitution volumes were chosen so that results were calculated from the middle portion (0.25 to 2.5 pmoles) of the standard curve (range, 0.1 to 5.0 pmoles). Recoveries after reconstitution were consistently quantitative. Data are expressed as pmoles cAMP/mg protein.

Fatty acid analysis. After incubation of cells for 10 min with bradykinin (1 μg/ml), IBMX (1 mM), or

bradykinin plus IBMX, the medium was collected and the release of fatty acids was determined. Heneicosic acid (4 nmoles) was added to the incubation medium as internal standard. The medium was acidified to pH 2 with HCl and fatty acids were extracted using three 2-ml portions of hexane. The hexane extract was evaporated to dryness and the fatty acids were converted to methyl esters by treatment with diazomethane (recoveries through this procedure were > 97%). Fatty acid methyl esters were analyzed by gas–liquid chromatography using a Hewlett–Packard 5830 gas chromatograph equipped with dual 6 ft 2 mm i.d. glass columns and dual flame ionization detectors maintained at 250°. Columns were packed with 10% SP 2330 on 100/20 mesh Chromasorb WAW (Supelco, Bellefonte, PA), and the oven was heated in a linear fashion from 150° to 220° at 5°/min. Nitrogen was used as a carrier gas (30 cc/min), and the injection port was maintained at 260°. A standard mixture of fatty acid methyl esters was prepared from pure fatty acids (Supelco) and used to standardize retention times and response factors (relative peak area/mole).

Sheep seminal vesicle microsomes. Microsomes were prepared from sheep seminal vesicles (Wayne State University, Detroit, MI) and resuspended (approximately 10 mg protein/ml) in 0.1 M potassium phosphate buffer, pH 7.4, containing EDTA (1 mM). After preincubation for 5 min in the above buffer containing hemoglobin (0.1 mg/ml), phenol (2 mM) and tryptophan (1 mM), aliquots of seminal vesicle microsomes were incubated with saturating levels of labeled arachidonic acid (100 μg/ml + 1.5 μCi of ^{3}H) for 2.0 min at 37° in air. Various doses of IBMX were added to the preincubation. The reaction was stopped by addition of 0.7 ml of ice-cold methanol–formic acid (5:2, v/v). Radiolabeled compounds were extracted twice with ethyl acetate (2 vol.). Samples were evaporated to dryness and redissolved in hexane–diethyl ether (9:1, v/v) and applied to a 0.75 g silicic acid column. Unreacted arachidonic acid was eluted with 20 ml of this solvent. The sample vial was rinsed with, and hydroxy fatty acids [12-hydropy-5,8,10-heptadecatrienoic acid (HHT)] eluted with, 20 ml of hexane–diethyl ether (8:2). The sample vial was then rinsed with, and prostaglandins eluted with, 20 ml of ethyl acetate–methanol (9:1). Aliquots of each fraction were used to determine recoveries and percent conversion (corrections were made based on theoretical ^{3}H loss during formation of each compound). Approximately 80% of the initial amount of ^{3}H added was recovered in the products mentioned below. Losses (15–18%) could not be extracted from the incubation medium and probably represented, in part, ^3H_2O formed during the various bio-conversions. Essentially quantitative recovery of extracted label through the chromatographic procedures was achieved. Prostaglandin standards ([^{3}H]PGE$_2$, [^{3}H]PGF$_{2\alpha}$ and [^{3}H]6-keto-PGF$_{1\alpha}$), hydroxy fatty acid standards ([^{3}H]15-OH-arachidonic acid) and [^{3}H]arachidonic acid were used to validate the chromatographic procedures. In some cases, samples were also analyzed by high performance liquid chromatography using previously published procedures [21]. Data obtained by both procedures were comparable. The major product of these incubations was PGE$_2$ (>80% of products). Other labeled products included HHT (<10%) and PGF$_{2\alpha}$ (<15%).

Data analysis. Data are presented as mean ± S.E.M. and were analyzed by one-way analysis of variance followed by the Student–Newman–Keuls test in cases where more than two means were compared [22].

RESULTS

As we have shown previously [15, 16], both bradykinin and A23187 potently stimulated PGI$_2$ production in our cultured endothelial cells (Table 1). FSK was found to effectively activate adenylate cyclase in a dose-dependent manner (0.1 to 10 μM) leading to a 10-fold elevation in cellular cAMP content at 10 μM (Table 1). Neither bradykinin nor A23187 altered cAMP levels on their own or interfered with the actions of FSK. Even in the face of this greatly elevated intracellular cAMP, no reduction of PGI$_2$ production could be measured when FSK-treated cells were stimulated with either bradykinin or A23187 (Table 1). Similarly, at lower doses of FSK (0.1 and 1 μM), no inhibition of bradykinin-stimulated PGI$_2$ production was observed (data not shown). Thus, it is clear that intracellular cAMP has no regulatory role in hormone or ionophore-stimulated PGI$_2$ production in endothelial cells.

