

Inhibition of platelet-activating-factor-induced human platelet activation by prostaglandin D₂

Differential sensitivity of platelet transduction processes and functional responses to inhibition by cyclic AMP

Mark BUSHFIELD, Archibald McNICOL and D. Euan MACINTYRE*

Department of Pharmacology, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

It has been proposed that cyclic AMP inhibits platelet reactivity: (a) by preventing agonist-induced phosphoinositide hydrolysis and the resultant formation of 1,2-diacylglycerol and elevation of cytosolic free Ca²⁺ concentration ([Ca²⁺]_i); (b) by promoting Ca²⁺ sequestration and/or extrusion; and (c) by suppressing reactions stimulated by (1,2-diacylglycerol-dependent) protein kinase C and/or Ca²⁺-calmodulin-dependent protein kinase. We used the adenylate cyclase stimulant prostaglandin D₂ to compare the sensitivity to cyclic AMP of the transduction processes (phosphoinositide hydrolysis and elevation of [Ca²⁺]_i) and functional responses (shape change, aggregation and ATP secretion) that are initiated after agonist-receptor combination on human platelets. Prostaglandin D₂ elicited a concentration-dependent elevation of platelet cyclic AMP content and inhibited platelet-activating-factor (PAF)-induced ATP secretion [I₅₀ (concn. causing 50% inhibition) ~ 2 nM], aggregation (I₅₀ ~ 3 nM), shape change (I₅₀ ~ 30 nM), elevation of [Ca²⁺]_i (I₅₀ ~ 30 nM) and phosphoinositide hydrolysis (I₅₀ ~ 10 nM). A 2-fold increase in cyclic AMP content resulted in abolition of PAF-induced aggregation and ATP secretion, whereas maximal inhibition of shape change, phosphoinositide hydrolysis and elevation of [Ca²⁺]_i required a greater than 10-fold elevation of the cyclic AMP content. This differential sensitivity of the various responses to inhibition by cyclic AMP suggests that the mechanisms underlying PAF-induced aggregation and ATP secretion differ from those underlying shape change. Thus a major component of the cyclic AMP-dependent inhibition of PAF-induced platelet aggregation and ATP secretion is mediated by suppression of certain components of the activation process that occur distal to the formation of DAG or elevation of [Ca²⁺]_i.

INTRODUCTION

Human platelet responsiveness is controlled by the opposing actions of stimulatory and inhibitory second-messenger molecules. Ca²⁺ and 1,2-diacylglycerol (DAG) act synergistically to promote platelet activation (Michell, 1983), whereas 3',5'-cyclic adenosine monophosphate (cyclic AMP) acts as an inhibitory modulator (Feinstein *et al.*, 1981). Production of DAG and elevation of the cytoplasmic free Ca²⁺ concentration ([Ca²⁺]_i) could both occur as a consequence of agonist-induced phosphoinositide hydrolysis. The phospholipase C-catalysed hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] directly yields DAG and inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃], which can release Ca²⁺ from intracellular stores (O'Rourke *et al.*, 1985). However, in the presence of the normal physiological concentration of extracellular free Ca²⁺ (1 mM), agonist-induced elevation of [Ca²⁺]_i derives predominantly from Ca²⁺ influx (Hallam *et al.*, 1984). The role, if any, of phosphoinositide hydrolysis in this process remains to be determined (Berridge, 1984). Elevation of platelet cyclic AMP concentration by inhibitory platelet agonists (e.g. PGI₂, PGD₂ and adenosine) results in the suppression of platelet activation

(Feinstein *et al.*, 1981). Besides inhibiting the cellular responses (Hawiger *et al.*, 1980; Feinstein *et al.*, 1981) initiated after exposure to stimulatory agonists, the inhibitory effects of cyclic AMP are also evident on the transduction processes that link receptor occupancy to cellular activation (Kaser-Glanzmann *et al.*, 1977; Fox *et al.*, 1979; Feinstein *et al.*, 1983; Sano *et al.*, 1983; Lapetina, 1984).

