Calcium, calmodulin, and the production of prostacyclin by cultured vascular endothelial cells

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The production of prostacyclin (PGI$_2$) by cultured porcine aortic endothelial cells, in response to serum and the calcium ionophore A23187, was inhibited by TMB-8, an antagonist of intracellular calcium mobilization. The calcium-channel blocker methoxyverapamil (D600) inhibited serum-induced PGI$_2$ production in but had little effect on A23187-induced PGI$_2$ production. Calmodulin activity was detected in endothelial-cell lysates and was inhibited by the calmodulin antagonist W7, which also inhibited PGI$_2$ production in response to both agonists. Calcium and calmodulin appear to play an important role in mediating PGI$_2$ production by the vascular endothelium.

The production of prostacyclin (PGI$_2$), the potent vasodilator and inhibitor of platelet aggregation, by the vascular endothelium, is believed to contribute to the non-thrombogenic properties of the vessel wall (Moncada & Vane, 1979). Cultured endothelial cells from a wide variety of vessels have been shown to synthesize PGI$_2$ in response to a number of stimuli, such as the calcium ionophore A23187 and thrombin (Weksl et al., 1978; Czervionke et al., 1979). Recently, normal human serum has been shown to stimulate PGI$_2$ production by cultured endothelial cells, suggesting the existence of naturally occurring stimulators of PGI$_2$ release in the blood (Coughlin et al., 1980; Ritter et al., 1983; Seid et al., 1983).

The mechanism by which the synthesis of PGI$_2$ is promoted by these agents has not been fully determined, although there is evidence to suggest that the activation of both phospholipase A$_2$ (Thomas et al., 1981) and phospholipase C (Hong & Deykin, 1980) may be involved. Both these enzymes require calcium for activation (Knapp et al., 1977; Pickett et al., 1979; Rittenhouse-Simmons, 1979). There is considerable evidence to suggest that the production of thromboxane A$_2$ (TXA$_2$) in platelets is mediated by calcium and that the calcium-dependent regulatory protein calmodulin is involved (Wong & Cheung, 1979; Wong et al., 1980). The possibility thus arises that calcium might also play an important role in the production of prostacyclin by endothelial cells; however, there has only been one report so far investigating this possibility (Brotherton & Hoak, 1982).
We therefore set out to investigate the role of calcium and calmodulin in the synthesis of PGI₂ by porcine aortic endothelial cells in culture, in response to normal human serum and the calcium ionophore A23187. We used three agents known to antagonize either the action of calcium or calmodulin: methoxyverapamil (D600), a calcium-channel blocker (Allen, 1982); 8-(N,N-diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8), an inhibitor of intracellular calcium mobilization (Malagodi & Chiou, 1974; Charo et al., 1976; Gorman et al., 1979); and N-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide (W7), an inhibitor of calmodulin activity (Hidaka et al., 1978).

Materials

A23187 was obtained from Calbiochem (Behring) and TMB-8 from Aldwych Chemical Company (Dorset). Methoxyverapamil was obtained from Knoll Pharmaceuticals. Beef heart phosphodiesterase and pig brain calmodulin were from Boehringer Corporation, and collagenase (type I) was from Sigma Chemical Company. [3H]-6-keto PGF₁α and [3H]-cyclic AMP were obtained from Amersham International. Multiwell dishes were obtained from Falcon-Becton Dickinson and all other tissue-culture plastics from Sterilin. Tissue-culture reagents were from Gibco-Europe. Authentic 6-keto PGF₁α was a gift from Dr. J. Pyke, Upjohn Company Ltd. (Kalamazoo, Michigan, U.S.A.). W7 was a gift from Dr. M. G. M. Blackburn, Sheffield University, and antiserum to 6-keto PGF₁α was a gift from Professor R. G. G. Russell, Sheffield University.

Methods

Endothelial cells were isolated from pig aortas by collagenase digestion and maintained in monolayer culture as previously described (Seid et al., 1983).

For measurement of calmodulin activity the endothelial cells were grown to confluence in 25-cm² tissue-culture flasks. The medium was removed and the cells rinsed in phosphate-buffered saline. A cell extract was then prepared as previously described (Ollis et al., 1983). Calmodulin activity was measured by the ability of the cell extract to activate a calmodulin-dependent phosphodiesterase using the method previously described (MacNeil et al., 1982; Ollis et al., 1983). The amount of calmodulin activity present in the endothelial-cell lysates was determined by comparison with a calmodulin standard and expressed as μg of calmodulin per mg of cell protein.

