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Secretion of 5-hydroxytryptamine from electropermeabilised human platelets

Effects of GTP and cyclic 3′,5′-AMP

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Enhancement by thrombin of Ca^{2+}-dependent 5HT secretion in the absence of added GTP decreases as the time between electropermeabilisation and addition of thrombin is increased. No decrease occurs if thrombin is added with GTP. Observation of apparent GTP-independent receptor/phospholipase C coupling may result from the presence of bound GTP in the preparation. Enhancement by GTP of Ca^{2+}-dependent 5HT secretion occurs with a significant lag indicating an agonist-independent effect. Cyclic 3′,5′-AMP inhibits enhancement by GTP of Ca^{2+}-dependent 5HT secretion while having no effect on enhancement induced by GTPγS. Hence cyclic AMP may impair receptor/phospholipase C coupling by enhancing N_p GTPase activity.

Platelet; Ca^{2+}; GTP; Secretion; cyclic AMP; Thrombin

1. INTRODUCTION

Previous studies have shown that Ca^{2+}-dependent secretion of 5-hydroxytryptamine (5HT) from electropermeabilised human platelets is enhanced by addition of an excitatory agonist, e.g. thrombin. This effect is due to an agonist induced reduction in the Ca^{2+} concentration ($EC_{50}^{Ca^{2+}}$) required to obtain half-maximal 5HT secretion [1–3]. Addition of a synthetic 1,2-diacylglycerol which mimics the consequence of hydrolysis of phosphoinositides by phospholipase C also enhances Ca^{2+}-dependent 5HT secretion by decreasing $EC_{50}^{Ca^{2+}}$ [2]. Since thrombin induces 1,2-diacylglycerol production in the permeabilised preparation [3], effects of agonists and other factors on $EC_{50}^{Ca^{2+}}$ for 5HT secretion can therefore be used as indirect indicators of the activation status of phospholipase C. Addition of GTP potentiates the effect of excitatory agonists on $EC_{50}^{Ca^{2+}}$ while even more marked decreases in this parameter are observed on addition of a metabolically stable GTP analogue, such as GTPγS, in the absence of an excitatory agonist [4,5]. However, the decrease in $EC_{50}^{Ca^{2+}}$ induced by a synthetic 1,2-diacylglycerol is not potentiated by addition of GTP under comparable conditions [5]. Hence, the site of action of GTP is believed to be at or near the level of the receptor, and involvement of a nucleotide-binding protein (N_p) in receptor-phospholipase C coupling has been postulated in the platelet [4]. However, since a substantial decrease in $EC_{50}^{Ca^{2+}}$ was observed on addition of thrombin in the absence of added GTP, Haslam and Davidson [3] suggested that a receptor-phospholipase C coupling mechanism might exist which did not involve participation of the putative N_p protein.

Inhibitory agonists, e.g. prostaglandin I_2,

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decrease platelet responsiveness as a consequence of their ability to activate adenylate cyclase and hence increase intracellular cyclic 3',5'-AMP (cAMP) levels [6]. Previous studies using electropermeabilised platelets have shown that the decrease in the extent of Ca^{2+}-dependent 5HT secretion caused by addition of cAMP results at least in part from inhibition of polyphosphoinositide hydrolysis, but are inconsistent with a direct effect of cAMP on phospholipase C [7].

The studies described here were designed to answer several questions. First, does the decrease in $EC_{50}^{Ca^{2+}}$ which can be observed under some conditions on addition of GTP in the absence of an excitatory agonist have the properties expected for an agonist-independent response? Second, is the observation of an agonist-induced decrease in $EC_{50}^{Ca^{2+}}$ in the absence of added GTP really indicative of a GTP-independent pathway of receptor/phospholipase C coupling as has been suggested previously [4]? And third, what is the site of action of cAMP on the receptor/phospholipase C transmembrane coupling system?

