Regulation of platelet cytosolic free calcium by cyclic nucleotides and protein kinase C

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PAF elicits a rapid, concentration-dependent elevation of platelet cytosolic free calcium ($[Ca_f]$), measured by quin2. Elevation of $[Ca_f]$ is transient, and the rate of reversal increases with agonist concentration. Adenylate cyclase stimulants (PGI_2, PGD_2) and 8-bromo cAMP; a guanylate cyclase stimulant (sodium nitroprusside) and 8-bromo cGMP; and a protein kinase C stimulant (phorbol myristate acetate) block the elevation of $[Ca_f]$ induced by PAF, and accelerate its reversal. These results suggest that cAMP, cGMP and 1,2-diacylglycerol (DAG) could act as second messengers to regulate $[Ca_f]$ in platelets. As PAF is known to stimulate platelet phosphoinositide hydrolysis (ergo DAG formation) but fails to elevate platelet cAMP or cGMP, it is proposed that DAG, via activation of protein kinase C, may act as an endogenous modulator of platelet $[Ca_f]$: an action that contributes to the role of DAG as a bi-directional regulator of platelet reactivity.

Platelet *Calcium* *Cyclic AMP* *Cyclic GMP* *Phorbol ester* *Protein kinase C*

1. INTRODUCTION

Platelet activation is mediated by the synergistic interaction of Ca^{2+}-dependent and Ca^{2+}-independent 'pathways'. The former requires that the platelet stimulatory agonists elicit an elevation of the cytosolic free Ca^{2+} concentration, $[Ca_f]$, and the latter that stimulatory agonists evoke formation of 1,2-diacylglycerol (DAG), the endogenous activator of protein kinase C [1]. Elevation of $[Ca_f]$ and DAG formation result from a common receptor-activated transduction process: hydrolysis of inositol phospholipids – phosphatidylinositol (PtdIns), PtdIns 4-phosphate (PtdIns 4P) or more likely, PtdIns 4,5-bisphosphate (PtdIns 4,5P_2) [2]. The phospholipase C-catalysed hydrolysis of PtdIns 4,5P_2 yields DAG and inositol 1,4,5-trisphosphate (Ins P_3) which can mobilise Ca^{2+} from intracellular storage sites [3].

As in other cell types, there must exist within platelets homeostatic mechanisms for maintaining resting $[Ca_f]$ at or near 100 nM, some 10000-fold less than the free Ca^{2+} concentration in plasma. Moreover, platelet activation may be a reversible process [4], and the elevation of $[Ca_f]$ (and, incidentally, of DAG or phosphatidic acid) observed following the addition of thrombin, PAF, TxA_2-mimetics, ADP, vasopressin and 5HT to human platelets are not maintained [5]. Thus, besides the homeostatic processes that maintain resting $[Ca_f]$ at or near 100 nM, there must exist within platelets mechanisms for restoring elevated $[Ca_f]$ towards basal. In other cells, $[Ca_f]$ is regulated by systems that limit Ca^{2+} influx, enhance Ca^{2+} sequestration and promote Ca^{2+} extrusion [6]. Similar mechanisms may operate in platelets. Mitochondria and the dense tubular system have been implicated as Ca^{2+}-sequestering organelles, with the latter being of major importance. The isolated dense tubular system actively accumulates Ca^{2+} and contains a $(Ca^{2+}+Mg^{2+})$-ATPase [7]. Sequestration of Ca^{2+} within this

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organelle is augmented by cAMP [8] and inhibited by phenothiazines [9], putative calmodulin 'antagonists' which exert numerous effects including inhibition of protein kinase C [10]. The mechanisms underlying extrusion of platelet Ca^{2+} are less well characterised. In other tissues Ca^{2+} efflux is commonly mediated via $(Ca^{2+}+Mg^{2+})$-ATPase and/or Na^+/Ca^{2+} exchange [6]. However the presence of a plasma membrane $(Ca^{2+}+Mg^{2+})$-ATPase in platelets remains to be demonstrated, and Na^+/Ca^{2+} exchange does not operate in resting platelets [11], although it may well operate at elevated $[Ca_f]$.

