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Factors affecting dense and α-granule secretion from electropermeabilized human platelets: Ca^{2+}-independent actions of phorbol ester and GTPγS

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Electropermeabilized human platelets containing 5-hydroxy[^{14}C]tryptamine ([^{14}C]5-HT)[1] were suspended in a glutamate medium containing ATP and incubated for 10 min with (in various combinations) Ca^{2+} buffers, phorbol 12-myristate 13-acetate (PMA), guanine nucleotides, and thrombin. Release of [^{14}C]5-HT and β-thromboglobulin (βTG) were used to measure secretion from dense and α-granules, respectively. Ca^{2+} alone induced secretion from both granule types; half-maximal effects were seen at a −log [Ca^{2+}_{free}] (pCa) of 5.5 and maximal secretion at a pCa of 4.5, when ~80% of 5-HT and ~50% of βTG were released. Addition of PMA, guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS), GTP, or thrombin shifted the Ca^{2+} dose-response curves for secretion of both 5-HT and βTG to the left and caused small increases in the maximum secretion observed. These results suggested that secretion from α-granules, like that from dense granules, is a Ca^{2+}-dependent process stimulated by the sequential activation of a G-protein, phospholipase C, and protein kinase C (PKC). However, high concentrations of PMA and GTPγS had distinct effects in the absence of Ca^{2+} (pCa > 9); 100 nM PMA released ~20% of platelet 5-HT but little βTG, whereas 100 μM GTPγS stimulated secretion of ~25% of each. Simultaneous addition of PMA greatly enhanced these effects of GTPγS. Phosphorylation of pleckstrin in permeabilized platelets incubated with [γ-^{32}P]ATP was used as an index of the activation of PKC during secretion. In the absence of Ca^{2+}, 100 nM PMA caused maximal phosphorylation of pleckstrin and 100 μM GTPγS was ~50% as effective as PMA; neither GTPγS nor Ca^{2+} enhanced the phosphorylation of pleckstrin caused by 100 nM PMA. These results indicate that, although activation of PKC promoted secretion, GTPγS exerted additional stimulatory effects on secretion from both dense and α-granules that were not mediated by PKC. Measurement of [^{3}H]inositol phosphate formation in permeabilized platelets containing [^{3}H]phosphoinositides showed that GTPγS did not stimulate phosphoinositide-specific phospholipase C in the absence of Ca^{2+}. It follows that in permeabilized platelets, GTPγS can both stimulate PKC and enhance secretion via G-protein–linked effectors other than this phospholipase.

Introduction

Platelets possess three types of secretory organelle: dense granules containing nucleotides, 5-hydroxytryptamine (5-HT), and Ca^{2+}; lysosomes containing acid hydrolases; and α-granules, which are present in much larger numbers than the others and contain numerous proteins with diverse functions (Zucker and Nachmias, 1985). Secreted α-granule constituents include β-thromboglobulin (βTG), platelet factor 4, fibrinogen, thrombospondin, von Willebrand factor, fibronectin, factor V, platelet-derived growth factor, and protease nexin-II (Kaplan *et al.*, 1979; Kaplan, 1986; Sander *et al.*, 1983; Stenberg *et al.*, 1984; Wencel-Drake *et al.*, 1985; Van Nostrand *et al.*, 1990). Despite the importance of some of these proteins in platelet aggregation (fibrinogen and thrombospondin), adhesion of platelets to the subendothelium (von Willebrand factor, fibronectin), formation of the prothrombinase complex (factor V), various cell growth responses (platelet-derived growth factor, protease nexin-II), and chemotaxis (βTG and platelet factor 4), much less is known about the factors that regulate α-granule secretion than about dense granule secretion (Kaplan, 1986). However, release of dense granule constituents by aggregating agents such as ADP, epinephrine, arachidonate, and low concentrations of

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[1] Abbreviations: βTG, β-thromboglobulin; DAG, 1,2-diacylglycerol; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); 5-HT, 5-hydroxytryptamine; pCa, −log [Ca^{2+}_{free}]; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RIA, radioimmunoassay.

thrombin or collagen is associated with the release of α-granule proteins, and it is clear that the high thrombin and collagen concentrations needed to release lysosomal hydrolases are not required (Kaplan *et al.*, 1979). Indeed, there is evidence that low concentrations of aggregating agents may release a higher percentage of α-granule proteins than of dense granule 5-HT (Kaplan *et al.*, 1979), though others have found that α-granule secretion is slower than that from dense granules (Akkerman *et al.*, 1982).

Studies on electropermeabilized human platelets have provided detailed information on intracellular factors that regulate the secretion of dense granule 5-HT and of lysosomal enzymes. High concentrations of Ca^{2+} ions (−log [Ca^{2+}_{free}] [pCa] 5) promote secretion of 5-HT in the presence of ATP (Knight and Scrutton, 1980). Phorbol ester (Knight and Scrutton, 1984), thrombin (Haslam and Davidson, 1984a; Knight and Scrutton, 1984), and guanine nucleotides—especially guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) (Haslam and Davidson, 1984b; Knight and Scrutton, 1986)—cause a leftward shift in the Ca^{2+} dose-response curve and thus greatly potentiate secretion at Ca^{2+} concentrations in the physiological range (pCa 7 to 6). These results can be at least partly explained by a G-protein–mediated activation of phosphoinositide-specific phospholipase C and the formation of 1,2-diacylglycerol (DAG) that promotes secretion by stimulating protein kinase C (PKC) (Haslam and Davidson, 1984b,c; Knight and Scrutton, 1984, 1986; Culty *et al.*, 1988). The release of lysosomal enzymes from electropermeabilized platelets is also stimulated by Ca^{2+}, but in this case thrombin, guanine nucleotides, and a synthetic DAG have been shown to enhance secretion primarily by increasing the maximum response to Ca^{2+} rather than the sensitivity of the secretory mechanism to this ion (Knight *et al.*, 1984; Athayde and Scrutton, 1990). Apart from preliminary reports (Coorssen and Haslam, 1990; Peltola and Scrutton, 1990), comparable information on the factors regulating α-granule secretion is not yet available. We present here a detailed comparison of dense and α-granule secretion from permeabilized human platelets, which shows that the same factors promote secretion from both granule types, though not with equal effectiveness.

Studies on other permeabilized cells have suggested that guanine nucleotides can stimulate secretion by a mechanism additional to the sequential activation of phospholipase C and PKC (reviewed by Gomperts, 1990). Thus, the effects of GTPγS on secretion from neutrophils and HL60 cells differ from those observed after direct activation of PKC by phorbol 12-myristate 13-acetate (PMA) (Barrowman *et al.*, 1986; Stutchfield and Cockcroft, 1988). Moreover, GTPγS can induce secretion without formation of phosphoinositide breakdown products in permeabilized adrenal chromaffin cells (Bittner *et al.*, 1986), mast cells (Cockcroft *et al.*, 1987), and RINm5F cells (Vallar *et al.*, 1987). These studies led Gomperts and colleagues to propose that in many cells a GTP-binding protein (termed G_E), distinct from that mediating the activation of phospholipase C, may play an essential role in the exocytotic event (Barrowman *et al.*, 1986; Cockcroft *et al.*, 1987; Howell *et al.*, 1987; Gomperts, 1986, 1990). Initial interpretations of studies with permeabilized platelets did not support a role for G_E in dense granule secretion, because GTPγS appeared to have little effect in the absence of Ca^{2+} ions (Haslam and Davidson, 1984b; Knight and Scrutton, 1986). However, in the present study, we have reexamined this question and have demonstrated that GTPγS, acting either alone or more potently in combination with PMA, can induce a Ca^{2+}- and phospholipase C-independent secretion of both dense and α-granule constituents. Our results, together with a recent report that GTPγS can stimulate Ca^{2+}-independent secretion of lysosomal enzymes from permeabilized platelets (Athayde and Scrutton, 1990), suggest that secretion of platelet granule constituents is not fundamentally different from exocytosis in cells in which a role for G_E has been postulated.

