Differential effects of G-protein activators on 5-hydroxytryptamine and platelet-derived growth factor release from streptolysin-O-permeabilized human platelets

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In this paper we have used streptolysin O (SLO)-permeabilized human platelets to examine the G-protein(s) that control Ca^{2+}-independent secretion from α and dense-core granules. As shown for electropermeabilized platelets, Ca^{2+} alone stimulated a concentration-dependent increase in 5-hydroxytryptamine (5-HT) (dense-core-granule marker) and platelet-derived growth factor (PDGF) (α-granule marker) release from the SLO-permeabilized cells. The EC_{50} values for Ca^{2+}-dependent 5-HT and PDGF release were 5 μM and 10 μM respectively. Guanosine 5′-[γ-thio]triphosphate (GTP[S]) (100 μM) stimulated Ca^{2+}-independent release from both α and dense-core granules. In contrast, AlF_4^- had no effect on Ca^{2+}-independent release from either α or dense-core granules. Neither GTP[S] nor AlF_4^- appeared to have a significant effect on Ca^{2+}-dependent release from α and dense-core granules. GTP[S] can activate both heterotrimeric and low-molecular-mass G-proteins, whereas AlF_4^- activates only heterotrimeric G-proteins. Our results, therefore suggest that secretion in the human platelet is regulated by a small G-protein. Both GTP[S]- and Ca^{2+}-dependent secretion were effected by extending the time between permeabilization with SLO and stimulation of secretion. GTP[S]-stimulated secretion from α and dense-core granules decreased rapidly after permeabilization. In contrast, Ca^{2+}-dependent 5-HT and PDGF release ran down at a much lower rate. These observations indicate that GTP[S] and Ca^{2+} act through parallel pathways to stimulate secretion from SLO-permeabilized platelets.

INTRODUCTION

Previous studies on electropermeabilized human platelets have examined the factors that regulate release of 5-hydroxytryptamine (5-HT) and β-thromboglobulin from dense-core and α granules respectively. Ca^{2+} alone stimulated release from both the dense-core and α granules [1]. Guanosine 5′-[γ-thio]triphosphate (GTP[S]), a slowly hydrolysable analogue of GTP, shifted the Ca^{2+} dose–response curve for release of 5-HT and β-thromboglobulin to the left [1,2]. A similar leftward shift in the Ca^{2+} dose–response curves for release from α and dense-core secretory granules was produced by activation of protein kinase C (PKC) by phorbol esters [1,3]. As addition of GTP[S] to electropermeabilized platelets activates PKC [1], the effect of GTP[S] on the Ca^{2+}-sensitivity for dense-core- and α-granule release may, in part, be explained by the activation of PKC via generation of diacylglycerol (DAG) by a GTP[S]-activated phospholipase C.

GTP[S] can also induce Ca^{2+}-independent secretion from α and dense-core granules [1]. Several studies have suggested that Ca^{2+}-independent GTP[S]-induced secretion may be due to activation of phospholipase D (PLD) [4]. In the absence of Ca^{2+}, GTP[S] stimulated the formation of PLD-induced phosphatidic acid (PA) [4]. In addition, there is an apparent correlation between the ability of bis-(*o*-aminophenoxy)ethane-*NNN′N′*-tetra-acetic acid (BAPTA) to inhibit GTP[S]-stimulated/Ca^{2+}-independent dense-core-granule release and PA formation [5]. Finally, ethanol inhibits both GTP[S]-stimulated PLD activity and dense-core-granule release [4]. All these observations are correlative, since there is no evidence for a direct link between PLD activity and secretion.

At present the G-protein that stimulates Ca^{2+}-independent secretion in platelets is unknown. Platelets express a number of heterotrimeric and low-molecular-mass G-proteins [6,7]. Studies in secretory cells have demonstrated that heterotrimeric and Ras-like small G-proteins can modulate regulated exocytosis. Heterotrimeric G-proteins are known to both potentiate and inhibit Ca^{2+}-dependent regulated exocytosis. The molecular identities of two of these proteins have been determined recently. Using G-protein-specific inhibitory peptides and antibodies, G_o has been shown to inhibit secretion in adrenal chromaffin cells [8], whereas G_{i3} stimulated exocytosis in Mast cells [9]. A number of small G-proteins have been linked with the control of vesicle-mediated protein transport [10]. One in particular, Rab3, has been implicated in the control of regulated exocytosis [11]. Interestingly, several laboratories have recently shown that the low-molecular-mass G-proteins, Arf and Rho, can modulate PLD activity in cells [12–14]. These results suggest the possibility that a small G-protein induces Ca^{2+}-independent secretion in human platelets by stimulating PLD activity.

