Guanine nucleotides decrease the free [Ca^{2+}] required for secretion of serotonin from permeabilized blood platelets Evidence of a role for a GTP-binding protein in platelet activation

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Human platelets containing granule-bound [^{14}C]serotonin were permeabilized, equilibrated at 0° C with ATP and with various Ca^{2+} buffers and guanine nucleotides, and then incubated at 25° C with or without a stimulatory agonist. Ca^{2+} alone induced the ATP-dependent secretion of [^{14}C]serotonin (50% at a p*Ca* of 5.1) but the sensitivity of secretrion to Ca^{2+} was greatly enhanced by guanine nucleotides [6-fold by 100 μM GTP, 100-fold by 100 μM guanyl-5′-yl imidodiphosphate and > 500-fold by 100 μM guanosine 5′-*O*-(3-thiotriphosphate)] or by stimulatory agonists (10-fold by 2 units thrombin/ml and 4-fold by 1 μM 1-*O*-octadecyl-2-*O*-acetyl-*sn*-glyceryl-3-phosphorylcholine). When both GTP and a stimulatory agonist were added, they had synergistic effects on secretion. Cyclic GMP and GMP acted similarly to GTP. The effects of all these guanine nucleotides were inhibited by guanosine 5′-*O*-(2-thiodiphosphate), whereas those of stimulatory agonists were not. Our results demonstrate the presence in platelets of guanine nucleotide-dependent and independent mechanisms regulating the sensitivity of secretrion to Ca^{2+}.

Guanine nucleotide *Calcium* *Thrombin* *Secretion* *Platelet*

1. INTRODUCTION

Abundant evidence has accumulated to indicate that Ca^{2+} plays an important role as an intracellular mediator of the secretory responses of the blood platelet. Thus, Ca^{2+} ionophores stimulate platelet degranulation [1,2], platelets permeabilized by high-voltage electric discharges secrete granule contents in response to added Ca^{2+} [3,4] and studies with the fluorescent indicator, quin2, have demonstrated increases in [Ca^{2+}_{free}] in response to stimuli such as thrombin [5] and platelet-activating factor [6]. In platelets [7], as in other cells [8,9], Ca^{2+} mobilization by physiological stimuli appears to be closely associated with the receptor-activated hydrolysis of phosphatidylinositol 4,5-bisphosphate to 1,2-diacylglycerol and inositol 1,4,5-trisphosphate. In some cell types, the latter product has been directly implicated in the release of Ca^{2+} from the endoplasmic reticulum into the cytosol [10,11]. The former also acts as a signal molecule by enhancing the Ca^{2+} sensitivity of protein kinase C [12], which in platelets phosphorylates a 47-kDa protein in a reaction closely associated with the secretory process [13–15]. Experiments with intact platelets have indeed suggested that Ca^{2+} acts synergistically with diacylglycerol to promote secretion [16] and this has been demonstrated directly in studies with permeabilized platelets [17–19]. In the latter system, thrombin increased the sensitivity of the secretion of serotonin to Ca^{2+} by 10-fold, an effect that was associated with stimulation of

Abbreviations: p*Ca*, $-\log[Ca^{2+}_{free}]$; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); 1-octadecyl-2-acetyl-G-3-PC, 1-*O*-octadecyl-2-*O*-acetyl-*sn*-glyceryl-3-phosphorylcholine (synthetic platelet-activating factor)

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diacylglycerol formation and enhanced phosphorylation of P47 [19]. The preservation of these receptor-mediated effects in permeabilized platelets permits investigation of low-M_r factors involved in receptor action. Here, we have investigated the possibility that guanine nucleotides may play a role in platelet activation. This study was prompted by the observation that the binding of agonists to hepatic α_1-adrenergic receptors, which are linked to Ca^{2+} mobilization, is regulated by guanine nucleotides [20] and a report that incorporation of these nucleotides into mast cells can promote secretion of histamine [21].

