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Role of Ca^{2+} and cyclic AMP in the regulation of the production of prostacyclin by the vascular endothelium

(thrombin/ionophore A23187/prostaglandin endoperoxide H_2/cyclic AMP phosphodiesterase/adenylate cyclase)

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ABSTRACT **Incubation of primary monolayer cultures of human umbilical vein endothelial cells with buffer, thrombin (0.5 unit/ml), ionophore A23187 (10 μM), arachidonic acid (20 μM), or prostaglandin H_2 (PGH_2) (4 μM) resulted in prostacyclin (PGI_2) production in nanomolar quantities to the extent of 36 ± 2, 276 ± 13, 485 ± 32, 533 ± 22, and 532 ± 22, respectively, as measured by radioimmunoassay of 6-keto-$PGF_{1\alpha}$. Preincubation of the endothelium with 1 mM 8-(*N,N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate, an antagonist of cytoplasmic Ca^{2+}, or with 4 mM 1-methyl-3-isobutylxanthine (MIX), an inhibitor of cyclic nucleotide phosphodiesterase activity, blocked PGI_2 release induced by thrombin or A23187, decreased arachidonic acid-induced release by ≈50%, but had no effect on PGH_2-induced release. Radioimmunoassay of cAMP in the endothelium showed that the basal level (1.85 ± 0.14 pmol of cAMP per 4.5 × 10^5 cells) was increased by an average of 3.9-fold with 4 mM MIX. PGI_2 (0.4 μM) had no significant effect on cAMP levels in the absence of MIX, but caused a 2-fold increase with 4 mM MIX. The findings suggest that: (*i*) the stimulation of PGI_2 biosynthesis is mediated by Ca^{2+}, (*ii*) increased cAMP inhibits PGI_2 production, and (*iii*) cAMP phosphodiesterase activity modulates PGI_2-induced increases in the intracellular concentration of cAMP.**

Prostacyclin (PGI_2) is a potent vasodilator and inhibitor of platelet aggregation that is believed to play an important role in the maintenance of the nonthrombogenic surface properties of the endothelial lining of blood vessels (1, 2). Cultured vascular endothelium, vascular smooth muscle, and—to a lesser degree—skin fibroblasts have the capacity to synthesize PGI_2 (3, 4). Stimuli that have been reported to induce the synthesis and release of PGI_2 from monolayer cultures of human umbilical vein endothelial cells include thrombin, trypsin, the ionophore A23187, sodium arachidonate, and platelet-derived endoperoxides (5, 6).

Insight into the mechanism by which stimuli induce PGI_2 release from the endothelium is provided by the present understanding of the regulation of prostaglandin (PG) and thromboxane synthesis in platelets and in other cells (7). The rate-limiting step in PG synthesis is thought to be the release of arachidonic acid from membrane phospholipid pools by phospholipases (8). Because Ca^{2+} is an activator of both phospholipase A_2 (9, 10) and phospholipase C (11, 12), the induction of PG biosynthesis is likely to be mediated by an increase in the cytoplasmic concentration of this cation. In platelets there is compelling evidence that cAMP stimulates the active transport of free Ca^{2+} out of the cytosol (13, 14), thereby inhibiting PG and thromboxane biosynthesis (15). PGI_2 is a potent activator of platelet adenylate cyclase (16, 17) and recently has been reported to stimulate adenylate cyclase activity in human umbilical vein endothelial cells (18).

Evidence is presented in this paper that suggests that: (*i*) the stimulation of PGI_2 biosynthesis from human umbilical vein endothelium is mediated by Ca^{2+}, (*ii*) increased cAMP inhibits PGI_2 production, and (*iii*) cAMP phosphodiesterase activity modulates PGI_2-induced increases in the intracellular concentration of cAMP.