In the present study we have described a streptolysin O (SLO)-permeabilized platelet preparation that we have used to characterize the nature of the G-proteins that regulate Ca^{2+}-independent secretion from α and dense-core granules. As previously described for electropermeabilized platelets [1], addition of Ca^{2+} induced release from α and dense-core granules. Neither GTP[S] nor

Abbreviations used: BAPTA, bis-(*o*-aminophenoxy)ethane-*NNN′N′*-tetra-acetic acid; DAG, diacylglycerol; PLD, phospholipase D; GTP[S], guanosine 5′-[γ-thio]triphosphate; 5-HT, 5-hydroxytryptamine; LDH, lactate dehydrogenase; PA, phosphatidic acid; PDGF, platelet-derived growth factor; PKC, protein kinase C; SLO, streptolysin O.

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AlF_4^- significantly shifted the Ca^{2+} dose–response curves for release from either α or dense-core granules. GTP[S], but not AlF_4^-, stimulated Ca^{2+}-independent release from α and dense-core granules. Controls comparing the influence of GTP[S] and AlF_4^- on DAG release showed that, under the experimental conditions used, AlF_4^- was active and equipotent to GTP[S]. As AlF_4^- only activates heterotrimeric G-proteins [15] (unlike GTP[S] which activates both heterotrimeric and small G-proteins) our finding that AlF_4^- cannot mimic the GTP[S] effect on granule release indicates that secretion in the human platelet may be directly controlled by a small G-protein.

MATERIALS AND METHODS

SLO was purchased from Murex Diagnostics, NorCross, GA, U.S.A. GTP[S], ATP and EGTA were obtained from Boehringer Mannheim, Indianapolis, IN, U.S.A. [^{3}H]Arachidonic acid (3 mCi/mmol), [γ-^{32}P]ATP (600 Ci/mmol) and [^{32}P]phosphorus were purchased from Dupont–NEN Boston, MA, U.S.A. The platelet-derived growth factor (PDGF) RIA kit and [^{3}H]5-HT were purchased from Amersham Corp., Arlington Heights, IL, U.S.A. All other biochemicals were supplied by Sigma Chemical Co., St. Louis, MO, U.S.A.

Platelet preparation

Whole blood was collected from healthy volunteers who did not ingest aspirin for 10 days, and platelet-rich plasma was prepared after collection by centrifugation at 150 ***g*** for approx. 15 min at room temperature. All manipulations were carried out at room temperature as previously described [16]. Briefly, platelet-rich plasma was treated for 5 min with 10 ng/ml prostaglandin I_2 and then pelleted by centrifugation at 800 ***g*** for 15 min. The platelet pellet was resuspended at 5×10^{10} platelets/ml in platelet-poor plasma by gentle trituration with a polyethylene transfer pipette, and labelled as described below. In those experiments in which the human platelets were not prelabelled with [^{3}H]5-HT the platelets were immediately filtered through a 2.5 cm $\times$ 20 cm Sepharose 4B-CL column equilibrated with Tyrode's–Hepes buffer containing 134 mM NaCl, 12 mM $NaHCO_3$, 3 mM KCl, 0.35 mM NaH_2PO_4, 1 mM $MgCl_2$, 5.6 mM dextrose, 3.5 g/l BSA and 10 mM Hepes, pH 7.4. The platelets were then incubated for 10 min at 25 °C prior to use.

[^{3}H]5-HT labelling of platelets

Human platelets (5×10^{10}/ml) were incubated at 30 °C for 1.5 h with 0.2 μCi/ml [^{3}H]5-HT. The [^{3}H]5-HT-labelled platelets were then gel filtered as described above.

DAG mass determinations

At the times indicated in the text, lipids were extracted into the organic phase as described by Bligh and Dyer [17]. DAG mass was quantified by the DAG kinase method as described by Preiss et al. [18].