Adenylate cyclase stimulants inhibit platelet reactivity and suppress agonist-induced elevation of $[Ca_f]$ [12]. This is taken as evidence that cAMP can regulate platelet Ca^{2+} homeostasis. However, other endogenous modulators of Ca^{2+} homeostasis may exist. Agents that stimulate cGMP formation inhibit platelet reactivity, but the mechanism(s) underlying this effect are poorly understood [13]. Tumour-promoting phorbol esters (e.g., phorbol 12-myristate 13-acetate, PMA), which mimic the effects of endogenous DAG in the activation of protein kinase C, inhibit agonist-induced elevation of $[Ca_f]$ in platelets and other cells [14,15]. Here,

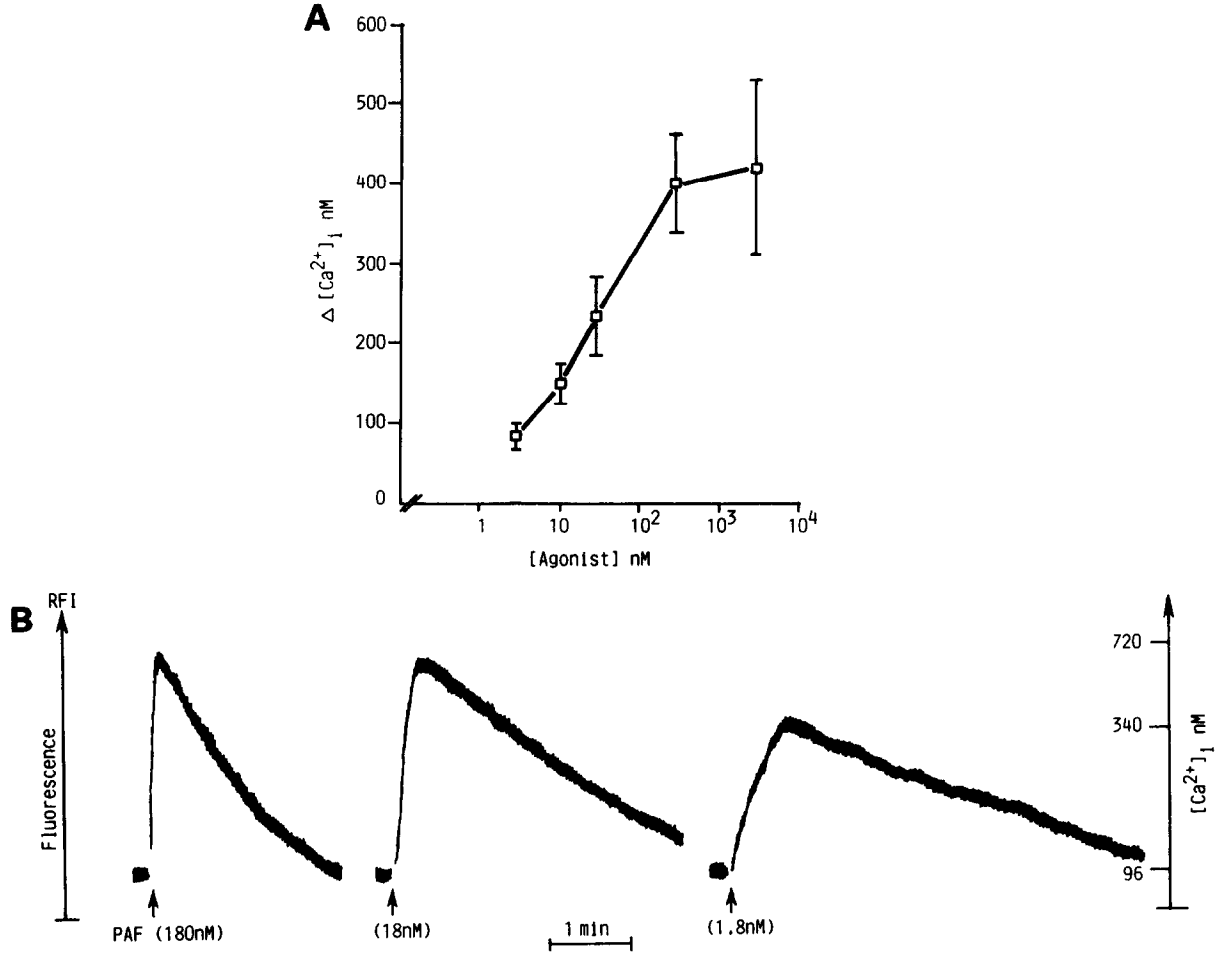

Fig.1. PAF-induced elevation of platelet $[Ca_f]$. Quin2-labelled human platelets were stimulated with PAF at the concentrations indicated. Changes in $[Ca_f]$ were calculated from the observed changes in dye fluorescence. (A) Concentration-response relationship: results, expressed as increase in $[Ca_f]$ above basal (90 nM) are mean values ± SD of 6–9 observations using platelets from different donors. (B) Fluorescence records: the data shown are typical of 4 experiments using platelets from different donors.

using adenylate cyclase stimulants (PGI_2, PGD_2) and 8-bromo cAMP, a guanylate cyclase stimulant (sodium nitroprusside, NaNP) and 8-bromo cGMP, and a protein kinase C stimulant (PMA), we investigated the possible role(s) of cAMP-mediated, cGMP-mediated and protein kinase C-mediated processes in regulating resting [Ca_f] and PAF-induced elevation of [Ca_f] in human platelets.

2. EXPERIMENTAL

Platelet cytosolic free Ca^{2+} was monitored by using the fluorescent quinoline dye, quin2, essentially as in [16].

