Protein kinase C-dependent and Ca^{2+} -dependent mechanisms of secretion from streptolysin O-permeabilized platelets: effects of leakage of cytosolic proteins

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Human platelets containing dense granules labelled with 5-hydroxy[¹⁴C]tryptamine ([¹⁴C]5-HT) were permeabilized by exposure to streptolysin O (SLO) in the presence of 4 mM [γ -³²P]ATP. Addition of either 100 nM phorbol 12-myristate 13-acetate (PMA) or of Ca^{2+} (pCa 5) at the same time as SLO induced secretion of dense-granule [¹⁴C]5-HT and the phosphorylation of pleckstrin by protein kinase C (PKC). Ca^{2+} also induced phosphorylation of myosin P-light chains. Guanosine 5'-[γ -thio]-triphosphate (GTP[S], 100 μ M) did not stimulate secretion from SLO-permeabilized platelets in the absence of Ca^{2+} (pCa > 9), but greatly potentiated secretion in the presence of low PMA (10 nM) or low Ca^{2+} (pCa 6). However, GTP[S] did stimulate myosin P-light-chain phosphorylation in the absence of Ca^{2+} , an effect that was associated with morphological changes, including granule centralization. Inhibition of PKC and of pleckstrin phosphorylation by Ro 31-8220 blocked secretion induced by PMA or by GTP[S] and PMA in the absence of Ca^{2+} , but did not

prevent the GTP[S]-induced phosphorylation of myosin P-light chains or secretion induced by Ca^{2+} at pCa 5. When the time period between exposure of platelets to SLO and challenge at pCa > 9 with PMA or with GTP[S] and PMA was increased, there were rapid and parallel decreases in the secretion and pleckstrin phosphorylation responses, which were lost after 3–5 min. In contrast, the responsiveness of secretion to Ca^{2+} (pCa 5) or to GTP[S] and Ca^{2+} (pCa 6) persisted for at least 10 min after exposure of platelets to SLO, although the ability of pleckstrin to undergo phosphorylation was still lost after 3–5 min. Both PKC and pleckstrin were undetectable within platelets after 5 min exposure to SLO. The results suggest that the loss of responsiveness to PMA or to GTP[S] and PMA is attributable to the leakage of PKC (and possibly pleckstrin) from the platelets, whereas secretion stimulated by Ca^{2+} or by GTP[S] and Ca^{2+} utilizes membrane-associated Ca^{2+} - and GTP-binding proteins and occurs independently of PKC activation.

INTRODUCTION

Numerous studies have shown that the ability of physiological agonists, such as thrombin, to stimulate a maximal secretion of dense and α -granule constituents from intact platelets requires both an increase in cytosolic Ca^{2+} and activation of protein kinase C (PKC) [1,2]. The mechanisms by which this is achieved have been analysed in some detail using electroporeabilized platelets in which the intracellular concentrations of Ca^{2+} and other small molecules can be precisely controlled without disruption of the physical process of secretion [3]. These studies have shown that the sensitivity of secretion to Ca^{2+} is greatly enhanced by activation of PKC [4,5]. In addition, use of this experimental system has led to the recognition of at least two distinct roles for GTP-binding proteins in secretion from platelets [5], as found in experiments with other permeabilized cells [6]. Thus GTP or its more stable analogue, guanosine 5'-[γ -thio]-triphosphate (GTP[S]) is required for the G-protein- and Ca^{2+} -dependent activation of phospholipase C in permeabilized platelets [7], which results in the stimulation of PKC by 1,2-diacylglycerol [8]. Secondly, experiments with electroporeabilized platelets have shown that GTP[S] acts synergistically with PKC when this enzyme is stimulated by phorbol 12-myristate 13-acetate (PMA), to induce a Ca^{2+} -independent secretion of granule constituents [5]; this effect is associated with the activation of phospholipase D, but not of phospholipase C [8,9]. GTP[S] also potentiated Ca^{2+} -induced secretion from permeabilized platelets after protein kinase activities had been

inhibited with staurosporine [8]. Thus results with electroporeabilized platelets have indicated that an optimal stimulation of secretion of 5-hydroxytryptamine (5-HT) from platelet dense granules or of β -thromboglobulin from α -granules can be achieved with any two of GTP[S], PMA and Ca^{2+} [8]. In the latter work the role of PKC was readily defined by monitoring the phosphorylation of its major platelet substrate, pleckstrin [5,8].

Further analysis of the mechanisms involved in the actions of these stimuli would be facilitated by the use of platelets permeabilized by a mechanism that permits the leakage, identification and reconstitution of proteins that contribute to the regulation of secretion. This approach has been applied successfully to several other cell types and has led to the identification of varied proteins that may participate in the regulation of secretion, including annexin II [10] and the catalytic subunit of cyclic AMP-dependent protein kinase [11] in adrenal chromaffin cells, phosphatidylinositol transfer protein in PC12 and HL60 cells [12,13], phosphatidylinositol 4-phosphate 5-kinase in PC12 cells [14] and the low- M_r GTP-binding protein ARF in HL60 cells [13]. One of the more successful methods of permeabilization used in these studies has been treatment of cells with streptolysin O (SLO) which, by interaction with cholesterol, generates large variable-sized pores that permit the egress and entry of proteins [15,16]. This method has been used with platelets in one study [17], in which GTP[S] induced a marked Ca^{2+} -independent secretion of granule constituents and failed to enhance the Ca^{2+} -sensitivity of secretion. These results differ from those obtained in previous work with

Abbreviations used: PKC, protein kinase C; SLO, streptolysin O; 5-HT, 5-hydroxytryptamine; PMA, phorbol 12-myristate 13-acetate; GTP[S], guanosine 5'-[γ -thio]triphosphate; ECL, enhanced chemiluminescence.