2. MATERIALS AND METHODS

2.1. *Materials*

[*side chain*-2-^{14}C]Serotonin (55 mCi/mmol) was from Amersham (Oakville, Ontario). Gpp(NH)p, GTPγS and GDPβS were obtained from Boehringer (Mannheim) and ATP (prepared by phosphorylation of adenosine and therefore essentially GTP-free), GTP, GMP, cyclic GMP and Arg^8-vasopressin from Sigma (St. Louis, MO). Human thrombin (3200 NIH units/mg protein) was from Calbiochem-Behring (La Jolla, CA). 1-Octadecyl-2-acetyl-G-3-PC was a gift from Dr H.R. Baumgartner of F. Hoffmann-La Roche (Basel) and U-46619 [(15*S*)-hydroxy-11α,9α-(epoxymethano)prosta-5*Z*,13*E*-dienoic acid] was provided by Dr J.E. Pike of the Upjohn Co. (Kalamazoo, MI).

2.2. *Preparation of suspensions of permeabilized platelets*

Human platelets in plasma containing ACD anticoagulant were incubated at 37°C with sufficient [^{14}C]serotonin to incorporate about 0.05 μCi of $^{14}C/10^9$ platelets. They were then washed and finally suspended at 1–2 × 10^9/ml in Ca^{2+}-free Tyrode's solution containing 5 mM 1,4-piperazinediethanesulphonic acid and 0.35% bovine serum albumin (final pH 6.5). After addition of 5 mM Na-EGTA (pH 6.5), 2-ml samples (at 20–23°C) were placed in a chamber formed by stainless-steel electrodes 0.2 cm apart and subjected to 10 electric discharges from a 4.5 μF capacitor charged at 3 kV. The permeabilized platelets were then cooled to 4°C, applied to a column of Sepharose CL-4B and eluted with a medium (pH 7.4) containing 12.5 mM $MgCl_2$ and the K^+ salts of glutamic acid (160 mM), Hepes (20 mM), EGTA (2.5 mM) and EDTA (2.5 mM) (buffer A). The eluate was then diluted with buffer A containing ATP to give a final ATP concentration of 5 mM and a platelet count of 0.45 × 10^9/ml. This suspension was stored at 0°C and used for up to 2 h. Further details are given in [19].

2.3. *Incubations*

Samples (80 μl) of suspensions of permeabilized platelets were mixed with 20 μl buffer A containing additions thought to act at intracellular sites, such as $CaCl_2$ and guanine nucleotides. The $CaCl_2$ required to give particular p*Ca* values was calculated as in [22] and the pH of $CaCl_2$ solutions adjusted so that a final pH of 7.4 was obtained. These mixtures were first equilibrated for 15 min at 0°C to ensure that additions penetrated the platelets fully [19]. Then, buffer A (1 μl) containing any stimulatory agonist was added and the final mixtures were incubated for 10 min at 25°C. Incubations were usually terminated by addition of 0.5 ml of 0.15 M KCl containing 1.8% (w/v) paraformaldehyde and 6 mM EDTA and the [^{14}C]serotonin released was determined [19]. The ^{14}C in supernatants from samples equilibrated for 15 min at 0°C with buffer A only (about 5% of total platelet ^{14}C) was subtracted in calculation of the percentage (R) of platelet [^{14}C]serotonin released by various additions. To determine the sensitivity of the secretion of [^{14}C]serotonin from permeabilized platelets to added Ca^{2+} buffers, the p*Ca* at which release of 50% of the total platelet ^{14}C occurred was calculated from the linear regression of $\log[R/100-R]$ on p*Ca*. Only values of R between 10 and 90% were used. Some incubations were terminated by centrifugation without additions to determine whether lactate dehydrogenase (assayed as in [23]) was released.

3. RESULTS

In this study, in which permeabilized platelets were equilibrated with GTP-free ATP, Ca^{2+} released 50% of platelet [^{14}C]serotonin at a p*Ca* of 5.11 ± 0.03 (mean ± SE from 10 experiments), a slightly lower value than observed in [19]. However, addition of an optimal dose of thrombin (2 units/ml) still increased the sensitivity of the

platelets to Ca^{2+} by about 10-fold to give 50% secretion at a p*Ca* of 6.13 ± 0.11 (mean ± SE from 8 experiments). Guanine nucleotides exerted effects qualitatively similar to those of thrombin, causing displacement of the Ca^{2+} log dose-response curve to the left (fig.1). Whereas GTP had a maximal effect at 100 μM that was slightly weaker than that of thrombin, the metabolically stable GTP analogues, Gpp(NH)p and GTPγS, were more effective than thrombin when present at 10 μM or above (fig.2). At 100 μM, Gpp(NH)p increased the Ca^{2+} sensitivity of the platelets by about 100-fold, though no secretion was observed in the absence of added Ca^{2+} (fig.1,2). This concentration of GTPγS enhanced Ca^{2+} sensitivity >500-fold and caused substantial release of [^{14}C]serotonin without added Ca^{2+} (fig.1). However, addition of a further 10 mM EGTA decreased this response, indicating that trace amounts of endogenous Ca^{2+} were required. Other experiments showed that 100 μM GTPγS did not release lactate dehydrogenase from permeabilized platelets, indicating that this nucleotide selectively promoted the secretion of granule constituents.

