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Guanine nucleotides inhibit agonist-stimulated arachidonic acid release in both intact and saponin-permeabilized human platelets

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The effects of guanine nucleotides on arachidonic acid (AA) release were studied in intact and saponin-permeabilized human platelets. While GTP[S] itself caused a stimulation of AA release in permeabilized cells, GTP[S], GDP[S], GTP, ATP and other nucleotides inhibited AA release in response to thrombin and other agonists in intact, as well as permeabilized platelets. Inhibition of agonist-stimulated AA release by nucleotides was partially attenuated by addition of ADP, and was abolished by prior stimulation of platelets to discharge the ADP-containing dense granules. These results suggest: (i) that released ADP plays an important contributory role in agonist-stimulated platelet AA release, and (ii) that guanine nucleotides can modulate platelet activation through an extracellular action which is distinct from their effects on G-proteins.

Guanine nucleotides; G-protein; Arachidonic acid release; (Human platelet)

1. INTRODUCTION

A number of investigators have recently provided evidence that AA release may be under the control of one or more guanine nucleotide-binding regulatory proteins (G-proteins) in human platelets. The evidence can be summarized as follows: the G-protein activators fluoroaluminate and GTP[S] stimulate AA release when added to platelets [1–4], and the G-protein inhibitors GDP[S] and pertussis toxin inhibit AA release in response to the physiological agonist thrombin [4].

During studies using permeabilized human platelets, we observed that thrombin-stimulated AA release is profoundly inhibited not only by GDP[S], but surprisingly also by GTP[S], GTP, GDP, ATP and many other nucleotides. The present study was undertaken to determine the mechanism by which exogenous nucleotides can inhibit agonist-induced platelet responses.

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Abbreviations: AA, arachidonic acid; CP/CPK, creatine phosphate plus creatine phosphokinase; FAF-BSA, fatty acid-free bovine serum albumin; GDP[S], guanosine-5′-*O*-(2-thiodiphosphate); GppNHp, guanylylimidodiphosphate; GTP[S], guanosine-5′-*O*-(3-thiotriphosphate)

2. EXPERIMENTAL

Human platelet suspensions were prelabeled with [^{3}H]AA as previously described [5] and gel-filtered through Sepharose 2B columns equilibrated with a modified Tyrode-Hepes medium composed of 134 mM NaCl, 2.9 mM KCl, 4 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 1 mM $MgCl_2$, 5 mM dextrose, 0.2 mg/ml FAF-BSA and 10 mM Hepes. All incubation media, including those which contained added nucleotides, were adjusted to pH 7.40 at 37°C. The final platelet density was $1–2 \times 10^8$/ml. Platelet samples (0.5 ml) were preincubated with nucleotides and/or the ADP scavenger CP/CPK for 5 min prior to addition of thrombin or other agonists; incubations were then terminated after an additional 5 min. For experiments utilizing degranulated platelets, a procedure similar to that reported by Kinlough-Rathbone et al. [6] was followed. Washed platelets (10^9/ml) were suspended in medium containing 1 mM EGTA and no added divalent cations. Platelets were then treated either with 1 μM A23187 for 5 min, or with 1 U/ml thrombin for 2 min at 23°C followed by addition of 2 U/min hirudin and 10 nM prostacyclin. Control experiments revealed that these conditions resulted in maximal (80–90%) discharge of dense granule contents, as assessed by measuring the release of [^{3}H]serotonin from prelabeled platelets. Degranulated platelets

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were then washed, labeled with [^{3}H]AA, and gel-filtered as described above. For studies with saponin-permeabilized platelets, [^{3}H]AA-prelabeled cells were gel-filtered into a medium composed of 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM dextrose, 0.5 mg/ml FAF-BSA and 20 mM Hepes. Incubations were begun by diluting 50 μl of concentrated (1–2 × 10^9 cells/ml) platelet suspension into 450 μl of a medium composed of 140 mM KCl, 1 mM $MgCl_2$, 1 mM dextrose, 0.5 mg/ml FAF-BSA, 20 mM Hepes, 12 μg/ml saponin and any desired pretreatments. Following a 5 min period to allow complete permeabilization of platelets, stimuli were added and incubations were terminated after an additional 5 min. Release of [^{3}H]AA and metabolites into the medium was assayed as described previously [5]. All data shown are representative of at least four separate platelet preparations which gave closely similar results.

Nucleotides were purchased from Boehringer Mannheim or Sigma, trypsin (TPCK-treated) was from Sigma. All other materials were from sources described previously [5,7].