In order to determine the relationship between elevation of cyclic AMP content and the degree of inhibition of distinct platelet responses, we examined the effects of PGD₂ on shape change, aggregation, ATP secretion, elevation of [Ca²⁺]_i and stimulation of [³²P]PtdA formation (an index of phosphoinositide hydrolysis and DAG formation) induced in human platelets by submaximal concentrations of PAF.

EXPERIMENTAL

Methods

Blood was obtained from healthy volunteers who denied taking drugs known to affect platelet function, and platelet-rich plasma (PRP) was prepared (Pollock *et al.*, 1984). In most cases (except phospholipid studies) the

Abbreviations used: DAG, 1,2-diacylglycerol; [Ca²⁺]_i, cytosolic free Ca²⁺ concentration; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate; PG, prostaglandin; PtdA, phosphatidic acid; PAF, platelet-activating factor (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine); PRP, platelet-rich plasma; I₅₀, concentration causing 50% inhibition.

* To whom reprint requests and correspondence should be addressed.

PRP was incubated with quin2 acetoxymethyl ester (10–15 μM ; 30–40 min; 37 °C). Quin2-labelled platelets were separated from plasma containing extraneous dye by gel filtration on columns of Sepharose 2B equilibrated with a modified Hepes-buffered Tyrode's solution (Pollock *et al.*, 1984). The quin2 content of the platelets was around 1 mmol/litre of cell water. Immediately before use the external free Ca^{2+} concentration was adjusted either to 1 mM (by addition of CaCl_2) or to about 1–10 nM (by addition of EGTA). $[\text{Ca}^{2+}]_i$ was measured as described previously (MacIntyre *et al.*, 1985). Shape change, aggregation and ATP secretion were monitored in parallel with $[\text{Ca}^{2+}]_i$ by using subsamples of the quin2-loaded gel-filtered platelets. Platelet shape change was monitored photometrically in stirred (700 rev./min) platelet suspensions (0.6 ml; 37 °C) by using a Mallin clinical aggregation recorder. In these studies, to decrease the extracellular $[\text{Ca}^{2+}]$ and hence prevent both platelet aggregation and Ca^{2+} influx, EGTA (4 mM) was added to each platelet sample 60 s before agonist addition. Aggregation and ATP secretion were monitored simultaneously in stirred (700 rev./min) platelet samples (0.45 ml; 37 °C) by using a Chronolog Corp. luminescence aggregometer. Incubations were for 90 s with PGD_2 or vehicle [saline (154 mM-NaCl)] before the addition of a submaximal concentration of PAF. The optimal PAF concentration (i.e. that which elicited around 70% of maximum response) was determined at the start of each experiment and varied from 18 to 180 nM-PAF.

Phospholipid studies were carried out essentially as described previously (Pollock *et al.*, 1984). PGD_2 or saline was added for 90 s and reactions were initiated by addition of PAF (18 nM). After 30 s, reactions were terminated (Pollock *et al.*, 1984) and lipids were extracted, dried at 40 °C under N_2 , separated by two-dimensional t.l.c. (Yavin & Zutra, 1977) and detected by exposure to iodine vapour. The spots corresponding to PtdA were scraped into vials and counted for radioactivity in a liquid-scintillation counter. Note that under the conditions of these experiments, changes in $[\text{Ca}^{2+}]_i$ reflect changes in PtdA concentration (Holmsen *et al.*, 1984) and the DAG precursor of PtdA is most probably derived via hydrolysis of phosphoinositides (MacIntyre & Pollock, 1983).

In most cases, for the estimation of cyclic AMP concentration, subsamples of platelets were removed from the fluorimeter or aggregometer cuvettes, or in phospholipid studies from samples processed in parallel, except that $[\text{Ca}^{2+}]_i$ was omitted, before the addition of PAF and were added to 2 vol. of ethanol. After addition to ethanol, the samples were vortex-mixed, left at room temperature for 5 min, centrifuged (10 min; 500 g; room temperature) and the supernatant removed and evaporated at 60 °C. The dried samples were resuspended in 50 mM-sodium acetate buffer, pH 6.2, and the cyclic AMP content was determined by radioimmunoassay (Brooker *et al.*, 1979).