cAMP and cGMP were extracted from platelets using ethanol. Extracts were evaporated to dryness, reconstituted in Na-acetate buffer (50 mM, pH 6.2) and acetylated using acetic anhydride:triethylamine (1:2, v/v). Authentic standards were similarly treated, and the cAMP and cGMP contents of platelet extracts were estimated by radioimmunoassay using iodinated tracers [17].

Adenosine 3′,5′-cyclic phosphoric acid 2′-*O*-succinyl-3-[^{125}I]iodotyrosine methyl ester (600 Ci/mmol) and guanosine 3′,5′-cyclic phosphoric acid 2′-*O*-succinyl-3-[^{125}I]iodotyrosine methyl ester (600 Ci/mmol) were obtained from Amersham International, Amersham, England. PGI_2, PGD_2, PAF and PMA were obtained and dissolved as described [14,16]. Other reagents were dissolved in iso-osmotic saline.

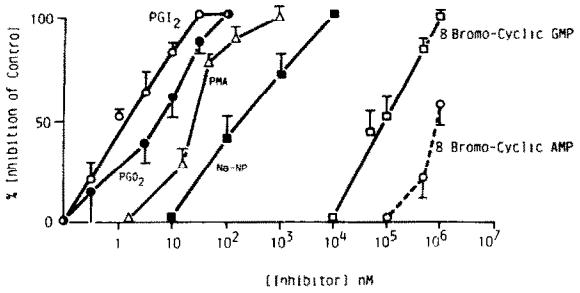

Fig.2. Inhibition of PAF-induced elevation of [Ca_f]. Quin2-labelled human platelets were pre-incubated with PGI_2, PGD_2, NaNP, PMA, 8-bromo cAMP, 8-bromo cGMP or appropriate vehicle, before the addition of a sub-maximal concentration of PAF (10–100 nM). Results (mean ± SD, n = 3–9) are expressed as percent inhibition of the control (vehicle + PAF) response.

3. RESULTS

Resting [Ca_f] in human platelets was 90 ± 3 nM (mean ± SE, n = 46). PAF induced a rapid, concentration-dependent elevation of [Ca_f] to a maximum of around 400–700 nM in different experiments. The concentration-response relationship is depicted in fig.1A and typical fluorescence records from a single experiment are shown in fig.1B. Note that as the concentration of agonist increases, the rate and extent of the resultant changes in fluorescence ([Ca_f]) also increase until a maximum value is attained. Thereafter the fluorescence (and [Ca_f]) declines towards the resting value. The rate of decline of fluorescence (and [Ca_f]) varies with the concentration of agonist: the response elicited by a high concentra-