3. RESULTS AND DISCUSSION

As shown in fig.1, the addition of G-protein activator GTP[S] to saponin-permeabilized platelets elicited a modest stimulation of [^{3}H]AA release. The stimulatory effect of GTP[S] was not seen in nonpermeabilized cells. Surprisingly, the G-protein inhibitor GDP[S] also caused a slight, but consistent stimulation of [^{3}H]AA release, whereas GTP was without effect. We predicted that if the platelet agonist thrombin activates AA release through a thrombin receptor/G-protein interaction, then GTP or GTP[S] might potentiate the action of submaximal concentrations of thrombin in permeabilized cells. However, all three guanine nucleotides substantially inhibited thrombin-stimulated [^{3}H]AA release at thrombin concentrations above 0.5 U/ml, and no potentiation was evident at lower concentrations (fig.1). Nucleotides had no apparent effect of their own on intact (nonpermeabilized) platelets up to the highest concentration tested, 3 mM (not shown).

[^{3}H]AA release stimulated by thrombin and other platelet agonists (trypsin, collagen) in intact cells was also inhibited by guanine nucleotides, ATP and other nucleotides as shown in table 1. Half-maximal inhibition of the response to 1 U/ml thrombin was attained at 306 ± 74 μM ATP (mean ± SE from 5 experiments) and the thrombin response was 85–95% inhibited at 3 mM ATP. Nucleosides, phosphate and pyrophosphate (1 mM) were not inhibitory (not shown). The fact

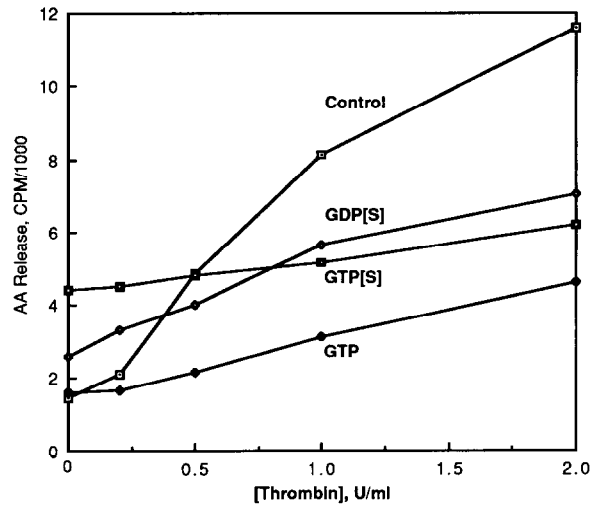

Fig.1. Effects of guanine nucleotides on thrombin-stimulated [^{3}H]AA release in saponin-permeabilized platelets. Nucleotide concentrations were: GTP[S], 10 μM; GDP[S], 100 μM; GTP, 1 mM. Experimental conditions are described in section 2. Data are means from duplicate determinations which varied by less than 4%.

that nucleotides, which are highly charged, hydrophilic compounds, can inhibit responses in intact cells implies that their site of action is extracellular. Platelets are known to possess cell surface receptors for the weak platelet agonist ADP, and ATP is a competitive antagonist at this unique purine receptor (review [8]). Upon stimulation, platelets release ADP and other substances stored in dense granules, thus providing a feedback stimulatory pathway which may amplify the effect of the primary stimulus. Although platelet activation by the strong agonist thrombin is generally thought to be largely independent of the actions of released ADP, the present findings raise the possibility that released ADP plays a significant role in the stimulation of [^{3}H]AA mobilization by thrombin, and that nucleotides may inhibit the thrombin response through blockade of the ADP receptor.

To explore this possibility we tested the following three criteria. First, if nucleotides inhibit thrombin-stimulated [^{3}H]AA release by blocking the action of released ADP, then the enzymatic removal of released ADP should inhibit the thrombin response in a manner similar to nucleotides. Fig.2 shows that the ADP scavenger CP/CPK in-

Table 1

Inhibitory effect of nucleotides on agonist-stimulated AA release

Agonist	Nucleotide	AA release (cpm)	% inhibition
None	none	532 ± 12	–
Thrombin (1 U/ml)	none	11924 ± 296	–
	GTP	6456 ± 65	48
	GTP[S]	5024 ± 443	61
	GppNHp	8468 ± 176	30
	GDP	7208 ± 293	41
	GDP[S]	4959 ± 255	61
	GMP	11939 ± 155	0
	ATP	3570 ± 90	73
	UTP	5367 ± 123	58
	CTP	4199 ± 31	68
Trypsin (10 μg/ml)	none	10752 ± 611	–
	ATP	3387 ± 376	72
	GTP	5784 ± 114	49
	GDP[S]	5423 ± 78	52
Collagen (40 μg/ml)	none	7827 ± 577	–
	ATP	2465 ± 392	74

