The Effect of Pharmacologic Inhibition of Phospholipid Methylation on Human Platelet Function

By Sanford J. Shattil, John A. Montgomery, and Peter K. Chiang

Human platelets are capable of synthesizing their major membrane phospholipid, phosphatidylcholine, by a methylation pathway. This involves the sequential transfer of methyl groups from S-adenosyl-l-methionine (AdoMet) to phosphatidylethanolamine, and in the process, AdoMet is converted to S-adenosylhomocysteine (AdoHcy). The activity of this methylation pathway is decreased upon stimulation of platelets by various agonists. We inhibited methylation reactions pharmacologically to see whether this inhibition plays any role in the process of platelet activation. Two inhibitors of AdoHcy hydrolase, 3-deaza-adenosine and 3-deaza-(±)aristeromycin (500 µM each), were effective in increasing platelet levels of AdoHcy and preventing turnover of AdoMet. Also, these compounds were equipotent in inhibiting platelet phospholipid methylation. However, while 3-deaza-adenosine potentiated platelet aggregation and 14C-serotonin release induced by epinephrine or adenosine diphosphate (ADP) (p < 0.01), 3-deaza-aristeromycin had no such effect. Neither compound affected platelet responses to thrombin or collagen. Inhibition of methylation reactions was not the only biochemical effect of 3-deaza-adenosine since it also blunted significantly the elevation of platelet cyclic adenosine monophosphate (AMP) levels induced by prostaglandin E1 (p < 0.02). Therefore, these studies demonstrate that inhibition of platelet phospholipid methylation, per se, has no discernible effect on the function of human platelets. The methylation pathway, though active in platelets, does not appear to be primarily involved in membrane events responsible for platelet activation.

Activation of platelets by agonists involves a number of biochemical and biophysical events within the platelet membrane that culminate in the platelet responses, shape change, aggregation, and secretion. Those membrane-associated processes that may participate in platelet activation include the binding of an agonist to its receptor, interaction of the receptor with components of the adenylate cyclase complex, conformational changes, proteolysis or phosphorylation of membrane proteins, synthesis of thromboxane A2, changes in membrane fluidity, and translocation of calcium ions across membranes.

In a number of mammalian cells, a change in the steady-state concentration of certain phospholipids may modulate many of these membrane events. In the platelet, for example, thromboxane A2 formation is initiated by the release of arachidonic acid from phosphatidylinositol and phosphatidycholine. Furthermore, platelet stimulation is associated with a transient increase in the minor phospholipid—phosphatidic acid. This phospholipid is ionophoretic, and its increase could mediate the translocation of calcium.

Still another example of a change in phospholipids during cell activation is perturbed synthesis of phosphatidylcholine via a methylation pathway. This pathway involves the sequential N-methylation of phosphatidylethanolamine to form phosphatidylcholine. An increase or decrease in the rate of phospholipid methylation has been proposed as a mechanism of signal transduction in rat and human erythrocyte membranes and in rabbit and guinea pig peritoneal leukocyte membranes. Recently, we demonstrated that human platelets are also capable of synthesizing phosphatidylcholine by the methylation pathway. Moreover, upon stimulation of platelets with agonists such as epinephrine, ADP, or thrombin, an immediate decrease in synthesis of phosphatidylcholine via this pathway is observed.

The conversion of phosphatidylethanolamine to phosphatidylcholine involves the transfer of methyl groups from S-adenosylmethionine (AdoMet) to phosphatidylethanolamine by the action of one or more membrane-associated methyltransferases. In the process, AdoMet is converted to S-adenosylhomocysteine (AdoHcy). In turn, AdoHcy is converted to adenosine and homocysteine by the enzyme, AdoHcy hydrolase. Pharmacologic inhibition of this enzyme results in an increase in the concentration of AdoHcy within the cell. An increase in AdoHcy in turn inhibits methylation reactions. We took advantage of this by incubating platelets with two different inhibitors of AdoHcy hydrolase, 3-deaza-adenosine (3-deaza-Ado) and 3-deaza-(±)aristeromycin (3-deaza-Ari). We asked whether these compounds would inhibit platelet phospholipid methylation and whether this would affect platelet function.
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MATERIALS AND METHODS

Materials

3-Deaza-Ado and 3-deaza-Ari* were synthesized as described previously. L-[Methyl-3H]methionine (12-15 Ci/mole), L-[35S]methionine (1314 Ci/mole), and [methyl-3H]choline chloride (49.5 mCi/mole) were from New England Nuclear, Boston, Mass. L-Epinephrine and ADP were from Sigma Chemical Company, St. Louis, Mo. Bovine thrombin was from Parke-Davis, Detroit, Mich.; collagen (calf skin, acid soluble) from Worthington Biochemicals, Freehold, N.J.; PGE, from Upjohn Company, Kalamazoo, Mich.; fibrinogen (Kabi AB) from Kabi Blood Products Division, Stockholm, Sweden. All phospholipid standards were from Supelco Company, Bellefonte, Pa., except for egg phosphatidylcholine (Grand Island Biochemical Company, Grand Island, N.Y.), phosphatidyl-N-monomethylethanolamine, and phosphatidylinositol (Avanti Biochemicals Inc., Birmingham, Ala.).