MATERIALS AND METHODS

Materials. Thrombin, bovine topical, was from Parke, Davis; A23187, from Calbiochem–Behring; arachidonic acid, from Nu Chek Prep (Elysian, MN); 1-methyl-3-isobutylxanthine (MIX), neutral alumina WN-3, and Dowex AG 50W-4X, from Sigma; and cAMP ^{125}I-radioimmunoassay kit, from New England Nuclear. 8-(*N,N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate (TMB-8) was a gift from R. Gorman (Upjohn). Prostaglandin H_2 (PGH_2) was biosynthesized according to the method of Gorman *et al.* (19). The sources of other supplies used are described elsewhere (20–23); other reagents were of analytical grade.

Endothelial Cell Cultures. Primary cultures of human endothelial cells from umbilical veins were prepared by a modification (21) of the method of Jaffe *et al.* (24). Confluent endothelial cell monolayers containing approximately 4.5 × 10^5 cells per 24-mm-diameter well of a 12-well culture plate (Linbro) were used 4 days after plating.

Incubation Conditions. To measure the production of PGI_2 by endothelial cell monolayers, the culture medium was aspirated and the monolayers were rinsed twice with 0.75 ml of Hanks' balanced salt solution (without $NaHCO_3$) buffered to pH 7.4 with 15 mM Hepes (Hanks/Hepes buffer). After rinsing, 0.5 ml of Hanks/Hepes buffer alone or with the indicated test reagent was layered on each monolayer. In some experiments endothelial cell monolayers were treated with TMB-8 or MIX prior to exposure to stimulatory agents. After aspirating the culture medium and rinsing the monolayers with Hanks/Hepes buffer, 0.45 ml of buffer containing TMB-8 or MIX was layered on the monolayers which were then preincubated at 37°C without rocking for 4 min and 10 min, respectively. At the end of the preincubation period, 0.05 ml of Hanks/Hepes buffer alone or with the indicated test reagent (at 10-fold the final concentration) was added to the monolayers to give a total volume of 0.5 ml. The monolayers were then incubated at 37°C for 5 min on a rocker platform, after which the incubation medium was aspirated into a disposable plastic transfer pipette (Bio-Rad) and frozen at −80°C prior to radioimmunoassay of 6-keto-$PFG_{1\alpha}$, the stable degradation product of PGI_2.

Radioimmunoassay of 6-Keto-$PGF_{1\alpha}$. The radioimmunoassay of 6-keto-$PGF_{1\alpha}$ is described elsewhere (22). Assay detection limits are 0.3 pmol of 6-keto-$PGF_{1\alpha}$ per ml, and 50% in-

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Abbreviations: TMB-8, 8-(*N,N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate; MIX, 1-methyl-3-isobutylxanthine; ASA, acetylsalicylic acid; PG, prostaglandin; PGI_2, prostacyclin.

hibition is obtained with 3.3 pmol of 6-keto-$PGF_{1\alpha}$ per ml. This assay has 4% crossreactivity with $PGF_{2\alpha}$, 2% with $PGF_{1\alpha}$, 1.6% with PGE_2, and <1% with PGE_1, PGD, or PGD_2.

Extraction and Purification of cAMP. cAMP was extracted from endothelial cell monolayers by adding 1.0 ml of ice-cold 5% (vol/vol) trichloroacetic acid to each well. Approximately 4000 cpm of [2,8,-^{3}H]cAMP was added to each well to monitor the recovery of cAMP during the purification procedure. After 10 min on ice, the extracts were aspirated from each well into a disposable plastic transfer pipette and stored therein at −80°C. cAMP in the extracts was isolated by a modification (25) of the method of Krishna *et al.* (26). Prior to radioimmunoassay, cAMP in the samples was acetylated. Each sample was assayed in duplicate at two dilutions.

Statistical Analysis. Statistical significance was determined by Student's *t* test. A *P* value of <0.05 was selected to denote statistical significance between groups. Unless indicated otherwise, all data represent a minimum of three separate experiments, each carried out in triplicate.