2. MATERIALS AND METHODS

Washed and aspirin-treated human platelets were incubated with [^{3}H]5HT as in [2] before being suspended in a glycine/K glutamate medium at pH 6.6 containing 4 mM $MgATP^{2-}$ and 0.4 mM EGTA, as described [2], or in this medium at pH 7.4. The pH 6.6 solution contained 2 mM Mg^{2+}, and the pH 7.4 solution contained 4 mM Mg^{2+}. When the Ca^{2+} concentration in the pH 7.4 solution was raised by addition of CaEGTA/EDTA buffers the total Mg concentration was adjusted to keep the Mg^{2+} concentration at approx. 4 mM. Platelets suspended in the pH 6.6 medium were rendered permeable by 10 exposures to a 20 $kV \cdot cm^{-1}$ field ($\tau = 30\ \mu s$) at 20°C (the '20°C preparation') as described in [8]. Aliquots of the permeabilised suspension were then incubated at 20°C for the times indicated in the figure legends before being challenged to secrete by addition of aliquots (75 μl) of the cell suspension to 35 μl of the glycine-based medium containing the appropriate Ca^{2+} buffer and other additives as indicated in the appropriate figure legends. The cell density in the final incubation medium was in the range $1\text{–}2 \times 10^8$ platelets/ml. After a further 15 min at 20°C secretion was terminated by addition of 100 μl of the suspension medium containing 0.1 M BAPTA and the platelets removed by centrifugation at $8000 \times g$ for 2 min. Aliquots (75 μl) of the supernatant fraction were added to 3.5 ml Cintran T liquid scintillation cocktail (BDH) and the ^{3}H content estimated using an LKB Wallac liquid scintillation spectrometer. The total cellular ^{3}H content was obtained by addition of aliquots (75 μl) of the platelet suspension to the liquid scintillation cocktail, and the extent of secretion was expressed as a percentage of the total ^{3}H content.

Platelets suspended in the pH 7.4 medium were rendered permeable as described above for the pH 6.6 medium but at 2°C (the '2°C preparation'). Aliquots of this permeabilised platelet suspension, held at 2°C, were added to aliquots of the glycine suspension medium containing the appropriate Ca^{2+} buffer and the additives as indicated in the figure legend. After equilibration at 2°C for the times as described in the figure legends, the cells were induced to secrete by a temperature jump to 20°C. This temperature increase was achieved by immersing the samples for 13 s in a 37°C water bath. Cells were then incubated at 20°C for 15 min (unless otherwise indicated in the figure legend) and secretion was terminated by immersing the samples in ice and immediately adding 100 μl of ice-cold glycine suspending medium containing 0.1 M BAPTA. Platelets were then removed and the extent of secretion estimated as described above for the 20°C preparation. The data points shown are the mean of three measurements with the error bars indicating the SE. The Ca^{2+} concentrations were calculated using the stability constants given in [7]. GTPγS was obtained from Boehringer-Mannheim; GTP (ATP-free), cAMP and human thrombin from Sigma, and BAPTA from BDH.

3. RESULTS AND DISCUSSION

Previous studies [3,4] have shown that addition of GTP in the absence of an added excitatory agonist, e.g. thrombin, decreases the $EC_{50}^{Ca^{2+}}$ for 5HT secretion when the studies are performed at pH 7.4 using a preparation that had been maintained at 2°C during electropermeabilisation and

gel filtration. This effect of GTP on the Ca^{2+} activation curve is not, however, observed when the studies are performed at pH 6.6 with a cell preparation which had been electropermeabilised at 20°C and then used without further treatment [5,6]. We have previously shown that the incubation temperature is the critical factor for maintenance of the apparent agonist-independent effect of GTP [7]. However, the effect of GTP in $EC_{50}^{Ca^{2+}}$ observed in the absence of an added excitatory agonist might result from contamination of the preparation with such an agonist, e.g. thrombin. This possibility can be tested by examining the time course of 5HT secretion since if the enhancement of the response by GTP is truly agonist-independent it would be expected [9] to occur with a significant lag which reflects slow exchange of GTP for GDP on the putative N_p protein involved in receptor/phospholipase C coupling. Such an experiment performed using the 2°C preparation is shown in fig.1. 5HT secretion initiated by a temperature jump to 20°C after addition and equilibration with 3 or 10 μM Ca^{2+} at 2°C occurs without a detectable time lag (fig.1A) whereas a clear delay of 30–60 s is observed when this response is induced at low Ca^{2+} concentrations in the presence of saturating concentrations of either GTP (fig.1B) or GTPγS (fig.1C). Previous studies have shown that such a delay is not observed when 5HT secretion is induced at low Ca^{2+} concentrations by addition of GTP in the presence of thrombin [7]. Further evidence specifically excluding the possibility that contamination by thrombin might account for the ability of GTP to decrease $EC_{50}^{Ca^{2+}}$ is provided by the observation (not shown) that this effect of GTP is not decreased by addition of hirudin, a specific, high-affinity inhibitor of thrombin [10]. Enhancement of Ca^{2+}-dependent 5HT secretion by GTP added in the absence of an excitatory agonist is concentration-dependent with an $EC_{50}^{Ca^{2+}}$ (3 μM) (fig.2) comparable to that determined previously in studies conducted in the presence of thrombin using the 20°C preparation [7].