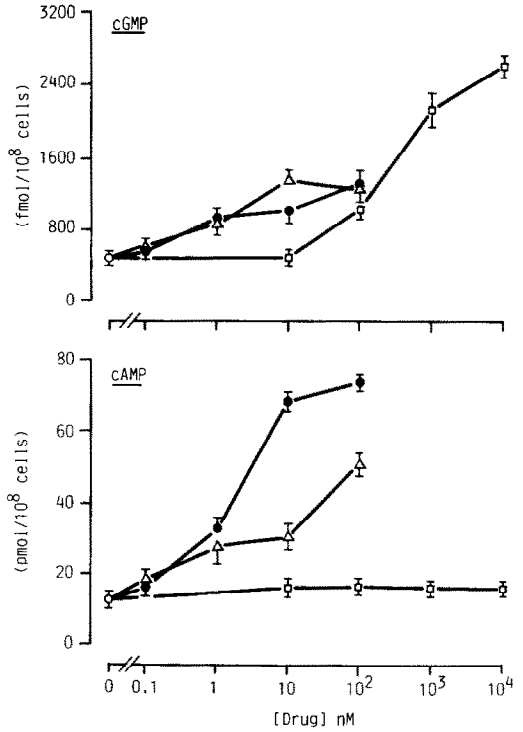

Fig.3. Effects of PGI_2, PGD_2 and NaNP on platelet cAMP and cGMP. Platelets preincubated (5 min, 37°C) with the phosphodiesterase inhibitor, isobutylmethylxanthine (100 μM), were then incubated (2 min, 37°C) with PGI_2, PGD_2 or NaNP at the concentrations indicated. Platelet cAMP and cGMP were measured by radioimmunoassay. Results are mean values ± SE, n = 4–8. (○, control; ●, PGI_2; △, PGD_2; □, NaNP).

tion of agonist decays more rapidly than the response elicited by a lower concentration of agonist. Addition of EGTA (to chelate extracellular Ca^{2+}, and thus abolish Ca^{2+} influx), at the peak of the PAF response, did not affect the rate of decline of fluorescence (not shown).

Addition of PGI_2 (<1 μM), PGD_2 (<1 μM), NaNP (<10 μM) or PMA (<1 μM) to platelets did not alter the basal $[Ca_f]$. However the elevation of $[Ca_f]$ induced by a sub-maximal concentration of PAF (10–100 nM), was inhibited in platelets pretreated with PGI_2, PGD_2, NaNP or PMA (2 min, 37°C) and with 8-bromo cAMP or 8-bromo cGMP (5 min, 37°C) (fig.2). When tested over the active concentration range, PGI_2 and PGD_2 elicit a marked elevation of platelet cAMP and a small elevation of platelet cGMP: NaNP induced a marked elevation of platelet cGMP but did not elevate platelet cAMP (fig.3).

The possible role of endogenous cAMP, cGMP and DAG in mediating the reversal of the PAF-induced elevation of $[Ca_f]$ was investigated: (i) by adding PGI_2, NaNP and PMA at the peak of the fluorescence signal and monitoring the rate of decay of the fluorescence; and (ii) by measuring changes in platelet cAMP and cGMP with time following PAF addition to platelets. Fig.4 indicates that all 3 agents accelerated the decline in fluorescence (and of $[Ca_f]$). No changes in the basal levels of platelet cAMP (12 ± 1 pmol/10^8 cells, mean ± SE, n = 8) or platelet cGMP (315 ± 30 fmol/10^8 cells, mean ± SE, n = 8) were observed for up to 5 min following the addition of PAF (<100 nM) to platelets.

4. DISCUSSION

PAF elicits a rapid, concentration-dependent elevation of $[Ca_f]$ in human platelets. This elevation of $[Ca_f]$, derived predominantly (>80%) via influx of intracellular Ca^{2+} [5], is reversible, and the rate of decline varies with the agonist concentration. That this decline occurs even in the continued presence of the agonist suggests that the receptors undergo rapid desensitisation and that normal homeostatic mechanisms restore $[Ca_f]$ towards basal values. As the rate of decline of fluorescence (i.e. $[Ca_f]$) is unaltered by addition of EGTA at the peak of the quin2 signal, it is probable that the decline in $[Ca_f]$ reflects sequestration and/or extrusion of Ca^{2+} rather than inhibition of persisting Ca^{2+} influx and/or mobilisation of internal Ca^{2+}.