For incubation with test materials, cells were passaged into multi-well dishes and grown to confluence. The growth medium was removed and the cells washed three times in 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes)-buffered Dulbecco's modified minimal essential medium (DMEM). The cells were then incubated with or without calcium antagonists in Hepes-buffered DMEM containing 0.1% BSA in a volume of 250 μl for 5 min at room temperature. This pre-incubation medium was then removed and replaced with fresh medium containing either the PGI₂-stimulating agent: serum (20%) or A23187 (5 μM) alone or in combination with the calcium or
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calmodulin antagonist in a final volume of 500 μl. This incubation was performed at 37°C under constant rotation on a Luckham plate shaker for 1 h. The supernatant medium was then removed and the amount of PGI₂ generated was measured by radioimmunoassay (RIA) for the stable metabolite 6-keto PGF₁α using the method previously described (Seid et al., 1983). The results are expressed as pmol of 6-keto PGF₁α per mg of cell protein. Each experiment was performed 4 times and the data from one representative experiment are shown.

D600 and A23187 were stored at -20°C as stock solutions of 10 mM in dimethyl sulphoxide (DMSO). W7 was stored at -20°C as a stock solution of 10 mM in water and TMB-8 was made up freshly before use in Hepes-buffered DMEM.

Results

The effects of the calcium ionophore A23187 on the production of 6-keto PGF₁α by endothelial cells are shown in Fig. 1. There was a dose-related increase in 6-keto PGF₁α production in response to A23187 over a concentration range of 0.1-10 μM. The highest concentration used was 10 μM; however in subsequent experiments to investigate the effect of calcium and calmodulin antagonists, 5 μM A23187 was used, which was found to give 13.9 ± 2.7 fold stimulation over basal (mean ± S.E.M., n = 3).

![Graph showing the effect of A23187 on 6-keto PGF₁α production](image_url)

Fig. 1. The effect of the calcium inophore A23187 on 6-keto PGF₁α production by endothelial cells. Each value represents the mean ± S.E.M. of triplicate wells of cells in a representative experiment.
The effects of the calcium-channel blocker D600 on PGI₂ production in response to serum and A23187 are shown in Fig. 2. Serum-stimulated PGI₂ production was inhibited by D600 in a dose-related manner and at 100 μM the mean inhibition was $51 \pm 2.8\%$ (mean ± S.E.M., n = 4). In contrast the effect of D600 on A23187-stimulated PGI₂ production was less marked. D600 had little or no effect until high concentrations were reached; in 3 out of 4 experiments there was some inhibition at 100 μM ($31.2 \pm 7.8\%$, mean ± S.E.M., n = 3).

TMB-8 was found to have a similar effect on both serum- and A23187-stimulated PGI₂ production, as shown in Fig. 3. Stimulation of

![Graph A](image)

Fig. 2. The effect of D600 on the production of 6-keto PGF₁₀α by cultured endothelial cells in the presence of (A) serum (○) and (B) A23187 (▲) and on basal production (○). Each point represents the mean ± S.E.M. of triplicate wells of cells. A single representative experiment for each of A and B is shown.
Fig. 3. The effect of TMB-8 on the production of 6-keto PGF1α by cultured endothelial cells in the presence of (A) serum (●) and (B) A23187 (▲) and on basal production (○). Each point represents the mean ± S.E.M. of triplicate wells of cells. A single representative experiment for each of A and B is shown.

PGI2 production by both agonists was inhibited by TMB-8 in a dose-related manner. The amount of inhibition achieved by TMB-8 at the highest concentration used, 500 μM, was also similar for both agonists; 85.6 ± 3.3% for serum and 78.0 ± 7.9% for A23187 (mean ± S.E.M., n = 4).

Heat-stable extracts of cultured endothelial cells were found to contain calmodulin activity as measured by activation of a calmodulin-dependent phosphodiesterase, parallel with authentic purified calmodulin (Fig. 4a). The amount of calmodulin in endothelial-cell extracts taken as a mean of 6 separate preparations was 2.4 ± 0.69 μg of calmodulin per mg of cell protein (mean ± S.E.M., n = 6). Activation of phosphodiesterase by endothelial-cell extracts was inhibited by the calmodulin antagonist W7 in a dose-related manner, and with an IC50 of 19.7 ± 1.7 μM (mean ± S.E.M., n = 3) (Fig. 4b). Similarly the production of PGI2 in response to serum and A23187 was
Fig. 4. (A) Activation of calmodulin-dependent phosphodiesterase by calmodulin standard (●) and by endothelial-cell extract (■) in an incubation volume of 100 μl.