Studies of Platelet Phospholipid and Cyclic AMP Metabolism

Venous blood from normal volunteers taking no medication was anticoagulated with 0.013 M trisodium citrate. Platelet-rich plasma was obtained, and in some experiments labeled with 3H-serotonin, as described previously. Platelets were separated from plasma by centrifuging cells through silicone oil, counting 3H in the platelet pellet, and correcting for trapped extracellular space using 381H-albumin.

To assess the effect of inhibitors of AdoHcy hydrolase on phospholipid metabolism, washed platelets were incubated with radiolabeled methionine or choline for 30 min at 37°C, at which time various concentrations of 3-deaza-Ado or 3-deaza-Ari were added and incubations carried out for an additional 30 min. Incubations were stopped with an equal volume of 3-deaza-Ado or 3-deaza-Ari were retained along with cyclic AMP after exhaustive extraction with ether. This introduced a source of error in the cyclic AMP radioimmunoassay (New England Nuclear, Boston, Mass.) because the concentration of these analogues present in the incubations cross-reacted with and were detectable by the cyclic AMP antibody. Therefore, prior to radioimmunoassay, cyclic AMP in platelet extracts was separated from the 3-deaza compounds by applying 1 ml of extract onto a 0.6 x 0.5 cm column of Dowex 50 W-X8 (H+) and eluting with deionized water. In this system, cyclic AMP was recovered in the first 10 ml of eluate, whereas 3-deaza-Ado and 3-deaza-Ari were retained. Cyclic AMP recoveries ranged from 85% to 96%.

Platelet AdoMet and AdoHcy

Platelets were separated from plasma, washed, and resuspended in buffer to 3 x 10^10 platelets/ml as for the phospholipid experiments above. Platelets were pulse-labeled by adding 0.5 μCi of 35S-methionine (380 μM final concentration) per milliliter of platelets and incubating for 30 min at 37°C. They were then centrifuged at 2000 g for 10 min at 22°C, reincorporated with methionine was decanted and the platelet pellet was resuspended in incubation buffer to the original volume. 3-Deaza-Ado, or 3-deaza-Ari, or an equal volume of 0.155 M NaCl was added, and the platelets incubated again at 37°C. At various times thereafter, 2-ml aliquots were removed and placed into tubes containing 0.22 ml of 50% sulfosalicylic acid. After standing at 25°C for 15 min, tubes were centrifuged for 10 min at 4°C at 2000 g and the supernatant stored at –70°C. Within 3 days, AdoMet and AdoHcy were isolated by high-performance liquid chromatography, and the radioactivity in each fraction determined.

Platelet Shape Change, Aggregation, and 3H-Serotonin Release

Platelet-rich plasma was gel-filtered in Tyrode's buffer and the platelet count adjusted to 3 x 10^10/ml. Platelets were not grossly activated by this procedure as evidenced by their continued ability to "swirl" and the lack of spontaneous microscopic aggregation. 3-Deaza-Ado or 3-deaza-Ari or an equal volume of 0.155 M NaCl was added and the mixtures incubated in capped polystyrene tubes for 30 min at 37°C without stirring. At 30 min, 0.45 ml of platelets were added to 1.5 ml capacity cuvettes containing 50 μl of purified fibrinogen and calcium chloride (final fibrinogen concentration, 200 μg/ml; calcium, 0.5 mM). Platelets were stirred at 1000 rpm at 37°C, activated by adding 5–10 μl of an agonist (epinephrine, ADP, thrombin, or collagen), and the extent of platelet aggregation recorded. The pH of all platelet samples ranged from 7.5 to 7.6. Reactions were terminated at 5 min with 100 mM formaldehyde and 5 mM EDTA and 3H-serotonin release measured. Platelet shape change was measured in the presence of 4 mM EDTA (to inhibit aggregation) in a Gilford spectrophotometer equipped with a stirrer and expanded scale recorder.