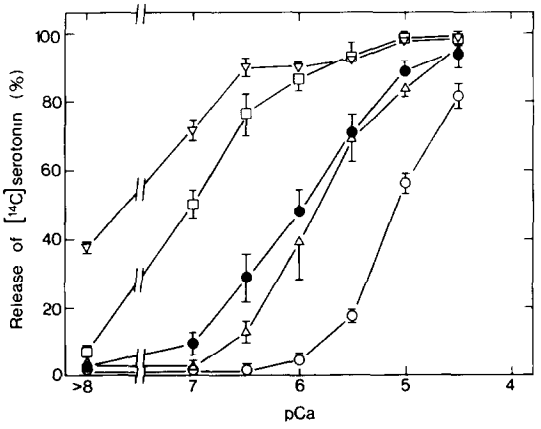


Fig.1. Effects of guanine nucleotides on the release of [^{14}C]serotonin from permeabilized platelets by buffered concentrations of Ca^{2+}_{free}. Samples of suspensions of permeabilized platelets were equilibrated for 15 min at 0°C with the indicated Ca^{2+} buffers and, variously, no other additions (○), 100 μM GTP (△), 100 μM Gpp(NH)p (□) or 100 μM GTPγS (▽). Thrombin (final concentration 2 units/ml) was then added to one group of control samples (●) and all were incubated for 10 min at 25°C. Release of [^{14}C]serotonin was measured. Values are means ± SE from 3 separate experiments.

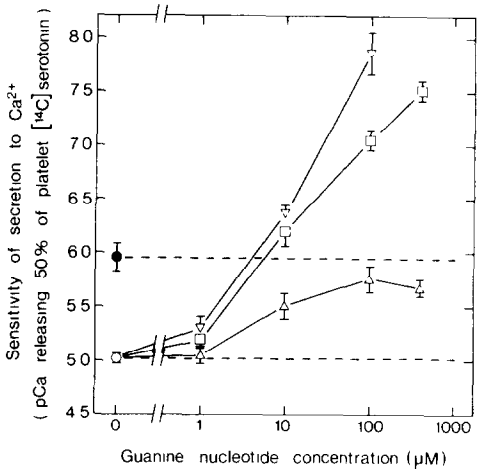
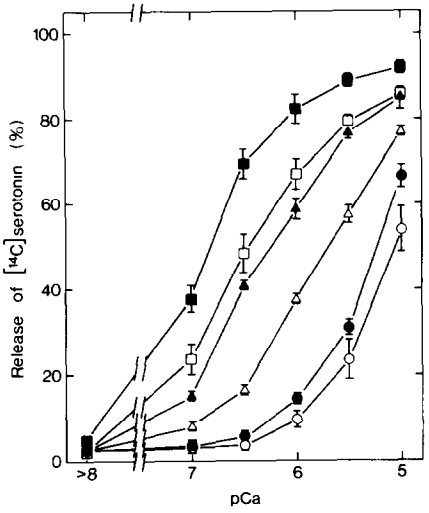


Fig.2. Effects of different concentrations of guanine nucleotides on the sensitivity to Ca^{2+} of the secretion of [^{14}C]serotonin from permeabilized platelets. Experiments were carried out as for fig.1 but with a range of concentrations of GTP (△), Gpp(NH)p (□) and GTPγS (▽). The p*Ca* causing release of 50% of platelet [^{14}C]serotonin was calculated (see section 2.3) for each guanine nucleotide concentration studied and for controls without (○) and with (●) 2 units thrombin/ml. Mean values ± SE from 3 separate experiments are shown.

Moreover, 100 μM GTPγS released <2% of the ^{14}C content of intact platelets labelled with [^{14}C]serotonin, when these platelets were isolated and incubated in a manner identical to permeabilized platelets. This result shows that GTPγS acts at intracellular sites.