We have also examined the relationship between the effects of thrombin and GTP on Ca^{2+}-dependent 5HT secretion under conditions (20°C preparation, pH 6.6) where no response is observed to GTP in the absence of thrombin. Figs 3 and 4 demonstrate that the results obtained de-

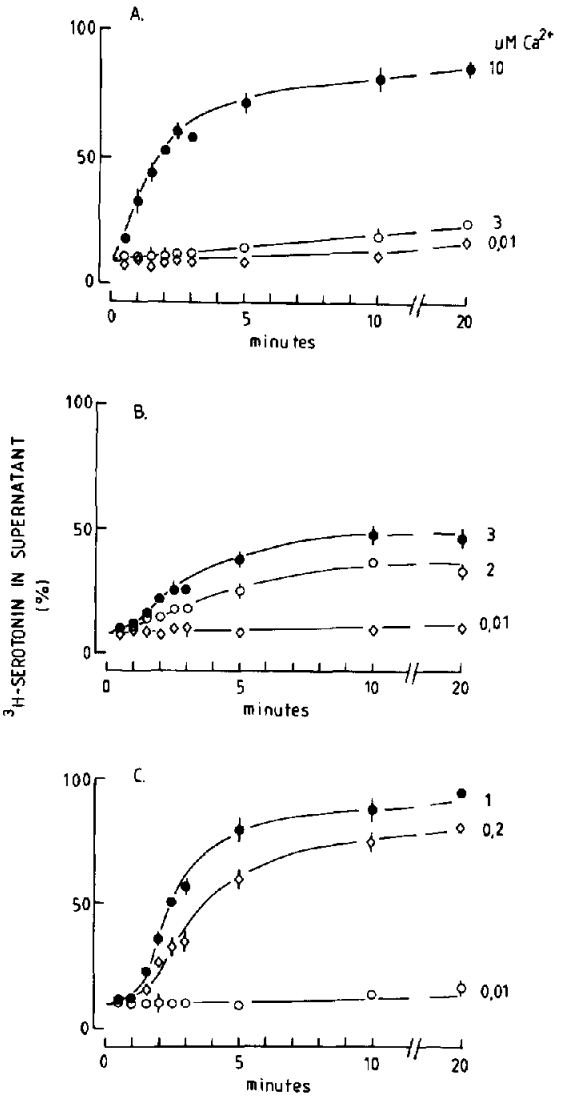

Fig.1. Time course of [^{3}H]5HT secretion induced by (A) Ca^{2+}, (B) Ca^{2+} in the presence of GTP, and (C) Ca^{2+} in the presence of GTPγS. Platelets suspended in the pH 7.4 medium were rendered permeable at 0°C and immediately incubated at 0°C with (A) no addition, (B) 100 μM GTP, or (C) 50 μM GTPγS for 2 min. Aliquots (100 μl) of cell suspension were then added to 6.6 μl of an appropriate Ca^{2+} buffer to give a final Ca^{2+} concentration of (A) 0.01 μM (◇), 3 μM (○), 10 μM (●); (B) 0.01 μM (◇), 2 μM (○), 3 μM (●); and (C) 0.01 μM (○), 0.2 μM (◇), and 1 μM (●). After 5 min at 0°C the temperature was rapidly raised to 20°C and at the various times shown the secretory response was terminated and the ^{3}H content of the supernatant fraction determined as described in section 2.

