Effect of various excitatory agonists on the secretion of 5-hydroxytryptamine from permeabilised human platelets induced by Ca^{2+} in the presence or absence of GTP

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Addition of GTP markedly enhances the ability of thrombin to cause a leftward shift in the Ca^{2+} dose/response curve for 5-hydroxytryptamine secretion from permeabilised human platelets. Little effect is observed on addition of GTP in the absence of thrombin. Neither ADP nor adrenaline, in the presence or absence of GTP, causes such a shift, whereas 5-hydroxytryptamine does so to a small extent but only in the presence of GTP. The leftward shift in the Ca^{2+} dose/response curve induced by 12-*O*-tetradecanoylphorbol-13-acetate or 1-oleyl-2-acetylglycerol is not enhanced by addition of GTP. The thrombin concentration required for half-maximal enhancement of the responce to Ca^{2+} is markedly reduced by addition of GTP. The results support the postulate that the effects of excitatory agonists in this system correlate with their ability to activate phospholipase C and provide further evidence for a role for GTP in signal transduction between the receptor and phospholipase C.

Platelet *Ca^{2+}* *GTP* *Excitatory agonist* *Secretion*

1. INTRODUCTION

Platelet permeabilised by multiple exposures to an intense electric field secrete the contents of their amine storage granules and a proportion of the contents of their lysosomes on addition of micromolar concentrations of Ca^{2+} [1]. Addition of thrombin causes a shift to the left in the dose/response curve for secretion from the amine storage granules induced by Ca^{2+} in this preparation without increasing the maximal extent of this response [2,3]. A similar but smaller shift results from addition of vasopressin, 11,9-epoxymethanoprostaglandin H_2 (U-46619) or platelet-activating factor (PAF) [4,5]. Addition of thrombin enhances the maximal extent of lysosomal secretion induced by Ca^{2+} without causing a significant shift in the dose/response curve [6]. Addition of thrombin also increases 1,2-diacylglycerol production in permeabilised platelets in the presence of Ca^{2+} concentrations which approximate those characteristic of the resting intact cell [7,8]. Furthermore, the effects of thrombin on secretion from the permeabilised platelet can be mimicked by addition of synthetic activators of protein kinase C, e.g., 1-oleyl-2-acetylglycerol, 12-*O*-tetradecanoylphorbol-13-acetate [2,6]. Phorbol esters which are ineffective as activators of protein kinase C are also unable to enhance the effects of Ca^{2+} on secretion from permeabilised platelets [6]. These data suggest that the effects of thrombin, and possibly also of vasopressin, PAF and U-46619, on secretion from this preparation result from the ability of these agonists to activate phospholipase C [9] and hence to cause 1,2-diacylglycerol production from surface membrane phosphoinositides. They also indicate a role for protein kinase C in the signal transduction sequence.

Recently a guanine nucleotide binding protein has been implicated in receptor-phospholipase C coupling on the basis that addition of micromolar concentrations of GTP enhance the stimulation of 5-hydroxytryptamine (5HT) secretion caused by thrombin or PAF in the presence of non-saturating

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concentrations of Ca^{2+} and that such stimulation can also be observed on addition of non-hydrolysable analogues of GTP in the absence of an excitatory agonist [4,5].

To test these postulates further we have examined the effect, in the permeabilised platelet system, of excitatory agonists which have little, if any, capacity to stimulate phospholipase C in the intact platelet [9]. We have also further characterised the effect of GTP on the properties of the response to thrombin.

2. MATERIALS AND METHODS

Blood was obtained by antecubital venepuncture from healthy human volunteers and platelets isolated in a glycine/K glutamate medium (pH 6.6) as described previously [6]. The medium contained either Mg ATP^{2-} or Mg CTP^{2-} and either EGTA or bis(*O*-amonophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid (BAPTA) as specified in the appropriate figure legend.