Materials

PGD_2 was a gift from Dr. J. E. Pike (Upjohn). PAF was purchased from Bachem, Bubendorf, Switzerland, and quin2 acetoxymethyl ester from Lancaster Synthesis, Morecambe, Lancs., U.K. $[\text{Amersham}]$ cyclic AMP (adenosine 3',5'-cyclic phosphoric acid 2'-O-succinyl-3- $[\text{Amersham}]$ iodotyrosine methyl ester) was from Amersham

International, and carrier-free $[\text{Amersham}]$ was supplied by the Regional Isotope Dispensary, Western Infirmary, Glasgow, Scotland, U.K. EGTA was obtained from Sigma. All other reagents were of the highest purity available and were obtained as previously described (Pollock *et al.*, 1984).

RESULTS

In confirmation of previous reports (MacIntyre & Pollock, 1983; Fouque & Vargaftig, 1984; Hallam *et al.*, 1984), preliminary studies established that PAF (1–180 nM) elicited, in a concentration-dependent manner, platelet shape change, aggregation, ATP secretion, stimulation of $[\text{Amersham}]$ formation and elevation of $[\text{Ca}^{2+}]_i$. The latter effect was decreased by around 80% in the presence of EGTA (4 mM), indicating that PAF-induced elevation of $[\text{Ca}^{2+}]_i$ comprises two components: influx of external Ca^{2+} and mobilization of internal Ca^{2+} (results not shown).

The basal level of platelet cyclic AMP (6.9 ± 0.4 pmol/ 10^8 cells; mean \pm S.E.M., $n = 6$) was augmented in a concentration-dependent manner after exposure to PGD_2 (Fig. 1a). PGD_2 also caused concentration-dependent inhibition of PAF-induced aggregation, ATP secretion, shape change, $[\text{Amersham}]$ formation and elevation of $[\text{Ca}^{2+}]_i$ monitored both in the presence and in the absence of EGTA (Figs. 1b–1g). PGD_2 was more potent as an inhibitor of PAF-induced ATP secretion (I_{50} around 2 nM) and aggregation (I_{50} about 3 nM) than of $[\text{Amersham}]$ formation (I_{50} about 10 nM), shape change (I_{50} about 30 nM) and elevation of $[\text{Ca}^{2+}]_i$ (I_{50} about 30 nM). Mobilization of internal Ca^{2+} (i.e. monitored in the presence of 4 mM-EGTA) and the elevation of $[\text{Ca}^{2+}]_i$ that results from both influx and mobilization (i.e. monitored in the presence of 1 mM-extracellular Ca^{2+}) were equally susceptible to inhibition by PGD_2 (Figs. 1f and 1g). The degree of inhibition of the various PAF-induced responses could be correlated with the rise in cyclic AMP content elicited by PGD_2 in the same (or parallel samples for $[\text{Amersham}]$ formation) platelet samples (Fig. 2). PAF-induced ATP secretion and aggregation were very susceptible to small increments in the cyclic AMP content, both being abolished by a doubling in the cyclic AMP content (Fig. 2). However, $[\text{Amersham}]$ formation, shape change and elevation of $[\text{Ca}^{2+}]_i$ were less susceptible: maximal inhibition of these responses did not occur until the cyclic AMP content had been elevated by more than 10-fold (Fig. 2).