Statistics

Mean values are expressed ± SEM. The difference between means was examined by Student's t test for paired data.

RESULTS

Effect of 3-Deaza-Adenosine on Platelet Phospholipid Methylation

Washed platelets incubated at 37°C with 0.15 μM L-[methyl-3H]methionine take up this radiolabel.
Platelets preincubated for 30 min with 3-deaza-Ado (500 μM) also took up l-[methyl-1H]-methionine at a rate identical to that of control platelets (data not shown). In separate experiments, platelets were pulse-labeled with l-[15S]methionine and the turnover of 35S-labeled AdoMet and AdoHcy measured. The labeled AdoHcy in control platelets turned over with a half-time of approximately 16 min and AdoMet with a half-time of about 14 min (Table 1). In contrast, in pulse-labeled platelets subsequently incubated with 500 μM 3-deaza-Ado, the amount of radiolabeled AdoHcy actually increased over 30 min, and the amount of AdoMet remained constant (Table 1). As in other cells incubated with 3-deaza-Ado, by 30 min we also detected the accumulation of 34,600 35S cpm/108 platelets in a chromatographic fraction that comigrated with authentic 3-deaza-AdoHcy. Thus, 3-deaza-Ado affected the metabolism of platelet AdoMet in a manner expected from its known action as an inhibitor and substrate of AdoHcy hydrolase. We were unable to measure the pool sizes of AdoMet and AdoHcy, and therefore, their specific activities, because the amount of these compounds in 108 platelets was too low to be quantitated by the assay used.

3-Deaza-Ado inhibited platelet phospholipid methylation in a concentration- and time-dependent manner. Platelets were preincubated with [methyl-1H]methionine for 30 min, at which time 3-deaza-Ado was added and the incorporation of methyl-3H into platelet phospholipids over the subsequent 30 min determined. 3-Deaza-Ado (500 μM) caused a 33% decrease in the amount of methyl-3H incorporated into platelet phospholipids. Some inhibition of methylation could be detected at 3-deaza-Ado concentrations as low as 0.05 μM (Table 2). In time course experiments, phospholipid methylation was inhibited significantly only after 20 min incubation of platelets with 3-deaza-Ado (500 μM), and the inhibition was maintained for at least 60 min. Therefore, in all subsequent studies we incubated 3-deaza-Ado with platelets for 30 min.

3-Deaza-Ado (500 μM) inhibited the incorporation of methyl-1H specifically into the methylated phospholipid intermediates, phosphatidyl-N-monomethylethanolamine, phosphatidyl-N,N-dimethylethanolamine, as well as into the final reaction product, phosphatidylycholine (Fig. 1). Radioactivity incorporated into

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### Table 1. Effect of 3-Deaza-Adenosine and 3-Deaza-Aristeronycin on AdoHcy and AdoMet in Platelets Pulse-Labeled With 35S-Methionine

<table>
<thead>
<tr>
<th></th>
<th>AdoHcy (35S cpm/4 x 10^8 Platelets)</th>
<th>AdoMet (35S cpm/4 x 10^8 Platelets)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>3-Deaza-Ado (500 μM)</td>
<td>3-Deaza-Ar (500 μM)</td>
</tr>
<tr>
<td>Minutes</td>
<td>Control</td>
<td>3-Deaza-Ado</td>
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### Table 2. Concentration-Dependent Inhibition of Platelet Phospholipid Methylation by 3-Deaza-Adenosine

<table>
<thead>
<tr>
<th>3-Deaza-Ado (M)</th>
<th>Percent Inhibition of Methyl-3H Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x 10^8</td>
<td>9.4 ± 2.9*</td>
</tr>
<tr>
<td>5 x 10^-7</td>
<td>14.4 ± 3.6</td>
</tr>
<tr>
<td>5 x 10^-6</td>
<td>18.4 ± 3.9</td>
</tr>
<tr>
<td>5 x 10^-5</td>
<td>25.0 ± 5.1</td>
</tr>
<tr>
<td>5 x 10^-4</td>
<td>32.8 ± 5.0</td>
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</tbody>
</table>

*Mean ± SEM of 5 experiments. Platelets incubated without 3-deaza-Ado incorporated into their phospholipids 1.20 ± 0.06 pmol methyl-3H/10^8 platelets.

