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INHIBITION BY GDPβS OF AGONIST-ACTIVATED PHOSPHOLIPASE C IN HUMAN PLATELETS REQUIRES CELL PERMEABILIZATION

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SUMMARY: The inhibition by guanosine 5'-[β-thio]diphosphate (GDPβS) of phospholipase C was compared in intact and saponin-permeabilized human platelets in order to assess whether effects of GDPβS on phospholipase C activation unrelated to guanine nucleotide binding function were occurring. GDPβS exhibited no effect on phospholipase C activity, monitored by phosphatidic acid formation, in intact platelets that were unstimulated or stimulated with 0.5 U/ml thrombin or 20 nM ONO-11113 (a stable thromboxane A_2 analogue). However, GDPβS did cause a marked decrease in the activity of phospholipase C in saponin-permeabilized platelets. Thus GDPβS is a viable tool for studying the role of G-proteins in transducing receptor-mediated activation of phospholipase C in platelets.

Agonist-induced activation of phospholipase C in human platelets causes the hydrolysis of phosphatidylinositol 4,5-bisphosphate producing inositol 1,4,5-triphosphate and diacylglycerol (1,2), which are generally recognized as important second messengers (3). Transduction of receptor occupancy to activation of phospholipase C is thought to be mediated by a guanine nucleotide binding protein, G_p (4). Much of the evidence implicating a role for G_p in hormone induced stimulation of phospholipase C has been provided using permeabilized platelets and the guanine nucleotide analogues GDPβS or GTPγS (5,6). GDPβS and GTPγS have proven to be useful tools in permeabilized cells and cell fractions for probing the relationship of GTP-binding proteins to phospholipase C activation (7). These guanine nucleotide analogues are unable to penetrate the plasma membrane and thus cannot exert their effects via G_p on intact cells, since G proteins are considered to be located at the cytoplasmic side of the plasma membrane (8). Further, guanine nucleotides and analogues are without effect on phospholipase C activity once the enzyme has been freed of G_p (9). Recently, however, it was reported that GDPβS (150 μM-3 mM) inhibits agonist-induced aggregation, Ca^{2+} mobilization and granule secretion in intact platelets (10). The authors concluded that the effects of GDPβS are therefore non-specific. The issue of whether results obtained with GDPβS are unrelated to the role of G proteins is one of some concern. To date, the inhibitory effects of GDPβS constitute the only evidence that receptor-

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directed agonist stimulation of phospholipase C is achieved through the mediation of a G protein. We addressed the question of whether non-specific effects of GDPβS are observed at concentrations necessary to inhibit the activation of phospholipase C. To this end, we have studied GDPβS effects on phospholipase C activity for both intact and permeabilized platelets.

MATERIALS AND METHODS

Preparation of Platelets. Human platelet-rich plasma, free of erythrocytes, was prepared fresh daily from 1 unit of blood donated by healthy individuals as described (11) in the presence of 0.5 μM-PGE_1 (Upjohn Company, Kalamazoo, MI, USA) and 1 mM aspirin. Platelets were obtained by centrifugation of the platelet-rich plasma 1200 x g for 5 minutes. The platelet pellet was resuspended in 5 ml of Buffer A containing 123.2 mM NaCl, 12.2 mM Na_3Citrate, 5.6 mM dextrose, 2.0 mM HEPES, 2.8 mM KCl, 8.9 mM $NaHCO_3$, 0.86 mM $MgCl_2$, 6.6 mM creatine phosphate, 45 units/ml creatine phosphokinase, 10.0 mM Trizma base (Sigma), pH 6.5. Platelets were incubated with 0.625 mCi/ml [^{32}P]P_i (New England Nuclear, 8500-9120 Ci/mmol) at 37°C for 60 minutes followed by sedimentation at 1200 x g for 90 seconds. The pellet was resuspended in 25 ml of buffer containing 140 mM NaCl, 20 mM HEPES, 1 mM EDTA, pH 7.1 and allowed to stand for 15 minutes. Afterward, the platelets were sedimented at 1200 x g for 5 minutes and resuspended to 1.75 x 10^9/ml in Buffer A, pH 7.1, without creatine phosphate and creatine phosphokinase.

