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PRELIMINARY COMMUNICATIONS

5'-METHYLTHIOADENOSINE AND 2',5'-DIDEOXYADENOSINE BLOCKADE OF THE INHIBITORY EFFECTS OF ADENOSINE ON ADP-INDUCED PLATELET AGGREGATION BY DIFFERENT MECHANISMS

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The phenomenon of aggregation of human platelets is complex and may be induced by such diverse chemical substances as ADP, collagen, thrombin, epinephrine, arachidonic acid, etc. (1). Factors involved include membrane receptors, the cyclic nucleotides, calcium and the prostaglandin system (1-9). Especially intriguing are recently discovered methylations of membrane phospholipids in mast cells (10) and carboxymethylations of membrane proteins that occur after addition of an aggregating agent, thrombin (11). This laboratory has examined the mechanisms by which adenosine (Ado) and its analogs inhibit ADP-induced aggregation and has described the structure-activity relationships of adenosine-type compounds (12,13). The findings are in general agreement with the concept that Ado analogs that stimulate adenylate cyclase and raise cAMP levels inhibit aggregation whereas those that block adenylate cyclase do not (14-16). Inhibitors of adenylate cyclase such as 2',5'-dideoxyadenosine (DDA) (17) and 9-(tetrahydro-2-furyl)adenine (SQ22536) (18) can reverse the inhibitory effects of Ado and prostaglandin E_1 (PGE_1) (18-20). The question arose whether the naturally occurring Ado derivative, 5'-deoxy-5'-methylthioadenosine (MTA, formed from S-adenosylmethionine during polyamine synthesis) could affect platelet aggregation (21). It was found that MTA compares in activity to DDA as an inhibitor of the effects of adenosine-type compounds on ADP-induced aggregation. Therefore, the behavior of MTA and DDA on the adenylate cyclase activity in partially purified preparations of human platelet membranes was compared. A preliminary report of these findings has been presented (22).

Methods

Fresh human blood was collected in 0.1 vol. of 3.8% sodium citrate. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were obtained by differential centrifugation (13). Platelet aggregation was measured by the turbidometric method of Born (23). The preparation of washed platelet membranes and the assay of adenylate cyclase were performed as described by Steer and Wood (24) except that caffeine was excluded from the assay mixture. Two hundred microliters of reaction mixture contained Tris-HCl buffer (pH 8.0), 30 mM; $MgCl_2$, 25 mM; creatine phosphate, 5 mM; creatine phosphokinase, 8-10 units; and [α-^{32}P]-ATP, 200 μM (1-1.5 x 10^6 cpm/assay). The reaction mixture was equilibrated at 30° for 2-3 min in a shaking water bath and the reaction was started by the addition of 30 μl of the platelet membrane preparation containing 5-10 μg of protein. At zero time, 5 and 10 min, the reactions were stopped by the addition of 0.2 ml of a solution containing ATP (4 mM), cAMP (1 mM) and sodium dodecylsulfate (2%). Fifty microliters containing [^{3}H]cAMP (8,000-10,000 cpm) was added to each assay tube to monitor [^{32}P]cAMP recovery and the mixtures were placed in a boiling water bath for 3 min. After addition of 0.8 ml of water to each tube, cAMP was purified using Dowex (AG 50W-X4) and alumina columns as described by Salomon et al. (25). The final eluates (4 ml) from the alumina columns were mixed with scintillation fluid and counted for [^{32}P] and [^{3}H]. Protein concentrations were determined by the method of Lowry et al. (26).

Results

Figure 1 shows the effects of 2-fluoroadenosine (F-Ado) in the presence of DDA, MTA and p-nitrobenzylthioinosine (NBMPR) on ADP-induced platelet aggregation. F-Ado (8 μM), which inhibits strongly, is non-inhibitory when MTA or the known adenylatecyclase inhibitor, DDA, is added simultaneously with F-Ado. However, if PRP is preincubated for 5 min with F-Ado and then with MTA or DDA, the inhibitory effect of F-Ado is not abolished (data not shown). Preincubation of PRP for 5 min with a potent nucleoside transport inhibitor, NBMPR (18 μM), does not alter the effects of MTA plus F-Ado (Fig. 1), suggesting that the behavior of these analogs involves interactions with the platelet membrane. The inhibitory effects of Ado, 2-chloroadenosine and N^6-phenyladenosine on ADP-induced platelet aggregation are

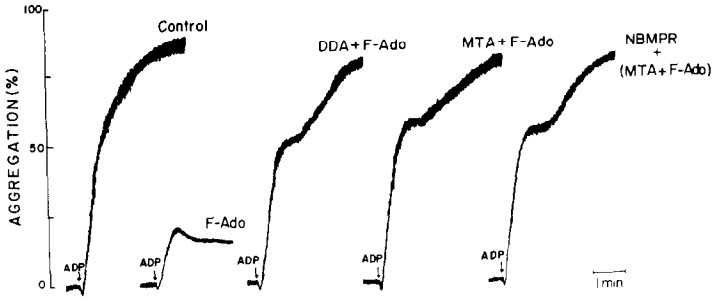

Figure 1. Effect of 2-fluoroadenosine (F-Ado) in the presence of 2',5'-dideoxyadenosine (DDA), 5'-methylthioadenosine (MTA) and p-nitrobenzylthioinosine (NBMPR) on ADP-induced aggregation. Platelet-rich plasma was incubated with F-Ado, 5 min; DDA + F-Ado, 5 min; MTA + F-Ado, 5 min; or NBMPR, 5 min, then MTA + F-Ado, 5 min. After incubation ADP was added to induce aggregation. The final concentrations of various substances were: ADP, 4 μM; F-Ado, 8 μM; DDA, 100 μM; MTA, 100 μM; and NBMPR, 18 μM.