RESULTS

The effects of thrombin, the divalent cation ionophore A23187, arachidonic acid, and the PG endoperoxide PGH_2 on the synthesis and release of PGI_2 from endothelial cell monolayers are shown in Table 1. The concentration at which each agent stimulated maximal production of PGI_2 was determined and used for all subsequent experiments. On the average, the amount of PGI_2 released in response to A23187, arachidonic acid, or PGH_2 was about twice that obtained upon stimulation with thrombin. Although the experiments in Table 1 were carried out in the presence of extracellular Ca^{2+} (1.26 mM $CaCl_2$), the amount of PGI_2 released in response to thrombin and A23187 was decreased by an average of about 40% and 60%, respectively, in Ca^{2+}-free Hanks/Hepes buffer with 0.5 mM EGTA.

The effect of the cytoplasmic Ca^{2+} antagonist, TMB-8, on PGI_2 release is shown in Table 2. Preincubation of endothelial cell monolayers with TMB-8 resulted in a total inhibition of both thrombin and A23187-induced release of PGI_2, no inhibition of PGH_2-induced release, and a partial inhibition of arachidonic acid-induced release. The inhibitory effect of TMB-8 was immediately reversible upon rinsing the monolayers with Hanks/Hepes buffer prior to addition of the stimulatory agent.

To investigate a possible role of cAMP in the regulation of PGI_2 production by the vascular endothelium, the effect of the cyclic nucleotide phosphodiesterase inhibitor, MIX, on both PGI_2 release and cAMP accumulation was determined. As measured by radioimmunoassay, the total intracellular concentration of cAMP in endothelial cell monolayers was 1.85 ± 0.14 pmol per 4.5×10^5 cells (mean ± SEM; n = 23 experiments). Preincubation of the monolayers for 10 min with 1 or 4 mM MIX increased cAMP levels by factors of 2.7 ± 0.3 and 3.9 ± 0.4, respectively (mean ± SEM; n = 12 experiments). The production of PGI_2 in response to stimulation by either thrombin or A23187 was partially inhibited by 1 mM MIX but was blocked by 4 mM MIX (Table 3). In contrast, both concentrations of MIX had a partial inhibitory effect on arachidonic acid-induced release, whereas neither concentration had a significant effect on PGH_2-induced release (Table 3).

The effects of thrombin, A23187, arachidonic acid, PGH_2, and PGI_2 on cAMP levels in endothelial cell monolayers preincubated with and without MIX are shown in Fig. 1*A*. In the absence of MIX, none of the agents had a significant effect on cAMP accumulation. However, each agent markedly potentiated the increase in cAMP resulting from preincubation with 1 mM MIX. The inability to detect the stimulation of cAMP production in the absence of MIX indicates that the cAMP phosphodiesterase activity of the vascular endothelium maintains a low, steady-state level of cAMP. In the presence of 4 mM MIX, cAMP accumulation was stimulated by arachidonic acid, PGH_2, and PGI_2 but not by thrombin or A23187. The lack of an effect of thrombin and A23187 on cAMP levels in monolayers preincubated with 4 mM MIX suggests that the potentiation of cAMP accumulation was due to the effects of PGI_2 released from the endothelium.

Therefore endothelial cell monolayers were preincubated for 30 min with 100 μM acetylsalicylic acid (ASA) to inhibit fatty acid cyclooxygenase activity and, hence, the synthesis of PGI_2 and other PGs from arachidonic acid. No measurable PGI_2 was released from ASA-treated monolayers in response to stimulation by buffer alone, thrombin, A23187, or arachidonic acid; however, PGH_2-induced release of PGI_2 was unaffected (data not shown). Fig. 1*B* shows that preincubation with ASA abolished the potentiation of cAMP accumulation observed in response to stimulation by thrombin, A23187, and arachidonic acid, but had no effect on either PGH_2- or PGI_2-dependent increases in cAMP production.