Platelets were rendered permeable by 10 exposures to an intense electric field (20 $kV \cdot cm^{-1}$, τ 1 30 μs) as described previously [6]. Aliquots (50 μl) of the permeabilised platelet suspension were then added to 50 μl of buffer containing the additions as indicated in the figure legends together with 8 μl of Ca EGTA to give a final EGTA concentration of 15 mM and the calculated Ca^{2+} concentration as indicated in the figure. The platelet count in the final incubation was in the range 2–4 $\times$ 10^8 cells/ml. When the resulting suspension contained only EGTA the pH remained at 6.6 and Ca^{2+} concentrations were calculated using equilibrium constants of $10^{-5.928}$ (Ca EGTA), $10^{-1.445}$ (Mg EGTA), $10^{-3.538}$ (Ca ATP^{2-}) and $10^{-3.675}$ (Mg ATP^{2-}). When the cell suspension had been permeabilised in the presence of BAPTA however, addition of Ca EGTA caused the pH to shift to 6.7 and the Ca^{2+} concentrations of such incubation systems were calculated using equilibrium constants of $10^{-6.959}$ (Ca BAPTA), $10^{-1.77}$ (Mg BAPTA), $10^{-6.127}$ (Ca EGTA), $10^{-1.362}$ (Mg EGTA), $10^{-3.50}$ (Ca ATP^{2-}) and $10^{-3.64}$ (Mg ATP^{2-}).

After incubation at 20°C for a time (minutes) specified in the figure legends, 0.1 ml of 0.1 M BAPTA in the glycine/K glutamate buffer (pH 6.6) was added to reduce the calculated Ca^{2+} concentration to less than 10^{-9} M and the platelets were then removed by centrifugation at 8000 $\times$ *g* for 2 min. The ^{14}C content of the supernatant fraction was estimated and expressed as a % of total ^{14}C content in the cell suspension as described previously [6].

BAPTA was obtained from BDH, and ADP, adrenaline bitartrate, 5-hydroxytryptamine creatinine sulphate, thrombin, ATP, CTP and GTP from Sigma.

3. RESULTS

Haslam and Davidson [4] have reported that addition of GTP to permeabilised platelets causes a significant increase in the extent of 5HT secretion observed at non-saturating concentrations of Ca^{2+} as well as enhancing the effect of thrombin on Ca^{2+}-dependent 5HT secretion. We have been able to duplicate the synergistic interaction between thrombin and GTP in our system but observe only a small effect of 10 μM GTP when added alone (fig.1a). No additional stimulation of secretion is found if the concentration of GTP is increased to 100 μM or if the time of incubation with GTP is increased (not shown). Further evidence for an effect of GTP on the properties of the response to thrombin has been obtained by measurement of the extent of [^{14}C]5HT secretion as a function of thrombin concentration at a series of different Ca^{2+} concentrations and in the presence (b) and absence (a) of 10 μM GTP (fig.2). These data demonstrate that the presence of GTP causes a 5–10-fold decrease in the thrombin concentration which is required to induce half-maximal 5HT secretion in the presence of non-saturating [Ca^{2+}], associated with a noticeable steepening of the dose/response curve (fig.2). No effect of either thrombin or of GTP is observed at the lowest calculated Ca^{2+} concentration used (0.01 μM). In the presence or absence of GTP, increases in calculated [Ca^{2+}] to 0.4, 0.7 and 2.0 μM also progressively decrease the thrombin concentration required to induce half-maximal response, although this effect is less marked than that caused by GTP (fig.2). Comparison of panels (a) and (b) of fig.2 also confirms that addition of 10 μM GTP in the absence of thrombin causes only a minimal increase in the ex-