PA determination

PA content was determined essentially as described previously by Pai et al. [19]. Briefly, platelets were labelled for 2 h at 30 °C with [^{3}H]arachidonic acid (5 μCi/ml) prior to filtration through Sepharose 4B-CL. At the indicated times phospholipids were extracted as described by Bligh and Dyer [17] and separated on Silica gel G plates with ethyl acetate/iso-octane/acetic acid/water (110/50/20/100, by vol.). The lipids were identified by using authentic standards and the spot containing PA was scraped off and quantified by liquid scintillation counting. In several experiments PA from unlabelled platelets was isolated as described above and then quantified by measuring inorganic phosphate after digestion of the PA as described by Dittmer and Wells [20].

Secretion assay

[^{3}H]5-HT radiolabelled platelets were diluted approx. 5-fold in permeabilization buffer (139 mM potassium glutamate, 20 mM Pipes, pH 6.6) to a final concentration of 1×10^8 platelets per ml. The platelet suspension was then divided into 100 μl aliquots, to which was added 100 μl of permeabilization buffer containing 1.0 unit/ml SLO. Since AlF_4^- stimulated secretion in intact platelets, the cell suspension was incubated with SLO for 2 min at 37 °C to ensure that maximal permeabilization was obtained prior to stimulation. Secretion was induced by the addition of 200 μl of permeabilization buffer (8 mM EGTA, 10 mM MgATP, 2 mM $MgCl_2$, sufficient $CaCl_2$ to give the desired free Ca^{2+} concentration) and either 200 μM GTP[S] or 100 μM AlF_4^-. The amount of $CaCl_2$ required to give the required free Ca^{2+} concentration was calculated using a computer program described previously [21]. The permeabilized platelets were incubated at 37 °C for 10 min and secretion terminated by rapidly placing the tubes in an ice bath. After 5 min at 4 °C, the platelets were pelleted by centrifugation at 12000 ***g*** for 1 min and the supernatants quickly removed for determination of [^{3}H]5-HT and PDGF release.

Measurement of secretion from dense-core and α granules

Secretion of [^{3}H]5-HT was quantified by counting 300 μl of supernatant in scintillant. PDGF released into the supernatant was quantified using a commercial RIA assay sold by Amersham International. Total platelet [^{3}H]5-HT and PDGF contents were determined by measuring [^{3}H]5-HT and PDGF released after lysis of platelets by addition of 1 vol. of 0.1 % (w/v) Triton X-100. Secretion levels of 5-HT and PDGF were expressed as percentages of cellular [^{3}H]5-HT or PDGF released above basal levels (i.e. in 1 nM Ca^{2+}). Basal release for [^{3}H]5-HT ranged between 4 and 8 % of total cellular [^{3}H]5-HT; no basal release of PDGF was detected.

RESULTS

Permeabilization of human platelets with SLO

To quantify SLO-induced platelet permeabilization, we measured the release of the cytosolic marker lactate dehydrogenase (LDH). As shown in Figure 1, maximal LDH release (84–86 %) was obtained with 0.4–0.6 unit/ml SLO. To assess whether the SLO was also permeabilizing the secretory granules we monitored radiolabelled 5-HT (a dense-core-granule marker) release. [^{3}H]5-HT release was found to mirror LDH release, reaching a maximum at 0.4–0.6 unit/ml SLO. This indicates that the 5-HT released probably reflects the emptying of a cytosolic pool which remains after labelling of the cells, not discharge from SLO-lysed dense-core granules. This hypothesis is supported by the fact that transmission electron micrographs of the permeabilized platelets showed no evidence of granule lysis (P. J. Padfield, N. Galvin and J. J. Baldassare, unpublished work). A concentration of 0.5 unit/ml SLO was used in all subsequent experiments.

Influence of Ca^{2+} on release from α and dense-core granules

Ca^{2+} induced concentration-dependent stimulation of 5-HT (dense-core-granule marker) and PDGF (α-granule marker)