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electropermeabilized platelets. We have now carried out a study with SLO-permeabilized platelets in which the relationship between secretion induced by GTP[S] and the phosphorylation of platelet proteins was examined, both before and after leakage of cytosolic proteins. The results indicate that activation of PKC is required for GTP[S] to induce a Ca^{2+} -independent secretion of 5-HT, and that GTP[S] can also potentiate the effects of Ca^{2+} by a PKC-independent mechanism. In addition, we found that GTP[S] induced a Ca^{2+} - and PKC-independent phosphorylation of platelet myosin P-light chains. Some of these results have been published in a preliminary form [18].

EXPERIMENTAL

Materials

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (3000 Ci/mmol) was obtained from Du Pont Canada Inc. (Mississauga, ON, Canada). 5-Hydroxy-[*side chain*- ^{14}C]tryptamine ($[\text{14C}]\text{5-HT}$) (55 mCi/mmol), horseradish peroxidase-linked donkey anti-rabbit IgG and enhanced chemiluminescence (ECL) reagents were from Amersham Canada Ltd. (Oakville, ON, Canada). SLO was supplied by Wellcome Diagnostics (Wellcome Foundation, Dartford, Kent, U.K.). GTP[S] and staurosporine were from Boehringer Mannheim Canada (Laval, PQ, Canada) and PMA and heparin from Sigma (St. Louis, MO, U.S.A.). Anti-pan PKC antibody was purchased from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.) and PVDF membrane (Immobilon-P) from Millipore (Canada) Ltd. (Mississauga, ON, Canada). Ro 31-8220 [19] was a gift from Dr. P. D. Davis of Roche Products (Welwyn Garden City, Herts., U.K.). Other materials were from sources listed previously [5].

Isolation and washing of human platelets

Blood (200 ml) was obtained from healthy donors who had taken no drugs, including aspirin, for at least 7 days and was collected into citric acid/sodium citrate/dextrose ('ACD') anticoagulant [20]. Platelet-rich plasma was obtained by centrifugation at $140 g_{av}$ for 15 min at room temperature. The platelets were concentrated by centrifugation of the latter at $1160 g_{av}$ for 15 min at room temperature and resuspension in 10 ml of plasma. They were then incubated for a total of 20 min at 37°C with two additions of $0.75 \mu\text{M}$ $[\text{14C}]\text{5-HT}$ (total $0.8 \mu\text{Ci}$). The labelled platelets were isolated and resuspended in Ca^{2+} -free Tyrode's solution (37°C) containing 5 mM Pipes (pH 6.5), 0.35% (w/v) BSA, 30 μg of apyrase/ml and 50 units of heparin/ml and were washed twice more by centrifugation at $800 g_{av}$ and resuspension in the same medium without heparin [21]. The platelets were finally centrifuged at $385 g_{av}$ for 10 min at room temperature, resuspended in buffer A (120 mM sodium glutamate, 5 mM potassium glutamate, 20 mM Hepes, 2.5 mM EDTA, 2.5 mM EGTA, 3.15 mM MgCl_2 , adjusted to pH 7.4 with NaOH) at a concentration of 8×10^8 platelets/ml and were stored at 25°C until used (within 20–100 min).

Permeabilization of platelets with streptolysin O and assays of secretion and protein phosphorylation

Permeabilization of platelets was carried out by mixing platelet suspension ($50 \mu\text{l}$, 4×10^7 platelets) with an equal volume of buffer A containing SLO, ATP, sufficient CaCl_2 or additional EGTA to give the desired pCa value and any other additions. The final concentration of SLO was 0.4 unit/ml and that of ATP was 4 mM. Additional EGTA was included to obtain a pCa value > 9 (final concentration 12.5 mM) [5]. The compositions of Ca^{2+} buffers and pCa values were calculated using the binding constants given in [22]. PMA, Ro 31-8220 and staurosporine

were dissolved in DMSO and included with the above additions. The permeabilized platelets were incubated with additions for 5 min at 25°C and then mixed with the appropriate stopping solution (see below). In some experiments, platelets were permeabilized as described above, except that stimulatory additions (PMA, GTP[S], Ca^{2+} buffers) were added at specific times after the platelets were permeabilized. The incubations were then allowed to proceed for an additional 5 min. The secretion of $[\text{14C}]\text{5-HT}$ from platelet dense granules was determined in incubations stopped with 5 vol. of 0.15 M KCl containing 1.8% (w/v) paraformaldehyde and 6 mM EDTA (adjusted to pH 7.4 with KOH) and was expressed as a percentage of the total platelet $[\text{14C}]\text{5-HT}$ [23]. To determine protein phosphorylation, incubations were carried out as described above, but with the addition of 10 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. These incubations were terminated by the addition of 5 vol. of ice-cold 10% (w/v) trichloroacetic acid. The precipitated proteins were dissolved in electrophoresis sample buffer and separated by SDS/PAGE [24]. Labelled proteins were located by autoradiography and, in some experiments, areas of the gels containing pleckstrin and myosin P-light chains were excised and counted for ^{32}P radioactivity, which was measured as Čerenkov radiation in 0.01% (w/v) 4-methylumbelliferone.