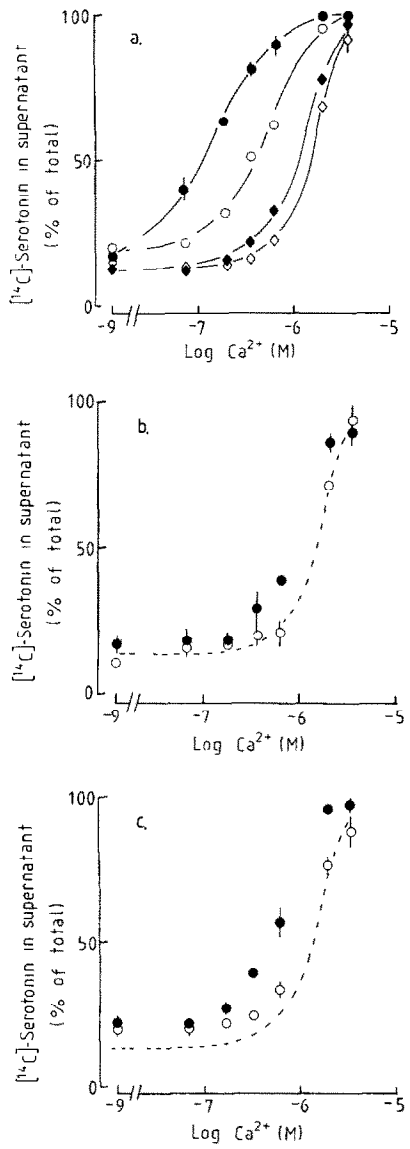

Fig.1. The effect of thrombin (a), adrenaline (b) and 5HT (c) on the Ca^{2+} dependence of [^{14}C]5HT release from permeabilised platelets in the presence and absence of GTP. Platelets suspended in a glycine-based medium (pH 6.6) [6], but containing 10 mM Na glutamate, 5 mM K glutamate, 2 mM Mg ATP^{2-} and 1 mM BAPTA were rendered permeable as described previously [6] and then incubated for 6–12 min at 20°C in the presence (closed symbols) or absence (open symbols) of 10 μM GTP. Aliquots of the cell suspension were then added to an equal volume of the suspension medium containing an appropriate Ca EGTA buffer plus thrombin (a), adrenaline (b) or 5HT (c). The final concentrations of the Ca EGTA buffer, thrombin, adrenaline and 5HT in the incubation medium were 15 mM, 0.6 units·ml^{-1}, 20 μM and 10 μM, respectively. Data for cells incubated in the absence of agonist are shown by ◇, ◆ and for cells incubated in the presence of agonist by ○, ●. After incubation for 10 min at 20°C the reaction was stopped by addition of excess BAPTA and the ^{14}C content of the supernatant fraction estimated as described in section 2. The data points shown are the mean of 3 determinations with the error bars indicating the SE. The dashed and dotted lines in (b) and (c) indicate the positions of the dose/response curves obtained in the absence of any addition and in the presence of thrombin + GTP as drawn on the basis of the data points shown in (a).

tent of [^{14}C]5HT secretion over the range 0.4–2.0 μM Ca^{2+} (calculated).

In contrast, adrenaline (20 μM), when added in the presence or absence of 10 μM GTP, causes no significant shift in the dose/response curve for [^{14}C]5HT secretion induced by Ca^{2+} (fig.1b). Similar results are obtained in the presence of 100 μM GTP or using adrenaline concentrations in the range 5–100 μM (not shown). Addition of 10 μM 5HT causes a slight shift in the Ca^{2+} activation curve but only when added in the presence of 10 μM GTP (fig.1c). However, as indicated by the dotted line, the effect is far less marked than that observed in the presence of thrombin (fig.1a). No additional shift is observed if the GTP concentration is increased to 100 μM (not shown). In fig.1b,c, the dashed line indicates the position of the dose/response curve obtained on addition of Ca^{2+} alone as taken from panel (a).

An identical system cannot be used to examine the effect of ADP on the properties of [^{14}C]5HT secretion from permeabilised platelets because the presence of $MgATP^{2-}$, which is required for the secretory process [10], is likely to inhibit binding of ADP to its receptor [11]. We have therefore performed studies in the presence of Mg CTP^{2-} instead of Mg ATP^{2-} since previous results have shown this nucleotide to be effective in supporting Ca^{2+}-dependent secretion both in the presence and absence of thrombin [6]. Fig.3 demonstrates that addition of 20 μM ADP to such a system has no effect on the dose/response curve for [^{14}C]5HT secretion induced by Ca^{2+}. Addition of 20 μM GTP together with ADP causes a small shift of the