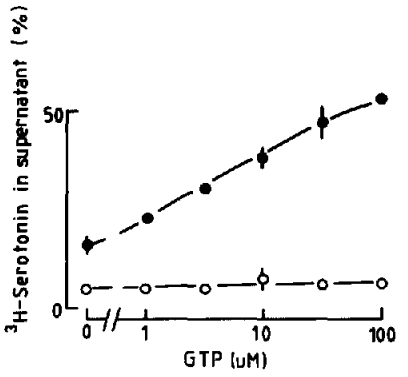

Fig.2. Relationship between the extent of [^{3}H]5HT secretion and GTP concentration. Platelets suspended in the pH 7.4 medium were rendered permeable at 0°C and immediately incubated for 10 min with the various concentrations of GTP as shown. Aliquots (100 μl) of the cell suspension were then challenged at 0°C with 6.6 μl of a Ca^{2+} buffer to give final Ca^{2+} concentrations of either 0.01 μM (○), or 2 μM (●). After a further 10 min at 0°C the temperature was rapidly raised to 20°C. After incubation at 20°C for 20 min the secretory response was terminated and the ^{3}H content in the supernatant determined as described in section 2.

pend very much on the experimental conditions employed, and on the time which elapses between the platelets being rendered permeable and being induced to secrete. If platelets are challenged with thrombin and Ca^{2+} shortly after being rendered permeable the $EC_{50}^{Ca^{2+}}$ is enhanced to such an extent that the presence of GTP has little additional effect on this parameter. As the time between permeabilisation and the challenge with Ca^{2+} is increased (fig.3B) the effect of thrombin on $EC_{50}^{Ca^{2+}}$

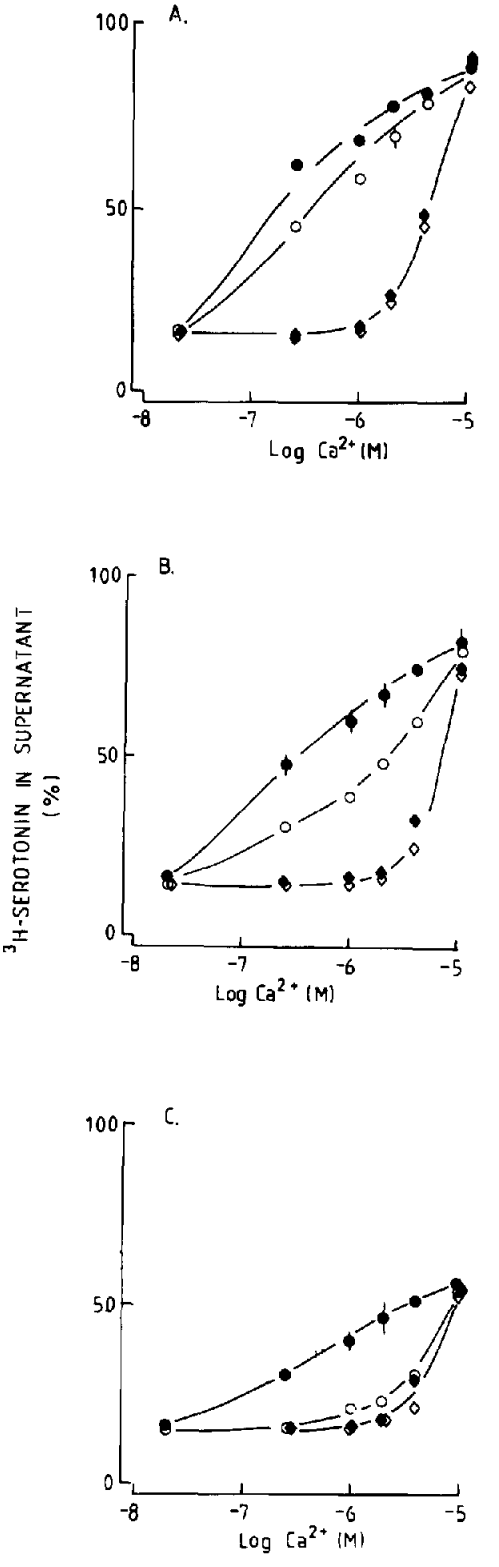

Fig.3. The effect of time of incubation at 20°C on the responsiveness of electropermeabilised platelets to thrombin in the presence and absence of GTP. Platelets in the pH 6.6 medium were rendered permeable at 20°C and immediately incubated with (closed symbols) or without (open symbols) 200 μM GTP. At various times later [(A) 5 min, (B) 20 min, (C) 90 min] aliquots of cell suspension (75 μl) were added to 25 μl of the glycine-based pH 6.6 medium containing CaEGTA to give the Ca^{2+} concentrations shown in the abscissa and with (○,●) or without (◇,◆) 5 nM thrombin. The secretory response was stopped after 15 min and the ^{3}H content of the supernatant fraction determined as described in section 2.