Measurement of the leakage of lactate dehydrogenase from SLO-permeabilized platelets

Samples ($50 \mu\text{l}$) of platelet suspension were mixed with $50 \mu\text{l}$ of additions in buffer A, including SLO, as above. After incubation, these samples were centrifuged at $15000 g$ for 20 s, using a microcentrifuge. Supernatant and pellet fractions were immediately separated and mixed with 2 ml of phosphate buffer (0.05 M KH_2PO_4 adjusted to pH 7.5 with KOH) containing 0.1% (w/v) Triton X-100. Lactate dehydrogenase was assayed as described in [25].

Analysis of the leakage of pleckstrin and PKC from SLO-permeabilized platelets by immunoblotting

Incubation mixtures (1 ml) containing EGTA to give a pCa > 9 , ATP and SLO were incubated at 25°C . At appropriate intervals, samples ($50 \mu\text{l}$) were centrifuged at $15000 g$ for 10 s, using a microcentrifuge. The supernatant and pellet fractions were immediately separated. Protein was then precipitated with 10% (w/v) trichloroacetic acid and subsequently dissolved in electrophoresis sample buffer. For immunodetection of pleckstrin, platelet proteins were separated by SDS/PAGE and electroblotted on to Immobilon-P using a transfer buffer [25 mM Tris/192 mM glycine/20% (v/v) methanol] containing 0.05% SDS. Blots were blocked with Tris-buffered saline (1.5 M NaCl/0.1 M Tris/HCl, pH 7.4) containing 0.1% (v/v) Tween-20 and 5% (w/v) non-fat milk powder, and probed with a rabbit polyclonal antibody raised against a peptide corresponding to the C-terminal 14 residues of pleckstrin. For immunodetection of PKC, proteins were separated by SDS/PAGE using 10% acrylamide, and electroblotted on to Immobilon-P using transfer buffer without SDS. Blots were blocked with Tris-buffered saline containing 0.1% (v/v) Tween-20 and 3% (w/v) milk powder and probed with an anti-pan PKC antibody. Immunoreactive proteins were detected by ECL using a donkey anti-rabbit IgG-horseradish peroxidase conjugate.

Electron microscopy of SLO-permeabilized platelets

After incubation for 5 min at 25°C with SLO and appropriate additions, 1.0 ml samples of platelet suspension (4×10^8 platelets)

were mixed with 4.0 ml of ice-cold fixative (2.5 % glutaraldehyde in 12 mM sodium cacodylate, pH 7.4). The platelets were fixed in this medium for 1 h at 0 °C, washed in 0.14 M cacodylate, pH 7.4, and postfixed with 1 % OsO_4 . After dehydration with graded ethanol concentrations and propylene oxide, the platelet pellet was infiltrated for 2 h with Spurr's resin (Marivac, Halifax, NS, Canada), which was then polymerized at 65 °C overnight. Ultrathin sections (70 nm) were cut on a Reichert (Vienna, Austria) Ultracut S microtome and stained with uranyl acetate and lead citrate (Marivac). Sections were viewed with a JEOL 1200 EX Biosystem electron microscope.

RESULTS

Factors affecting secretion from SLO-permeabilized platelets

Since previous studies have demonstrated that Ca^{2+} ions stimulate secretion from electroporabilized platelets and that the sensitivity of these platelets to Ca^{2+} is greatly enhanced by activation of protein kinase C [4] or addition of GTP[S] [5,26], we initially investigated the effects of these factors on secretion from SLO-permeabilized platelets. To facilitate comparison of the results with earlier work, the platelets were washed and resuspended at 25 °C in a Ca^{2+} -free medium containing glutamate as the principal anion [5,23]. However, it was necessary to use Na^+ rather than K^+ as the principal cation to prevent a gradual loss of [^{14}C]5-HT from the platelets prior to permeabilization. The concentration of SLO required for optimal permeabilization of the plasma membrane with minimal release of granule contents was determined to be 0.4 units/ml in platelet suspension containing 4×10^8 platelets/ml. Addition of SLO to these platelet preparations in the effective absence of Ca^{2+} (12.5 mM EGTA, pCa

Figure 1 Ca^{2+} -independent secretion of [^{14}C]5-HT from SLO-permeabilized platelets stimulated by PMA or by PMA with GTP[S]

Platelets labelled with [^{14}C]5-HT were permeabilized at a pCa value > 9 and incubated at 25 °C for 5 min in the presence of the indicated concentrations of PMA, either without GTP[S] (□) or with 100 μM GTP[S] (■). PMA and GTP[S] were added at the same time as SLO. The percentage of [^{14}C]5-HT released was then measured; values are means $\pm$ S.E.M. from three determinations.

Figure 2 Secretion of [^{14}C]5-HT and phosphorylation of pleckstrin and myosin light chains in SLO-permeabilized platelets stimulated by Ca^{2+} , Ca^{2+} with GTP[S] or Ca^{2+} with PMA

Platelets labelled with [^{14}C]5-HT were permeabilized in the presence of Ca^{2+} buffers adjusted to give the indicated pCa values with no other additions (●), 100 μM GTP[S] (▲) or 10 nM PMA (■). GTP[S] and PMA were added at the same time as SLO. (a) Incubations were carried out for 5 min at 25 °C before measurement of the percent [^{14}C]5-HT released; (b) and (c), parallel incubations were carried out in the presence of [$\gamma\text{-}^{32}\text{P}$]ATP and ^{32}P incorporation into pleckstrin (b) and myosin P-light chains (myosin P-LCs) (c) was determined. Values are means $\pm$ S.E.M. from three determinations (secretion) or means $\pm$ range from two determinations (protein phosphorylation).