As has been done by others, we studied the effect

Table 1. Effect of forskolin on cAMP levels and PGI$_2$ production in endothelial cells*

Condition	cAMP (pmoles/mg protein)	6-Keto-PGF$_{1\alpha}$ (ng/flask × 10 min)
Control	67.5 ± 3.3 (9)	15.0 ± 2.0 (17)
Bradykinin (1 μg/ml)	71.9 ± 3.5 (9)	148.6 ± 14.2† (17)
A23187 (0.2 μM)	76.4 ± 3.0 (4)	205.4 ± 12.3† (8)
FSK (10 μM)	840.2 ± 30.8† (9)	16.5 ± 2.6 (17)
Bradykinin + FSK	928.7 ± 31.6† (9)	168.4 ± 13.2† (17)
A23187 + FSK	932.9 ± 15.8† (4)	200.4 ± 11.7† (8)

* Data are given as mean ± S.E.M., N = numbers in parentheses.

† Values were significantly greater than control ($P < 0.01$). No significant difference was noted between bradykinin and bradykinin plus FSK or between A23187 and A23187 plus FSK.

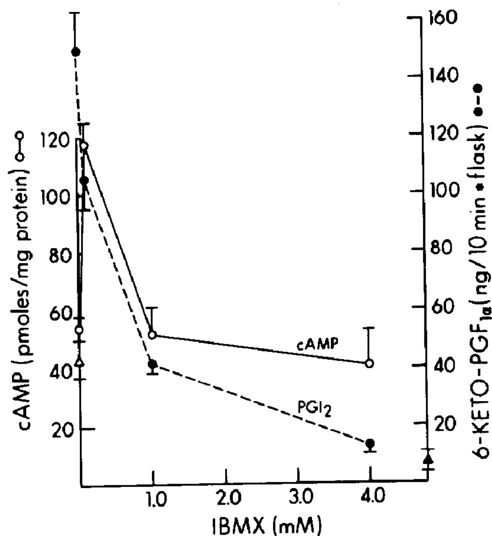

Fig. 1. Effect of IBMX on cAMP and PGI_2 production by bradykinin-stimulated aortic endothelial cells. Data are given as mean ± S.E.M., N = 6. Key: (△) basal cellular cAMP (no additions), and (▲) = basal PGI_2 production (no additions). At 1 mM IBMX, cAMP levels were significantly different from those with 0.1 mM IBMX ($P < 0.01$) and at 1 mM IBMX PGI_2 levels were reduced significantly from those at 0 mM IBMX ($P < 0.01$).

of a cyclic nucleotide phosphodiesterase inhibitor, IBMX, on PGI_2 production. In several published studies utilizing IBMX, elevations in intracellular cAMP have been assumed but not measured. In the present study, we found that cAMP levels were, in fact, not different from control (40.2 ± 2.1 pmoles/mg protein, N = 3) after incubating cells with 0.1 mM IBMX (45.3 ± 3.2 pmoles/mg), 1 mM IBMX (50.4 ± 10.2 pmoles/mg) or 4 mM IBMX (36.0 ± 6.2 pmoles/mg). Additionally, as can be seen in Fig. 1, IBMX elevated cAMP levels only when PGI_2 production was elevated by bradykinin (see 0.1 mM IBMX). Also, as is evident from Fig. 1, IBMX produced a dose-dependent inhibition of bradykinin-stimulated PGI_2 production by endothelial cells (from 151.8 ng to 41.4 ng or 72% at 1 mM IBMX, Fig. 1). Furthermore, IBMX inhibited basal PGI_2 production (data not shown). These effects were not due to elevated cAMP levels. As mentioned above, cAMP levels increased with low dose IBMX where inhibition of PGI_2 production was not pronounced. This presumably occurred through PGI_2 activation of adenylate cyclase and accumulation of intracellular cAMP in the presence of phosphodiesterase inhibitors.

To determine the site of inhibition of IBMX on PGI_2 synthesis, we initially studied the effect of this compound on fatty acid release from endothelial cells. From Table 2 it can be seen that IBMX partially blocked bradykinin-stimulated arachidonic acid release (32% inhibition) presumably by inhibition of a phospholipase. However, this degree of phospholipase inhibition did not account for the 72% inhibition of PGI_2 synthesis discussed earlier. No other significant differences were seen in the other fatty acids measured.