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Fig. 1. Effect of 500 μM 3-deaza-adenosine on the incorporation of radioactivity from l-[methyl-3H]methionine into individual platelet phospholipids. Open bars refer to control platelets incubated with radiolabeled methionine for a total of 60 min. Closed bars represent platelets incubated the final 30 min with 3-deaza-adenosine. Platelet lipids were extracted and individual phospholipids isolated by thin-layer chromatography as described in Materials and Methods. The only platelet phospholipids that incorporated radioactivity are those shown here. Lyso PC refers to lysophosphatidylcholine, PC to phosphatidylcholine, dimethyl PE to phosphatidyl-N,N-dimethylethanolamine, and monomethyl PE to phosphatidyl-N-monomethylethanolamine. This experiment is representative of three so performed.
phosphatidylcholine was inhibited by 60%, whether expressed in terms of cpm/10⁶ platelets or cpm/μg phosphatidylcholine phosphorus. In contrast, 3-deaza-Ado did not decrease the incorporation of 14C-choline into platelet phosphatidylcholine.

**Effect of 3-Deaza-Adenosine on Platelet Function**

3-Deaza-Ado (500 μM) by itself did not stimulate the aggregation or secretion of gel-filtered platelets. However, it consistently potentiated the functional effects of the agonists, epinephrine and ADP. Thus, in response to concentrations of epinephrine that caused minimal aggregation (less than 20%) and no 14C-serotonin release in control platelets, platelets incubated with 3-deaza-Ado underwent 74% ± 7.0% aggregation and 37% ± 5.0% 14C-serotonin release (Figs. 2 and 3). Potentiation was observed at 3-deaza-Ado concentrations as low as 0.5 μM. Similar results were obtained with ADP (Figs. 2 and 3). In contrast, 3-deaza-Ado had no effect on ADP-induced platelet shape change or on aggregation and serotonin release induced by thrombin (0.005–1.0 U/ml) or collagen (0.1–5.0 μg/ml).

The above studies demonstrate that 3-deaza-adenosine both inhibits phospholipid methylation and potentiates platelet function by ADP and epinephrine. However, they do not prove that the effect of 3-deaza-Ado on platelet function is caused by its effect on phospholipid methylation. Two additional experiments were carried out to support or reject such a causal relationship.

First, because 3-deaza-Ado's parent compound, adenosine, is known to affect platelet cyclic AMP levels, we measured this cyclic nucleotide. The level of cyclic AMP in control platelets increased 5-fold and 6.5-fold in response to 280 nM and 560 nM PGE₁, respectively (Fig. 4). In contrast, platelets preincubated with 3-deaza-Ado (500 μM) responded significantly less well than controls to PGE₁ (p < 0.02) (Fig. 4). Thus, inhibition of methylation reactions is not the only biochemical effect of 3-deaza-Ado on platelets.

**Effect of 3-Deaza-(±)Aristeromycin on Platelets**

Second, we incubated platelets with another compound, 3-deaza-Ari, which in preliminary studies was found to be a potent inhibitor of beef liver AdoHcy hydrolase (P. K. Chiang, unpublished observations). This compound was as potent as 3-deaza-Ado in inhibiting the turnover of platelet AdoHcy and AdoMet (Table 1) and in inhibiting phospholipid methylation (Fig. 5). The concentration-dependence of inhibition of methylation was similar to that shown for 3-deaza-Ado in Table 2. However, in sharp contrast to the potentiating effect of 3-deaza-Ado on platelet aggregation and secretion, 3-deaza-Ari had neither a potentiating nor an inhibitory effect on epinephrine or ADP-induced platelet aggregation or secretion (Fig. 6). Collagen- and thrombin-induced aggregation were similarly unaffected. Furthermore, unlike 3-deaza-Ado, 3-deaza-Ari had no effect on platelet cyclic AMP levels, either in the presence or absence of PGE₁.
Fig. 4. Effect of 500 μM 3-deaza-adenosine on platelet cyclic AMP levels. After washing platelets free of plasma, control platelets (open bars) and platelets with 3-deaza-adenosine (closed bars) were incubated without stirring for 30 min at 37°C. In some tubes, PGE1 was added 0.5 min before stopping the reactions with trichloroacetic acid and analyzing for cyclic AMP. Data represent the means ± SEM of 6 experiments.

DISCUSSION

We became interested in examining phospholipid methylation in platelets because, in other mammalian cells, perturbation of the methylation pathway by agonists has been linked to membrane intermediary events that may also participate in platelet activation. These include receptor-adenylate cyclase coupling, phospholipase activation, changes in membrane fluidity, and calcium translocation. In fact, our previous studies indicate that the synthesis of phosphatidylcholine by methylation is inhibited immediately upon the stimulation of platelets by epinephrine, ADP, or thrombin.