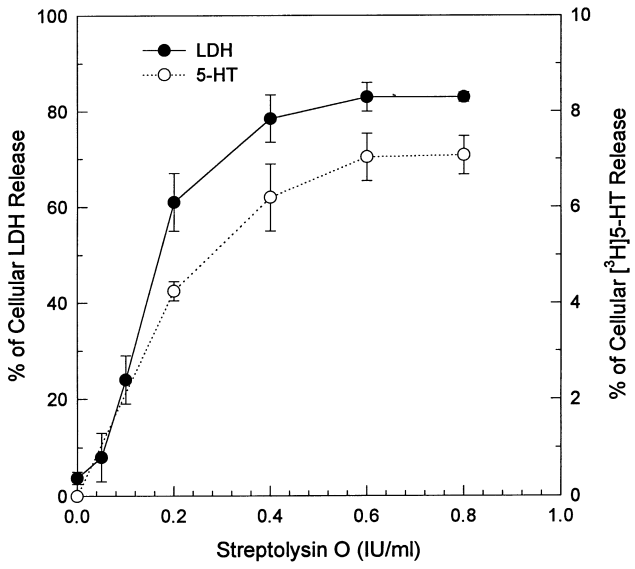

Figure 1 Effect of SLO concentration on LDH and [^{3}H]5-HT release

Platelets were labelled with [^{3}H]5-HT and then incubated at 37 °C with increasing concentrations of SLO. After 10 min the incubations were terminated and LDH and [^{3}H]5-HT release determined as described in the Materials and methods section. Release is expressed as a percentage of the total activity present in the cells before permeabilization. Results represent the means and the ranges of two independent experiments.

release (Figures 2a and 2b and Figures 3a and 3b). Maximal 5-HT release was obtained with 30 μM free Ca^{2+} and half-maximal release at 4–6 μM Ca^{2+}. The apparent EC_{50} for Ca^{2+}-dependent PDGF release, determined from a dose–response curve with maximal PDGF release at 100 μM Ca^{2+}, is 10 μM Ca^{2+}. The true EC_{50} will be higher than the apparent EC_{50} value calculated from these results, thus the Ca^{2+} dose–response curve for 5-HT release is therefore at least half an order of magnitude to the left of that for PDGF. This difference in Ca^{2+} sensitivity for secretion from α and dense-core granules has been observed previously in electropermeabilized platelets [1].

Influence of AlF_4^- and GTP[S] on granule release

GTP[S] (100 μM), a slowly hydrolysable analogue of GTP, stimulated Ca^{2+}-independent (1 nM Ca^{2+}) release of both 5-HT and PDGF. Addition of GTP[S] (100 μM) resulted in a small decrease (7.3 μM to 4.25 μM Ca^{2+}) in the EC_{50} for Ca^{2+}-dependent PDGF release but had no effect on the EC_{50} for Ca^{2+}-dependent 5-HT release (Figures 2a and 2b). However, AlF_4^- (50 μM) had no major effect on either 5-HT or PDGF release (Figures 3a and 3b). As seen in Figure 3(a), AlF_4^- stimulated a small increase in Ca^{2+}-independent (1 nM Ca^{2+}) 5-HT discharge, but this increase was found to be statistically insignificant. In contrast to AlF_4^-, which only activates heterotrimeric G-proteins, GTP[S] is known to stimulate both heterotrimeric and low-molecular-mass G-proteins [15]. Our findings would indicate that a small G-protein(s) can directly control both dense-core- and α-granule release from human platelets.

Controls for activity of AlF_4^-

To account for the possibility that AlF_4^- is inactive in the experimental buffers used for permeabilization, we compared the effects of AlF_4^- (50 μM) and GTP[S] (100 μM) on the formation

