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CYCLIC AMP INHIBITS PLATELET ACTIVATION INDEPENDENTLY OF ITS EFFECT ON CYTOSOLIC FREE CALCIUM

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SUMMARY: Stimulation of platelets with the ionophore A23187, thrombin, ADP or PAF-acether resulted in a rapid increase of cytosolic free Ca^{2+}, as measured with Quin-2, and in aggregation, 5HT secretion and - in the case of the first two agonists - thromboxane generation. PGI_2 and dibutyryl cyclic AMP inhibited all these responses, except in the case of A23187, in response to which the increase in Ca^{2+} was unaffected, although the other responses were inhibited. The inhibition of aggregation and secretion in response to the combination of thrombin and A23187 was indistinguishable from that in response to thrombin alone. It thus appears that cAMP inhibits these responses independently of its effect in lowering cytosolic free Ca^{2+}.

Platelet aggregation and secretion in response to thrombin and various other agonists involve the activation of parallel stimulus-response coupling mechanisms leading eventually to the Ca^{2+}-dependent phosphorylation of a 47 kDa protein (P47) by protein kinase C (1), and the calmodulin-Ca^{2+} - dependent phosphorylation of myosin light chains by myosin light chain kinase (2). The second messengers involved in these transduction mechanisms include 1,2-diacylglycerol (DG) and inositol 1,4,5-trisphosphate($InsP_3$), both of which are the products of hydrolysis of phosphatidylinositol 4,5-bisphosphate, derived from inositol lipids in the plasma membrane, by the action of phospholipase C. DG activates protein kinase C, greatly enhancing its Ca^{2+} sensitivity (4,5), while $InsP_3$ releases Ca^{2+} from intracellular

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Abbreviations: DG, 1,2-diacylglycerol; $InsP_3$, inositol 1,4,5-trisphosphate; cAMP, adenosine 3',5'-cyclic monophosphate; dBcAMP, dibutyryl cAMP; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; PGI_2, prostaglandin I_2, prostacyclin; PAF-acether, 1-O-hexadecyl-2-acetyl-sn-glyceryl-3-phosphoryl-choline; TPA, 12-O-tetradecanoylphorbol-13-acetate; PI, phosphatidylinositol.

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storage sites into the cytosol (6), making it available for protein phosphorylation and other reactions. Raised levels of cyclic AMP, such as may be produced by the inhibitory prostaglandins I_2, D_2 and E_1 (7-9), inhibits these activation mechanisms, and this inhibitory action has usually been attributed to its effect in lowering cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) (7,10,11). There is evidence, however, that cAMP also inhibits the breakdown of inositol lipids (12,13) and the activity of myosin light chain kinase (14). We show here that while the effects of PGI_2 and dBcAMP in inhibiting platelet activation by thrombin, ADP or PAF-acether and in reducing $[Ca^{2+}]_i$ occur in parallel, the inhibition of aggregation, secretion and thromboxane production in response to thrombin is independent of the effect on $[Ca^{2+}]_i$.

METHODS: Fresh blood from healthy volunteers, who denied taking aspirin for the previous two weeks, was drawn into plastic tubes containing 1/6 vol. of acid citrate dextrose (ACD) and centrifuged at room temperature for 15 minutes at 120 g. Platelet-rich plasma was incubated at 37 °C for 30' with 15 μM Quin-2 acetoxymethyl ester (Lancaster Synthesis Ltd) and 1 μCi/ml [^{14}C] 5HT (Amersham PLC). Platelets were pelleted at 1000 g for 15 minutes and resuspended in calcium-free Hepes buffer (pH 7.4) at approximately $1\text{-}1.2.10^8$ ml^{-1}. Before the experimental procedure, $CaCl_2$ or EGTA was added to give either a final extracellular concentration of 1 mM Ca^{2+} or a virtually Ca^{2+}-free medium.

Agonists and inhibitors were then added as 0.01 vol of the platelet suspension to the following final concentrations: Human thrombin (BPL, Elstree), 0.1 u/ml; ADP, 10 μM; PAF-acether, 20 nM; A23187, 200 nM; TPA, 20-200 nM; PGI_2, 1.0 pM-100 nM; dBcAMP, 0.1 - 2.0 mM.