> 9) led to the leakage of only 9 ± 1 % of the platelet [^{14}C]5-HT in 5 min incubations at 25 °C (mean $\pm$ S.E.M., 17 expts.), indicating that there was no loss of dense-granule integrity. In contrast, when Ca^{2+} at a final pCa of 5 was added with SLO, secretion of 78 ± 2 % of the platelet [^{14}C]5-HT was observed after 5 min (mean $\pm$ S.E.M., 15 expts.). Analysis of the time course of the action of Ca^{2+} at pCa 5 showed that secretion of [^{14}C]5-HT was almost maximal after incubation for 2 min. In subsequent experiments, 5 min incubations were used. Although addition of 100 nM PMA at the same time as SLO to platelets at a pCa > 9 caused secretion of 43 ± 3 % of [^{14}C]5-HT (mean $\pm$ S.E.M., nine expts.), addition of 10 nM PMA had little or no effect on secretion in the absence of Ca^{2+} (Figure 1). These effects were comparable with those caused by PMA in the absence of SLO. In contrast, addition of 100 μM GTP[S] did not induce secretion from intact platelets or from permeabilized platelets in the absence of Ca^{2+} ions. However, addition of 10 nM PMA and 100 μM GTP[S] provided a potent stimulus to secretion from

Table 1 Effect of the PKC inhibitor Ro 31-8220 on Ca^{2+} -independent secretion of [^{14}C]5-HT and the phosphorylation of pleckstrin and myosin light chains in SLO-permeabilized platelets

Platelets labelled with [^{14}C]5-HT were permeabilized at a pCa > 9, either with no other additions or in the presence of 100 μM GTP[S], 10 nM PMA or 100 μM GTP[S] and 10 nM PMA, in each case in the absence or presence of 3 μM Ro 31-8220. All additions were made at the same time as SLO. Incubations were for 5 min at 25 °C before measurement of the percentage of [^{14}C]5-HT released. Parallel incubations were carried out in the presence of [γ - ^{32}P]ATP, and ^{32}P incorporation into pleckstrin and myosin P-light chains (P-LCs) was determined. Values for secretion are means $\pm$ S.E.M. from three determinations (two with GTP[S] + PMA), whereas values for protein phosphorylation are means $\pm$ range from two determinations.

Additions	Release of [^{14}C]5-HT (% of total)	$10^{-3} \times ^{32}\text{P}$ incorporation into pleckstrin (c.p.m./ 10^9 platelets)	$10^{-3} \times ^{32}\text{P}$ incorporation into myosin P-LCs (c.p.m./ 10^9 platelets)
None	11 $\pm$ 0	1.0 $\pm$ 0.0	0.9 $\pm$ 0.1
Ro 31-8220	12 $\pm$ 0	0.9 $\pm$ 0.0	0.8 $\pm$ 0.0
GTP[S]	11 $\pm$ 0	3.0 $\pm$ 0.2	3.3 $\pm$ 0.0
GTP[S] + Ro 31-8220	13 $\pm$ 1	1.4 $\pm$ 0.0	3.0 $\pm$ 0.0
PMA	20 $\pm$ 5	5.7 $\pm$ 0.3	1.1 $\pm$ 0.4
PMA + Ro 31-8220	13 $\pm$ 1	1.7 $\pm$ 0.4	0.8 $\pm$ 0.0
GTP[S] + PMA	71 $\pm$ 5	11.6 $\pm$ 0.5	3.2 $\pm$ 0.4
GTP[S] + PMA + Ro 31-8220	12 $\pm$ 0	3.2 $\pm$ 0.1	2.8 $\pm$ 0.0

Figure 3 Effects of Ro 31-8220 and staurosporine on secretion of [^{14}C]5-HT and on the phosphorylation of pleckstrin and myosin light chains in SLO-permeabilized platelets stimulated by Ca^{2+} or by Ca^{2+} and GTP[S]

Samples of platelets labelled with [^{14}C]5-HT were mixed with Ca^{2+} buffers adjusted to give the indicated pCa values and 0.2% DMSO (●), 10 μM Ro 31-8220 (▲) or 2 μM staurosporine (■), either in the absence (a, b and c) or presence (d, e and f) of 100 μM GTP[S]. After incubation for 1 min at 25 °C, the platelets were permeabilized by addition of SLO and incubated for a further 5 min. The percent secretion of [^{14}C]5-HT (a and d) was then measured. Parallel incubations were carried out in the presence of [γ - ^{32}P]ATP and ^{32}P incorporation into pleckstrin (b and e) and myosin P-light chains (myosin P-LCs) (c and f) was determined. Values are means $\pm$ S.E.M. from three determinations (secretion) or means $\pm$ range from two determinations (protein phosphorylation).

permeabilized platelets, indicating that these compounds acted synergistically (Figure 1). Similarly, synergism was observed between the effects of Ca^{2+} and PMA and between those of Ca^{2+} and GTP[S] (Figure 2a). Thus, at pCa 6, relatively little secretion of [^{14}C]5-HT occurred with Ca^{2+} alone ($19 \pm 4\%$, mean $\pm$ S.E.M., 12 expts.), but marked secretion was observed when 10 nM PMA (61%, mean for two expts.) or 100 μM GTP[S] ($85 \pm 1\%$, mean $\pm$ S.E.M., 11 expts.) was also present. At a first approximation, 10 nM PMA increased the sensitivity of the platelets to Ca^{2+} by an order of magnitude and 100 μM GTP[S] by at least two orders of magnitude (Figure 2a).