Results

Effects of Ca^{2+} ions on secretion

We used [^{14}C]5-HT and βTG as markers for the secretion of platelet dense and α-granule constituents, respectively. Permeabilized human platelets contained 71 ± 3 μg of βTG/10^9 cells (mean ± SE, 17 determinations). Incubation of permeabilized platelets for 10 min at 25°C in the absence of added Ca^{2+} ions, or at a buffered pCa of 7, did not cause any release of either [^{14}C]5-HT or βTG. However, incubation with higher Ca^{2+} concentrations (pCa 6 to 4.5) led to secretion from both granule types (Figure 1). There was no consistent difference in the Ca^{2+} sensitivity of the release of [^{14}C]5-HT and βTG, in that the most marked effects of increasing Ca^{2+} concentrations were always obtained at pCa values between 6 and 5. In 10-min incubations, half-maximal secretion from both granule types required a pCa of 5.5 ± 0.1 (mean

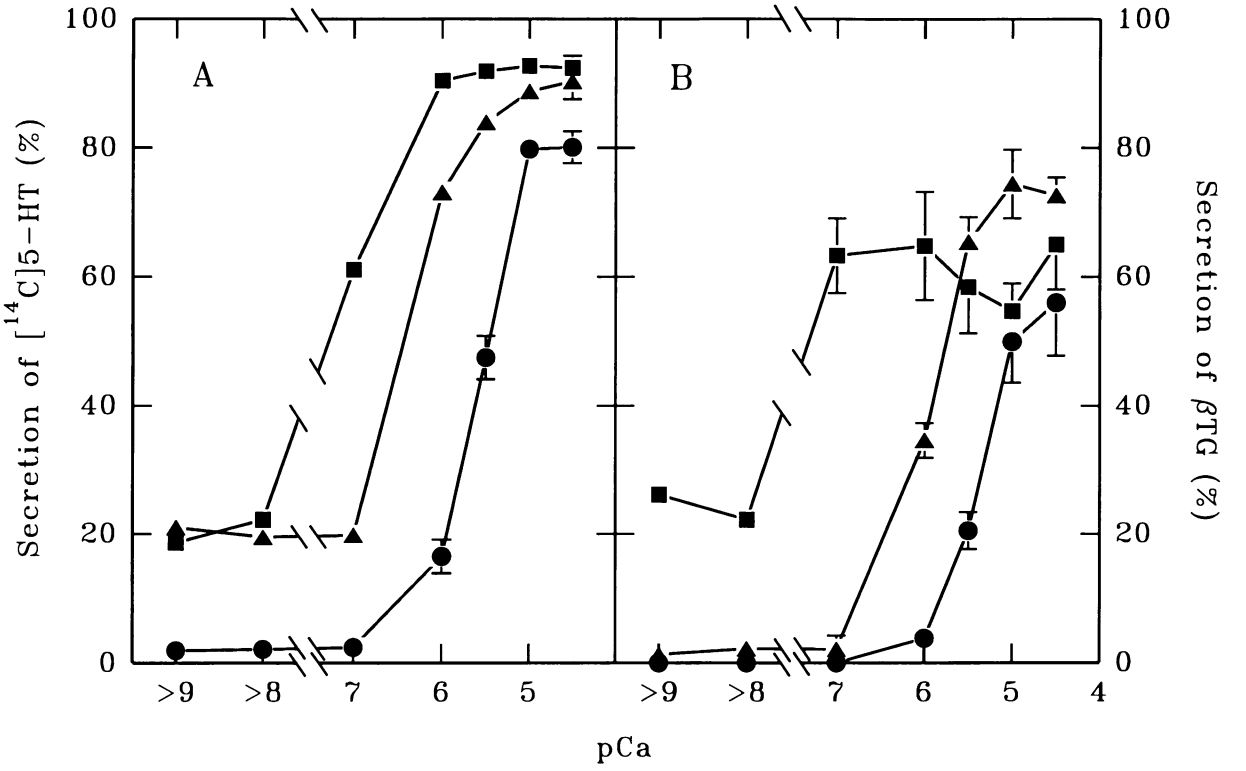

Figure 1. **Effects of PMA and of GTPγS on the secretion of [^{14}C]5-HT (A) and βTG (B) from permeabilized platelets at various buffered Ca^{2+} concentrations.** Samples of permeabilized platelets in a glutamate-based medium containing ATP (see Materials and methods) were equilibrated for 15 min at 0°C with the indicated Ca^{2+} buffers and either no other additions (●), 100 nM PMA (▲), or 100 μM GTPγS (■). The samples were then incubated for 10 min at 25°C and [^{14}C]5-HT and βTG secretion were then determined. Values are means ± SE from three identical incubation mixtures.

± SE, 5 experiments); maximal secretion, observed at a pCa of 4.5, amounted to 81 ± 3% of the platelet [^{14}C]5-HT and 50 ± 3% of platelet βTG (means ± SE). The latter value is significantly less than the former ($p < 0.001$). Higher Ca^{2+} concentrations (e.g., pCa 4) caused less secretion (not shown), probably as a result of Ca^{2+}-dependent proteolysis (Haslam and Davidson, 1984a). Although Ca^{2+} appeared to have very similar effects on the secretion of dense and α-granule constituents, the dose-response curves for βTG release were slightly but consistently steeper than those for [^{14}C]5-HT, as shown by a progressive increase in the ratio of βTG to [^{14}C]5-HT released between pCa values of 6 and 4.5 (Figure 2A). This suggests a minor difference in the regulation of secretion from the two granule types under these conditions. At a pCa of 5, in the absence of other additions, secretion of βTG was only slightly slower than that of [^{14}C]5-HT, with both approaching maximum values after 10–20 min (Figure 3, C and D).

Effects of PMA on secretion

A previous study with permeabilized platelets (Knight and Scrutton, 1984) showed that addition of PMA (16 nM) shifted the Ca^{2+} dose-response curve for secretion of [^{14}C]5-HT to the left and caused just detectable secretion of [^{14}C]5-HT in the absence of added Ca^{2+}. In the present work, we used a higher PMA concentration (100 nM), which was just sufficient to activate PKC optimally in permeabilized platelets (see later), and observed similar though somewhat more marked effects (Figure 1A). Thus, PMA caused 5- to 10-fold increases in the Ca^{2+} sensitivity of [^{14}C]5-HT secretion, significant release of [^{14}C]5-HT in the absence of Ca^{2+} (pCa > 8), and a small but significant increase in the secretion observed at pCa 4.5 ($p < 0.05$). To determine whether or not truly Ca^{2+}-independent release of [^{14}C]5-HT occurs, we also measured secretion in samples containing additional ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA, final concentration 12.5 mM, pCa > 9). Under these conditions, the release of [^{14}C]5-HT amounted to 22 ± 1% (mean ± SE, 5 experiments) and was no less than seen with 2.5 mM EGTA (pCa > 8). Thus, we were able to conclude that PMA can induce a significant Ca^{2+}-independent se-