becomes less marked. However if the cells are incubated in the presence of GTP over this period, the effectiveness of thrombin in reducing $EC_{50}^{Ca^{2+}}$ is maintained (fig.4). Most of our previous studies [5,7] have been performed with cells induced to secrete 15–30 min after being rendered permeable. Hence the results have indicated a partial dependence of the effect of thrombin on added GTP similar to that shown in fig.3B. If the platelets are incubated at 20°C for an even longer period (60–90 min) before being challenged with Ca^{2+}, thrombin now has little effect on $EC_{50}^{Ca^{2+}}$ (fig.3C). If, however, GTP was present during this preincubation period, addition of thrombin together with Ca^{2+} causes a marked decrease in $EC_{50}^{Ca^{2+}}$. If taken in isolation, fig.3A would suggest that the effect of thrombin on $EC_{50}^{Ca^{2+}}$ is GTP independent, fig.3B that a component of this effect of thrombin is independent of GTP whilst another component is GTP dependent, and fig.3C that thrombin has no effect on $EC_{50}^{Ca^{2+}}$ unless GTP is added. However, when taken together, the data suggest that tightly bound GTP is present in the system and mediates the ability of thrombin to decrease $EC_{50}^{Ca^{2+}}$ which on the basis of previous studies is an indicator of thrombin-induced activa-

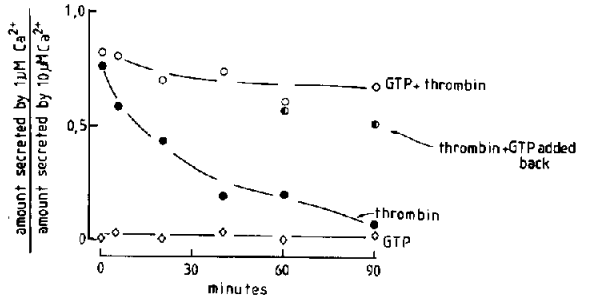

Fig.4. The decline in responsiveness to thrombin as a function of the time after electropermeabilisation. The experimental conditions are as described for fig.3. The ordinate is expressed as the amount of [^{3}H]serotonin released over 15 min as a result of raising Ca^{2+} from 0.1 to 1 μM, relative to the amount released by 10 μM Ca^{2+}. The abscissa is the time between electropermeabilisation and challenging the cells with Ca^{2+}. Cells incubated in the presence of 200 μM GTP and challenged with Ca^{2+} alone (◇), or with Ca^{2+} and thrombin (○); cells incubated in the absence of GTP and challenged with Ca^{2+} and thrombin (●); cells incubated in the absence of GTP and challenged after 60 and 90 min with Ca^{2+} together with both thrombin and 200 μM GTP (◐).