Shape change and aggregation were measured in a Payton dual channel aggregometer at 37 °C, stirring at 900 rev/min. Aggregation was recorded as the percentage change in light transmission 2 minutes after the addition of agonist.

Release of 5HT and thromboxane in the aggregometer cuvette were measured 2 min after the addition of the aggregating agent. Imipramine (2 μM) was added just before the aggregating agent to prevent re-uptake of secreted 5HT. 100 μl aliquots were transferred to microtubes containing 0.4 ml of 1.25% formaldehyde in isotonic saline and immediately centrifuged at 1400 g for one minute in a Beckman Microfuge; the 5HT in the supernatant was determined by scintillation counting, as a percentage of the total platelet 5HT. 200 μl aliquots were taken into ice-cold microtubes containing 0.4% EDTA and 5 μg/ml indomethacin in Hepes buffer (pH 7.4), immediately centrifuged as above and TXB2 measured in the supernatant by radioimmunological assay.

Cytoplasmic free Ca^{2+} was calculated from Quin 2 fluorescence at 37 °C, determined simultaneously with aggregation but in an unstirred system, using a Perkin Elmer LS-5 spectrofluorimeter with excitation at 339 nm and emission at 500 nm, as described by Hallam et al (15).

RESULTS AND DISCUSSION: In the presence of 1 mM extracellular Ca^{2+}, ADP and PAF-acether each stimulated a rapid rise of $[Ca^{2+}]_i$ from the resting level of 80-100 nM to 0.8 - 0.9 μM, while thrombin (0.1 u/ml) and A23187 (200 nM) raised $[Ca^{2+}]_i$ to somewhat higher levels (0.9 - 1.3 μM and 1.2 - 1.5 μM respectively). All these agonists also stimulated aggregation and 5HT secretion, though ADP and PAF-acether were less potent than thrombin or A23187. Thromboxane release was also stimulated by thrombin and A23187, but was not measured in response to the weaker agonists. These responses were inhibited in a dose-dependent manner by pre-incubation for 2 minutes with increasing concentrations of either PGI_2 or dBcAMP (Fig 1). Aggregation and secretion in response to PAF-acether were completely inhibited by 0.1 nM PGI_2, at which concentration a moderate rise of $[Ca^{2+}]_i$, to about 400 nM, was still attained (Fig 1D), but all responses to the other agonists were inhibited more closely in parallel, so that aggregation, secretion and thromboxane production were completely abolished only by those