The effects of equilibration of permeabilized platelets with GTP on the actions of thrombin and other agonists were investigated over a wide range of p*Ca* values. Low concentrations of GTP (e.g., 4 μM) that alone caused only small increases in the release of [^{14}C]serotonin from permeabilized platelets, stimulated secretion appreciably more in the presence of thrombin, particularly at p*Ca* values of 6–7 (fig.3). Determination of the p*Ca* values causing secretion of 50% of platelet [^{14}C]serotonin in 4 experiments of this type showed that 4 μM GTP caused an increase of 0.09 ± 0.05 (mean ± SE) in the absence of thrombin and of 0.52 ± 0.03 (mean ± SE) in the presence of 2 units thrombin/ml. Similar synergistic interactions between GTP and thrombin were observed when low concentrations of both were used (e.g., table 1,



expt.2) or when a weak thrombin stimulus (0.1 units/ml) was studied in the presence of 100 μM GTP. A high concentration of 1-octadecyl-2-ace-

Fig.3. Synergistic effects of GTP and aggregating agents on the sensitivity to Ca^{2+} of the secretion of [^{14}C]serotonin from permeabilized platelets. Samples of a suspension of permeabilized platelets were equilibrated for 15 min at 0°C with the indicated Ca^{2+} buffers and either no guanine nucleotide (open symbols) or 4 μM GTP (closed symbols). The samples were then incubated for 10 min at 25°C with, variously, no further additions (○, ●), 2 units thrombin/ml (□, ■) or 1 μM 1-octadecyl-2-acetyl-G-3-PC (△, ▲) and the release of [^{14}C]serotonin was measured. Values are means ± SE from 3 determinations in the same experiment.

Table 1

Effects of GDPβS on the potentiation of [^{14}C]serotonin release from permeabilized platelets by guanine nucleotides

Expt.	Additions	Release of [^{14}C]serotonin (%)	
		Without GDPβS	With 400 μM GDPβS
1	None	9 ± 1	8 ± 1
	GTP (4 μM)	11 ± 1	6 ± 1[b]
	GTP (100 μM)	46 ± 4	14 ± 2[b]
	Gpp(NH)p (100 μM)	85 ± 3	39 ± 4[b]
	GTPγS (10 μM)	66 ± 1	36 ± 5[b]
	GTPγS (100 μM)	93 ± 2	80 ± 1[b]
	Thrombin (2 units/ml)	66 ± 1	61 ± 4
	Thrombin (2 units/ml) + GTP (4 μM)	83 ± 2	64 ± 5[a]
	1-Octadecyl-2-acetyl-G-3-PC (1 μM)	38 ± 5	38 ± 5
	1-Octadecyl-2-acetyl-G-3-PC (1 μM) + GTP (4 μM)	61 ± 3	39 ± 5[a]
2	None	7 ± 0	7 ± 1
	GTP (10 μM)	20 ± 2	9 ± 0[b]
	GMP (10 μM)	22 ± 2	9 ± 1[b]
	Cyclic GMP (10 μM)	15 ± 2	10 ± 3
	Thrombin (0.2 units/ml)	19 ± 0	23 ± 1
	Thrombin (0.2 units/ml) + GTP (10 μM)	55 ± 3	27 ± 2[a]
	Thrombin (0.2 units/ml) + GMP (10 μM)	60 ± 1	27 ± 0[b]
	Thrombin (0.2 units/ml) + cyclic GMP (10 μM)	58 ± 0	30 ± 1[b]

[a] $p < 0.05$
[b] $p < 0.005$

Samples of suspensions of permeabilized platelets containing [^{14}C]serotonin were mixed with additions to give a final p*Ca* of 6.0 and the guanine nucleotide concentrations indicated. These mixtures were equilibrated for 15 min at 0°C. Thrombin or 1-octadecyl-2-acetyl-G-3-PC was then added to the samples indicated and all were incubated for 10 min at 25°C before measurement of the [^{14}C]serotonin released. Incubation mixtures contained 3.5 mM ATP (expt.1) or 4 mM ATP (expt.2). Results are means ± SE from 3 determinations in the same experiment. The significance of inhibition of the release of [^{14}C]serotonin by GDPβS was determined by Student's *t* test

tyl-G-3-PC (1 μM) increased the sensitivity of the secretion of [^{14}C]serotonin to Ca^{2+} about 4-fold (fig.3). Effects almost identical to those of 1-octadecyl-2-acetyl-G-3-PC were also obtained with 0.2 μM Arg^8-vasopressin and 10 μM U-46619, a prostaglandin endoperoxide analogue (not shown). Equilibration of permeabilized platelets with 4 μM GTP potentiated the effects of all 3 of these agonists at least as effectively as it did the action of thrombin (e.g., fig.3).