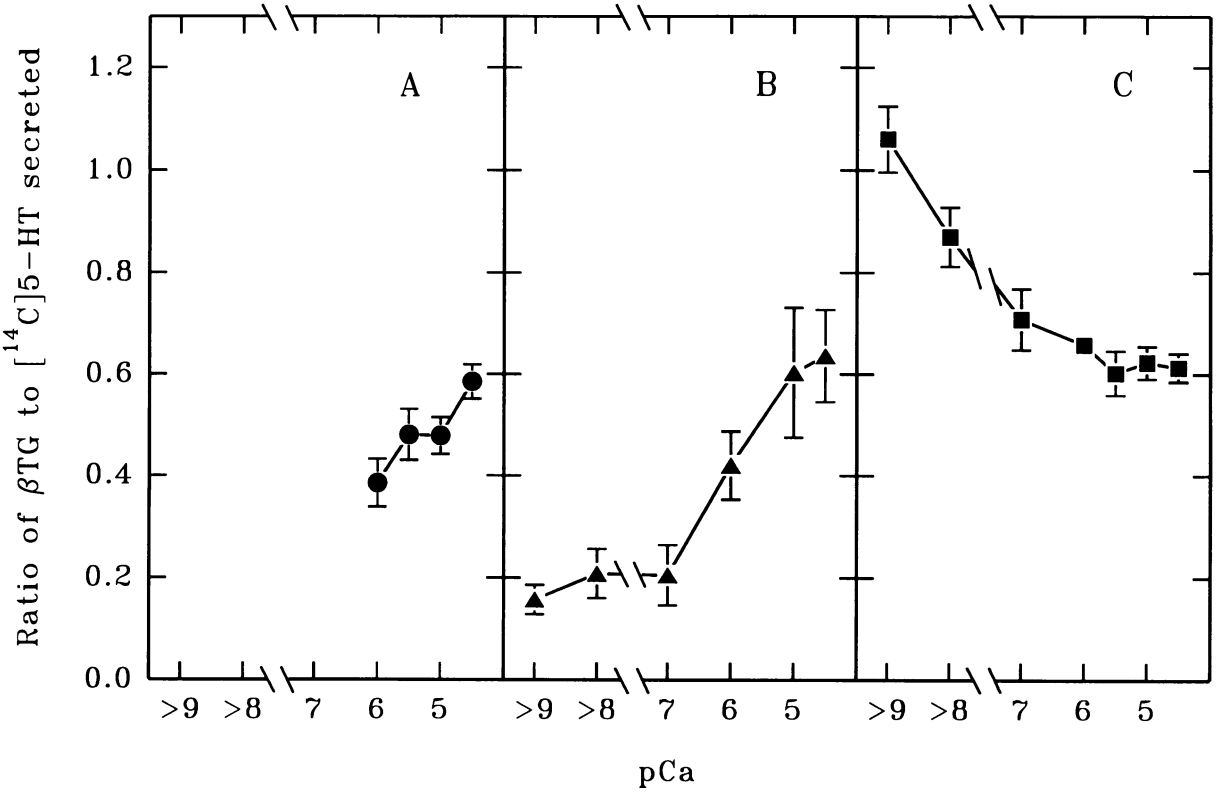

Figure 2. **Effects of GTPγS and of PMA on the relationship between the secretion of βTG and [^{14}C]5-HT from permeabilized platelets at various Ca^{2+} concentrations.** Permeabilized platelets were equilibrated and incubated with the indicated Ca^{2+} buffers and either no other additions (A), 100 nM PMA (B), or 100 μM GTPγS (C), as described for Figure 1. The percent secretion of βTG and of [^{14}C]5-HT were determined for each condition and expressed as a ratio. Values are means ± SE from 3 to 11 separate experiments. When secretion of βTG and [^{14}C]5-HT was negligible (A, pCa > 6), ratios were not calculated.

cretion of [^{14}C]5-HT from permeabilized platelets. Analysis of the time-course of [^{14}C]5-HT release showed that, at a pCa > 9, dense granule secretion continued for at least 20 min in the presence of PMA (Figure 3A). At a pCa of 5, secretion was much more rapid and, in the presence of PMA, was almost complete within 5 min (Figure 3C).

As observed for the secretion of [^{14}C]5-HT, incubation of permeabilized platelets with 100 nM PMA enhanced the Ca^{2+}-sensitivity of secretion of βTG and increased the maximum extent of secretion observed at a pCa of 4.5 (Figure 1B). However, 100 nM PMA caused very little secretion of βTG in the absence of Ca^{2+} ions. Thus, at a pCa > 9, the platelet βTG found in the supernatant after incubation for 10 min with 100 nM PMA amounted to only 3 ± 1% (mean ± SE, 5 experiments), and the ratio of βTG to [^{14}C]5-HT released (calculated as percentages of the platelet content) was only 0.16 ± 0.03 (mean ± SE). This ratio increased progressively as the Ca^{2+} concentration was increased from a pCa of 7 to 4.5, reaching a value of 0.64 ± 0.09 (Figure 2B), essentially the same as observed in the absence of PMA. In the presence of PMA, secretion of βTG was slightly delayed relative to that of [^{14}C]5-HT (Figure 3, C and D). These results imply a significant difference between the regulatory mechanisms responsible for the secretion of dense and α-granule constituents.

Effects of guanine nucleotides on secretion

Previous work with electropermeabilized platelets prepared as in the present study showed that 100 μM GTPγS greatly enhanced the Ca^{2+} sensitivity of the secretion of [^{14}C]5-HT and also caused some secretion in the absence of added Ca^{2+} (Haslam and Davidson, 1984b,c); small amounts of Ca^{2+} in the reagents or released from the platelets appeared to be partly responsible for the latter effect. We have now reinvestigated these findings and have compared [^{14}C]5-HT release with that of βTG (Figure 1).

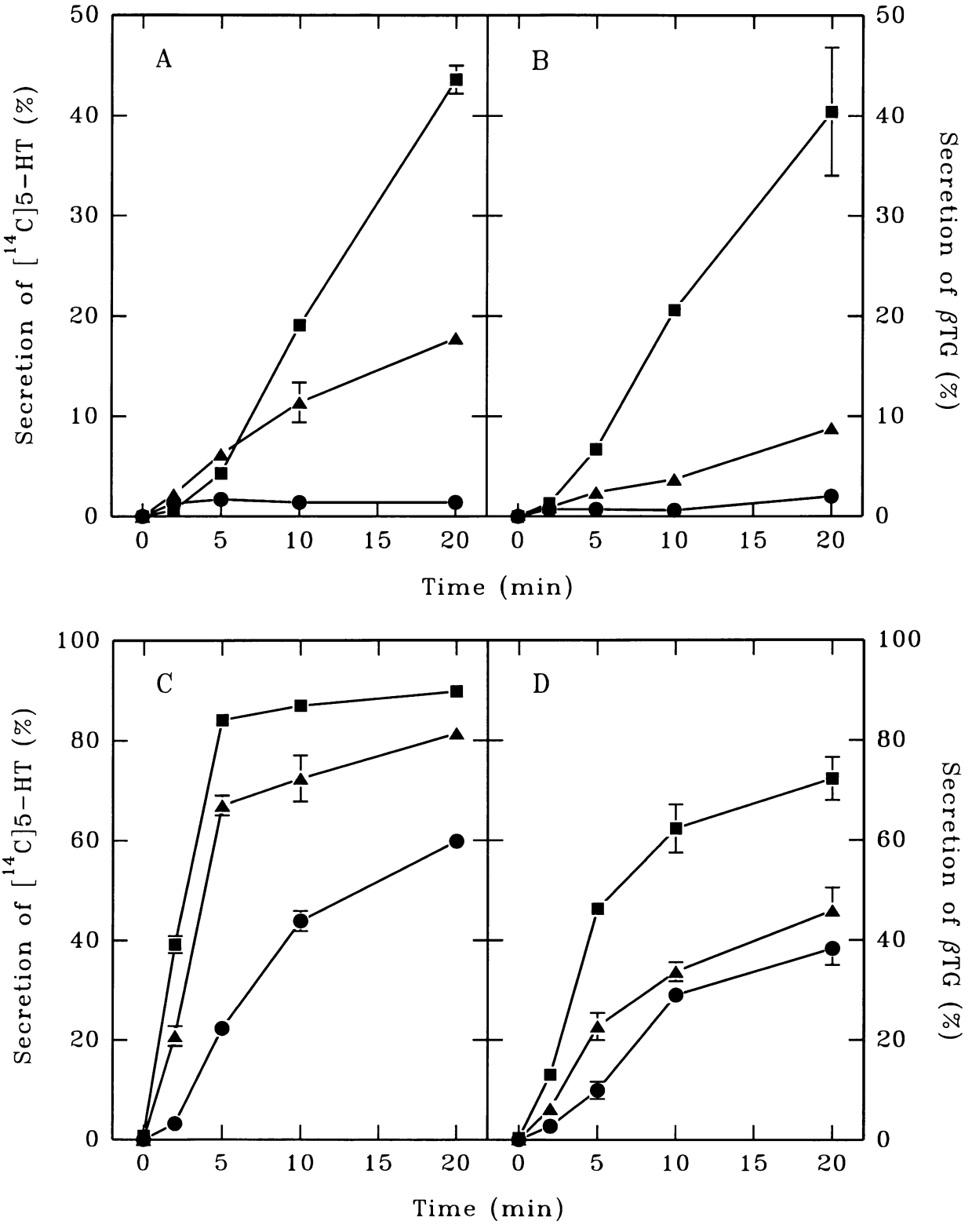

Figure 3. **Effects of PMA and of GTPγS on the time course of secretion of [^{14}C]5-HT (A and C) and βTG (B and D) from permeabilized platelets.** Experiments were carried out as for Figure 1 at pCa values of either >9 (A and B) or 5 (C and D); the period of incubation at 25°C was varied as indicated. Samples were equilibrated and incubated with no other additions (●), with 100 nM PMA (▲), or with 100 μM GTPγS (■). Values are means ± SE from three identical incubation mixtures.