DISCUSSION

PAF is believed to activate human platelets by mechanisms dependent upon DAG and elevated $[\text{Ca}^{2+}]_i$ and consequent upon receptor-linked phosphoinositide hydrolysis (MacIntyre *et al.*, 1985). PGD_2 causes elevation of platelet cyclic AMP concentration and inhibition of platelet function (Smith *et al.*, 1976; De Gaetano *et al.*, 1982), presumably mediated by cyclic AMP-dependent protein kinase. It has been proposed that elevated cyclic AMP concentrations inhibit platelet function by suppressing the production of the stimulatory second-messenger molecules Ca^{2+} (Feinstein *et al.*, 1983) and DAG (Lapetina, 1984). This effect could be mediated by inhibition of phospholipase C and prevention of receptor-stimulated $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis. Adenylate cyclase stimulants cause a decrease in the elevated $[\text{Ca}^{2+}]_i$

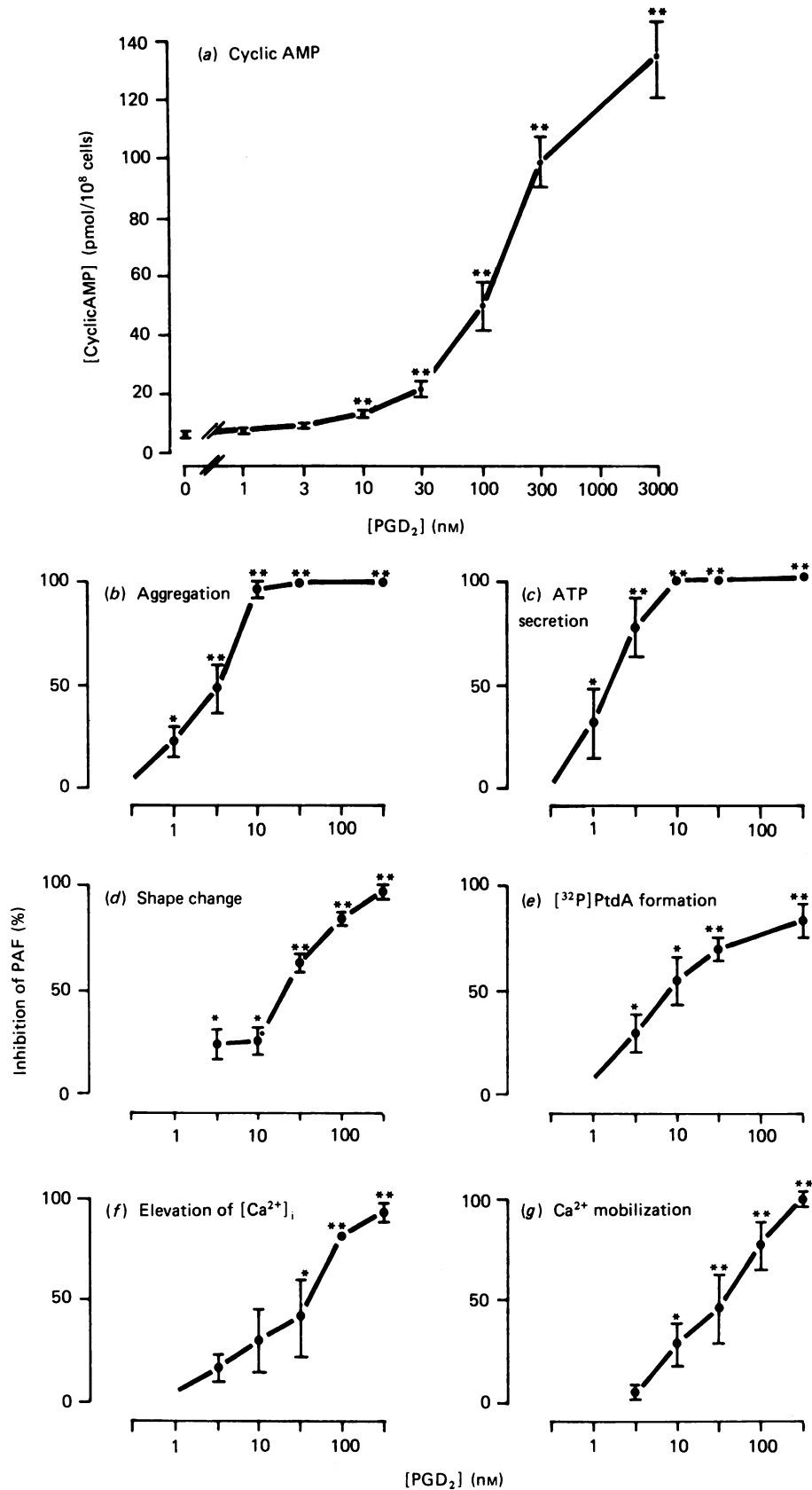


Fig. 1. Effects of PGD₂ on (a) platelet cyclic AMP content and (b-g) PAF-induced platelet responses