The extent of platelet permeabilization achieved with SLO in these experiments was monitored by measuring the release of lactate dehydrogenase into the medium. After incubation of platelets with SLO (0.4 units/ml) for 5 min at 25 °C, 71–76% of platelet lactate dehydrogenase was found in the suspending medium, regardless of whether the incubation mixtures contained no additions (pCa > 8), 100 nM PMA with or without 100 μM GTP[S] or Ca^{2+} at pCa 5. After 30 min incubation, 95–97% of platelet lactate dehydrogenase had diffused from the platelets. These results confirm that, at the concentration used, SLO caused full permeabilization of platelets and show that this was not affected by the presence of the stimuli investigated.

Relationships between protein phosphorylation and secretion of [^{14}C]5-HT in SLO-permeabilized platelets

Previous studies have investigated the phosphorylation of pleckstrin and of the P-light chains of myosin in electroporated platelets equilibrated with [γ - ^{32}P]ATP [5,23]. In the present work, platelets were permeabilized with SLO in the presence of [γ - ^{32}P]ATP and the ^{32}P incorporation into these proteins was determined after 5 min. Ca^{2+} caused concentration-dependent increases in the phosphorylation of both pleckstrin and myosin P-light chains, which were maximal at pCa 5 and decreased at higher Ca^{2+} concentrations (Figures 2b and 2c). Considerable phosphorylation of these proteins was also seen at pCa 6, despite the relatively low secretion of [^{14}C]5-HT. Addition of a low PMA concentration (10 nM) at the same time as SLO had little effect on the phosphorylation of myosin at any pCa value, but greatly stimulated the phosphorylation of pleckstrin at all pCa values

Figure 4 Effects of increases in the time between permeabilization of platelets and challenge with PMA or with PMA and GTP[S] on protein phosphorylation and secretion of [^{14}C]5-HT in the absence of Ca^{2+}

Platelets labelled with [^{14}C]5-HT were permeabilized with SLO at $\text{pCa} > 9$ in the presence of [$\gamma\text{-}^{32}\text{P}$]ATP and then preincubated for various periods before addition of vehicle (DMSO), PMA or both PMA and GTP[S]. Incubations were continued for 5 min at 25°C and ^{32}P incorporated into platelet proteins was then determined by SDS/PAGE and autoradiography. Lanes 1 and 2, permeabilized platelets were preincubated for 0 and 10 min respectively and then incubated for 5 min with no additions, apart from 0.2% DMSO; lanes 3–7, permeabilized platelets were preincubated for 0, 1, 3, 5 and 10 min respectively and then incubated with 100 nM PMA; lanes 8–12, permeabilized platelets were preincubated with SLO for 0, 1, 3, 5 and 10 min respectively and then incubated with 100 nM PMA and 100 μM GTP[S]. Parallel incubations were carried out in the absence of [$\gamma\text{-}^{32}\text{P}$]ATP for measurement of the percentage of platelet [^{14}C]5-HT secreted. Values shown below are means from three determinations.

from > 9 to 5. This phosphorylation of pleckstrin did not correlate with secretion (Figures 2a and 2b). At $\text{pCa} > 9$, addition of 100 μM GTP[S] caused only a small (15–20% maximal) increase in pleckstrin phosphorylation but increased the phosphorylation of the P-light chains of myosin to $41 \pm 4\%$ of the level seen at $\text{pCa} 5$ (mean $\pm$ S.E.M., six expts.; see also Figures 2b and 2c), suggesting that GTP[S] can induce protein phosphorylation by a mechanism distinct from those stimulated by PMA or Ca^{2+} . At $\text{pCa} 7$, GTP[S] enhanced the phosphorylation of both pleckstrin and myosin, as well as secretion. With higher Ca^{2+} concentrations ($\text{pCa} 5$), GTP[S] exerted no effect on myosin phosphorylation additional to that observed in the presence of Ca^{2+} or of Ca^{2+} and 10 nM PMA. These results suggest that phosphorylation of myosin P-light chains occurred at the same sites, whether the platelets were stimulated by GTP[S] at $\text{pCa} > 9$ or by Ca^{2+} at $\text{pCa} 5$.

To investigate the roles of protein phosphorylation in secretion from SLO-permeabilized platelets further, we studied the effects of Ro 31-8220, a selective inhibitor of PKC [19], and of staurosporine, a broad-spectrum protein kinase inhibitor. At $\text{pCa} > 9$, 3 μM Ro 31-8220 completely inhibited the stimulation of secretion caused by 10 nM PMA with 100 μM GTP[S] and, at the same time, markedly diminished the phosphorylation of pleckstrin induced by these compounds, whether they were added individually or in combination (Table 1). In contrast, Ro 31-8220 did not inhibit the phosphorylation of myosin P-light chains induced by GTP[S], whether PMA was present or not (Table 1). Experiments with a higher concentration of Ro 31-

Figure 5 Effects of increases in the time between permeabilization of platelets and challenge with high Ca^{2+} on secretion of [^{14}C]5-HT and protein phosphorylation

Platelets labelled with [^{14}C]5-HT were permeabilized with SLO at $\text{pCa} > 8$ in the presence of [$\gamma\text{-}^{32}\text{P}$]ATP, pre-incubated for various periods and then stimulated by addition of Ca^{2+} buffer giving a pCa value of 5. Incubations were continued for 5 min at 25°C and measurement of ^{32}P incorporation into platelet proteins was detected by SDS/PAGE and autoradiography. Lane 1, no preincubation; lanes 2–5, preincubation for 1, 3, 5, and 10 min respectively. Parallel incubations were carried out in the absence of [$\gamma\text{-}^{32}\text{P}$]ATP for the measurement of the percentage of platelet [^{14}C]5-HT secreted. Values are means from three determinations.