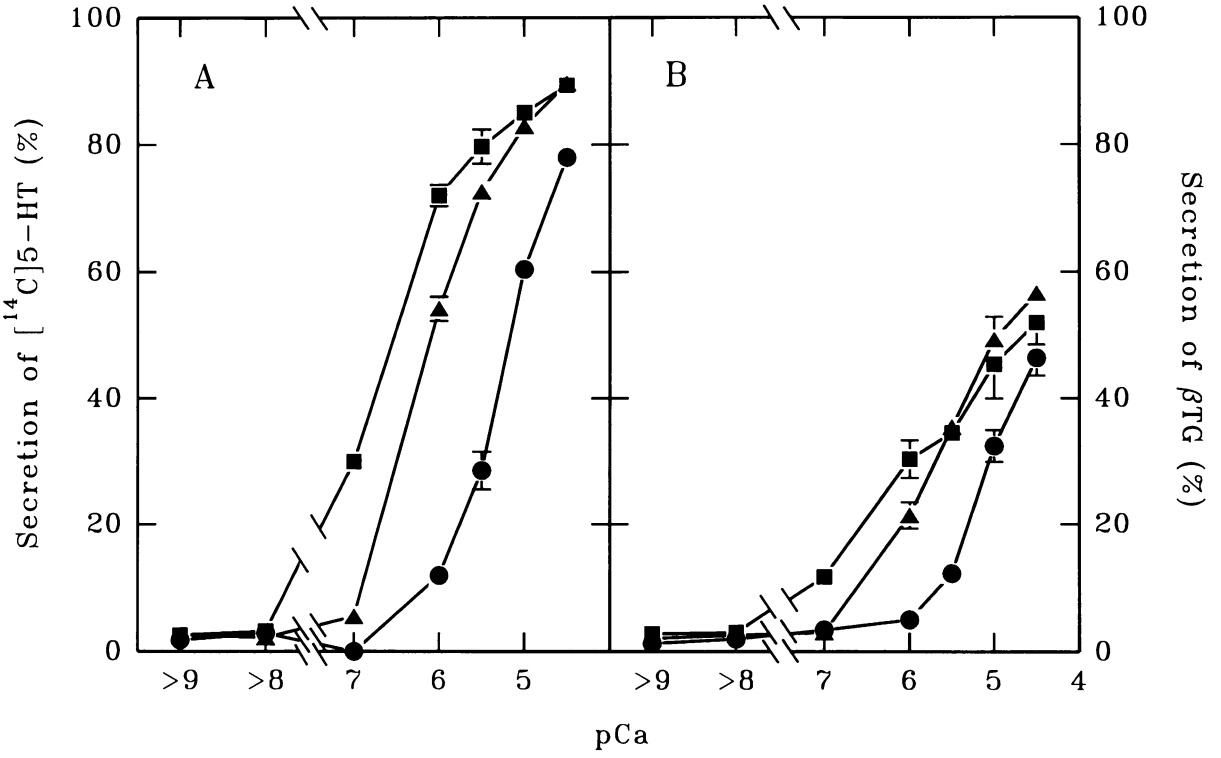

Figure 4. **Effects of GTP and of GTPγS on the secretion of [^{14}C]5-HT (A) and βTG (B) from permeabilized platelets at various buffered Ca^{2+} concentrations.** Samples of permeabilized platelet suspension were equilibrated for 15 min at 0°C with the indicated Ca^{2+} buffers and either no other additions (●), 100 μM GTP (▲), or 10 μM GTPγS (■). Samples were then incubated for 10 min at 25°C and [^{14}C]5-HT and βTG secretion were then determined. Values are means ± SE from three identical incubations.

We found that 100 μM GTPγS caused substantial secretion from both granule types in the presence of 12.5 mM EGTA (pCa > 9), amounting to 25 ± 2% of the platelet [^{14}C]5-HT and 26 ± 2% of platelet βTG (means ± SE, 10 experiments). These values were only slightly less than ([^{14}C]5-HT) or the same as (βTG) those obtained with 2.5 mM EGTA (pCa > 8). Thus, 100 μM GTPγS differed in its effects from 100 nM PMA, which induced Ca^{2+}-independent secretion mainly from the dense granules. GTPγS also enhanced the maximal secretion of [^{14}C]5-HT and of βTG, again observed at a pCa of 4.5, to values of 92 ± 3% and 57 ± 5%, respectively (means ± SE; 4 experiments). These results show that in the absence of Ca^{2+} ions, 100 μM GTPγS induced the secretion of a much higher percentage of the releasable βTG (49 ± 4%) than of the releasable [^{14}C]5-HT (27 ± 4%) (means ± SE, 4 experiments), indicating that the actions of GTPγS favor the secretion of α-granule constituents under these conditions. As the Ca^{2+} concentration was increased in the presence of 100 μM GTPγS, the ratio of βTG to [^{14}C]5-HT released decreased progressively to reach a value similar to that seen in the absence of GTPγS (Figure 2C). In the absence of Ca^{2+} ions, the GTPγS-induced secretion of both [^{14}C]5-HT and βTG proceeded slowly and almost linearly for 20 min, apart from an initial 2-min lag (Figure 3, A and B), whereas at a pCa of 5, secretion of both granule constituents was greatly accelerated, with that of [^{14}C]5-HT slightly preceding that of βTG, as also observed with PMA (Figure 3, C and D).

Ca^{2+}-independent secretion of [^{14}C]5-HT and βTG was not observed in the presence of either 10 μM GTPγS or 100 μM GTP. However, these additions enhanced the Ca^{2+}-sensitivity of secretion of both [^{14}C]5-HT and βTG and, to some extent, the maximum secretion obtained (Figure 4). As reported previously for [^{14}C]5-HT (Haslam and Davidson, 1984b), 10 μM GTPγS shifted the Ca^{2+} dose-response curve for βTG secretion further to the left than did 100 μM GTP and even promoted limited secretion at a pCa of 7. Thus, in the pCa range in which these concentrations of guanine nucleotides were effective (7 to 4.5), no significant dissociation of [^{14}C]5-HT and βTG secretion was apparent.

Effects of thrombin on secretion

Addition of an optimal dose of thrombin (2 U/ml; Haslam and Davidson, 1984a) increased the Ca^{2+}-sensitivity of secretion from both dense and α-granules to similar extents, but caused no release of [^{14}C]5-HT or βTG in the absence of Ca^{2+} ions (Figure 5). Thrombin also increased the maximum secretion of βTG and, to a lesser extent, of [^{14}C]5-HT. The effects of this concentration of thrombin (Figure 5) were quantitatively very similar to those of 100 μM GTP (Figure 4). Addition of a low GTP concentration (10 μM) with thrombin caused a further shift to the left of the Ca^{2+} dose-response curves for secretion, so that substantial effects were seen at a pCa of 7 (as previously reported for [^{14}C]5-HT by Haslam and Davidson, 1984b). However, no secretion of either granule constituent was detected in the complete absence of Ca^{2+} ions (Figure 5). These results suggest that addition of thrombin, or of thrombin with GTP, stimulated secretion of both dense and α-granule constituents by the same or similar Ca^{2+}-dependent mechanisms.