In the presence of 1 mM MIX, the release of 100–500 nM PGI_2 is induced by thrombin, A23187, arachidonic acid, and PGH_2 (Table 3); however, each agent stimulated significantly

Table 1. Stimulation of PGI_2 production

Stimulus	n	6-keto-$PGF_{1\alpha}$, nM
None	54	36 ± 2
Thrombin, 0.5 unit/ml	54	276 ± 13
A23187, 10 μM	25	485 ± 32
Arachidonic acid, 20 μM	22	533 ± 34
PGH_2, 4 μM	6	532 ± 22

Endothelial cell monolayers were incubated for 5 min at 37°C with rocking in the presence of each agent. Values are means ± SEM for the number of experiments (n) indicated.

Table 2. Inhibition of PGI_2 production by TMB-8

Stimulus	n	6-keto-$PGF_{1\alpha}$, nM	
		Control	TMB-8, 1 mM
None	8	43 ± 9	28 ± 6
Thrombin, 0.5 unit/ml	6	304 ± 37	33 ± 10
A23187, 10 μM	5	540 ± 59	29 ± 5
Arachidonic acid, 20 μM	4	658 ± 110	249 ± 13
PGH_2, 4 μM	3	544 ± 47	566 ± 74

Endothelial cell monolayers were preincubated for 4 min at 37°C without rocking in Hanks/Hepes buffer alone (control) or with buffer containing TMB-8. Stimulatory agents were added to the media and incubations were continued for 5 min. Values are means ± SEM for the number of experiments (n) indicated.

Table 3. Inhibition of PGI_2 production by MIX

Stimulus	n	6-keto-$PGF_{1\alpha}$, nM		
		Control	MIX, 1 mM	MIX, 4 mM
None	11	24 ± 2	13 ± 2	10 ± 1
Thrombin, 0.5 unit/ml	10	303 ± 33	133 ± 25	21 ± 5
A23187, 10 μM	9	526 ± 43	267 ± 36	30 ± 5
Arachidonic acid, 20 μM	9	463 ± 32	366 ± 24	231 ± 27
PGH_2, 4 μM	6	538 ± 30	485 ± 25	455 ± 46

Endothelial cell monolayers were preincubated for 10 min at 37°C without rocking in Hanks/Hepes buffer alone (control) or with buffer containing MIX. Stimulatory agents were added to the media and incubations were continued for 5 min. Values are means ± SEM for the number of experiments (n) indicated.