8220 (10 μM) or with staurosporine (2 μM) showed that these compounds completely inhibited any phosphorylation of pleckstrin caused by Ca^{2+} (Figure 3b) or by Ca^{2+} with GTP[S] (Figure 3e) and prevented secretion induced by Ca^{2+} at $\text{pCa} 6$ (Figure 3a) or by Ca^{2+} at $\text{pCa} 7$ with GTP[S] (Figure 3d). However, these compounds only slightly inhibited secretion induced by Ca^{2+} at $\text{pCa} 5$, whether GTP[S] was present or not. This indicates that high concentrations of Ca^{2+} can induce secretion of [^{14}C]5-HT by a mechanism that does not require activation of PKC. Although Ro 31-8220 and staurosporine had similar effects on pleckstrin phosphorylation and secretion, only staurosporine inhibited myosin P-light chain phosphorylation completely. Ro 31-8220 (10 μM) did not affect the phosphorylation of myosin P-light chains induced by GTP[S] (Figure 3f), but did partially inhibit that caused by Ca^{2+} . The latter component may represent phosphorylation attributable to the activation of PKC by Ca^{2+} -dependent mechanisms.

Effects of preincubation of platelets with SLO on their responsiveness to stimulation

A common characteristic of cells that have been permeabilized with SLO is a decrease in responsiveness to stimulation that is dependent on the length of preincubation with this permeabilizing agent (e.g. [27]). We have therefore studied the effects of increasing the time of preincubation of platelets with SLO on the secretion of [^{14}C]5-HT and protein phosphorylation caused by PMA, PMA with GTP[S], Ca^{2+} and Ca^{2+} with GTP[S] (Figures 4–6).

A 1 min preincubation with SLO abolished the ability of 100 nM PMA to stimulate Ca^{2+} -independent secretion, whereas

Figure 6 Effects of increases in the time between permeabilization of platelets and challenge with combinations of Ca^{2+} and GTP[S] on secretion of [^{14}C]5-HT and on the phosphorylation of pleckstrin and myosin light chains

Platelets labelled with [^{14}C]5-HT were permeabilized with SLO at $\text{pCa} > 8$ and preincubated at 25 °C for the indicated times before addition of Ca^{2+} buffers giving pCa values of 7 (○, ●) or 6 (□, ■) in the absence (open symbols) or presence (closed symbols) of 100 μM GTP[S]. (a) Incubations were continued for 5 min before measurement of the percentage [^{14}C]5-HT released. (b) and (c), parallel incubations were carried out in the presence of [γ - ^{32}P]ATP and ^{32}P incorporation into pleckstrin (b) and myosin P-light chains (myosin P-LCs) (c) was determined. Values are means $\pm$ S.E.M. from three determinations (secretion) or means $\pm$ range from two determinations (protein phosphorylation).

more than 3–5 min of preincubation was required to prevent pleckstrin phosphorylation (Figure 4). The much larger Ca^{2+} -independent secretion induced by addition of both PMA and GTP[S] and the associated phosphorylation of pleckstrin and myosin P-light chains (Figure 4) were also abolished when these reagents were added 3–5 min after SLO. Thus, in the absence of Ca^{2+} , platelets preincubated with SLO became rapidly insensitive to stimulation by PMA alone or by PMA with GTP[S].

The effects of increasing the period of preincubation of platelets with SLO on secretion and protein phosphorylation stimulated by Ca^{2+} at $\text{pCa} 5$ were quite different (Figure 5). The level of secretion obtained with Ca^{2+} decreased slightly over the first 3 min of preincubation with SLO, but was then maintained at a high level with preincubation periods up to at least 10 min (Figure 5). This was also true for myosin P-light chain phosphorylation. In contrast, the stimulation of pleckstrin phosphorylation by Ca^{2+} at $\text{pCa} 5$ was no longer detectable after

Figure 7 Time course of the leakage of pleckstrin and PKC from SLO-permeabilized platelets

Platelets were permeabilized at $\text{pCa} > 9$ and incubated at 25 °C. The incubation mixture was sampled immediately after addition of SLO (lane 1), and 1 min (lane 2), 3 min (lane 3), 5 min (lane 4), and 10 min (lane 5) after permeabilization. The platelets were sedimented, pellet and supernatant fractions were rapidly separated, and the protein in each fraction was analysed by SDS/PAGE and electroblotted on to Immobilon-P. Membranes were probed with polyclonal antibodies to pleckstrin (a) and PKC (b). Immunoreactive bands were detected by ECL.

a 3 min preincubation with SLO. Thus, secretion of [^{14}C]5-HT stimulated by Ca^{2+} at $\text{pCa} 5$ could occur in the complete absence of pleckstrin phosphorylation.