All nucleotides were present at 1 mM. Data are means ± range from duplicate determinations

hibits thrombin-stimulated [^{3}H]AA release, especially at low thrombin concentrations. The inhibitory nucleotide ATP caused little or no additional inhibition when added in the presence of CP/CPK, consistent with the belief that both inhibitors share a common mechanism of action, i.e. interference with released ADP. Similar inhibition was seen with apyrase as an ADP scavenger, and with trypsin as the platelet stimulus (not shown).

Second, if the nucleotides are acting as competitive inhibitors at the ADP receptor, then the inhibitory effects of nucleotides should be overcome by addition of exogenous ADP. As shown in fig.3, added ADP attenuated (by approx. 50%) the inhibition of thrombin-stimulated [^{3}H]AA release caused by GDP[S]. Similar attenuation was seen for ATP, GTP, GTP[S] and other inhibitory nucleotides (not shown). For these experiments, conditions were chosen such that ADP itself would have little effect of its own on [^{3}H]AA release. Thus, our washed platelet suspensions did not contain fibrinogen which is required for direct platelet responses to this weak agonist [9]. Also, ADP had little effect on the response to 1 U/ml thrombin alone (fig.3). However, with lower thrombin concentrations (0.01–0.1 U/ml) at which secretion of dense granule contents was found to be submaximal, we consistently observed an enhancement of thrombin-stimulated [^{3}H]AA release by 1–10 μM

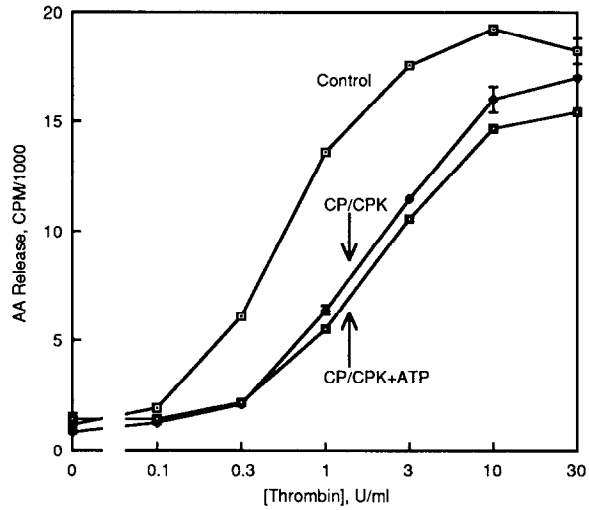

Fig.2. Inhibition of thrombin-stimulated [^{3}H]AA release by the ADP scavenger system, creatine phosphate (5 mM) plus creatine phosphokinase (10 U/ml). [ATP] was 1 mM. Data are means ± range (indicated by the bars unless smaller than the symbols) from duplicate determinations.

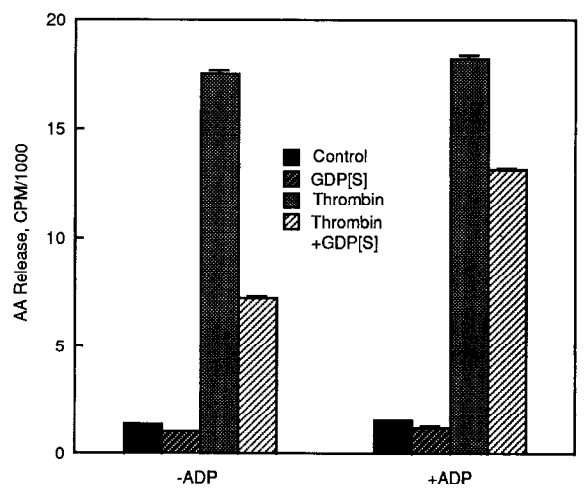

Fig.3. Attenuation by ADP of the inhibitory effect of GDP[S] on thrombin-stimulated [^{3}H]AA release. Drug concentrations were: thrombin, 1 U/ml; GDP[S], 1 mM; ADP, 100 μM. Increasing [ADP] to 1 mM did not result in a greater reversal of inhibition (not shown). Data are means (± SD) from triplicate determinations.

ADP under the same experimental conditions (not shown).

