Regulation of Platelet Arachidonic Acid Oxygenation by Cyclic AMP

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Intracellular cyclic adenosine monophosphate (AMP) levels regulate the generation of thromboxane by platelets by inhibiting the hydrolysis of arachidonic acid from membrane phospholipids. However, there is conflicting evidence regarding the role of cyclic AMP in the control of the subsequent oxygenation of arachidonic acid by cyclooxygenase. We studied the regulation of cyclooxygenase activity by agents that elevate platelet cyclic AMP (dibutyryl cyclic AMP and prostaglandins), measuring arachidonate-induced aggregation, O2 consumption, and malonaldehyde formation. In platelet-rich plasma, arachidonic acid oxygenation was partially inhibited by 0.1 mM and completely inhibited by 0.5 mM dibutyryl cyclic AMP. This inhibitory effect of cyclic AMP was absent in gel-filtered platelets suspended in buffer containing 0.5% albumin, and was progressively restored as plasma was added in increasing concentrations. Increasing the albumin concentration in platelet buffer suspensions likewise increased the ability of cyclic AMP to block the arachidonate-induced O2 burst and MDA production. We conclude that (1) the presence of plasma proteins is important in investigating platelet arachidonic acid metabolism, and (2) platelet cyclooxygenase activity is inhibited by cyclic AMP when examined in a plasma milieu or at least in the presence of physiologic albumin concentrations.

MATERIALS AND METHODS

Platelet Aggregation

Venous blood anticoagulated with 13 mM sodium citrate was centrifuged at 160 g for 10 min to obtain platelet-rich plasma (PRP). Aggregation was monitored using a standard nephelometric method, in which 0.4 ml of PRP or platelet suspension in buffer was stirred at 1000 rpm at 37°C in a Payton dual-channel aggregometer (Payton Associates, Inc., Buffalo, N.Y.). In all experiments, arachidonic acid was used as the aggregating stimulus. Blood was obtained from volunteers who had not ingested aspirin for at least 10 days prior to donation. Platelet counts were determined in a Model F Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.).

Gel Filtration

Platelets were separated from plasma by gel filtration by applying PRP to a 150 mm x 25 mm column of Sepharose 2B. The platelets were then eluted with a modified Tyrode's buffer containing 130 mM NaCl, 9 mM sodium bicarbonate, 6 mM dextrose, 10 mM sodium citrate, 10 mM Trizma base, 0.8 mM KH2PO4, 3 mM KCI, 0.9 mM MgCl2, 2 mM CaCl2 (unless otherwise stated), 0.5% albumin (unless otherwise stated), and 0.2% fibrinogen, pH 7.3. Fatty acids were removed from albumin by charcoal treatment as.

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Modulation of platelet intracellular cyclic AMP by thromboxane and prostaglandins and the sites of action of cyclic AMP in its inhibition of TXA2 generation. Solid lines represent stimulation; dashed lines represent inhibition.

Previously described, and final albumin concentrations were adjusted to 0.5% with protein determinations by the method of Lowry et al.

Oxygen Consumption

Polarographic measurements were made with a YSI Model 53 oxygen monitor equipped with a Model 5331 Clark-type oxygen sensor (Yellow Springs Instrument Co., Yellow Springs, Ohio). The electrode was fitted to a Gilson water-jacketed cell (OX-15253, Gilson Medical Electronics, Middleton, Wisc.) connected to a Lauda circulator (K-2/RD) maintained at 37°C. Concurrent tracings of arachidonic acid stimulated oxygen consumption and platelet aggregation could be made by connecting the outputs of both instruments to one dual-channel recorder. At 37°C, a 0.13 M NaCl solution was assumed to contain 400 natom O2/ml at equilibrium with room air. Full-scale deflection of the instrument was adjusted to correspond to this O2 content.

Malonaldehyde (MDA) Formation

Measurements of MDA, a direct breakdown product of the endoperoxides, were made using a fluorimetric assay. Protein was precipitated from platelet suspensions by the addition of an equal volume of 20% trichloroacetic acid (TCA) in 0.6 N HCl, followed by centrifugation at 8700 g for 15 min in a Beckman microfuge (Beckman, Palo Alto, Calif.). The protein-free supernatant was mixed with an equal volume of 0.53% thiobarbituric acid (TBA), heated at 70°C for 30 min, and cooled to room temperature. Fluorimetric measurements were made in a Perkin-Elmer MPF-44B fluorescence spectrophotometer (Perkin-Elmer, Norwalk, Conn.), with excitation and emission wavelengths of 510 nm and 553 nm, respectively. MDA standards were prepared by dilutions of malonaldehyde tetraethyl acetal in absolute ethanol with equal volumes of 0.2 N HCl, which were allowed to hydrolyze overnight.