To determine the effect of IBMX on cyclooxygenase and prostacyclin synthetase activity, we used arachidonic acid as substrate. We demonstrated that IBMX inhibited conversion of arachidonate to PGI_2 by intact cells in a dose-dependent manner (Fig. 2) (65% inhibition at 1 mM). To address the issue of specific enzyme inhibition, we prepared sheep seminal vesicle microsomes and incubated them with saturating levels of labeled arachidonic acid and various doses of IBMX. As shown in Table 3, IBMX inhibited cyclooxygenase activity in a dose-dependent manner in this microsomal system, reaching 65.2% at 1 mM. These data are virtually identical to those from endothelial cells and strongly suggest that the major inhibitory effect of IBMX in endothelial cells is on cyclooxygenase, although minor inhibitory effects on prostacyclin synthetase cannot be totally excluded.

DISCUSSION

In the present report, we have shown that forskolin, a direct activator of adenylate cyclase, greatly increased intracellular cAMP levels in endothelial cells even in the absence of phosphodiesterase inhibition. However, the large amount of cAMP generated was not effective in regulating either hormone

Table 2. Effect of IBMX on bradykinin-stimulated fatty acid release from endothelial cells*

	Fatty acid†					
	16:0	16:1	18:0	18:1	18:2	20:4
Control	4.87 ± 0.52	0.56 ± 0.03	2.40 ± 0.19	4.59 ± 0.18	0.87 ± 0.09	0.07 ± 0.02
IBMX (1 mM)	4.38 ± 0.10	0.43 ± 0.03	2.35 ± 0.06	4.76 ± 0.07	0.68 ± 0.02	0.11 ± 0.03‡
Bradykinin (1 μg/ml)	4.67 ± 0.15	0.71 ± 0.07	2.85 ± 0.16	5.26 ± 0.17	0.78 ± 0.05	0.88 ± 0.02§
Bradykinin + IBMX	5.47 ± 0.40	0.92 ± 0.14	2.94 ± 0.12	6.25 ± 0.35	0.77 ± 0.04	0.62 ± 0.06

* Data were analyzed by one-way analysis of variance followed by the Student–Newman–Keuls test. Values are expressed as nmoles of fatty acid, mean ± S.D., N = 4.

† Fatty acids are designated by the number of carbons: number of double bonds.

‡ Not different from control.

§ Greater than control ($P < 0.01$) and greater than bradykinin + IBMX ($P < 0.01$).

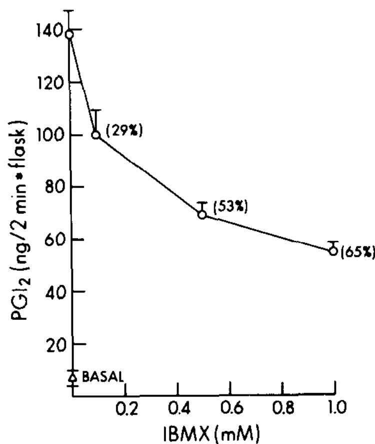

Fig. 2. Effect of IBMX on arachidonic acid metabolism by endothelial cells. Data are given as mean ± S.E.M., N = 4. Numbers in parentheses represent percent inhibition. Key: (△) basal PGI_2 production (no additions).

stimulated or ionophore (A23187) stimulated PGI_2 synthesis. Thus, in contradiction to a number of published reports [7–10, 13], we found no evidence for cAMP-mediated inhibition of prostaglandin synthesis in cultured endothelial cells. This difference most likely resulted from the use of cyclic nucleotide phosphodiesterase inhibitors to maintain elevated cAMP levels and the assumption by others that this was its only action. While IBMX has been widely used to inhibit cyclic nucleotide phosphodiesterase, it has been found to exhibit other activities including interference with adenosine at the purine receptor level [23] and with Ca^{2+} metabolism [24]. Thus, even when cAMP levels are documented, the use of IBMX as a phosphodiesterase inhibitor to infer cAMP-mediation of a process must be viewed cautiously.

In cultured endothelial cells, we observed that IBMX at doses commonly used to inhibit phosphodiesterase also effectively inhibited prostacyclin biosynthesis (IC_{50} between 0.5 and 0.8 mM). Since IBMX partially blocked both arachidonic acid release from cells and metabolism of released arachidonic acid to prostaglandins in both cells and seminal vesicle microsomes, IBMX-mediated inhibition of bradykinin-stimulated PGI_2 release was due to its actions at both phospholipase and cyclooxygenase. The mechanism for these effects is not known but is not due to elevated cAMP levels (Fig. 1). Thus, it appears that in endothelial cells cAMP does not inhibit prostaglandin synthesis but the phosphodiesterase inhibitor IBMX does.