Synergistic effects of PMA and GTPγS on secretion

The ability of GTPγS to induce secretion of βTG from permeabilized platelets in the absence of Ca^{2+} ions, a condition in which PMA had little effect, suggested that the action of the former compound might not be fully accounted for by the activation of PKC. We therefore studied the effects of increasing concentrations of GTPγS on Ca^{2+}-independent secretion in the absence and presence of an optimal concentration of PMA (100 nM). In 10-min incubations in the absence of PMA, the release of platelet [^{14}C]5-HT and βTG caused by GTPγS did not exceed 40%, even with 1 mM of the latter compound (Figure 6, A and B). Secretion of [^{14}C]5-HT and βTG caused by 100 μM GTPγS amounted to 70–80% of the maximum observed with 1 mM GTPγS in the absence of Ca^{2+}. However, when PMA was included in the incubation mixtures, the effects of all concentrations of GTPγS on the release of both [^{14}C]5-HT and βTG were enhanced (Figure 6, A and B). This effect was particularly striking with 10 μM GTPγS, which did not cause

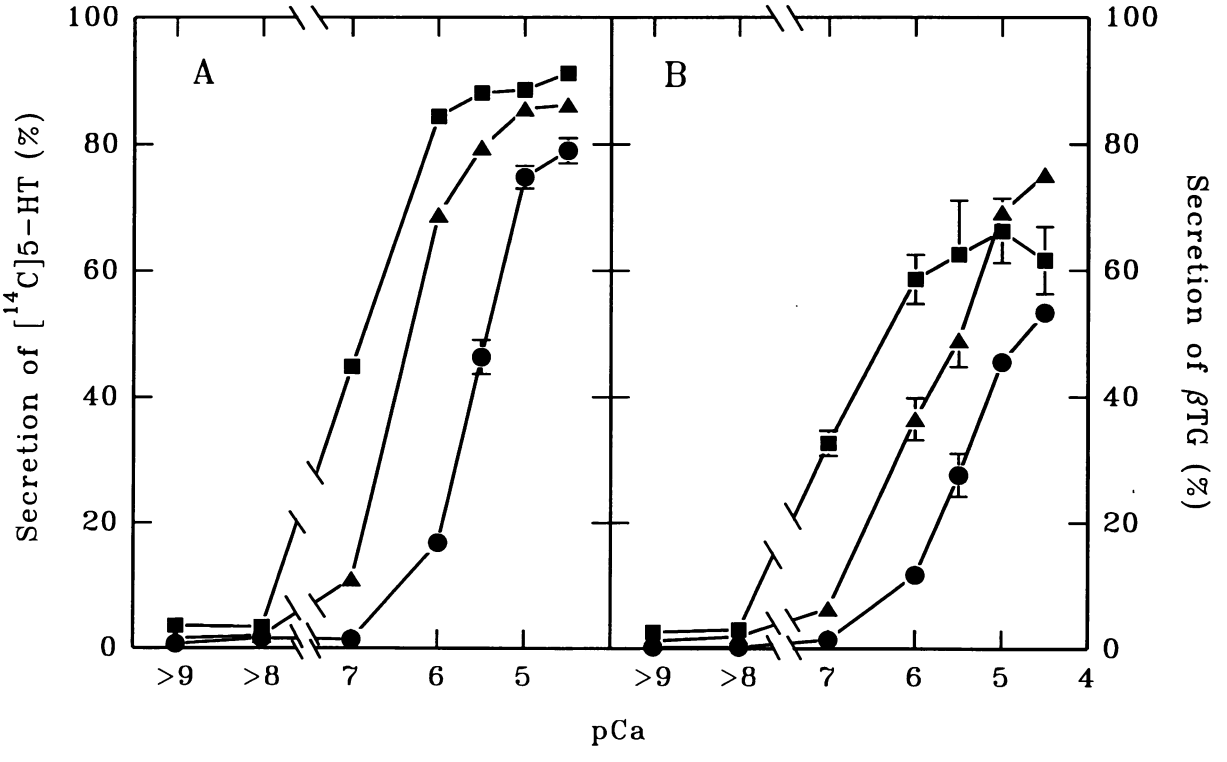

Figure 5. **Effects of GTP and thrombin on the sensitivity to Ca^{2+} of the secretion of [^{14}C]5-HT (A) and βTG (B) from permeabilized platelets.** Samples of permeabilized platelet suspension were equilibrated for 15 min at 0°C with the indicated Ca^{2+} buffers and were then incubated for 10 min at 25°C. Samples contained either no other additions (●) or 2 U of thrombin/ml added after equilibration with Ca^{2+} alone (▲) or with Ca^{2+} and 10 μM GTP (■). Secretion of both [^{14}C]5-HT and βTG were then determined. Values are means ± SE from three identical incubation mixtures.

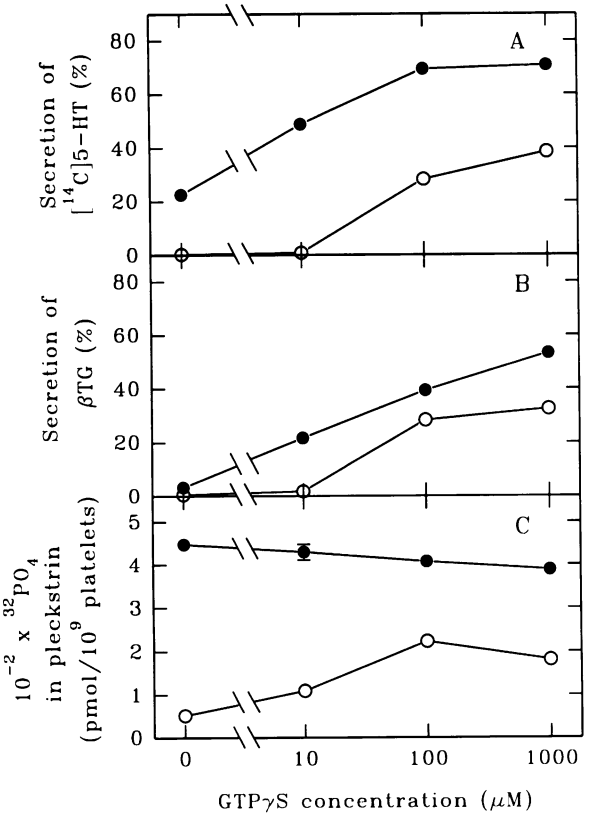

Figure 6. **Ca^{2+}-independent secretion of [^{14}C]5-HT and βTG from permeabilized platelets by different concentrations of GTPγS added without or with PMA; relationship to pleckstrin phosphorylation.** Samples of permeabilized platelets containing 12.5 mM EGTA (pCa > 9) were equilibrated for 15 min at 0°C with the indicated concentrations of GTPγS in the absence (○) or presence (●) of 100 nM PMA and were then incubated for 10 min at 25°C. The secretion of [^{14}C]5-HT (A) and βTG (B) were measured in three identical samples; mean values ± SE are shown. Parallel duplicate incubations were carried out with permeabilized platelet suspension containing [γ-^{32}P]ATP for measurement of the phosphorylation of pleckstrin (C); mean values ± range are shown.

any secretion by itself. These supra-additive effects of GTPγS and an optimal concentration of PMA provide further evidence that GTPγS must exert its effects, at least in part, by a mechanism other than activation of PKC.

Relationship between protein phosphorylation and secretion

The phosphorylation of pleckstrin, the major substrate of PKC in platelets (Imaoka *et al.*, 1983; Sano *et al.*, 1983; Tyers *et al.*, 1988), was used as an index of the extent to which this enzyme was activated during secretion. As expected, incubation of permeabilized platelets with 100 nM PMA in the presence of [γ-^{32}P]ATP caused a marked increase in the ^{32}P-labeling of pleckstrin (Figures 6C and 7A). In the absence of Ca^{2+} ions, phosphorylation of pleckstrin amounted to 481 ± 28 pmol/10^9 platelets (mean ± SE, 3 experiments) and was not further increased by addition of GTPγS (Figure 6C) or Ca^{2+} ions (Figure 7A). Indeed, both GTPγS and high Ca^{2+} concentrations tended to decrease the phosphorylation of pleckstrin seen in the presence of PMA. Lower concentrations of PMA (20 and 50 nM) caused only slightly less phosphorylation than 100 nM (not shown), indicating that 100 nM was optimal. Addition of GTPγS alone in the absence of Ca^{2+} ions (pCa > 9) also caused a phosphorylation of pleckstrin amounting to 53 ± 2% of the maximum extent seen with PMA (mean ± SE, 3 experiments). Addition of Ca^{2+} ions slightly increased the phosphorylation of pleckstrin observed in the presence of GTPγS, but never to the level seen with PMA alone (Figure 7A).

As reported previously (Haslam and Davidson, 1984a), addition of Ca^{2+} ions alone to permeabilized platelets increased the phosphorylation of both pleckstrin and myosin light chain (Figure 7). Although phosphorylation of pleckstrin was optimal at a pCa of 4.5, reaching a level similar to that seen in the presence of GTPγS and Ca^{2+}, this reaction did not correlate well with secretion, in that substantial phosphorylation was seen at a pCa value as low as 6. Although PMA and, to a lesser extent, GTPγS enhanced the phosphorylation of myosin light chain at pCa values in the range >9–5, presumably as a result of the activation of PKC, the dominant factor regulating the phosphorylation of this protein was the Ca^{2+} concentration (Figure 7B). This contrasts with the largely Ca^{2+}-independent effects of PMA and GTPγS on the phosphorylation of pleckstrin (Figure 7A).