Plasma-free platelet suspensions were incubated with PGD₂ (90 s, 37 °C) at the concentrations indicated before the addition of PAF (18–180 nM) and/or removal of a sample for estimation of cyclic AMP content. Changes in (a) cyclic AMP content and inhibition of PAF-induced (b) aggregation, (c) ATP secretion, (d) shape change, (e) [³²P]PtdA formation, (f) elevation of [Ca²⁺]_i in the presence of 1 mM-[Ca²⁺]_o (extracellular free Ca²⁺ concentration) and (g) Ca²⁺ mobilization in the presence of EGTA (4 mM), were monitored as described under 'Methods'. Note that [Ca²⁺]_i changes in part (f) result from mobilization of internal Ca²⁺ stores and influx of external Ca²⁺, whereas those in (g) result from mobilization of internal Ca²⁺ stores alone. The results are mean values ± S.E.M. for between two and seven experiments, each performed in triplicate. The data (PGD₂ versus control) were analysed using Student's *t*-test: **P* < 0.05; ***P* < 0.005.

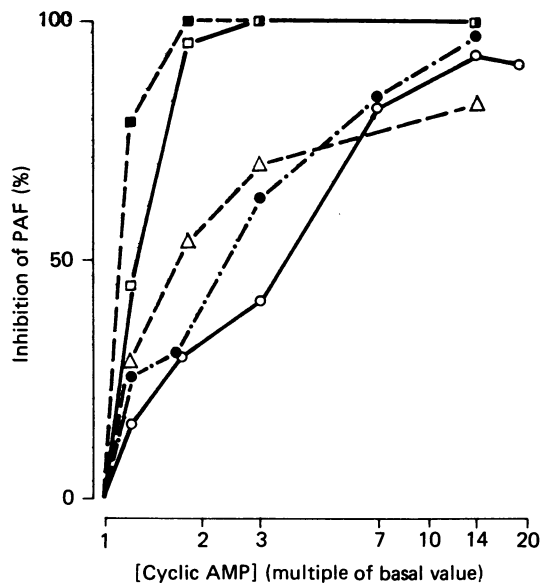


Fig. 2. Relationship between platelet cyclic AMP content and inhibition of PAF-induced platelet responses

The data are taken from Fig. 1 and show inhibition of PAF-induced ATP secretion (■), aggregation (□), [³²P]PtdA formation (△), shape change (●) and elevation of [Ca²⁺]_i (○). The basal cyclic AMP content was 6.9 ± 0.4 pmol/10⁸ platelets.

when added after stimulatory agonists (Feinstein *et al.*, 1983). This effect might be mediated by increased Ca²⁺ sequestration and/or extrusion (Feinstein *et al.*, 1983; Kaser-Glanzmann *et al.*, 1977). In addition, cyclic AMP-dependent protein kinase has been shown to inhibit platelet myosin-light-chain kinase activity, a Ca²⁺-activated enzyme (Hathaway *et al.*, 1981). Hence cyclic AMP can modify the rate of production and inactivation of the stimulatory second messengers and can antagonize their effects to prevent or reverse agonist-induced platelet activation.