Chemicals

Reagents were obtained from the following sources: bovine albumin (fraction V), TBA, and dibutyryl cyclic AMP from Sigma Chemical Company, St. Louis, Mo.; sodium arachidonate from Nu Chek-Prep, Elysian, Minn.; MDA tetraethyl acetal from ICN K&K Laboratories, Plainview, N.Y.; Sepharose 2B from Pharmacia, Uppsala, Sweden. PGE2 and PGI2 were kindly provided by Dr. John Pike, Upjohn, Kalamazoo, Mich.

RESULTS

Figure 2 shows concurrent tracings of platelet aggregation, the O2 burst, and simultaneous measurements of MDA production, following the addition of 0.5 mM arachidonate to platelet-rich plasma (PRP). Preincubation of PRP for 5 min with 0.1 mM dibutyryl cyclic AMP (dBcAMP), a lipophylic cyclic AMP

![Fig. 2. Concurrent tracings of oxygen consumption (upper panel) and aggregation (lower panel) of platelets in plasma. Simultaneous determinations of MDA production follow oxygen consumption tracings in upper panel. At the point of the arrow, 0.5 mM sodium arachidonate was added, following preincubation of PRP for 5 min at 37°C with 0.1 mEq/L and 0.5 mM dBcAMP. The initial slopes of the oxygen consumption tracings prior to the addition of arachidonate represent basal O2 consumption of stirred platelets. The figure is representative of 4 such experiments.](image-url)
analog, decreased both aggregation and O₂ consumption and reduced MDA production from 6.5 to 4.0 nmol/10⁹ platelets. Preincubation of PRP with 0.5 mM dBcAMP completely abolished arachidonate-induced aggregation and the associated O₂ burst and inhibited MDA generation by 94%. Comparable inhibition of aggregation, O₂ consumption, and MDA formation was also observed when platelets were preincubated with 1 μM PGI₁ or PGE₁, two prostaglandins that elevate endogenous platelet cyclic AMP. Preincubation of PRP with 0.5 mM dBcAMP could not completely inhibit the O₂ burst induced by a higher concentration (2.5 mM) of arachidonate (data not shown).

In order to better define the factors involved in the inhibition of platelet arachidonic acid oxygenation by cyclic AMP, platelets were separated from plasma proteins by gel filtration into buffer containing 0.5% albumin. As shown in Fig. 3, dBcAMP and PGE₂ were unable to inhibit the arachidonate-induced O₂ burst or MDA production following gel filtration into this buffer. However, the O₂ burst induced by a lower concentration of arachidonate (0.1 mM) could be inhibited by preincubation with dBcAMP in the same buffer (not shown).

To elucidate how plasma might permit cyclic AMP to inhibit the oxygenation of arachidonic acid, various concentrations of plasma were added to the gel-filtered platelets. As shown in Fig. 4, with increasing amounts of added plasma, there is a progressive diminution in both the O₂ burst and MDA formation. This probably reflects the plasma binding of exogenous arachidonate, resulting in progressively smaller amounts of free arachidonate being available to the platelets for oxygenation. When the gel-filtered platelets with added plasma were preincubated with 3 mM dBcAMP, the O₂ burst and MDA production could now be inhibited.

Albumin, a major constituent of plasma, avidly binds fatty acids and could reduce the amount of free arachidonate available to the platelet for oxygenation. This binding could also facilitate the inhibitory effect of cyclic AMP noted in plasma. Accordingly, the effect of albumin on arachidonic acid oxygenation was examined by comparing the formation of MDA in gel-filtered platelets in the presence of either 0.5% untreated albumin or the same concentration of delipidated albumin. As shown in Fig. 5, less MDA was generated in response to 0.5 mM dBcAMP in the presence of delipidated albumin. Furthermore, when the albumin concentration of the gel-filtered platelets was increased from 0.5% to 3.5%, the ability of dBcAMP to inhibit the O₂ burst and MDA production was restored. The degree of inhibition was comparable to that achieved by the addition of plasma noted in Fig. 4.

Figure 6 illustrates that the ability of dBcAMP to inhibit the arachidonate-induced O₂ burst was fully reestablished in the washed platelets when albumin in
Fig. 5. Effect of albumin on arachidonate-induced MDA production of washed platelets. Platelets were gel-filtered into buffer containing either 0.5% untreated albumin or 0.5% albumin delipidated by charcoal treatment. Platelets were stimulated with 0.5 mM arachidonate, aliquots were withdrawn at the indicated time intervals, and the reactions stopped by addition to equal volumes of 20% TCA in 0.6 N HCl.