The effects of preincubation of platelets with SLO on the synergistic actions of Ca^{2+} and GTP[S] were also investigated. Treatment of platelets with SLO for 3 min before addition of Ca^{2+} at $\text{pCa} 7$ with GTP[S] largely abolished secretion and the phosphorylation of pleckstrin and myosin P-light chains. Pleckstrin phosphorylation in response to addition of Ca^{2+} at $\text{pCa} 6$ with GTP[S] was also lost after a 3 min preincubation of platelets with SLO (Figure 6b), and myosin phosphorylation was suppressed after 5 min (Figure 6c). However, the secretion induced by Ca^{2+} at $\text{pCa} 6$ with GTP[S] decreased to only about half its initial value after 3 min preincubation with SLO and then remained at the same level after preincubations of up to 10 min (Figure 6a). Thus there were two components to the Ca^{2+} -dependent secretion of [^{14}C]5-HT, both of which were stimulated by GTP[S]. The first component decayed rapidly after permeabilization in parallel with the loss of pleckstrin phosphorylation, whereas the second component was maintained for at least 10 min and was independent of pleckstrin phosphorylation. Since SLO-permeabilized platelets remained responsive to the combination of Ca^{2+} at $\text{pCa} 6$ with GTP[S], whereas Ca^{2+} alone at $\text{pCa} 6$ had little effect, the target for GTP[S] must be retained in the platelets after permeabilization.

Leakage of pleckstrin and PKC from SLO-permeabilized platelets

The diameter of the pores formed by SLO in the plasma membrane of treated cells allows the leakage of cytosolic proteins [16,27]. The rate at which this occurs under the conditions of our experiments was studied at $\text{pCa} > 9$ for pleckstrin and PKC, which are largely confined to the cytosol of resting platelets.

Figure 8 Effects of GTP[S] and PMA on the ultrastructure of SLO-permeabilized platelets

Samples of platelet suspension (1.0 ml, 4×10^8 platelets) were incubated for 5 min at a $\text{pCa} > 9$ with the following additions made at the same time as SLO: (a) none; (b) 100 μM GTP[S]; (c) 100 nM PMA; (d) 100 μM GTP[S] and 100 nM PMA. The platelets were then fixed and processed for electron microscopy as described in the Experimental section.

Immunoblots of pleckstrin (Figure 7a) and PKC (Figure 7b) showed the time-dependent loss of these proteins from permeabilized platelets (pellet fractions) and their appearance in the surrounding medium (supernatant fractions). Although their recovery by acid precipitation of supernatant fractions may not be quantitative, a significant proportion of the pleckstrin and PKC detected by the antibodies was extracellular 1 min after permeabilization, and the loss of these proteins from the permeabilized platelets approached completion after 3–5 min. Thus both pleckstrin and PKC diffused from the platelets more rapidly than did the more massive lactate dehydrogenase.

Effects of GTP[S] and PMA on the ultrastructure of SLO-permeabilized platelets

Platelets that had been permeabilized by exposure to SLO for 5 min at $pCa > 9$ showed a marked decrease in staining under the electron microscope, consistent with the loss of cytosolic proteins (Figure 8a). However, the plasma membrane retained its overall integrity, as did the α -granules, the small mitochondria, occasional dense granules and the vesicles of the dense tubular system, all of which were widely scattered throughout individual platelets. Addition of 100 μM GTP[S] with SLO at $pCa > 9$ caused marked changes in the ultrastructure of the platelets, which developed numerous surface protrusions resembling filopodia or lamellipodia, and showed a marked centripetal movement of granules and other organelles into a contracted mass surrounded by densely staining protein (Figure 8b). A few granules were contiguous with large, possibly surface-connected, vesicles, but there was little sign of granule loss. In contrast, addition of 100 nM PMA at $pCa > 9$ did not lead to a contractile response, though after 5 min the scattered granules were much decreased in number; some of those remaining were close to the plasma membrane (Figure 8c). Incubation of platelets with SLO and both GTP[S] and PMA in the absence of Ca^{2+} led to an almost complete disappearance of granules, which were replaced by large vesicles within or immediately outside dense masses of stained protein (Figure 8d). Some of these vesicles had plausible surface connections. The appearance of these last platelets was not dissimilar from that of thrombin-treated intact platelets [28]. Addition of SLO with Ca^{2+} at $pCa 5$ caused ultrastructural changes (not shown) similar to those seen with GTP[S] and PMA in the absence of Ca^{2+} .

DISCUSSION

Comparison of the initial part of the present study with previous work using electroporated platelets [5,8,9,23,26] suggested that similar regulatory mechanisms were operating in the two permeabilized platelet preparations, but with some quantitative differences. The sensitivities of dense-granule secretion to Ca^{2+} in the two preparations were comparable and, in both, addition of PMA or GTP[S] enhanced Ca^{2+} responsiveness, the latter more effectively than the former. In both preparations, synergism was seen between PMA and GTP[S] in the absence of Ca^{2+} . However, in electroporated platelets, GTP[S] caused a limited (about 25%) Ca^{2+} -independent secretion of 5-HT in the absence of PMA [5], whereas no secretion was observed under these conditions in our experiments with SLO-permeabilized platelets. This is probably explained by the ability of GTP[S] to induce a substantial Ca^{2+} -independent activation of PKC in electroporated platelets [5], as indicated by a half-maximal phosphorylation of pleckstrin, whereas GTP[S] had much less effect on PKC activity in platelets permeabilized with SLO at $pCa > 9$. A similar mechanism may account for the marked

Ca^{2+} -independent secretion induced by GTP[S] in the SLO-permeabilized platelets used by Padfield et al. [17]. Protein phosphorylation was not monitored in the latter study, nor were PKC inhibitors used, but we suggest that PKC was either activated prior to permeabilization or was markedly stimulated by GTP[S]. It is noteworthy that, in preliminary experiments, we observed phosphorylation of pleckstrin and a subsequent Ca^{2+} -independent activation of secretion by GTP[S] in platelets cooled to 0 °C before addition of SLO, a condition known to stimulate intact platelets [29]. Prior activation of PKC could in part also explain why Padfield et al. [17] were unable to detect synergism between Ca^{2+} (at $pCa 7-6$) and GTP[S], since our experiments with Ro 31-8220 show that PKC activation plays a major role in the action of GTP[S] over this Ca^{2+} range. Finally, any unrecognized PKC activation may complicate interpretation of the effects of SLO permeabilization on the later responsiveness of platelets to GTP[S], since loss of PKC or its substrates may be difficult to distinguish from loss of a GTP-binding protein.