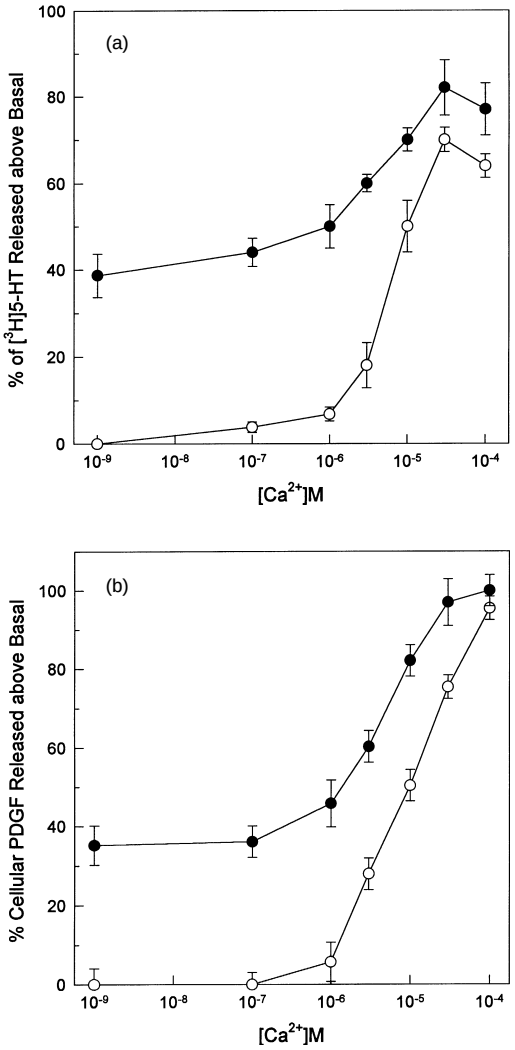

Figure 2 Effect of GTP[S] on the Ca^{2+} dose–response curves for (a) [^{3}H]5-HT and (b) PDGF release

[^{3}H]5-HT-labelled human platelets were permeabilized for 2 min with 0.5 unit/ml SLO. The cells were then challenged with increasing concentrations of Ca^{2+}, either alone (○) or in the presence of 100 μM GTP[S] (●). Secretion was terminated after 10 min and [^{3}H]5-HT (**a**) and PDGF (**b**) release determined. Results are expressed as percentages of cellular [^{3}H]5-HT or PDGF released above basal levels. Basal release was determined in the presence of 1 nM Ca^{2+}. The results represent the means ± S.E.M. from three independent experiments.

of DAG and PA at 1 nM and 1 μM free Ca^{2+}(Table 1). At 1 nM Ca^{2+}, neither GTP[S] nor AlF_4^- stimulated any detectable increase in DAG production. However, at 1 μM Ca^{2+} both AlF_4^- and GTP[S] stimulated an approximate 2-fold increase in DAG synthesis (Table 1). At 1 nM free Ca^{2+}, GTP[S] but not AlF_4^- stimulated a statistically significant increase in PA production, as measured by the increase in [^{3}H]arachidonate into PA. Similar increases in PA content were observed when PA levels were determined by measurement of inorganic phosphate in the PA fraction (results not shown). At 1 μM Ca^{2+}, GTP[S] and AlF_4^- both induced a 3.6–3.7-fold increase in PA synthesis (Table 1). These results demonstrate that at 1 μM Ca^{2+} both GTP[S] and AlF_4^- are equally active in stimulating increases in PA and DAG content. To demonstrate further that the EGTA present in the Ca^{2+}-free buffers did not inactivate the AlF_4^-, we next examined the effect of the addition of GTP[S] (100 μM) and AlF_4^- (50 μM)

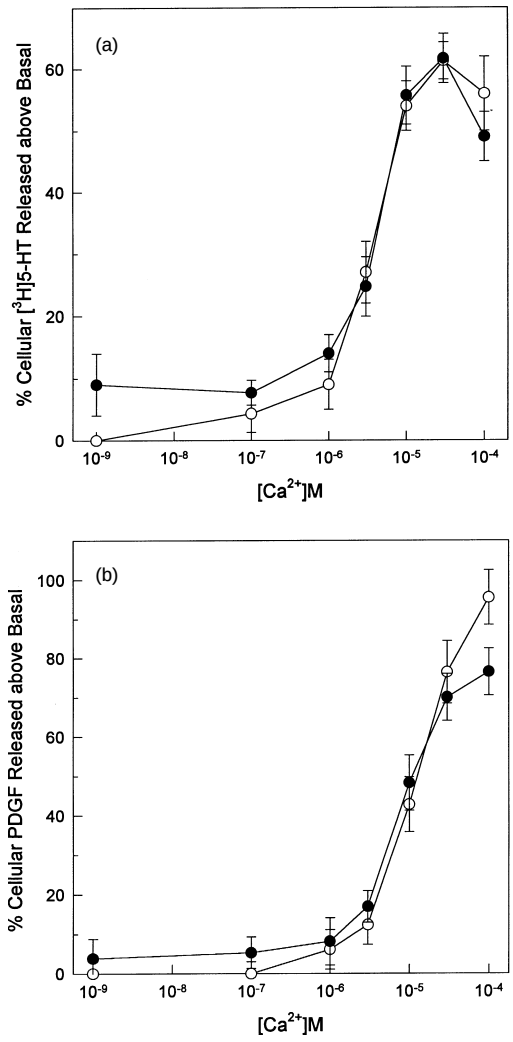

Figure 3 Effect of AlF_4^- on the Ca^{2+} dose–response curves for [^{3}H]5-HT (a) and PDGF (b) release

[^{3}H]5-HT-labelled human platelets were permeabilized for 2 min with 0.5 unit/ml SLO. The SLO-permeabilized platelets were then incubated with increasing concentrations of Ca^{2+}, either alone (○) or in the presence of 50 μM AlF_4^- (●). Secretion was terminated after 10 min and [^{3}H]5-HT (**a**) and PDGF (**b**) release determined. Results are expressed as percentages of cellular [^{3}H]5-HT or PDGF released above basal levels. Basal release was determined in the presence of 1 nM Ca^{2+}. The results represent the means ± S.E.M. from three independent experiments.

