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CONCENTRATIONS OF cAMP PHOSPHODIESTERASE INHIBITORS THAT BLOCK THE ADP-INDUCED SHAPE CHANGE OF RABBIT PLATELETS DO NOT INCREASE PLATELET cAMP.

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When the cyclic AMP (cAMP) concentration in platelets is increased, platelet functions are inhibited. It is generally assumed that inhibitors of cAMP phosphodiesterase inhibit platelet reactions by increasing cAMP, but this is not established. We have studied the effects of cAMP phosphodiesterase inhibitors on ADP-induced platelet shape change, nucleoside diphosphokinase (NDK) activity, and cAMP concentrations. Papaverine (0.08 mM), dipyridamole (0.2 mM), caffeine (10 mM), or theophylline (8 mM) prevented the shape change of washed rabbit platelets induced by 1 μM ADP. We showed previously that PGE_1, which increases cAMP, inhibits platelet NDK activity. Therefore we investigated the effects of cAMP phosphodiesterase inhibitors on platelet NDK activity and cAMP. At concentrations that prevented ADP-induced shape change, papaverine and dipyridamole had no effect on the formation of ^{14}C-ATP from ^{14}C-ADP by washed rabbit platelets. The methylxanthines partially inhibited NDK activity of washed rabbit platelets and of isolated membranes, probably due to the structural similarity between the adenine ring of ADP and these substances. However, it seems unlikely that these substances exert their inhibitory effects through interference with platelet NDK. None of these phosphodiesterase inhibitors increased platelet cAMP above basal levels, measured both by a protein binding assay and by prelabeling the platelet adenine nucleotides by incubation with ^{14}C-adenine. When adenylate cyclase was stimulated with PGE_1 (1.2 μM), the cAMP concentration was increased from 7.8 to 27.2 pmol/10^8 platelets, and in the presence of phosphodiesterase inhibitors, the cAMP concentration was greater than 50 pmol/10^8 platelets at 90 sec. Since the phosphodiesterase inhibitors by themselves had no detectable effect on cAMP at concentrations that inhibit ADP-induced shape change, it seems likely that they act through other mechanisms. Whether or not they all act in the same way remains to be determined.

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ROLES OF CYCLIC NUCLEOTIDES IN THE INHIBITION OF PLATELET FUNCTION BY NITROPRUSSIDE AND BY ASCORBATE.

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The effects of nitroprusside and of ascorbate on the collagen-induced aggregation of washed human platelets and on the associated release of dense granule constituents were correlated with their effects on platelet cyclic GMP and cyclic AMP, which were measured either by radioimmunoassays or prelabelling methods. Nitroprusside at concentrations from 1 to 400 μM increased platelet cyclic GMP from 6 to 100-fold (maximum value approx. 50 pmol/10^9 platelets) and at concentrations above 10 μM also increased cyclic AMP about 2-fold (maximum value approx. 36 pmol/10^9 platelets). Platelet cyclic GMP reached a peak after an incubation period inversely related to the nitroprusside concentration and then declined. Collagen, which increased platelet cyclic GMP about 2-fold, enhanced the effect of nitroprusside on cyclic GMP but not cyclic AMP. Freshly prepared ascorbate (10 mM) increased platelet cyclic GMP about 8-fold. Storage of the ascorbate at pH 7 or simultaneous addition of 5 μM $CuCl_2$ potentiated its action to give 15 to 20-fold increases in cyclic GMP and small increases in cyclic AMP. The results suggested that oxidation of the ascorbate was involved in these effects. In all the above studies, increases in platelet cyclic GMP greater than 6 to 10-fold were associated with measurable increases in cyclic AMP and with inhibitions of collagen-induced platelet responses that roughly correlated with the cyclic nucleotide changes. Addition of 100 μM 2',5'-dideoxyadenosine (an inhibitor of adenylate cyclase) blocked increases in platelet cyclic AMP but did not affect increases in cyclic GMP; this compound also decreased (but did not abolish) the inhibitory effects of nitroprusside and of ascorbate + $CuCl_2$. These findings suggested roles for both cyclic GMP and cyclic AMP in mediating the inhibitions of platelet function by nitroprusside and ascorbate.

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PHOSPHORYLATION OF PLATELET PROTEINS IN RESPONSE TO ACTIVATION BY PHORBOL 12-MYRISTATE 13-ACETATE.

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We have investigated the ^{32}P-labelling of platelet proteins in response to 5uM to 10uM phorbol 12-myristate 13-acetate (PMA) which triggers pseudopod formation and aggregation but an atypical release without granule centralization by a contractile gel. Total platelet protein samples resolved on polyacrylamide-sodium dodecyl sulfate gels showed greater than 3 fold increases sustained over a 15 minute time course in the ^{32}P-labelling of 260,000; 40,000; and 20,000 apparent molecular weight peptides. While similar increases in ^{32}P-labelling are observed with other activators, such as thrombin, arachidonate, and A23187, peak phosphorylation routinely occurs between 30 to 60 seconds followed by an aggregation dependent dephosphorylation to less than 50% of peak levels between 3 to 5 minutes. The cytoskeletal cores which remain after 1% Triton X-100 extraction of platelets activated by typical stimuli contain mostly actin, myosin, and actin-binding protein. The presence in this cytoskeletal core most of the ^{32}P-label associated with the 260,000 and 20,000 molecular weight peptides suggests that these phosphopeptide are the 260,000 molecular weight actin binding protein, and the 20,000 molecular weight myosin light chain subunits. Cytoskeletal cores prepared from PMA activated platelets still contain greater than 90% of the ^{32}P-labelled 260,000 molecular weight peptide but less than 20% of the ^{32}P-labelled 20,000 molecular weight peptide, most of which is found in the solubilized fraction. These results suggest that the lack of granule centralization by a contractile gel is due to a disruption of actin-myosin interaction even though the myosin light chain is phosphorylated. This effect seems to be specific in that actin-binding protein - actin interaction believed to be responsible for pseudopod formation is still present in PMA activated platelets.

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FLUORIDE, AN INTERNALLY ACTING PLATELET ACTIVATOR, CAUSES PHOSPHORYLATION OF PLATELET PHOSPHATIDIC ACID AND 20K AND 47K PROTEINS.

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The effects of fluoride, which is transported into platelets in order to induce secretion, are compared with known effects of thrombin, which acts via external sites. Thus, the changes related to transmission of signal through the platelet membrane will not be common to the two activators, only those changes which are subsequent to the internal triggering of platelet activation. Human platelets were prepared by collection in EDTA and washing in saline-EDTA or by gel filtration of citrated platelet-rich plasma. The two methods gave similar results. Platelets prelabeled in plasma with ^{32}P and them separated were incubated at 37°C with 10 mM fluoride at pH 7.4, and samples removed at intervals. (1) The protein was precipitated with $HClO_4$, then solubilized by sonication with SDS buffer and the protein bands separated by acrylamide slab gel electrophoresis. The 20K and 47K bands showed 100 to 200% increase in label, with maximum at 8 min incubation (50% secretion) and a great increase seen already at 3 min incubation, where little secretion is observed. (2) Samples were extracted with chloroform-methanol, evaporated to dryness under N_2, redissolved in chloroform and applied on thinlayer silica gels on aluminum plates. Two different systems for separating phosphatidic acid (PA) were used. No significant increase in ^{32}P radioactivity was seen in PA the first 3 min. The label at 20 min was 3x that at 8 min. Thus the labeling related to contractile events, a late step in secretion, precedes the labeling of PA, suggesting that the major part of this labeling is not related to the initial phase of platelet activation.

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