Fig. 6. Cyclic AMP inhibition of arachidonate-induced O₂ burst and MDA production of washed platelets in 3% albumin. Platelets were preincubated with or without 3 mM dBcAMP for 5 min at 37°C, followed by the addition of 0.5 mM arachidonate at the point of the arrow. Experiments were performed in the presence of 3 mM calcium (upper panel) or in the absence of calcium (lower panel). The figure is representative of 4 such experiments.

The regulation of prostaglandin biosynthesis by cyclic AMP is of broad biologic significance. It has been demonstrated in a number of tissues such as the brain, thyroid, and ovaries, as well as in fat cells, Graafian follicles, and neuroblastoma, glioma, and fibroblast cell lines. It is of interest to note that in these systems, unlike in the platelet, cyclic AMP generally stimulates rather than inhibits prostaglandin production. The intracellular level of cyclic AMP in the platelet, which is critical in modulating its activation, is largely determined by a balance of platelet-derived (e.g., thromboxane A₂, PGD₂) and external (e.g., PGI₂) metabolites of arachidonic acid. Increased intracellular cyclic AMP has been shown to inhibit the formation of platelet prostaglandins and thromboxane, but as in other cells, the sites where cyclic AMP exerts its control have not been fully defined.

Cyclic AMP has been shown to block the release of arachidonic acid from membrane phospholipids, but its effect on the subsequent oxygenation of arachidonic
acid by cyclooxygenase has been a matter of controversy. Some previous reports have supported our findings, while others have been unable to demonstrate an inhibitory role of cyclic AMP in arachidonic acid oxygenation. The reason for these discrepancies is difficult to explain. However, all of the previous studies evaluated the effect of cyclic AMP on cyclooxygenase by measuring thromboxane B2, a stable derivative of thromboxane A2. The choice of thromboxane B2 as an indicator of thromboxane A2 synthesis when the incubation is performed in plasma is complicated by the covalent binding of thromboxane A2 and endoperoxide by serum albumin. Furthermore, such steady-state determinations of an end-product of arachidonic acid transformation do not take into account the possible effect of cyclic AMP on intermediate processes and reaction rates. Lindgren et al. have recently shown that cyclic AMP may alter the affinity of cyclooxygenase for arachidonic acid: inhibition of thromboxane B2 formation by cyclic AMP was found at low, but not at high, substrate (arachidonic acid) concentrations, suggesting a $K_m$ change of the cyclooxygenase. Here we have shown, by recording its effect on substrate-induced oxygen consumption, that cyclic AMP can directly inhibit platelet cyclooxygenase activity. These observations were confirmed by measuring MDA, which is released stoichiometrically with 12l-hydroxy 5,8,10-heptadecatenoic acid (HHT) upon fragmentation of the direct endoperoxide products of arachidonic acid oxygenation.

It has been suggested that washed platelet preparations may not be a suitable model for investigating arachidonic acid oxygenation. Our studies emphasize the potential importance of plasma proteins in the control of platelet arachidonate metabolism. When examined in a plasma milieu, or in the presence of physiologic albumin concentrations, platelet cyclooxygenase activity clearly appears to be regulated by intracellular cyclic AMP levels. However, the effect of plasma on permitting cyclic AMP to inhibit platelet cyclooxygenase may not be simply to limit arachidonic acid availability. Normal plasma contains an endogenous protein inhibitor of the prostaglandin synthetase system, which has been thought to act as a regulator of prostaglandin synthesis. It is possible that cyclic AMP facilitates the activity of this endogenous inhibitor and that this cofactor is lost during gel filtration.

Plasma contains only very small amounts of free arachidonic acid, and platelets normally do not utilize exogenous arachidonic acid for prostaglandin and thromboxane synthesis. The physiologic significance of our experimental observations may therefore be questioned. However, not only arachidonate-induced but also thrombin-induced platelet aggregation and thromboxane production have been shown to be dependent on the free fatty acid content of albumin in the surrounding medium. This suggests that endogenous arachidonic acid utilization may also be modulated by extracellular albumin concentration. Arachidonic acid may bind to plasma albumin after being hydrolyzed from membrane phospholipids.

We conclude that cyclic AMP levels in the platelet regulate both the release of free arachidonic acid from endogenous membrane phospholipid pools and the subsequent oxygenation step that generates the prostaglandin endoperoxides. Since fatty acid cyclooxygenase is a key regulatory enzyme in prostaglandin metabolism, it will be of interest to investigate its control by cyclic nucleotides in other cell systems, particularly vascular endothelial cells.

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