Table 1 Effect of AlF_4^- and GTP[S] on DAG formation in SLO-permeabilized platelets

Human platelets were permeabilized for 2 min with 0.5 unit/ml SLO. The SLO-permeabilized platelets were incubated for 10 min and DAG quantified as described in the Materials and methods section. The results represent the means ± S.E.M. from three independent experiments.

Conditions	DAG (mol%)	[^{3}H]PA content (c.p.m./10^9 platelets)
1×10^{-9} M Ca^{2+}	0.159 ± 0.025	894 ± 127
1×10^{-9} M Ca^{2+} + GTP[S]	0.156 ± 0.018	2327 ± 154
1×10^{-9} M Ca^{2+} + AlF_4^-	0.161 ± 0.023	796 ± 203
1×10^{-6} M Ca^{2+}	0.163 ± 0.031	2194 ± 316
1×10^{-6} M Ca^{2+} + GTP[S]	0.339 ± 0.018	11096 ± 518
1×10^{-6} M Ca^{2+} + AlF_4^-	0.297 ± 0.035	10992 ± 628

Table 2 Effect of EGTA on AlF_4^-- and GTP[S]-stimulated PA formation and 5-HT release in SLO-permeabilized platelets

Human platelets were permeabilized for 2 min with 0.5 unit/ml SLO in the presence or absence of EGTA. The SLO-permeabilized platelets were incubated for 10 min and PA and 5-HT quantified as described in the Materials and methods section. The results represent the means ± S.E.M. from three independent experiments.

Conditions	[^{3}H]PA formation (c.p.m./10^9 platelets)	[^{3}H]5-HT release[a](%)
+ EGTA		
1×10^{-9} M Ca^{2+}	909 ± 271	7.8 ± 5.9
1×10^{-9} M Ca^{2+} + GTP[S]	3749 ± 519	41.8 ± 7.3
1×10^{-9} M Ca^{2+} + AlF_4^-	1217 ± 361	13.2 ± 1.3
− EGTA		
1×10^{-9} M Ca^{2+}	1015 ± 447	13.9 ± 3.2
1×10^{-9} M Ca^{2+} + GTP[S]	3915 ± 725	50.9 ± 11.1
1×10^{-9} M Ca^{2+} + AlF_4^-	1187 ± 328	20.8 ± 7.2

[a] [^{3}H]5-HT release is presented as percentage of total cellular [^{3}H]5-HT.

on dense-core-granule release and PA production at nominally 1 nM free Ca^{2+} in EGTA-free buffers. Due to Ca^{2+} contamination the concentration of free Ca^{2+} in the EGTA-free buffers most probably ranged between 1 nM and 100 nM. The exclusion of EGTA did not influence the magnitude of 5-HT release or PA formation stimulated by either GTP[S] or AlF_4^- (Table 2). In the absence of EGTA there was a small but statistically insignificant increase in the basal release of 5-HT. This release is probably in response to the Ca^{2+} present as a contaminant in the permeabilization buffers, or leakage of Ca^{2+} from intracellular stores. The level of Ca^{2+} must be less than 1 μM since, at 1 μM Ca^{2+}, we have shown that GTP[S] and AlF_4^- stimulate a 3.5–4.0-fold increase in PA formation, and at Ca^{2+} concentrations above 1 μM Ca^{2+} 5-HT release increases dramatically.