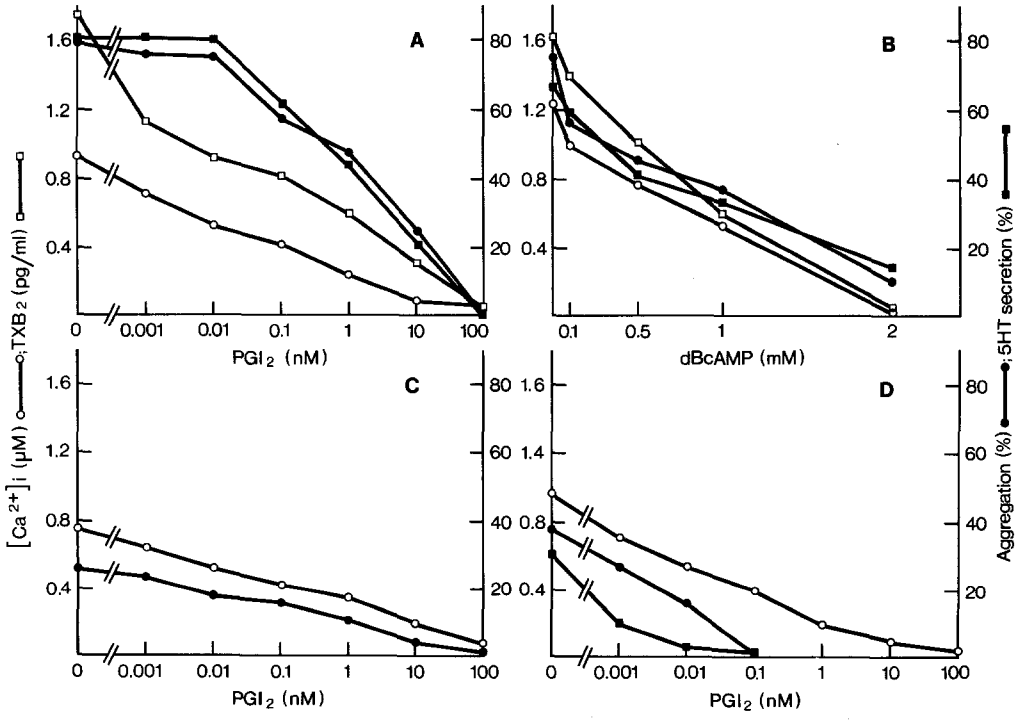

Fig 1 Effect of preincubation for 2 min with PGI_2 (A,C,D) or dBcAMP (B) on $[Ca^{2+}]_i$ (○—○), aggregation (●—●), 5HT secretion (■—■) and TXB_2 production (□—□) in response to thrombin (0.1 u/ml; A,B), 10 uM ADP (C) and 20 nM PAF-acether (D).

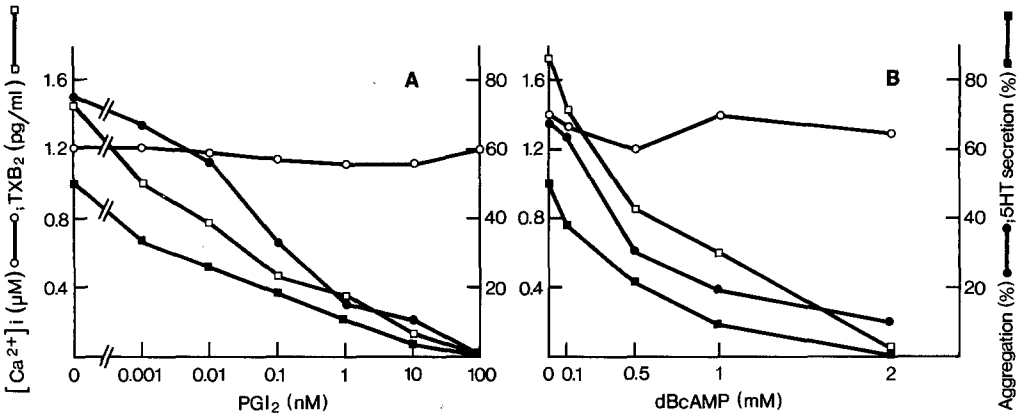

Fig 2 Effect of preincubation for 2 min with PGI_2 (A) or dBcAMP (B) on $[Ca^{2+}]_i$, aggregation, 5HT secretion and TXB_2 production in response to 200 nM A23187. Symbols as in Fig 1.

concentrations of inhibitors which also prevented increase of $[Ca^{2+}]_i$ above resting levels. With the ionophore A23187, the dose-dependent effect of both inhibitors on aggregation, 5HT secretion and thromboxane production was very similar to that with thrombin, but there was no effect on the Ca^{2+} response (Fig 2). TPA (20 - 200 nM) induced aggregation and secretion without any effect on $[Ca^{2+}]_i$, as previously reported (16), and these responses were not significantly inhibited by PGI_2 or dBcAMP (results not shown). In the presence of EGTA, $[Ca^{2+}]_i$ rose to 300-400 nM in response to thrombin and to about 200 nM in response to ADP and PAF, presumably as a result of mobilization from intracellular stores (17).

When 5-100 nM PGI_2 or 2 mM dBcAMP was added 60 seconds after thrombin, ADP or PAF-acether, when a maximum Ca^{2+} response had already been achieved, there was a rapid dose-dependent fall of $[Ca^{2+}]_i$ and arrest or reversal of aggregation. After A23187 (70-200 nM), there was an equally rapid reversal of aggregation but no change in $[Ca^{2+}]_i$.