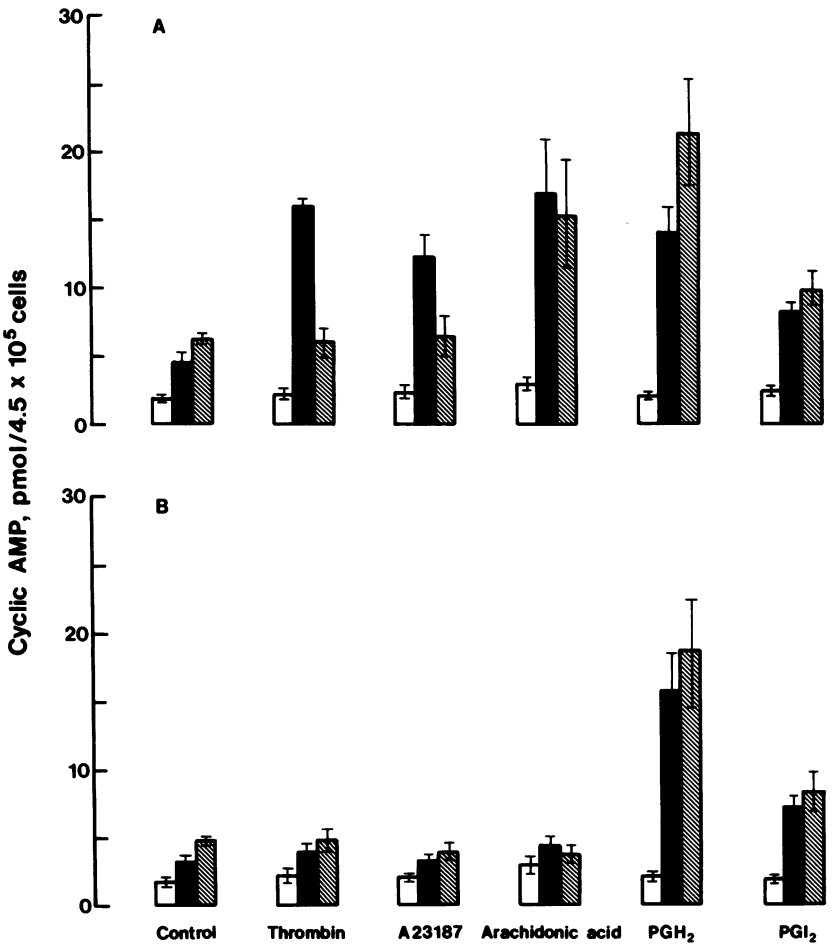

FIG. 1. Effect of PGI_2 production on the concentration of cAMP in endothelial cell monolayers. Endothelial cell monolayers were incubated for 30 min at 37°C without rocking in a 95% air/5% CO_2 atmosphere with culture medium alone (*A*) or culture medium with 100 μM ASA (*B*). Monolayers were rinsed twice with Hanks/Hepes buffer and then preincubated for 10 min at 37°C without rocking with buffer alone (□), with buffer containing 1 mM MIX (■), or with buffer containing 4 mM MIX (▨). Hanks/Hepes buffer alone (control) or buffer with thrombin (0.5 unit /ml), A23187 (10 μM), arachidonic acid (20 μM), PGH_2 (4 μM), or PGI_2 (0.4 μM) was added to the preincubation medium, and incubations were continued for 5 min at 37°C with rocking. Values are means ± SEM for three experiments.

greater increases in cAMP accumulation than that observed with 400 nM PGI_2 (Fig. 1*A*). On the average, 400 nM PGI_2 increased the intracellular concentration of cAMP in endothelial monolayers preincubated with 1 or 4 mM MIX by about 2-fold in nine experiments. Fig. 2*A* shows that increases in cAMP accumulation were not substantially greater upon stimulation of monolayers with PGI_2 concentrations as high as 10 μM. Maximal increases in cAMP levels were attained by 2 min of exposure to PGI_2 and remained elevated for about 10 min (Fig. 2*B*). These results suggest that, in addition to PGI_2, other PGs released from the vascular endothelium may stimulate cAMP accumulation.

DISCUSSION

By studying the effects of agents that act at discrete steps in the pathway of PGI_2 production, we have obtained evidence that, together with the current literature, has enabled us to propose a model for the regulation of the synthesis and release of PGI_2 from the vascular endothelium (Fig. 3). Lollar and Owen (27) recently reported that an active site-dependent receptor or binding site appears to be involved in thrombin-induced release of arachidonate and metabolites of arachidonate from human umbilical vein endothelial cell monolayers. Although the initial step of thrombin stimulation of PGI_2 production is not known, thrombin has been reported to stimulate the influx of $^{45}Ca^{2+}$ into bovine aortic endothelial cells (28). The stimulation of PGI_2 release by A23187 that both we and Weksler *et al.* (5) have observed suggests that the induction of PGI_2 biosynthesis may be mediated by an increase in the concentration of free Ca^{2+} in the cytoplasm. Moreover, the ability of thrombin and A23187 to induce PGI_2 release in the absence of extracellular Ca^{2+} indicates that these agents act by triggering the mobilization of Ca^{2+} from intracellular storage pools or binding sites. The total inhibition of both thrombin and A23187-stimulated release of PGI_2 by TMB-8, which has been shown to be a specific antagonist of intracellular Ca^{2+} (29, 30), lends further support to the supposition that Ca^{2+} is the primary intracellular mediator of the induction of PGI_2 biosynthesis in the vascular endothelium.