Time-dependent decay of GTP[S] and Ca^{2+}-dependent granule release

Next we examined the effect of the time of addition of 100 μM GTP[S] or 30 μM Ca^{2+}on 5-HT and PDGF release from SLO-permeabilized human platelets. Delaying the stimulation of secretion after permeabilization produced a time-dependent decline (run-down) in both responses (Figures 4a and 4b). The kinetics of the run downs for Ca^{2+}- and GTP[S]-dependent release were very different. The GTP[S]-dependent/Ca^{2+}-independent 5-HT and PDGF release responses declined very rapidly. By 5–10 min of permeabilization both responses were almost abolished. In contrast, the decline in the Ca^{2+}-dependent 5-HT and PDGF release responses was biphasic, consisting of an initial rapid drop followed by a slow gradual decline. This meant that at the later time points it was still possible to obtain Ca^{2+}-dependent release even though GTP[S]-dependent discharge had totally run down.

DISCUSSION

SLO was found to permeabilize human platelets efficiently without causing any apparent damage to the secretory vesicles. As previously reported for electropermeabilized platelets, addition of Ca^{2+} to SLO-permeabilized platelets resulted in a

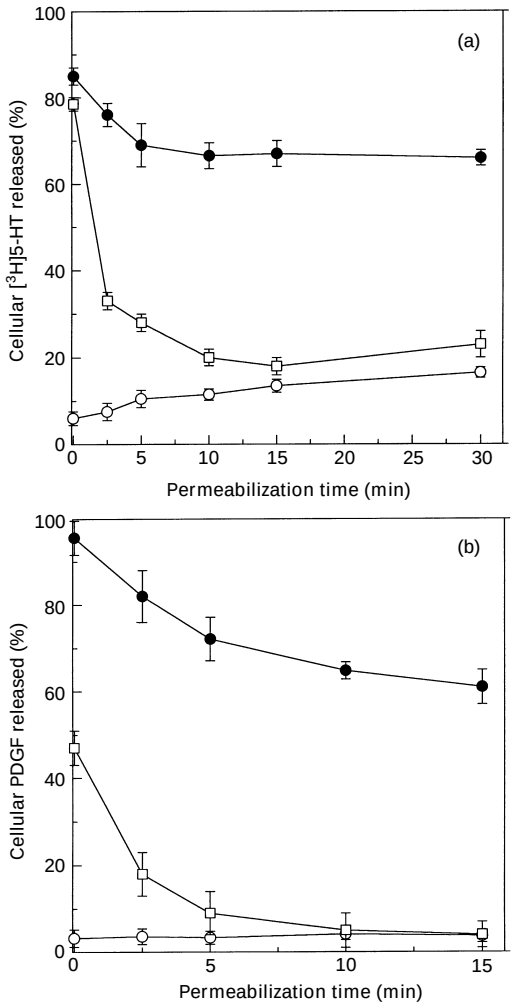

Figure 4 Effect of delaying stimulation after permeabilization on stimulated [^{3}H]5-HT (a) and PDGF (b) release

[^{3}H]5-HT-labelled human platelets were permeabilized with 0.5 unit/ml SLO. The SLO-permeabilized platelets were incubated at 37 °C for the indicated times before [^{3}H]5-HT and PDGF secretion were stimulated by addition of 1 nM Ca^{2+} (○), 1 nM Ca^{2+} + 100 μM GTP[S] (□), or 30 μM Ca^{2+} (●). Ten minutes after addition of the Ca^{2+} buffer the incubations were terminated and [^{3}H]5-HT (**a**) and PDGF (**b**) release were determined. The release responses are expressed as percentages of cellular [^{3}H]5-HT or PDGF. The results represent the means ± S.E.M. from three independent experiments.