(B) Inhibition by W7 of the activation of calmodulin-dependent phosphodiesterase by endothelial-cell extract, using a concentration of cell extract producing activity equivalent to 10-20 μg of calmodulin standard.

The dotted line represents calmodulin-independent phosphodiesterase activity. A representative experiment is shown and the same endothelial-cell extract is used for A and B.

inhibited by W7 in a dose-related manner (Fig. 5). At the highest concentration of W7 used, 100 μM, the amount of inhibition in both cases was over 50%, being 53.2 ± 7.8% with respect to serum and 68.0 ± 10% with respect to A23187 (mean ± S.E.M., n = 4).

Each of the three drugs tested had little or no effect on basal levels of PGI₂ production.
Discussion

Calcium acts as a mediator of a wide variety of cellular responses, many of which are now believed to be dependent upon the calcium-dependent regulatory protein calmodulin. The role of calcium in prostaglandin synthesis has been mainly studied in platelets (Pickett et al., 1977; Knapp et al., 1977; Gorman et al., 1979; Wong & Cheung, 1979). However, there has been one recent report to suggest that calcium may also play a role in the mediation of prostacyclin synthesis by the vascular endothelium (Brotherton & Hoak, 1982). The current study investigates the role of calcium in prostacyclin release from cultured aortic endothelial cells and explores the possibility that the effect of calcium in mediating prostacyclin release might, itself, be mediated by calmodulin.

Platelet secretion can occur in the absence of extracellular calcium (Feinman & Detwiler, 1974), is unaffected by the calcium-channel blocker D600, but is prevented by the inhibitor of intracellular calcium mobilization TMB-8 (Charo et al., 1976). Similarly, platelet production of TXA₂ has been shown to be independent of extracellular calcium but, again, can be inhibited by TMB-8 (Wong et al., 1980; Shore & Murphy, 1981). Likewise, the report of the calcium dependence of prostacyclin release from human umbilical-vein endothelial cells indicates that it is primarily intracellular calcium that is of importance (Brotherton & Hoak, 1982).
We have shown that the calcium-channel blocker D600 produced up to 50% inhibition of serum-stimulated prostacyclin production by endothelial cells but had little effect on prostacyclin release in response to A23187. This result might be explained by the fact that A23187 acts not only to transport calcium across cell membranes but has an intracellular site of action by mobilizing calcium from internal stores (Feinman & Detwiler, 1974; Babcock, 1976; Pickett, 1977; Thomas, 1981). It is unlikely that the calcium-channel blocker D600 would have any effect on this intracellular calcium mobilization. The apparent difference in the effect of D600 on serum- and A23187-stimulated PGI₂ production might indicate that there are different mechanisms acting. The serum factor, which is as yet undefined, may be acting via a cell-surface receptor, whereas A23187 appears to be entering the cell and exerting a direct effect on intracellular calcium stores.

The effect of TMB-8 obtained in the present study, in which there was a marked inhibition of prostacyclin production induced by either serum or A23187, would seem to confirm that it is mainly mobilization of intracellular calcium that is important in the regulation of prostacyclin production by endothelial cells. Nevertheless, the fact that D600 can inhibit, at least partially, serum-stimulated prostacyclin release indicates some requirement for extracellular calcium in the action of serum.

The activation of phospholipase A₂ in platelets has been found to be mediated by calmodulin since the addition of calmodulin to platelet membranes or intact platelets stimulated phospholipase A₂ activity and increased TXA₂ activity (Wong & Cheung, 1979; Wong et al., 1980). We have shown that calmodulin activity is present in endothelial-cell lysates and that this activity, like that of authentic calmodulin, can be inhibited by the calmodulin antagonist W7. We also found that the calmodulin antagonist W7 was equally potent in inhibiting prostacyclin release from endothelial cells stimulated by either serum or A23187.

In conclusion, therefore, prostacyclin production stimulated by serum and by the calcium inophore A23187 appears to be mediated by calcium; furthermore, our studies indicate that mobilization of intracellular calcium is more important than calcium flux across the endothelial cell membranes. In addition, we have further shown that calmodulin is present in endothelial-cell lysates and that the calmodulin antagonist W7 can inhibit prostacyclin release stimulated by both agonists used in the study. These results therefore suggest that calmodulin may mediate the important role of calcium in the regulation of PGI₂ production by vascular endothelial cells.

At this stage we can only speculate on the site of action of calcium and calmodulin in PGI₂ synthesis. Based on previous studies in platelets it is most likely to be at the stage of arachidonic acid release by the action of one or more phospholipases.

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References