In platelets, arachidonic acid can be released from either phosphatidylcholine by the action of phospholipase A_2 (31, 32, 33) or from phosphatidylinositol by the coupled activity of phospholipase C and diglyceride lipase (11, 12). Ca^{2+} has been shown to activate both phospholipase A_2 (9, 10, 31) and phospholipase C (11, 12). The membrane phospholipid source of arachidonic acid in the vascular endothelium has not yet been identified; however, our data suggest that endothelial phospholipase(s) is likewise dependent on Ca^{2+} for activity. Although the mechanism by which Ca^{2+} activates phospholipases is not known, Wong and Cheung (34) have presented preliminary evidence suggesting that the stimulation of human platelet

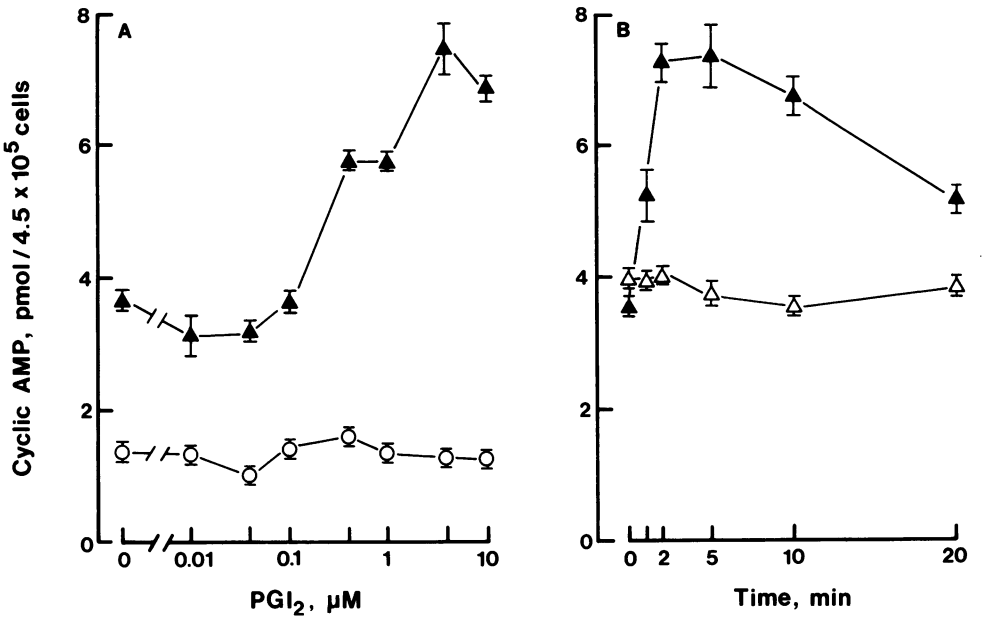

FIG. 2. (*A*) Dose–response relationship for the stimulation of cAMP accumulation by PGI_2. Endothelial cell monolayers were preincubated for 10 min at 37°C without rocking in Hanks/Hepes buffer alone (○) or in buffer containing 1 mM MIX (▲). PGI_2 was added to the preincubation media at the concentrations indicated and incubations were continued for 5 min at 37°C with rocking. (*B*) Time course of PGI_2 stimulation of cAMP accumulation. Endothelial cell monolayers were preincubated for 10 min at 37°C without rocking in Hanks/Hepes buffer with 1 mM MIX. Hanks/Hepes buffer alone (△) or buffer plus 0.4 μM PGI_2 (▲) was added to the preincubation media and incubations were continued for the times indicated. Values are means ± SEM of triplicate determinations from one representative experiment.

phospholipase A_2 by Ca^{2+} may be mediated by calmodulin.

The availability of free cytoplasmic Ca^{2+} in platelets appears to be regulated by the intracellular concentration of cAMP. It has been suggested that cAMP acts primarily by stimulating the active transport of Ca^{2+} out of the platelet cytosol, thereby inhibiting platelet function as well as phospholipase activity (10, 11, 35). We have investigated the role of cAMP in the regulation of PGI_2 biosynthesis by inhibiting cyclic nucleotide phosphodiesterase activity with MIX which produces a dose-dependent increase in the intracellular concentration of cAMP. The inhibition of thrombin and of A23187-induced release of PGI_2 by elevated levels of cAMP that we observed suggests that PGI_2 production by the vascular endothelium may be under bidirectional control by Ca^{2+} and cAMP as defined by Berridge (36). On the basis of our findings, we can only speculate upon the most likely mechanism of action of cAMP in the regulation of PGI_2 release from the vascular endothelium. The inhibition of thrombin- and A23187-induced production of PGI_2 by elevated levels of cAMP suggests that cAMP indirectly inhibits phospholipase A_2 or C activity (or both) in the endothelium by pro-