The mechanism by which guanine nucleotides enhanced secretion was investigated by using GDPβS, a competitive inhibitor of the interactions of GTP with guanine nucleotide-binding proteins [24,25]. Simultaneous equilibration with 400 μM GDPβS almost completely blocked all the actions of GTP on permeabilized platelets and roughly halved the potentiation of secretion by 100 μM Gpp(NH)p or 10 μM GTPγS (table 1, expt.1). A weaker inhibition of the effects of 100 μM GTPγS was observed. Although GDPβS blocked the enhancement of secretion by GTP added prior to thrombin or 1-octadecyl-2-acetyl-G-3-PC, it did not inhibit the potentiation of secretion caused by the latter agonists alone (table 1). This finding suggests the existence of both guanine nucleotide-dependent and independent mechanisms that regulate the sensitivity of secretion to Ca^{2+}.

Low concentrations of GMP and cyclic GMP (e.g., 10 μM) were found to exert stimulatory effects on the secretion of [^{14}C]serotonin in both the presence and absence of thrombin that were almost identical with those caused by the same concentrations of GTP (table 1, expt.2). The actions of the former guanine nucleotides were also inhibited by GDPβS.

4. DISCUSSION

Our results show that guanine nucleotides, acting at one or more intracellular sites, enhance the Ca^{2+} sensitivity of the secretory mechanism of the blood platelet. Both with respect to the relative activities of the GTP derivatives tested (GTPγS > Gpp(NH)p $\gg$ GTP) and the inhibition of their actions by GDPβS, our observations are strongly reminiscent of the effects of these compounds on the N_s and N_i proteins mediating activation and inhibition of adenylate cyclase, respectively [24–28]. It is therefore probable that a similar guanine nucleotide-binding protein with GTP-ase activity plays a role in platelet activation. Participation of a GTP-binding protein in platelet activation also offers a possible explanation of the stimulation of platelet degranulation by F^- [29]. The author in [21] has proposed that such a protein may directly regulate Ca^{2+} channels in mast cells. Thus, he found that incorporation of metabolically stable guanine nucleotides into permeabilized mast cells, which were then resealed, only stimulated secretion of histamine in the presence of extracellular Ca^{2+}. However, this mechanism cannot account for the effects of these compounds on permeabilized platelets, which were equilibrated with Ca^{2+} buffers. A more plausible explanation is suggested by our finding that the action of thrombin alone, which closely resembled that of GTP, appears to be mediated by an enhanced formation of diacylglycerol and the subsequent activation of protein kinase C[17,19]. We therefore suggest that a guanine nucleotide-binding protein may play a role in the receptor-mediated stimulation of phosphoinositide breakdown to diacylglycerol in platelets. This hypothesis is supported by the synergistic effects of GTP and stimulatory agonists and by preliminary results indicating that guanine nucleotides stimulate diacylglycerol formation in permeabilized platelets [17]. This mechanism could also account for the results in [21], if another product of phosphoinositide metabolism, such as 1,4,5-inositol trisphosphate, promoted Ca^{2+} entry into the mast cells. However, it is important to note that the failure of GDPβS to block the enhancement of Ca^{2+} sensitivity by agonists in the absence of added GTP, suggest that an additional GTP-independent mechanism of receptor action exists in platelets.

As also noted in [18], we found that low concentrations of cyclic GMP can potentiate secretion of [^{14}C]serotonin from permeabilized platelets, particularly in the presence of thrombin. However, since the same concentrations of either GMP or GTP had very similar effects and the actions of all 3 compounds were blocked by GDPβS, cyclic GMP probably acts through the formation of GTP within the permeabilized platelets, rather than by a specific cyclic GMP-dependent mechanism, as suggested in [18]. As Gpp(NH)p and GTPγS are poor substrates for guanylate cyclase compared with GTP [30], cyclic GMP formation could not ac-

count for the relative activities of these compounds on permeabilized platelets. In any case, the available evidence [31,32] suggests that cyclic GMP has inhibitory rather than stimulatory effects in intact platelets.

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