Activation of phosphoinositide-specific phospholipase C

Previous studies in this laboratory have shown that GTPγS stimulates the release of [^{3}H]inositol phosphates from [^{3}H]phosphoinositides by phospholipase C in permeabilized platelets incubated at a pCa of 7 and that this effect was much diminished in the absence of added Ca^{2+} ions (pCa > 8) (Culty *et al.*, 1988). We have now shown that no significant [^{3}H]inositol phosphate formation could be detected in the presence or absence of 100 μM GTPγS, when the Ca^{2+} ion concentration was further reduced by additional EGTA (pCa > 9) (Table 1). This finding suggests

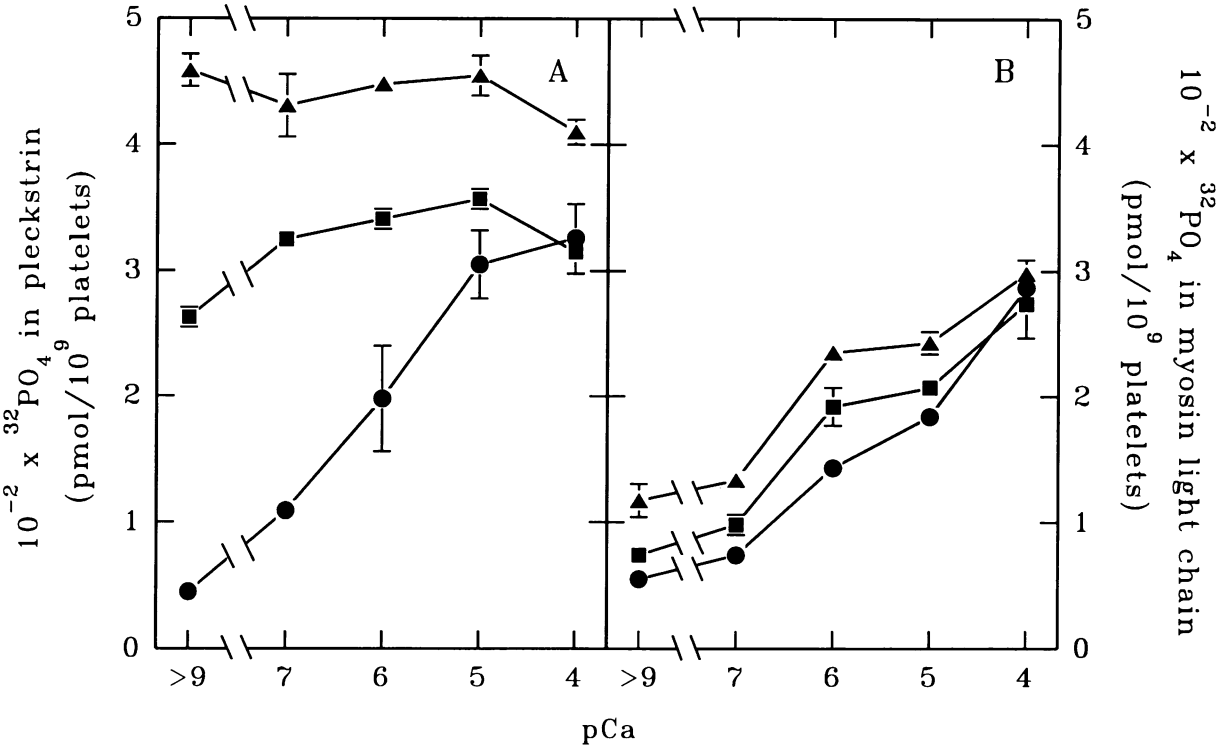

Figure 7. **Effects of Ca^{2+}, PMA, and GTPγS on the phosphorylation of pleckstrin (A) and myosin light chain (B) in permeabilized platelets.** Samples of permeabilized platelet suspension containing [γ-^{32}P]ATP were equilibrated for 15 min at 0°C with the indicated Ca^{2+} buffers and either no other additions (●), 100 nM PMA (▲), or 100 μM GTPγS (■). The samples were then incubated for 10 min at 25°C before the addition of trichloroacetic acid and analysis of ^{32}P-labeled proteins by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Areas of gels containing pleckstrin and myosin light chain were cut out and counted for ^{32}P. Values are means ± range from duplicate incubation mixtures.

that the Ca^{2+}-independent activation of PKC by GTPγS was not mediated by DAG released from phosphoinositides. This conclusion is supported by the finding that, although there was trace formation of [^{3}H]inositol phosphates at pCa 7 in the absence of GTPγS (Table 1), the associated phosphorylation of pleckstrin (Figure 7A) was much less than observed with GTPγS at a pCa > 9, when no phosphoinositide-specific phospholipase C activity was detected. The enhanced Ca^{2+}-independent secretion of 5-HT and βTG caused by addition of GTPγS in the presence of PMA (Figure 6) was also not associated with any [^{3}H]inositol phosphate formation; indeed, PMA inhibited the stimulation of [^{3}H]-inositol phosphate formation caused by GTPγS at a pCa of 7 (Table 1).

Discussion

We selected βTG as the most appropriate marker for α-granule secretion because many of the others, including platelet factor 4, bind extensively to the platelet surface (Stenberg *et al.*, 1984; Capitanio *et al.*, 1985), usually by Ca^{2+}-dependent mechanisms (George and Onofre, 1982). Our results show that secretion of βTG from electropermeabilized platelets, as monitored by release of βTG into the platelet supernatant, was stimulated by the addition of Ca^{2+} ions in a manner very similar to the secretion of dense granule 5-HT. Thus, half-maximal secretion was seen at a pCa of 5.5 in both instances. However, of the total βTG in permeabilized platelets (~70 μg/10^9 platelets), only 50–60% could be released at an optimal Ca^{2+} concentration (pCa 4.5), the exact value depending on whether or not a second stimulus was present. The Ca^{2+}-stimulated release of an even lower percentage of platelet factor 4 from permeabilized platelets has recently been reported (Peltola and Scrutton, 1990). This maximum secretion of βTG should be compared with the much more complete release of dense granule 5-HT (80–90%). However, the βTG released in our experiments was similar to the maximum secreted from intact platelets in response to thrombin (38 μg/10^9 platelets; Akkerman *et al.*,

Table 1. Effects of GTPγS and PMA on total [^{3}H]inositol phosphate formation in permeabilized platelets incubated in the presence and absence of Ca^{2+} ions

	[^{3}H]inositol phosphates (dpm/10^9 platelets)	
Additions	pCa > 9	pCa 7
None	−250 ± 187	508 ± 211
PMA (100 nM)	−166 ± 183	129 ± 211
GTPγS (100 μM)	31 ± 192	11 008 ± 434
GTPγS (100 μM) + PMA (100 nM)	−15 ± 197	4 894 ± 247

Platelets labeled with both [^{3}H]inositol and [^{14}C]5-HT were permeabilized, as described under Materials and methods. Samples were equilibrated for 15 min at 0°C with the additions indicated at the designated pCa values and were then incubated for 10 min at 25°C before extraction and measurement of [^{3}H]inositol phosphates. Amounts found in the suspension of permeabilized platelets before equilibration of the samples (1333 ± 182 dpm/10^9 platelets) were subtracted. Values given are means ± SE of the difference from three identical samples. The secretion of [^{14}C]5-HT at a pCa > 9 (not shown) was similar to that illustrated in Figure 6.

1982) or released from platelets by physical methods of disruption (Rink *et al.*, 1983; Krishnamurthi *et al.*, 1986; Coorssen and Haslam, unpublished). This suggests that platelets contain a form of βTG that is not readily secreted. Preliminary experiments suggest that this βTG is membrane-bound, because it is not released by freezing and thawing followed by sonication, but can then be extracted partly by 2 M KCl, as well as by detergent (Coorssen and Haslam, unpublished). It is possible that this pool of βTG is the source of that found on the plasma membrane after stimulation of platelets with thrombin (Stenberg *et al.*, 1984).