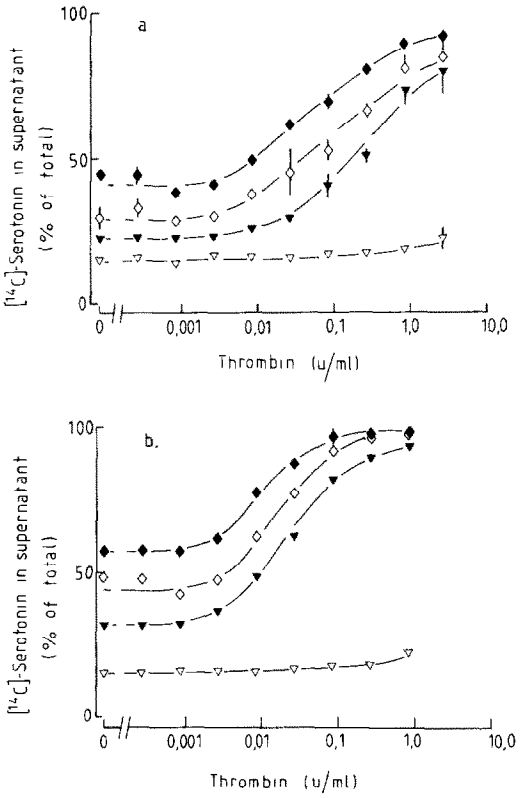

Fig.2. The effect of addition of GTP on the dose/response curve for [^{14}C]5HT release from permeabilised platelets by thrombin determined at several fixed calculated Ca^{2+} concentrations. Platelets suspended in the glycine-based medium (pH 6.6) [6] but containing 2 mM $MgATP^{2-}$ and 1 mM BAPTA were rendered permeable as described previously and then incubated for 15–23 min at 20°C in the absence (a) or presence (b) of 10 μM GTP. Aliquots of these cell suspensions were then added to an equal volume of the suspension medium containing a Ca EGTA buffer and thrombin. The final concentrations of Ca EGTA and thrombin were 15 mM and as indicated in the figure, respectively. The final calculated Ca^{2+} concentrations were <0.01 μM (▽); 0.4 μM (▼), 0.7 μM (◇) and 2 μM (◆). After incubation for 15 min at 20°C the reaction was stopped by addition of BAPTA and the ^{14}C content of the supernatant fraction estimated as described in section 2. The data points are the mean of 3 determinations with the error bars showing the SE.

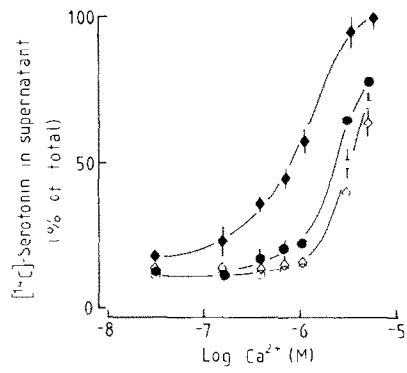

Fig.3. The effect of ADP on the Ca^{2+} dependence of [^{14}C]5HT release from permeabilised platelets in the presence and absence of GTP. Platelets suspended in the glycine-based medium (pH 6.6) [6] but containing 10 mM Na glutamate, 5 mM K glutamate, 0.5 mM EGTA and 5 mM Mg CTP^{2-} were rendered permeable as described previously [6] and then incubated for 5 min at 20°C in the absence or presence of 20 μM GTP. Aliquots of the cell suspension were added to an equal volume of the suspending medium also containing an appropriate Ca EGTA buffer and where indicated ADP or thrombin. The final concentrations of the Ca EGTA buffer, ADP and thrombin in the incubation system were 15 mM, 20 μM and 0.6 units·ml^{-1}, respectively. After incubation for 10 min at 20°C the reaction was stopped by addition of excess BAPTA and the ^{14}C content of the supernatant fraction determined as described in section 2. The data points are the mean of 3 determinations with the error bars indicating the SE. The symbols indicate respectively incubation with Ca EGTA alone (◇), Ca EGTA + ADP (○), Ca EGTA + ADP + GTP (●), Ca EGTA + thrombin (◆).

dose/response curve to the left but this effect is no greater than that observed for addition of GTP alone (fig.1a). Addition of thrombin, however, causes a very significant leftward shift in the dose/response curve which approaches that observed in the system containing Mg ATP^{2-} (fig.1a,3). Hence the nature of the effect of thrombin on Ca^{2+}-induced [^{14}C]5HT secretion is not altered by this nucleotide substitution.