In the present study we have shown that small rises in cyclic AMP content (up to a 100% increase) induced by PGD₂ greatly inhibited PAF-induced aggregation and ATP secretion, with lesser inhibition of [³²P]PtdA formation and only marginal inhibitions of shape change or elevation of [Ca²⁺]_i. These results indicate that small rises in cyclic AMP content results in inhibition of some platelet functional responses (aggregation and secretion), but have relatively little effect upon the putative underlying transduction processes, phosphoinositide hydrolysis and elevation of [Ca²⁺]_i. These findings are consistent with cyclic AMP not only acting to prevent the formation or increase the inactivation of Ca²⁺ and DAG, but also acting at a later stage in the activation process to oppose the intracellular actions of Ca²⁺ and DAG. These latter effects could be mediated by inhibition of Ca²⁺- or DAG-dependent phosphorylation reactions or by inhibitory phosphorylation of the same target proteins that are acted upon by the 'stimulatory' kinases. These observations are in accord with a recent report by Pannocchia & Hardisty (1985) in which PGI₂ and dibutyryl cyclic AMP were shown to inhibit platelet functions with little or no inhibition of elevation of [Ca²⁺]_i. However, those authors made no attempt to

correlate the observed effects with the level of platelet cyclic AMP.

Clearly PAF-induced shape change was much less susceptible to small rises in cyclic AMP content than were aggregation and ATP secretion. Similar results were found when PGI₂ was used as the inhibitory platelet agonist (results not shown). The reasons for this discrepancy are unknown, but it might suggest that the mechanisms underlying PAF-induced aggregation and secretion differ from those that govern shape change. It is possible that shape change could be more dependent upon the absolute levels of the stimulatory second messengers, since inhibition of PAF-induced shape change paralleled inhibition of elevation of [³²P]PtdA formation and [Ca²⁺]_i. A recent report has demonstrated identical I₅₀ values for cyclic AMP-dependent inhibition of ADP-induced aggregation and shape change (Steen & Holmsen, 1984). However, those studies may not be directly comparable, since both the stimulatory (ADP) and inhibitory (PGE₁, adenosine) agonists used differed from those employed in the present study. Indeed, there is evidence that different agonists show differential sensitivity to inhibition by cyclic AMP (Krishnamurthi *et al.*, 1984; Rink & Sanchez, 1984).

Human platelets contain a single population of receptors for PGD₂ that are coupled to adenylate cyclase activation and consequent elevation of the platelet cyclic AMP content, to approx. 100-fold the basal level (Smith *et al.*, 1976; Siegl *et al.*, 1979). The differential sensitivity of the various PAF-induced platelet responses to inhibition by PGD₂ reflects, in part, the 'receptor reserve' of the system: that is, a low degree of PGD₂ receptor occupancy, which elicits a modest increment in platelet cyclic AMP content (100%), is sufficient to abolish PAF-induced aggregation and ATP secretion, whereas a higher degree of receptor occupancy that elicits a moderate increase in cyclic AMP content (10-fold) is required to abolish shape change, phosphoinositide hydrolysis and Ca²⁺ flux. The effects of cyclic AMP are thought to be mediated by cyclic AMP-dependent protein kinase, of which two types exist in platelets (Nimmo & Cohen, 1977; Salama & Haslam, 1984). Evidence in numerous cell types would indicate that only small increments in cyclic AMP concentration are necessary to stimulate maximally cyclic AMP-dependent protein kinase (Flockhart & Corbin, 1982). If this is also the case in human platelets, as is indicated by our preliminary findings (M. Bushfield & D. E. MacIntyre, unpublished work), then the bulk of the cyclic AMP generated by platelets in response to adenylate cyclase stimulants is not coupled to activation of cyclic AMP-dependent protein kinases. This could indicate that cyclic AMP and/or cyclic AMP-dependent protein kinase are compartmentalized within platelets, and that the major cyclic AMP compartment is redundant in terms of regulating platelet reactivity. Indeed, there is evidence for compartmentalization of cyclic AMP and cyclic AMP-binding proteins in platelets (Lyons, 1980; Hashimoto, 1983).

The reasons underlying the differential sensitivity of the various platelet responses to increase in cyclic AMP concentration and the role of cyclic AMP-dependent protein kinase in the control of platelet responsiveness require further investigation. We suggest that a major component of inhibition of PAF-induced platelet activation by cyclic AMP-dependent processes is mediated by suppression of certain aspects of the activation process

that occur distal to the formation or elevation of the stimulatory second messengers, Ca^{2+} and DAG.

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