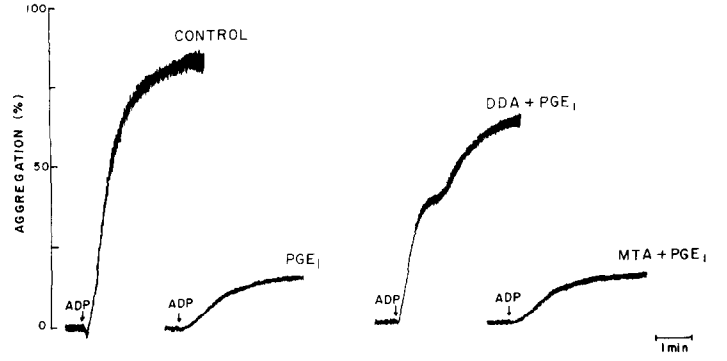

Figure 2. Effect of PGE_1 in the presence of DDA and MTA on ADP-induced aggregation. Platelet-rich plasma was incubated for 5 min with PGE_1; DDA + PGE_1; or MTA + PGE_1 and ADP was added to induce aggregation. The concentration of PGE_1 was 1 μM; those of ADP, DDA and MTA were the same as in the experiments of Fig.1. DDA or MTA was added to PRP 1 min before incubation with PGE_1 for 5 min.

Table 1. Effect of 2',5'-dideoxyadenosine and 5'-methylthioadenosine on platelet adenylate cyclase*

Nucleosides	Concentration (μM)	Per cent change in enzyme activity
2',5'-Dideoxyadenosine	20	- 65
	100	- 80
5'-Methylthioadenosine	20	+ 2
	100	+ 5
	200	- 5

* Enzyme assays were carried out in duplicate or triplicate as described in Methods. Adenylate cyclase activity in the control samples (without addition of any nucleosides) was 114 ± 12 $pmoles \cdot min^{-1} \cdot (mg\ protein)^{-1}$.

also abolished by both MTA and DDA. Dose-response experiments indicate that MTA and DDA are approximately equal in their ability to reverse the effects of F-Ado. Both compounds at 20 μM concentrations reduced the effects of F-Ado (8 μM) by 50 percent.

Figure 2 shows the effects of DDA and MTA on PGE_1 inhibition of ADP-induced platelet aggregation. The inhibitory effect of PGE_1 is greatly reduced by pretreatment with DDA but is not affected by MTA. This suggested that MTA and DDA act through different mechanisms and that MTA may not inhibit platelet adenylate cyclase. Table 1 shows the effect of DDA and MTA on adenylate cyclase activity of washed preparations of human platelet membranes. DDA strongly inhibits adenylate cyclase, whereas MTA has no effect. Continuing studies with these platelet membranes will examine in detail the effects of DDA and MTA on the actions of various substances that affect adenylate cyclase, e.g. PGE_1, Ado and its analogs.

Discussion

These findings indicate that the naturally occurring Ado analog, MTA, can overcome the inhibitory effects of Ado and related compounds on ADP-induced platelet aggregation, and although it compares in activity to DDA (a potent inhibitor of adenylate cyclase), it clearly acts by a different mechanism. This difference is shown by the capacity of DDA but not of MTA to overcome the blocking effects of PGE_1. Consistent with these observations performed with intact platelets are findings with partially purified platelet membrane-bound adenylate cyclase where DDA is a potent inhibitor but MTA is without effect. The membrane preparations employed in these studies were 10-fold or more higher in specific activity than those used by others (17,18,27). These membrane preparations were washed four times prior to assay in order to eliminate soluble proteins such as the phosphodiesterases and other soluble factors that might influence adenylate cyclase activity. One may speculate on the relation of these observations to the recently described transmethylation reactions that affect membrane-mediated cellular responses. Recent reports indicate that conversion of phosphatidylethanolamine to phosphotidylcholine via membrane-bound methyltransferases of mast cells results in the formation of arachidonic acid (a precursor of prostaglandins synthesis) and promotion of Ca^{++} influx (10). Also, rapid carboxymethylation of platelet membrane proteins is induced by addition of thrombin (11). It is possible that membrane-associated transmethylation reactions such as these are key components of cellular signals in response to specific stimuli. Interestingly, the MTA analog S-isobutyryl-3-deazaadenosine inhibits the methylation of phospholipids in mast cells. Thus, one may ask whether naturally formed MTA or Ado and its analogs play a role in these methylation reactions.

A question to be explored is the possible role of MTA formed in large amounts in tumor cells during polyamine synthesis. MTA phosphorylase has a low K_m (9 μM) for this substrate (28) and normally only low levels of intracellular MTA are detectable. However, if this enzyme is inhibited or lacking, MTA efflux might occur with effects on surrounding tissues. Since blood platelets play a role in the establishment of tumor metastases, one wonders whether production of MTA by these cells can facilitate platelet aggregation by overcoming inhibitions caused by Ado.

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