Phosphatidic Acid Formation. Diacylglycerol formed as a consequence of phospholipase C activation is converted in the platelet to phosphatidic acid, which is therefore used as a monitor of phospholipase C activity. Washed platelets (100 μl; 1.75 x 10^8) were preincubated at 37°C with 3.4 volumes of Buffer B containing 120 mM KCl, 4 mM $MgCl_2$, 25 mM NaCl, 1 mM NaH_2PO_4, 1 mM EGTA, 280 μM Ca^{+2}, 0.5 mM ATP, 15 mM HEPES, pH 7.1, with or without 15 μg/ml saponin, for 8.75 minutes. ATP was included for saponin-permeabilized platelets in order to maintain pools of phosphatidylinositol 4,5-bisphosphate, but was present, as well, for intact platelet preparations to maintain comparable conditions. Saponin permeabilization was carried out on platelets which were not prelabeled with [^{32}P]P_i but were instead exposed to [^{32}P]-γ-ATP (10 μCi/ml) (12). After the initial 8.75 minutes, GDPβS (0-200 μM; Boehringer Mannheim) was added, followed 15 seconds later by the addition of buffer (intact platelets) or [^{32}P]-γ-ATP (saponin-permeabilized platelets). Buffer, α-thrombin (0.5 U/ml; a gift from Dr. Ken Mann) or thromboxane A_2 analogue ONO-11113 (20 nM; ONO Pharmaceutical Co., LTD., Osaka, Japan) was added 1 minute after the addition of [^{32}P]-γ-ATP or buffer. The entire reaction volume equaled 0.5 ml. The reaction was allowed to proceed for varied periods and quenched with 3.75 volumes of $CHCl_3$/MEOH/HCl (1:2:0.02, v/v/v). The phases were split by the addition of 1 volume of H_2O and 1 volume of $CHCl_3$. The [^{32}P]phosphatidic acid in the $CHCl_3$ phase was quantitated as described previously (11) and expressed as the mean ± the range of duplicate points.

RESULTS

Intact platelets were compared in parallel with saponin-permeabilized platelets with respect to GDPβS effects on phosphatidic acid accumulation. Intact platelets utilized endogenous [^{32}P]ATP to form [^{32}P]phosphatidic acid, whereas permeabilized platelets utilized the [^{32}P]γ-ATP provided exogenously. GDPβS (100 μM) had no effect on phospholipase C activity in intact platelets as measured by [^{32}P]phosphatidic acid formation, under basal conditions, or after stimulation with either thrombin or ONO-11113 (Fig. 1A). The saponin-