The present studies further emphasize the importance of PKC in the regulation of secretion from platelets. First, a selective inhibitor of PKC, Ro 31-8220 [19], largely prevented secretion of 5-HT induced by PMA and GTP[S] at $pCa > 9$ or that induced by Ca^{2+} or Ca^{2+} with GTP[S] over a physiological range of Ca^{2+} ion concentrations ($pCa 7-6$). These results correlate with the effectiveness of this compound as an inhibitor of secretion in intact platelets [2]. Secondly, SLO caused a rapid loss of PKC and its major substrate, pleckstrin, from platelets that correlated with the run-down in responsiveness of secretion and pleckstrin phosphorylation to these stimuli. Although this and previous evidence of a role for PKC in the secretion seen with low Ca^{2+} concentrations is very strong, the results do not establish that phosphorylation of pleckstrin mediates the action of PKC. Multiple minor substrates of PKC, any one of which could mediate its actions, can be resolved by SDS/PAGE. Indeed, several recent studies on the interactions of pleckstrin and its PH domains with other proteins indicate that, after phosphorylation, it may exert inhibitory effects on signal transduction by binding to phosphatidylinositol 4,5-bisphosphate [30], by sequestering the $\beta\gamma$ -subunits of G-proteins [31,32] and by stimulating inositol trisphosphate degradation [33]. However, an additional stimulatory effect on secretion is not precluded by any of these studies. It should now be possible to determine directly the roles of PKC and pleckstrin in secretion of platelet granule constituents by reconstituting SLO-permeabilized platelets with these proteins. Although additional proteins involved in secretion may simultaneously leak from the cells, the stability of secretion induced by Ca^{2+} and GTP[S] at $pCa 6-5$ suggests that the basic mechanism of exocytosis is retained in SLO-permeabilized platelets.

The mechanisms of action of GTP[S] in facilitating PKC-dependent and Ca^{2+} -dependent secretion from platelets require further clarification. Our observation that GTP[S] stimulated secretion in the presence of 10–100 nM PMA, which induces a marked to maximal phosphorylation of pleckstrin, confirms our previous conclusion [5–8] that GTP[S] acts by mechanisms additional to stimulation of the phospholipase C–PKC axis. We have previously provided evidence that GTP[S] may exert some of its effects by stimulating phospholipase D activity [8,9], but the present study suggests an additional action. Thus GTP[S] induced major changes in the ultrastructure of SLO-permeabilized platelets in the absence of Ca^{2+} or of any activation of PKC. These changes included the formation of filopodium or lamellipodium-like protrusions of the plasma membrane and a centripetal movement of granules, which appeared to be trapped in a mass of contractile protein filaments. This result corresponds closely to the initial effects of stimuli which induce platelet

aggregation without secretion [28,34]. Previous studies have suggested that the centripetal movement of granules is a contractile process mediated by platelet myosin [28,35]. It was therefore of interest that GTP[S] induced a Ca^{2+} -independent phosphorylation of myosin P-light chains in SLO-permeabilized platelets. This effect was not seen in electroporated platelets [5], perhaps because of the greater activation of PKC by GTP[S]. Recently, evidence has been obtained that GTP[S] can enhance myosin phosphorylation in smooth muscle by activation of Rho-kinase, which both inhibits myosin phosphatase [36] and directly phosphorylates myosin light chains [37]. Our results suggest that similar mechanisms may operate in platelets. The role of myosin phosphorylation in secretion is not yet clear, but it is possible that close contact between granules and the membranes of the surface-connected canalicular system facilitates membrane fusion [28,35]. Nevertheless, our results show that phosphorylation of myosin is neither sufficient (as with GTP[S] alone), nor necessary (as with PMA alone) for secretion to occur and additional actions of GTP[S] are likely. In mast cells, a comparable centripetal redistribution of actin filaments induced by GTP[S] appears to require both heterotrimeric and low- M_r GTP-binding proteins (Rac and Rho) [38], whereas secretion may involve independent effects on the latter proteins [39].

In one important respect, our results are similar to those obtained by Padfield et al. [17]. Thus, in both studies, Ca^{2+} -induced secretion was resistant to the loss of cytosolic proteins caused by SLO. Our results also show that secretion of 5-HT induced by Ca^{2+} at pCa 6–5 proceeded in the complete absence of detectable PKC, indicating that, despite the physiological importance of PKC in secretion, the enzyme is not an obligatory component of the exocytotic mechanism. Although the major part of the stimulation of secretion by GTP[S] at pCa values from 7 to 6 was attributable to activation of PKC and was subject to run-down after permeabilization, GTP[S] potentiated secretion at pCa 6–5 in the absence of PKC activity, whether the enzyme was inhibited by Ro 31-8220 or allowed to leak from the cells. It is unlikely that myosin phosphorylation has a significant role in this action of GTP[S], since Ca^{2+} alone caused a marked and persistent stimulation of myosin light chain phosphorylation, as well as a contractile response. Our results show that, whatever mechanism underlies this effect of GTP[S], it is mediated by a GTP-binding protein that is not readily lost from the platelets after permeabilization.

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