Incubation of permeabilized platelets with thrombin, particularly in the presence of GTP, with low concentrations of GTPγS (e.g., 10 μM), or with PMA, all shifted the Ca^{2+} dose-response curve for the release of βTG to the left, either into or beyond the physiological range (pCa 7 to 6), as previously reported for dense granule 5-HT (Haslam and Davidson, 1984a,b; Knight and Scrutton, 1984, 1986). Thus, the regulation of the secretion of α-granule constituents from permeabilized platelets resembles much more closely that of dense granule constituents than that of lysosomal hydrolases, since in the latter case, the above stimuli enhance the maximum response to Ca^{2+} rather than the Ca^{2+} sensitivity of secretion (Knight *et al.*, 1984; Athayde and Scrutton, 1990). Because thrombin and guanine nucleotides activate phosphoinositide-specific phospholipase C in permeabilized platelets (Brass *et al.*, 1986; Culty *et al.*, 1988), enhance the formation of DAG (Haslam and Davidson, 1984a,c; Brass *et al.*, 1986), and promote the phosphorylation in these cells of the PKC substrate pleckstrin (Haslam and Davidson, 1984a; Figure 7), the similar effects of these stimuli and of PMA on secretion from permeabilized platelets can plausibly be explained by the activation of PKC. However, high concentrations of GTPγS (e.g., 100 μM) were far more effective in promoting the release of 5-HT and particularly of βTG than was 100 nM PMA, which stimulated the phosphorylation of pleckstrin to the maximum extent possible in permeabilized platelets and always to a higher level than was observed with GTPγS. Moreover, although Ca^{2+} enhanced the phosphorylation of pleckstrin, presumably by activating PKC, this effect did not correlate well with secretion in either a previous study (Haslam and Davidson, 1984a) or the present work. This is not to say that PKC is unimportant. The potentiating effects of PMA and synthetic DAG (Knight and Scrutton, 1984; Athayde and Scrutton, 1990) and preliminary results showing that complete inhibition of PKC markedly reduces but does not abolish the effects of GTPγS and Ca^{2+} on secretion (Haslam and Davidson, 1990) both indicate that PKC plays a major role in secretion of platelet granule constituents. It is, nevertheless, clear that both GTPγS and Ca^{2+} ions promote secretion of dense and α-granule constituents by mechanisms additional to the activation of PKC.

Thus, as in other cells (Gomperts, 1990), there appear to be three major factors involved in the regulation of secretion from platelets, namely Ca^{2+} ions, guanine nucleotide, and PKC. Our results indicate that these factors exert independent as well as interacting effects with significantly different consequences for the secretion of dense and α-granule constituents. Since the ratio of the maximum percentages of βTG and 5-HT secreted was ~0.6 (at pCa 4.5), whether or not PMA or GTPγS was present (Figure 2), departures from this ratio under conditions of suboptimal stimulation are indicative of selective secretion from one or the other granule type. By this criterion, low Ca^{2+} concentrations (pCa 7 to 6) tended to favor secretion from dense granules when either no other stimulus or PMA was present, but induced the parallel secretion of both dense and α-granule constituents when GTPγS was present. However, the clearest dissociation of dense and α-granule secretion was obtained in the effective absence of Ca^{2+} ions. When no $CaCl_2$ was added to the

permeabilized platelets, only limiting pCa values could be calculated, since the Ca^{2+} present depended on reagent contamination and the extent to which platelet Ca^{2+} was released into the medium. However, even with the most conservative assumptions (see Materials and methods), pCa values in incubation mixtures (pH 7.4) containing no $CaCl_2$ and either 2.5 or 12.5 mM EGTA were >8 or >9, respectively. Because little difference in secretion was observed under these two conditions with any stimulus (see Figure 1), we believe that such effects can reasonably be described as Ca^{2+}-independent. In particular, PMA induced the slow Ca^{2+}-independent secretion of dense granule 5-HT, as has also been found by others (Knight and Scrutton, 1984; Athayde and Scrutton, 1990). However, PMA caused little release of βTG under these conditions, suggesting that activation of PKC is an insufficient stimulus for α-granule secretion. In intact platelets, PMA has been reported to stimulate the secretion of both dense and α-granule constituents (Rink *et al.,* 1983; Krishnamurthi *et al.,* 1986). The reason for this discrepancy is not known, but it is possible that additional factors play a role in the intact system. In the absence of Ca^{2+}, 100 μM GTPγS differed markedly from 100 nM PMA in its effects on secretion from permeabilized platelets, causing release of both βTG and 5-HT, but preferentially of the former (ratio over 1.0, Figure 2C). Selective secretion of platelet factor 4 in response to GTPγS has also recently been noted (Peltola and Scrutton, 1990). Our results also showed that GTPγS stimulated the phosphorylation of pleckstrin in the absence of Ca^{2+}, but much less effectively than PMA. This again indicates that GTPγS has effects, particularly on the secretion of βTG, that cannot be accounted for by the activation of PKC, a conclusion that is further strengthened by the enhanced stimulation of secretion by GTPγS in the presence of an optimal concentration of PMA. Synergism between GTPγS and PMA in the absence of Ca^{2+} was also observed with respect to the secretion of β-glucuronidase from permeabilized HL60 cells (Stutchfield and Cockcroft, 1988).

The differences that we have observed between the effects of PMA and GTPγS are not sufficient to establish that the latter acts through a GTP-binding protein coupled to an effector other than phosphoinositide-specific phospholipase C, because the diacylglycerol formed by this enzyme could well potentiate secretion through effects additional to the activation of PKC. It is, therefore, crucial that we have been able to show for the first time in platelets that GTPγS induces secretion under conditions in which there is no detectable phosphoinositide breakdown. In this regard, our results are similar to those obtained in experiments with permeabilized adrenal chromaffin cells (Bittner *et al.,* 1986) and RINm5F cells (Vallar *et al.,* 1987). Secretion has also been dissociated from phospholipase C activity by neomycin in streptolysin O–permeabilized mast cells (Cockcroft *et al.,* 1987), studies of which have provided the best evidence that a specific GTP-binding protein, G_E, mediates exocytosis (Gomperts, 1986, 1990). Although secretion from permeabilized mast cells required the simultaneous presence of Ca^{2+} and GTPγS, when the principal anion present was Cl^- (Howell *et al.,* 1987), these stimuli exerted independent effects very similar to those we obtained in platelets, when the anion was glutamate (Churcher and Gomperts, 1990). Thus, there may be no essential difference between exocytosis from platelets and from cells in which a role for a hypothetical G_E has been postulated.

Although the mechanism by which GTPγS stimulates secretion from permeabilized platelets in the absence of Ca^{2+} ions remains obscure, it is associated with the phosphorylation of pleckstrin. The function of pleckstrin is still unknown (Tyers *et al.,* 1988), but phosphorylation of this protein does indicate that activation of PKC has occurred (Sano *et al.,* 1983). Platelets have, in fact, been shown to contain an unidentified isozyme of PKC that can phosphorylate pleckstrin in the absence of Ca^{2+} (Tsukuda *et al.,* 1988), and current results show that PKC is essential for the Ca^{2+}-independent secretion of dense and α-granule constituents, caused by GTPγS (Coorssen, Davidson, and Haslam, unpublished). These findings imply that, even in the absence of Ca^{2+}, GTPγS acts on a G-protein–coupled effector enzyme that generates a lipid second messenger capable of stimulating PKC. Because GTPγS also has an essential action that is not accounted for by activation of PKC, it is possible that a product of the same effector enzyme stimulates secretion by another mechanism, perhaps by acting as a membrane fusogen. Alternatively, GTPγS may stimulate Ca^{2+}-independent secretion through two different GTP-binding proteins. Candidate effector enzymes that could be involved in the formation of activators of PKC and possibly of fusogenic lipids in the absence of Ca^{2+} include a form of phospholipase C that utilizes substrates other than the phosphoinositides, phospholipase A_2 and phospholipase D. A phosphatidylcholine