concentration-dependent release from both α and dense-core granules, the approximate EC_{50} for Ca^{2+} for each response is 10 μM and 5 μM respectively. These values are higher than those previously obtained by Haslam and co-workers [1] using electropermeabilized platelets. This lack of agreement between our results and those of Haslam and co-workers may reflect the differences in the permeabilization techniques and experimental protocols employed. In this study platelets were permeabilized for 2 min prior to stimulation of secretion by addition of either Ca^{2+}, GTP[S] or AlF_4^-. In contrast to platelets permeabilized by electroporation, platelets treated with SLO rapidly lose their cytosolic proteins. Therefore, during the 2 min prior to addition of Ca^{2+}, GTP[S] or AlF_4^-, cytosolic components that regulate the Ca^{2+} sensitivity of the release response may be lost from the SLO-permeabilized platelets. This loss of cytosolic components may also account for the inability of GTP[S] to produce a decrease in the EC_{50} for Ca^{2+} for dense-core-granule release, as has previously been reported in electropermeabilized platelets [1]. The GTP[S]-induced increase in the Ca^{2+} sensitivity for granule release was thought to be due to DAG activation of PKC [1]. In this study we have shown that AlF_4^- and GTP[S] both stimulate increases in DAG. However, neither AlF_4^- nor GTP[S] induced a significant leftward shift in the Ca^{2+} dose–response curves for either α- or dense-core-granule release. Addition of phorbol 12-myristate 13-acetate (100 nM) to the SLO-permeabilized platelets caused a significant decrease in the EC_{50} for Ca^{2+} for release from α and dense-core granules (P. Padfield and J. J. Baldassare, unpublished work). These results suggest that the inability of AlF_4^- and GTP[S] to influence the Ca^{2+} sensitivity of granule secretion in the SLO-permeabilized cells is not due to the loss of the PKC pathway and, furthermore, suggests that in electroporated cells the GTP[S]-induced increase in Ca^{2+} sensitivity for secretion is not due to PKC activation, but to some other as yet unidentified mechanism.

The finding that GTP[S], but not AlF_4^-, can stimulate Ca^{2+}-independent (1 nM Ca^{2+}) release from α and dense-core granules indicates that a low-molecular-mass G-protein regulates secretion from both types of granule. The ineffectiveness of AlF_4^- is not a result of chelation of AlF_4^- by EGTA, since the exclusion of EGTA from the experimental buffers did not significantly alter the magnitude of the responses obtained with either GTP[S] or AlF_4^-. In addition, at 1 μM Ca^{2+} similar increases in AlF_4^-- and GTP[S]-stimulated DAG and PA formation were observed. The low-molecular-mass G-proteins most often linked with the control of regulated exocytosis are Rab isotypes, in particular Rab3. Modulation of Rab3 isoform levels, either by introduction of antisense oligonucleotides (anterior pituitary cells) [22] or by overexpression of native or mutated forms of the protein (chromaffin cells) [23] has led to inhibition of regulated exocytosis, both in intact and permeabilized cells. Recent studies [24] have demonstrated that platelets express Rab isoforms: Rab1, Rab3b, Rab6 and Rab8, and that the presence of thrombin, a potent inducer of secretion, resulted in the phosphorylation of Rab3b, Rab6 and Rab8. In addition to the Rab proteins platelets also express a number of other small G-proteins including Rap1B [25], Rap2B [26] and Rho [27]. Therefore, the Ca^{2+}-independent GTP[S]-induced response that we have observed may be mediated by one of a number of small G-proteins.

Haslam and Coorssen [4] found a strong correlation between PA formation from PLD-induced phosphatidylcholine hydrolysis and secretion of 5-HT. GTP[S] and phorbol ester added to electropermeabilized human platelets stimulated Ca^{2+}-independent PA formation. Addition of ethanol or BAPTA resulted in inhibition of PLD activity concomitant with loss of 5-HT secretion. Our observation that in the presence of 1 μM Ca^{2+} both AlF_4^- and GTP[S] stimulate a 3.5–4.0-fold increase in PA, although only GTP[S] stimulates 5-HT release, suggests that in human platelets PA formation is not involved in the control of secretion. However, further work is required to confirm this observation. Finally, the results of the run-down studies, shown in Figures 4(a) and 4(b), demonstrate that after 15–20 min of permeabilization it is possible to obtain Ca^{2+}-dependent release even though GTP[S]-dependent discharge had almost run down. This would indicate that Ca^{2+} and GTP[S] act through parallel pathways to stimulate release. In addition, our finding that Ca^{2+}-dependent PDGF or 5-HT release did not run down is very different from digitonin-permeabilized chromaffin cells [28] or SLO-permeabilized pancreatic acini [29] where the Ca^{2+}-dependent secretory response ran down rapidly after permeabilization. This suggests that the Ca^{2+}-dependent secretory response

in human platelets is not dependent upon diffusible cytosolic factors.

In summary, using SLO-permeabilized cells we have demonstrated that GTP[S] but not AlF_4^- can stimulate release from both α and dense-core granules. Controls showed AlF_4^- to be active and equipotent to GTP[S]. The disparity in the activities of AlF_4^- and GTP[S] indicate that a Ras-like small G-protein potentially controls α- and dense-core-granule secretion in the human platelet. This protein does not appear to stimulate discharge by activation of PLD as has been previously proposed.

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