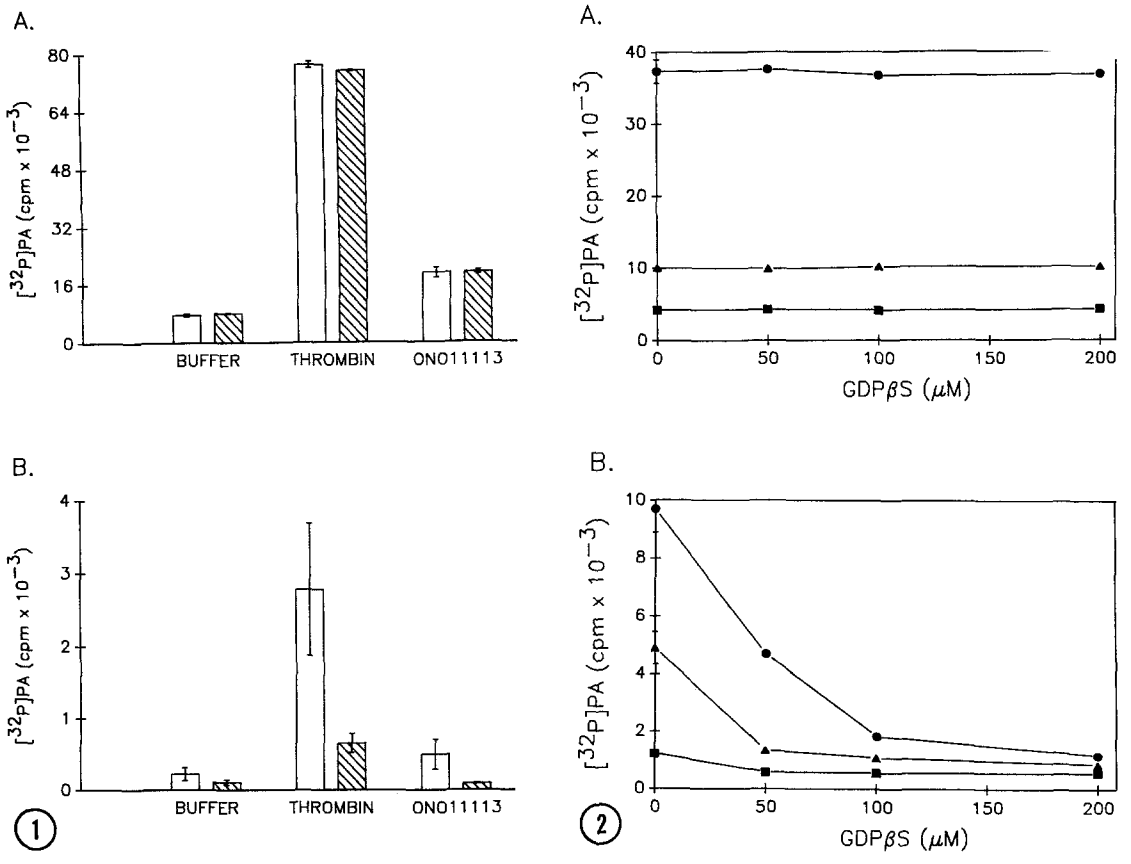

Figure 1. Panel A. [^{32}P]Phosphatidic acid formed in intact platelets in response to 2.5 minutes exposure to buffer, 0.5 U/ml thrombin or 20 nM ONO-11113. Bars indicate the amount of [^{32}P]phosphatidic acid formed in the presence (stripped bars) or absence (open bars) of 100 μM GDPβS.

Panel B. [^{32}P]Phosphatidic acid formed in saponin-permeabilized platelets in response to 2.5 minutes exposure to buffer, 0.5 U/ml thrombin or 20 nM ONO-11113. Bars indicate the amount of [^{32}P]phosphatidic acid formed in the presence (stripped bars) or absence (open bars) of 100 μM GDPβS.

Figure 2. Panel A. [^{32}P]Phosphatidic acid formed in intact platelets in response to 2.5 minutes exposure to buffer (closed squares), 0.5 U/ml thrombin (closed circles) or 20 nM ONO-11113 (closed triangles), in the presence of increasing amounts of GDPβS.

Panel B. [^{32}P]Phosphatidic acid formed in saponin-permeabilized platelets in response to 2.5 minutes exposure to buffer (closed squares), 0.5 U/ml thrombin (closed circles) or 20 nM ONO-11113 (closed triangles), in the presence of increasing amounts of GDPβS.

permeabilized platelets, however, showed a marked inhibition of phospholipase C activity in response to either agonist in the presence of GDPβS (Fig. 1B).

GDPβS (up to 200 μM) had no effects on phospholipase C activity in the intact platelets exposed to buffer, thrombin or ONO-11113 (Fig. 2A). In contrast, GDPβS at a concentration of 50 μM produced virtually complete inhibition of ONO-11113-induced [^{32}P]phosphatidic acid accumulation, and half-