It thus appears that PGI_2 and dBcAMP inhibit the activation of platelets by A23187 without affecting the concentration of cytosolic free Ca^{2+}. Since the dose-response curves for inhibition of aggregation, 5HT secretion and thromboxane generation were very similar for thrombin and A23187, the question arose

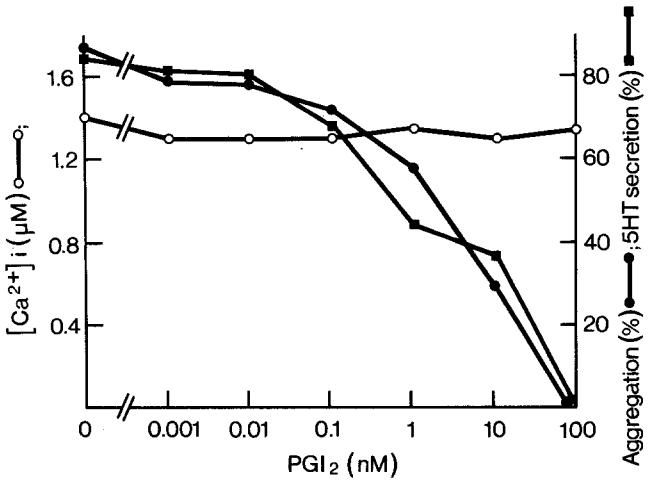

Fig 3 Effect of preincubation for 2 min with PGI_2 on $[Ca^{2+}]_i$, aggregation and 5HT secretion in response to thrombin (0.1 u/ml) + 200 nM A23187. Symbols as in Fig 1.

whether the inhibition of these responses to thrombin were also independent of the simultaneous reduction in $[Ca^{2+}]_i$. Platelets were therefore preincubated with PGI_2 or dBcAMP for 2 minutes before stimulation with the combination of A23187 (200 nM) and thrombin (0.1 u/ml). There was no effect on $[Ca^{2+}]_i$ at any inhibitor concentration, but the inhibitory effects of PGI_2 (Fig 3) and dBcAMP (not shown) on aggregation and 5HT secretion were indistinguishable from those in response to this concentration of thrombin alone. Similar results were obtained whether the agonists were added simultaneously or sequentially in either order.

These findings thus indicate that the inhibitory effect of cAMP on platelet aggregation, secretion and thromboxane generation is not solely due to its action in lowering $[Ca^{2+}]_i$. Indeed from comparison of Figs 1A and 3 it can be argued that the inhibition of thrombin-induced aggregation and secretion is entirely independent of the cytosolic free Ca^{2+} concentration, while Fig 1D shows that the effects of PAF-acether were inhibited at PGI_2 concentrations which still allowed a moderate increase in $[Ca^{2+}]_i$, equivalent to that derived from release of intracellular stores alone. It should be noted, however, that even the highest concentrations of PGI_2 or dBcAMP used did not reduce $[Ca^{2+}]_i$

below its normal resting level of 80-100 nM, though they did inhibit the release from intracellular stores as well as the influx of Ca^{2+}, as previously reported (18).

Since TPA-induced secretion and aggregation were not significantly inhibited by raised cAMP concentrations, it may be inferred that the Ca^{2+}-independent inhibitory effects we have observed do not involve the phosphorylation of P47 by protein kinase C, but either an earlier step in the activation process or a parallel mechanism. The generation of DG from PI (10,13) and the regulation of myosin light chain kinase by cAMP-dependent protein kinase (14) are candidate sites for such inhibitory activity. The latter effect of cAMP is not Ca^{2+}-dependent (14) but there is as yet no clear evidence whether the observed inhibition of DG generation by cAMP depends on a reduction in the free Ca^{2+} available to the phospholipase C, or indeed whether this enzyme is the target for inhibition. It has been shown, however, that DG formation in response to thrombin occurs as rapidly in the presence of 340 nM $\left[Ca^{2+}\right]_i$, derived from intracellular stores, as at the higher concentrations achieved from influx when 1 mM Ca^{2+} is present in the medium (19). Although these findings do not exclude the dependence of DG formation on low $\left[Ca^{2+}\right]_i$, they suggest that inhibition of the pathway by cAMP might well be Ca^{2+}-independent. It would seem appropriate to examine the effect of cAMP on DG formation in the presence of A23187.

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