The present studies demonstrate that the AdoHcy hydrolase inhibitors, 3-deaza-adenosine and 3-deaza-aristeromycin, are effective inhibitors of phospholipid methylation in human platelets. 3-Deaza-Ado is also capable of potentiating platelet aggregation and secretion induced by certain agonists such as epinephrine and ADP, but not by others such as collagen or thrombin. However, 3-deaza-Ado not only inhibits platelet phospholipid methylation, but it blunts the increase in platelet cyclic AMP levels induced by PGE1. In contrast, 3-deaza-Ari has no demonstrable effect on platelet function or cyclic AMP metabolism. Taken together, these data strongly suggest that inhibition of platelet phospholipid methylation, per se, plays no role in mediating agonist-induced aggregation or secretion of human platelets. Thus, the decrease in phospholipid methylation observed after the addition of agonists appears to be a result of, rather than causally involved in, platelet activation.

Another possible interpretation of our results is that inhibition of phospholipid methylation indeed potentiates platelet aggregation or secretion but that this potentiation is not observed in the case of 3-deaza-Ari because of some coincidental and independent inhibitory effect of this compound on aggregation and...
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secretion. This complicated interpretation appears very unlikely since 3-deaza-Ari by itself did not elevate platelet cyclic AMP and was never observed at any concentration to inhibit agonist-induced platelet aggregation or secretion.

3-Deaza-Ari was as potent as 3-deaza-Ado as an inhibitor of phospholipid methylation. Since 3-deaza-Ari had no effect on platelet function, the potentiating effect of 3-deaza-Ado on platelet function must not be due to its effect on methylation. The mechanism of the potentiating effect of 3-deaza-Ado remains unclear. Adenosine may affect platelet function by competing with ADP at a surface receptor, by stimulating or inhibiting adenylate cyclase, and by becoming incorporated into platelet nucleoside and nucleotide pools. 3-Deaza-Ado does not become incorporated into nucleoside or nucleotide pools. However, like adenosine, 3-deaza-Ado could interact with ADP at the platelet surface or with enzymes involved with cyclic AMP metabolism. In platelets as in other cells, it is also capable of interacting with homocysteine to form a novel congener, 3-deaza-S-adenosylhomocysteine, which itself might influence cell function. Thus, there are several loci at which 3-deaza-Ado might influence platelet function unrelated to its inhibition of phospholipid methylation.

We do not know the mechanism by which 3-deaza-Ado limits the platelet's cyclic AMP responses to PGE\textsubscript{1}. Zimmerman et al. have shown that 3-deaza-Ado increases the level of AdoHcy in mouse lymphocytes. This affects both adenylate cyclase and cyclic AMP phosphodiesterase in these cells, resulting in enhanced cyclic AMP responses to PGE\textsubscript{1}. On the other hand, AdoHcy decreases the response of rat retina adenylate cyclase to dopamine. Whatever the mechanism of the effect on cyclic AMP, these studies and our own emphasize the need for caution in concluding that a functional effect of 3-deaza-Ado on a cell is specifically related to its inhibition of methylation reactions. In addition, the effect on cyclic AMP may not completely account for 3-deaza-Ado's potentiation of platelet aggregation. Pharmacologic inhibition of adenylate cyclase, by itself, does not cause or potentiate platelet aggregation.

Homocysteine thiolactone is known to augment the inhibitory effect of 3-deaza-Ado on methylation reactions. As a result, an augmentation by homocysteine thiolactone of 3-deaza-Ado's functional effects on a cell has been taken as support for those functional effects being caused by inhibition of methylation. We found that homocysteine thiolactone also augments the inhibition of platelet phospholipid methylation by 3-deaza-Ado (S. J. Shattil and P. K. Chiang, unpublished observations). However, homocysteine thiolactone, by itself, inhibited platelet aggregation without inhibiting phospholipid methylation. Therefore, we were unable to use this compound unambiguously in aggregation studies with 3-deaza-Ado.

Synthesis of phosphatidylcholine by transmethylation is a quantitatively minor renewal pathway for platelet membrane phosphatidylcholine. The present study does not address and cannot exclude some as yet undefined role for phospholipid methylation in resting human platelets or in the function of platelets from other species. In this regard, the phosphatidylcholine synthesized by methylation in rabbit platelets is relatively rich in arachidonic acid compared to that synthesized via the choline pathway. Nonetheless, our studies establish that neither a stimulation nor an inhibition of phospholipid methylation appears to be causally involved in the activation of human platelets.

ACKNOWLEDGMENT

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The effect of pharmacologic inhibition of phospholipid methylation on human platelet function

SJ Shattil, JA Montgomery and PK Chiang