phospholipase C that is Ca^{2+} independent and is activated by GTPγS has been described in liver membranes (Irving and Exton, 1987) but has not so far been reported in platelets. A GTPγS-activated phospholipase A_2 is present in platelet membranes but appears to require Ca^{2+} (Akiba *et al.,* 1989; Silk *et al.,* 1989). Finally, we have recently shown that rabbit platelet membranes contain a phospholipase D activity that is stimulated by GTPγS in the absence of Ca^{2+}, though this ion does enhance enzyme activity (Haslam *et al.,* 1990; Van der Meulen and Haslam, 1990). In all cases, PMA treatment appears to potentiate the actions of GTPγS on these enzymes, which would be consistent with the effect of PMA on GTPγS-induced secretion from permeabilized platelets. Each of these potential effector enzymes can generate, directly or indirectly, DAG and/or arachidonate, both of which can activate platelet PKC in the absence of Ca^{2+} (Tsukuda *et al.,* 1988). Moreover, DAG (Siegel *et al.,* 1989; Coorssen and Rand, 1990), arachidonate (Meers *et al.,* 1988), and the immediate product of phospholipase D action, phosphatidate (Sundler and Papahadjopoulos, 1981; Leventis *et al.,* 1986), are all potentially fusogenic. However, exogenous phospholipase C does not stimulate secretion from permeabilized adrenal chromaffin cells, despite substantial DAG formation (Eberhard *et al.,* 1990). Moreover, secretion has been dissociated from phospholipase A_2 activation in permeabilized mast cells (Churcher *et al.,* 1990). For these reasons, activation of phospholipase D may be the most promising candidate. A role for phospholipase D–generated phosphatidate or its metabolites in exocytosis from mast cells has recently been proposed (Gruchalla *et al.,* 1990), and it may be significant that activation of this enzyme is particularly prominent in neutrophils (Billah *et al.,* 1989), the cell in which a phospholipase C–independent effect of GTPγS on secretion was first detected (Barrowman *et al.,* 1986).

Materials and methods

Materials

Radioimmunoassay (RIA) kits for βTG, [*side-chain*-2-^{14}C]5-HT (55 mCi/mmol) and ACS scintillant were from Amersham (Oakville, Ont.). [γ-^{32}P]ATP was from Du Pont Canada (Mississauga, Ont.). GTPγS was obtained from Boehringer Mannheim Canada (Laval, Que.). ATP (prepared by phosphorylation of adenosine), GTP, PMA, piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), L-glutamic acid, and bovine serum albumin (fraction V) were from Sigma (St. Louis, MO). Human α-thrombin (2700 U/mg) was kindly provided by Dr. J.W. Fenton II of the New York State Department of Health (Albany, NY). Other materials were from sources listed in Haslam and Davidson (1984a) and Culty *et al.* (1988).

Labeling and permeabilization of human platelet suspensions

Human platelets were isolated, labeled with [^{14}C]5-HT, and washed, as described in Haslam and Davidson (1984a). Labeled platelets—in Ca^{2+}-free Tyrode's solution (pH 6.5) containing 5 mM PIPES, 0.35% (w/v) bovine serum albumin, and 5 mM EGTA—were electropermeabilized in a 0.2-cm-wide chamber by 10 discharges from 4.5-μF capacitors charged at 3 kV; this procedure yielded permeabilized platelets with a sensitivity to Ca^{2+} identical to that described by Haslam and Davidson (1984a). As previously, the permeabilized platelets were 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 potassium salts of glutamic acid (160 mM), HEPES (20 mM), EGTA (2.5 mM), and EDTA (2.5 mM) (buffer A). Immediately after elution, the platelet suspension was diluted with buffer A containing ATP to give 3–5 × 10^8 platelets/ml (final ATP, 5 mM) and stored at 0°C until used (within 2 h).

Incubations

Samples (160 μl) of permeabilized platelet suspension were mixed with 40 μl of buffer A containing any guanine nucleotide and the $CaCl_2$ required to give a particular pCa value (Haslam and Davidson, 1984b). PMA or vehicle (0.4 μl of dimethylsulfoxide) was added at the same time as the platelets to prevent the PMA from coming out of solution. These mixtures were equilibrated for 15 min at 0°C before transfer to 25°C and further incubation for up to 20 min. Thrombin, if present, was added in 2 μl of buffer A at the time of transfer to 25°C. Incubations were terminated by addition of 200 μl of ice-cold buffer (pH 7.4) containing EDTA (35 mM), glutamate (80 mM), and HEPES (20 mM) and immediate centrifugation at 12 000 × *g* for 1 min. Supernatants were quickly recovered for determination of the platelet [^{14}C]5-HT and βTG released.

The concentration of $CaCl_2$ required to give the desired pCa values in the EGTA/EDTA/$MgCl_2$ buffer system used were calculated according to Fabiato and Fabiato (1979). The pH values of the $CaCl_2$ solutions were adjusted so that a final pH of 7.4 was obtained after mixing with the other additions. The [Ca^{2+}_{free}] in incubation mixtures containing no added $CaCl_2$ cannot be determined accurately, but, assuming that the total platelet Ca^{2+} (80 nmol/mg protein; Feinstein and Fraser, 1975) was released into the medium during incubations and that reagent contamination cannot exceed 20 μM Ca^{2+}, we calculate that the pCa value under these conditions must be >8. To verify that this [Ca^{2+}_{free}] has no functional effect, we increased the EGTA concentration in some incubation mixtures from 2.5 to 12.5 mM, which decreases the [Ca^{2+}_{free}] sixfold and, on the above assumptions, gives a pCa value >9 in the absence of added $CaCl_2$.

Measurement of secretion from dense and α-granules

Release of platelet [^{14}C]5-HT was measured by counting 200 μl of supernatant in ACS scintillant; βTG in the supernatant was determined by RIA. Total platelet [^{14}C]5-HT and βTG were determined in permeabilized platelets lyzed by addition of 0.01 vol. of 10% (w/v) Triton X-100. The secretion of each of these granule constituents was expressed as a percentage of the total present in the permeabilized platelet suspension after subtraction of that found in supernatant

from permeabilized platelets that had not undergone equilibration or incubation. The latter amount was never more than 5% of the total [^{14}C]5-HT and 1% of the total βTG in the permeabilized platelets.

The RIA procedure described by Amersham was modified to permit 250 assays/kit. The assay volume was reduced from 450 to 90 μl, comprising 40 μl of ^{125}I-labeled βTG, 40 μl of anti-βTG serum, and 10 μl of βTG standard or of a dilution of platelet supernatant or lysate. Centrifugation of the $(NH_4)_2SO_4$-precipitated antigen-antibody complex was carried out at 12 000 × *g* for 2 min, and the pellet was then washed with an equivalent amount of $(NH_4)_2SO_4$ solution. Both βTG standards and dilutions of experimental samples were adjusted to contain 0.001% Triton X-100. Failure to include Triton X-100 in assays of supernatant samples reduced the values of βTG obtained.

Phosphorylation of platelet proteins

To measure protein phosphorylation in permeabilized platelets, the methods of Haslam and Davidson (1984a) were used. Suspensions of permeabilized platelets (containing 5 mM ATP) were mixed with [γ-^{32}P]ATP to give 100 μCi/ml. Samples (final volume 0.2 ml) were equilibrated and incubated with appropriate additions, as described above, and incubations were then terminated by addition of 1.0 ml of 10% (w/v) trichloroacetic acid. Platelet protein was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Imoaka *et al.*, 1983). After autoradiography, the regions of the gel containing pleckstrin (apparent M_r, 47 000) and the phosphorylated light chain of myosin (apparent M_r, 20 000) were cut out and counted for ^{32}P, which was measured as Cerenkov radiation in 0.01% (w/v) 4-methylumbelliferone. Incorporation of ^{32}P into both pleckstrin and myosin light chain was expressed as pmol/10^9 platelets.

Measurement of [^{3}H]inositol phosphate formation

In some experiments, platelets were labeled with both [^{3}H]inositol and [^{14}C]5-HT before permeabilization, to permit measurement of phosphoinositide-specific phospholipase C activity. Labeling with [^{3}H]inositol and measurement of the total formation of [^{3}H]inositol phosphates in permeabilized platelets were carried out essentially as described by Culty *et al.* (1988). However, the [^{3}H]inositol phosphates were eluted from Dowex-1 together, rather than individually, using 1.2 M ammonium formate in 0.1 M formic acid. LiCl was not included in these assays, because previous work showed that this salt decreased rather than increased the total recovery of [^{3}H]inositol phosphates in this system (Culty *et al.*, 1988).

Statistics

Incubations for measurement of secretion (or [^{3}H]inositol phosphate formation) were performed in triplicate and incubations for measurement of protein phosphorylation in duplicate; values from individual experiments are given as mean ± SE or as mean ± range, respectively. Experiments were repeated three or more times and pooled results are given as mean ± SE; the significance of differences was then determined by two-sided paired *t* tests.

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