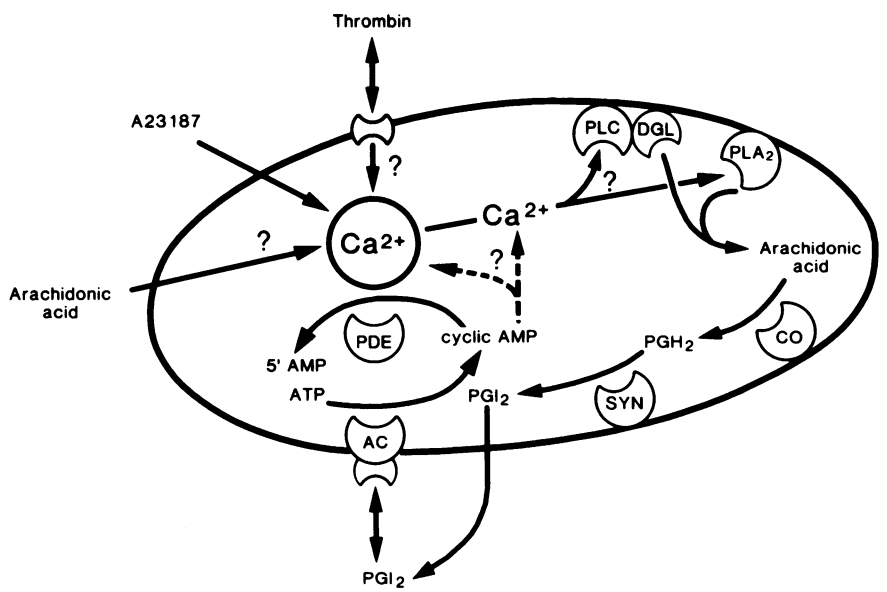

FIG. 3. Schematic representation of some of the mechanisms that may be involved in the regulation of PGI_2 production by the vascular endothelium. PLA_2, phospholipase A_2; PLC, phospholipase C; DGL, diglyceride lipase; CO, fatty acid cyclooxygenase; SYN, PGI_2 synthetase; AC, adenylate cyclase; PDE, cAMP phosphodiesterase. Stimulatory pathways are indicated by solid lines and inhibitory pathways by broken lines.

moting the removal of Ca^{2+} or by blocking the action of Ca^{2+}.

The marked stimulation of cAMP accumulation by PGI_2 detected only in the presence of MIX confirms similar observations recently reported by Schafer *et al.* (37) and by Hopkins and Gorman (18). Other agents that have been reported to increase intracellular cAMP concentrations in cultured vascular endothelial cells only in the presence of a cyclic nucleotide phosphodiesterase inhibitor include PGE_1, PGE_2, isoproterenol (18, 37), norepinephrine, phenylephrine, acetylcholine, and 5-hydroxytryptamine (38). The requirement for a cyclic nucleotide phosphodiesterase inhibitor to permit detection of the stimulation of adenylate cyclase activity by PGI_2 and other agonists suggests that a low steady-state level of cAMP is maintained in normal, intact vascular endothelium by the activity of cAMP phosphodiesterase. Thus, cAMP phosphodiesterase may modulate a negative-feedback mechanism involving the activation of adenylate cyclase by PGI_2 and other agonists of adenylate cyclase. Drugs and other agents that inhibit cAMP phosphodiesterase activity of the vascular endothelium may similarly decrease the capacity of the vessel wall to produce PGI_2 in response to thrombogenic stimuli.

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