Addition of GTP has no effect on the leftward shift of the Ca^{2+} dose/response curve for [^{14}C]5HT release induced by substances which directly activate protein kinase C without involvement of a surface membrane receptor. This is shown for TPA in fig.4. Similar results have been obtained for OAG (not shown). The EC_{50} value for Ca^{2+} is obtained as 1.3 μM in the absence of other additions and as 0.39 μM in the presence of 16 nM TPA ± 20 μM GTP. In the same experiment, addition of GTP markedly enhanced the

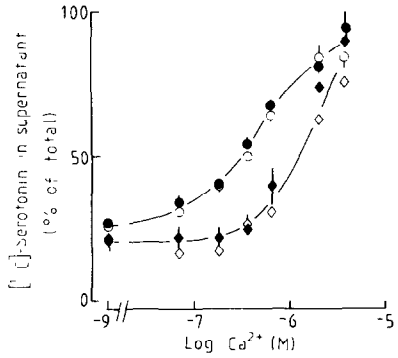

Fig.4. The effect of TPA on the Ca^{2+} dependence of [^{14}C]5HT release from permeabilised platelets in the presence and absence of GTP. Platelets suspended in the glycine-based medium (pH 6.6) [6] but containing 2 mM Mg ATP^{2-}, and 1 mM BAPTA were rendered permeable as described previously [6] and then incubated for 10 min at 25°C. After incubation for a further 7 min in the presence or absence of 20 μM GTP, aliquots of the cell suspension were then added to an equal volume of suspending medium containing appropriate Ca EGTA buffers and also, where indicated, TPA. The final concentrations of the Ca EGTA buffer and TPA were 15 mM and 16 nM, respectively. After incubation for 15 min at 20°C the reaction was stopped by addition of excess BAPTA and the ^{14}C content of the supernatant fraction determined as described in section 2. The data points are the mean of 3 determinations with the error bars indicating the SE. The symbols indicate respectively incubation with Ca EGTA (◇), Ca EGTA + GTP (◆), Ca EGTA + TPA (○), and Ca EGTA + TPA + GTP (●).

leftward shift of the Ca^{2+} dose/response curve caused by addition of thrombin (cf. fig.1a). The EC_{50} for Ca^{2+} was obtained as 0.4 μM in the presence of 0.5 unit·ml^{-1} thrombin and as 0.09 μM in the presence of 0.5 unit·ml^{-1} thrombin + 20 μM GTP.

4. DISCUSSION

Our studies provide further evidence in support of the postulate [2,7] that excitatory agonists enhance the ability of Ca^{2+} to induce amine storage granule secretion from permeabilised platelets as a consequence of activation of phospholipase C and the resultant production of 1,2-diacylglycerol. Agonists such as ADP and adrenaline which have little, if any, ability to activate phospholipase C (as indicated by agonist-stimulated production of 1,2-diacylglycerol [12] or phosphatidate [9]) also fail to enhance Ca^{2+}-dependent [^{14}C]5HT secretion from permeabilised platelets (fig.1b,3). 5HT, which is weakly effective as an agonist for phosphatidate synthesis [9], causes a similar weak enhancement of Ca^{2+}-dependent [^{14}C]5HT secretion (fig.1c). This response is much smaller than that observed on addition of thrombin (fig.1a) which has a much higher efficacy for both 1,2-diacylglycerol synthesis [12,13] and for phosphatidate [9] synthesis. The results previously obtained for the effects of vasopressin, PAF and U-46619 also accord with this correlation since these agonists cause both phosphatidate synthesis [9] and enhancement of Ca^{2+}-dependent [^{14}C]5HT secretion [4,5] at a level which is intermediate between that observed on stimulation by thrombin and by 5HT.

Our data are also consistent with the proposed role for GTP in mediating maximally effective coupling between phospholipase C and surface membrane receptors for excitatory agonists which have the capacity to activate this enzyme [4] (fig.1), and provide further support for this model by demonstrating that no effect of GTP is observed when Ca^{2+}-mediated secretion is enhanced by compounds, e.g., TPA, which interact directly with an intracellular target (fig.4). Although the leftward shift in the Ca^{2+} dose/response curve observed in the presence of TPA or OAG (EC_{50} Ca^{2+} = 0.4 μM) is smaller than that achieved on addition of thrombin + GTP (EC_{50} Ca^{2+} = 0.09 μM) (fig.1a,4), the failure to observe the expected equivalence may be due to the use of non-saturating levels of synthetic and exogeneously added analogues which are in any case likely to be less effective than natural 1,2-diacylglycerol generated in situ. Furthermore, the effect of GTP in steepening the thrombin dose/response curve accords with predictions based on analogy with receptor–N-protein interaction in the adenylate cyclase system, although it is not predicted on the basis of the analogy that addition of GTP would enhance the affinity for thrombin (fig.2) [14].

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