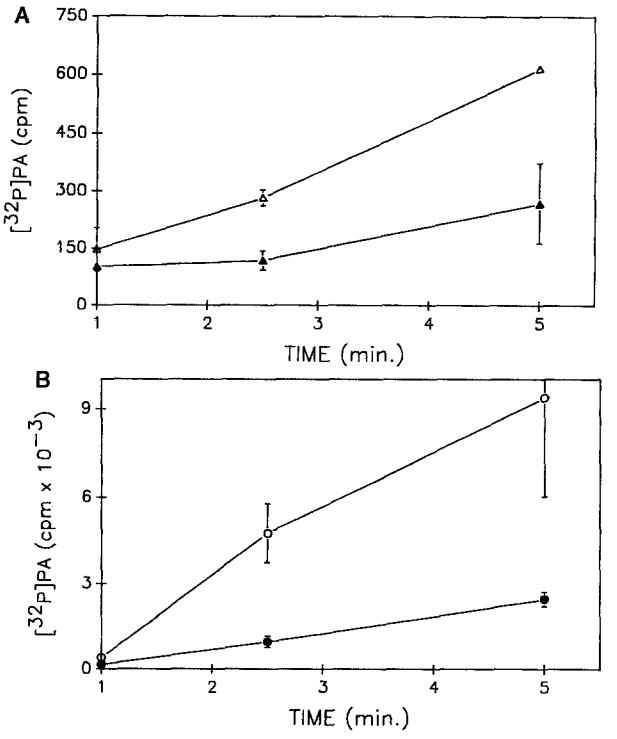

Figure 3. Panel A. [^{32}P]Phosphatidic acid formed in saponin-permeabilized platelets in response to 20 nM ONO-11113 for varied amounts of time. Platelets were incubated as in Figure 1B, but for varied periods with agonist in the absence (open triangles) or presence (filled triangles) of 100 μM GDPβS.

Panel B. [^{32}P]Phosphatidic acid formed in saponin-permeabilized platelets exposed to thrombin for varied amounts of time. Platelets were incubated as in Figure 3A, substituting 0.5 U/ml thrombin for ONO-11113 in the absence (open circles) or presence (filled circles) of GDPβS (100 μM).

maximal inhibition of thrombin-induced effects (Fig. 2B). More than 95% inhibition was observed for both agonists at 200 μM GDPβS.

The inhibitory effects of GDPβS on [^{32}P]phosphatidic acid accumulation in permeabilized platelets persisted through a more extended period of stimulation with ONO-11113 (Fig 3A) or thrombin (Fig 3B). Incubations beyond that shown were not performed, due to complications associated with protease activity in saponin-permeabilized platelets (Rittenhouse, unpublished observations).

DISCUSSION

We have provided evidence that permeabilization of human platelets is necessary in order for inhibition by up to 200 μM GDPβS of receptor-mediated phospholipase C activity to occur. It has been reported that GDPβS at various concentrations can inhibit agonist-induced platelet aggregation (150 μM GDPβS), Ca^{2+} mobilization (1.5 mM GDPβS) and granule secretion (1.5 mM GDPβS) in intact platelets (10). These authors also observed that 3 mM GDPβS inhibited thrombin-induced [^{32}P]P_i labeling of the "45 kDa" protein in intact platelets.

All of these changes are associated with phospholipase C activation, insofar as inositol 1,4,5-triphosphate promotes Ca^{2+} mobilization, and diacylglycerol, with consequent protein kinase C activation, promotes secretion and phosphorylation of "45 kDa" protein. Since our data indicate that GDPβS, at concentrations that optimally inhibit phospholipase C activation in permeabilized cells, has no such effect on intact platelets, we conclude that the reported nonspecific effects of GDPβS on Ca^{2+} mobilization, secretion, and protein phosphorylation are associated with the high concentrations of GDPβS employed. High concentrations (an order of magnitude greater than those that we have used) of GDPβS may become inhibitory to phospholipase C in intact platelets.

Our data are consistent with the notion that GDPβS, at the low concentrations employed here, exerts its effects primarily by binding to the G-protein coupled to phospholipase C, thereby preventing GTP from binding. Without the binding of GTP to this G-protein there can be no activation of the G-protein and subsequent activation of phospholipase C (13,14). GDPβS was unable to exert any inhibitory effect on phospholipase C activity in intact platelets presumably because it was unable to interact with the G-protein involved in modulating phospholipase C activity. Thus, it still remains that GDPβS is a viable tool for examining the role of GTP-binding proteins in transducing receptor stimulation of phospholipase C in permeabilized platelets. Furthermore, these data support the growing evidence which suggests that receptor-coupled phosphoinositide breakdown in platelets is governed by a GTP-binding protein.

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