Table 3. Effect of IBMX on fatty acid cyclooxygenase activity*

	% Conversion†	% Inhibition
Control	9.93 ± 0.21	
0.1 mM IBMX	9.91 ± 0.09	0
0.5 mM IBMX	6.70 ± 0.59	32.5
1.0 mM IBMX	3.46 ± 0.36	65.2

* Sheep seminal vesicle microsomes were preincubated for 5 min with buffer or buffer containing IBMX. Labeled arachidonic acid (100 μg + 1.5 μCi of ^3H) and the incubation was continued for 2.0 min. The reaction was stopped and samples were analyzed as described in Methods. Data are given as mean ± S.D., N = 3.

† Conversion to total cyclooxygenase products (approximately 9% HHT, 10% PGF_2 and 81% PGE_2).

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REFERENCES

1. R. L. Czervoinke, J. B. Smith, G. L. Fry, J. C. Hoak and D. L. Haycraft, *J. clin. Invest.* **63**, 1089 (1979).
2. S. L. Swartz, G. H. Williams, N. K. Hollenberg, L. Levine, R. G. Dluhy and T. J. Moore, *J. clin. Invest.* **65**, 1257 (1980).
3. J. J. Huttner, E. T. Gwebu, R. V. Panganamala, G. E. Milo, D. G. Cornwell, H. M. Sharma and J. C. Geer, *Science* **197**, 289 (1977).
4. A. Hassid, *Am. J. Physiol.* **243**, C205 (1982).
5. R. F. Irvine, *Biochem. J.* **204**, 3 (1982).
6. R. S. Kent, S. L. Diedrich and A. R. Whorton, *J. clin. Invest.* **72**, 455 (1983).
7. M. Minker, N. Stanford, M-Y. Chi, G. J. Roth, A. Raz, P. Needleman and P. W. Majerus, *J. clin. Invest.* **59**, 449 (1977).
8. E. G. Lapetina, C. J. Schmitger, K. Chandrabase and P. Cuatrecasas, *Biochem. biophys. Res. Commun.* **76**, 828 (1977).
9. B. Adler, M. A. Gimbrone, Jr., A. I. Schaffer and R. I. Handin, *Blood* **58**, 514 (1981).
10. N. K. Hopkins and R. R. Gorman, *J. clin. Invest.* **67**, 540 (1981).
11. L. K. Lim, N. H. Hunt, R. D. Eichner and M. I. Weidemann, *Biochem. biophys. Res. Commun.* **114**, 248 (1983).
12. A. I. Schafer, M. A. Gimbrone, Jr. and R. I. Handin, *Biochem. biophys. Res. Commun.* **96**, 1640 (1980).
13. A. F. A. Brotherton and J. C. Hoak, *Proc. natn. Acad. Sci. U.S.A.* **79**, 495 (1982).
14. K. B. Seamon and J. W. Daly, *J. biol. Chem.* **256**, 9799 (1981).
15. A. R. Whorton, C. E. Willis, R. S. Kent and S. L. Young, *Lipids* **19**, 17 (1984).
16. A. R. Whorton, S. L. Young, J. L. Data, A. Barchowsky and R. S. Kent, *Biochim. biophys. Acta* **721**, 79 (1982).
17. E. A. Jaffe, R. L. Nachman, C. G. Becker and C. R. Minick, *J. clin. Invest.* **52**, 2745 (1983).
18. A. R. Whorton, B. J. Sweetman and J. A. Oates, *Analyt. Biochem.* **98**, 455 (1979).
19. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
20. A. L. Steiner, C. W. Parker and D. M. Kipnis, *J. biol. Chem.* **247**, 1106 (1972).
21. A. R. Whorton, K. Carr, M. Smigel, L. Walker, K. Ellis and J. A. Oates, *J. Chromat.* **163**, 64 (1979).
22. G. W. Snedecor and W. G. Cochran, *Statistical Methods.* University of Iowa Press, Ames, IA (1967).
23. C. Londos, J. Wolff and D. M. F. Cooper, in *Purinergic Receptors* (Ed. G. Burnstock), p. 287. Chapman & Hall, New York (1981).
24. T. N. Wells and J. G. Hardman, *Adv. Cyclic Nucleotide Res.* **8**, 119 (1977).