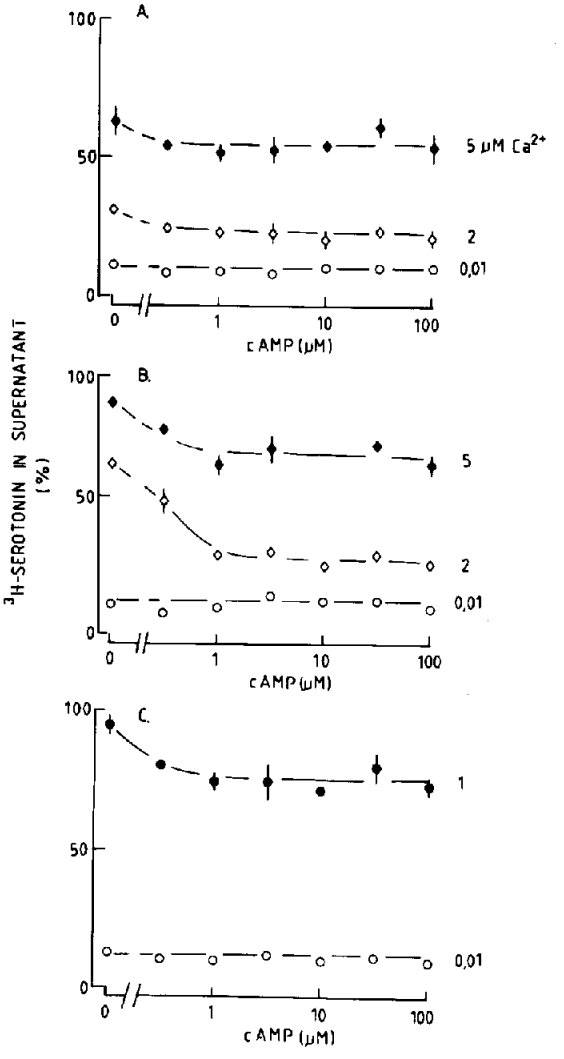

Fig.5. The effect of cyclic AMP on [^{3}H]5HT secretion induced by Ca^{2+} in the presence or absence of GTP and GTPγS. Platelets suspended in the pH 7.4 medium were rendered permeable at 2°C and incubated with 2 μM IBMX together with the various concentrations of cAMP as indicated. After 6 min 35 μl of cell suspension were added to 35 μl of the glycine medium containing similar levels of cAMP and the appropriate Ca^{2+} buffer either alone (A), or together with GTP or GTPγS, to give a final concentration of (B) 100 μM GTP, (C) 20 μM GTPγS. After 2 min the cells were rapidly warmed to 20°C. After 15 min incubation at 20°C secretion was terminated and the ^{3}H content of the supernatant fraction estimated as described in section 2. In (A,B) the final Ca^{2+} concentrations were 0.01 μM (○), 2 μM (◇), and 5 μM (◆); and in (C) 0.01 μM (○), and 1 μM (●).

tion of phospholipase C [2–4]. Prolonged incubation at 20°C is required to remove, or degrade, tightly bound GTP and hence reveal the absolute requirement for this nucleotide in receptor/phospholipase C coupling. Our observations therefore suggest that no GTP-independent receptor-phospholipase C coupling pathway [3] is likely to exist. In cells which have been incubated for 60–90 min in the absence of GTP the ability of thrombin to decrease $EC_{50}^{Ca^{2+}}$ can be restored simply by adding back the nucleotide. Hence the protective effect observed on incubation with GTP (figs 3,4) may be due to maintenance of the required concentration of this nucleotide in the system. However, we cannot exclude the possibility that the presence of GTP during the preincubation period also stabilises the transmembrane coupling machinery.

We have further used the 2°C preparation to obtain insight into the mechanism by which an increase in cAMP may inhibit 5HT secretion from electropermeabilised platelets. In this preparation the component of the secretory response induced by addition of 0.1 mM GTP alone in the presence of 2 μM Ca^{2+} is markedly inhibited by addition of cAMP. Fig.5B shows that addition of 1 μM cAMP completely abolishes the decrease in $EC_{50}^{Ca^{2+}}$ induced by addition of GTP. We have previously shown, using the 20°C preparation, that addition of 5 μM cAMP abolishes the enhancement of Ca^{2+}-sensitive 5HT secretion induced by thrombin in the presence of GTP [7]. In contrast, this concentration of cAMP has little effect on the extent of 5HT secretion induced either by Ca^{2+} alone (fig.5A) or by GTPγS in the presence of a low concentration of Ca^{2+} (fig.5C). These latter results are comparable to those obtained with the 20°C preparation [7]. The finding that the GTP-sensitive component of the secretory response observed in the absence of thrombin is inhibited by cAMP, whereas the GTPγS component is not, suggests that the GTPase activity of the putative N_p protein may be modified by the presence of cAMP. The results shown in fig.5 together with those obtained previously [7] are consistent with the postulate that cAMP inhibits agonist-induced activation of phospholipase C by enhancing the GTPase activity of the N_p protein. The efficiency of receptor-phospholipase C coupling would therefore be diminished due to the decreased lifetime of the N_p-GTP complex. A similar suggestion has recently been made by Haslam [11].

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