And third, in degranulated platelets in which agonist-induced discharge of ADP can no longer occur, nucleotides should not be able to inhibit other agonist-induced responses if they are acting simply as ADP antagonists. Fig.4 depicts a typical experiment in which platelets were degranulated by prior stimulation with the Ca^{2+} ionophore A23187, labeled with [^{3}H]AA and then challenged with thrombin. In striking contrast to nondegranulated cells in which nucleotides and CP/CPK were inhibitory (figs 1–3, table 1), a complete loss of inhibition or even a potentiation of thrombin-stimulated [^{3}H]AA release by nucleotides and CP/CPK was seen in degranulated platelets (fig.4). Note in fig.4 that ADP enhanced the stimulatory effect of 1 U/ml thrombin in degranulated platelets as opposed to nondegranulated platelets (fig.3), further supporting a role for released ADP in the thrombin response. In some experiments, platelets were degranulated by thrombin pretreatment. Fig.5 shows that in this preparation, GDP[S] and ATP no longer inhibit trypsin-stimulated [^{3}H]AA release in either intact or saponin-permeabilized cells.

Taken together, these results suggest that the inhibitory effect of GDP[S] and other nucleotides on agonist-stimulated AA release is exerted, at least in part, through a competitive blockade of the platelet surface ADP receptor. Nucleotide-induced inhibition was not restricted to AA release as the agonist response, but was seen also for thrombin- or trypsin-stimulated serotonin secretion and [^{3}H]arachidonyl diacylglycerol formation (not shown). Following the completion of this study, a paper by Krishnamurthi et al. [10] appeared which reported an inhibition of agonist-stimulated aggregation, serotonin secretion, Ca^{2+} mobilization and protein phosphorylation by guanine nucleotides in human platelets. It is therefore clear that the entire range of platelet responses can be suppressed by exogenous nucleotides, including GDP[S], at concentrations which are commonly used for experiments in permeabilized cells. Thus, it is doubtful that GDP[S] can be of value as a selective G-protein inhibitor in human platelets. Furthermore, these studies provide direct evidence for the importance of released ADP as a contributing factor in platelet activation.

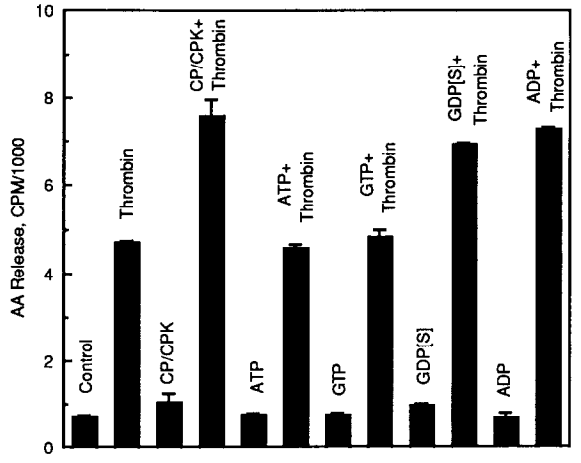

Fig.4. Thrombin (1 U/ml)-stimulated [^{3}H]AA release in A23187-degranulated platelets. See section 2 for experimental details. [ADP] was 10 μM; concentrations of other pretreatments are given in the legends for table 1 and fig.2. Data are means (± SD) from triplicate determinations.

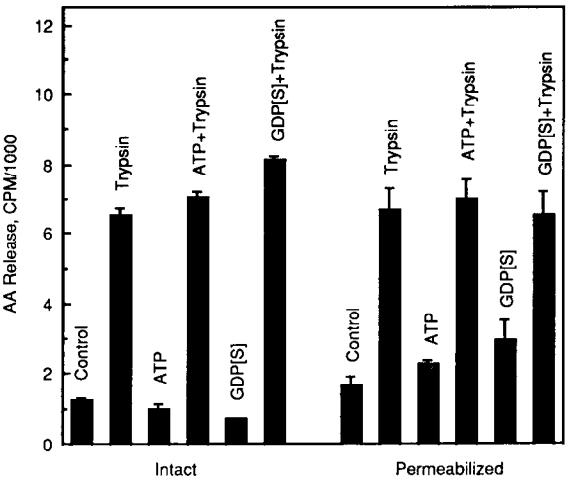

Fig.5. Trypsin (10 μg/ml)-stimulated [^{3}H]AA release in thrombin-degranulated platelets. Nucleotide concentrations were 1 mM. Data are means (± SD) from triplicate samples.

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