8-Bromo cAMP and agents that stimulate adenylate cyclase; 8-bromo cGMP and agents that stimulate guanylate cyclase, and tumour-promoting phorbol esters, all result in inhibition of the elevation of $[Ca_f]$ induced by PAF. Such an ac-

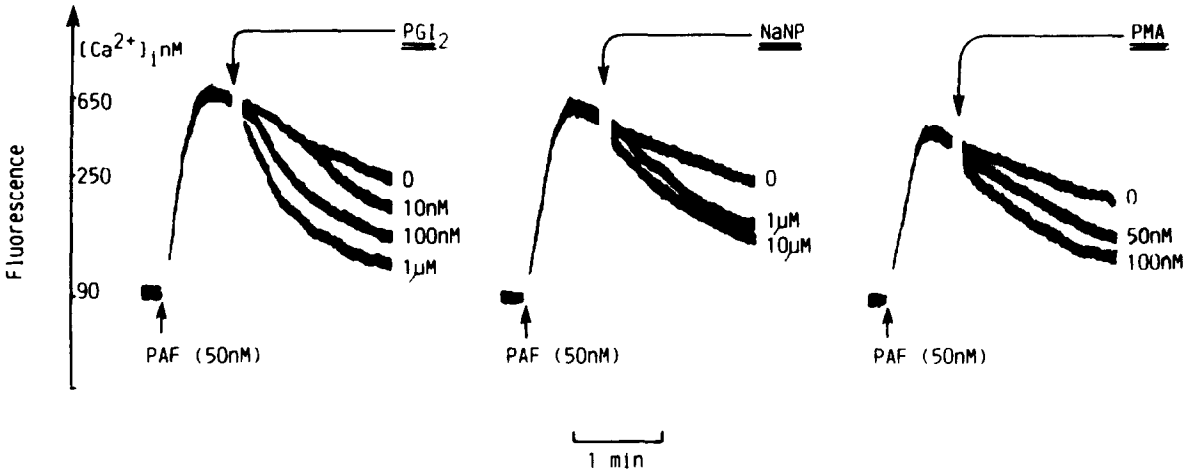

Fig.4. Reversal of PAF-induced elevation of $[Ca_f]$. PGI_2, NaNP and PMA at the concentrations indicated, or appropriate vehicle, were added to quin2-labelled human platelets at the peak of the fluorescence change elicited by PAF. Changes in $[Ca_f]$ were calculated from the observed changes in dye fluorescence. The data shown are typical of at least 4 experiments using platelets from different donors.

tion on $[Ca_f]$ may contribute to the inhibitory effects that cAMP, cGMP [13] and protein kinase C [18] exert on agonist-induced platelet activation. Suppression of agonist-induced elevation of $[Ca_f]$ may result from effects on processes that promote Ca^{2+} influx and/or mobilisation of internal Ca^{2+}, or on processes that promote Ca^{2+} sequestration and/or extrusion. PAF is a potent stimulus of human platelet phosphoinositide metabolism [6] (one of) the molecular mechanism(s) that mediates elevation of $[Ca_f]$ in platelets, as in other cells [2]. Consequently, cAMP-mediated, cGMP-mediated and PMA-mediated inhibition of PAF-induced Ca^{2+} flux may reflect inhibition of phospholipase C. In support of this, there is evidence that cAMP, cGMP and PMA can inhibit phosphoinositide metabolism in platelets [14,19,20]. Moreover when added at the peak of the elevation in $[Ca_f]$ elicited by PAF, PGI_2, NaNP and PMA, all accelerate the decline in fluorescence, and hence in $[Ca_f]$. The accelerated decline in the PAF-induced Ca^{2+} signal suggests that PGI_2, NaNP and PMA also affect processes involved in Ca^{2+} sequestration or extrusion. The mechanism(s) underlying Ca^{2+} efflux from platelets are ill understood, but apparently do not involve plasma membrane Ca^{2+}/Mg^{2+}-ATPase or Na^+/Ca^{2+} exchange [11], although there is evidence for the involvement of the plasma membrane glycoprotein IIb/IIIa complex [21]. Consequently, how, and if, Ca^{2+} efflux from platelets can be modulated by processes regulated by cAMP, cGMP or DAG remains an enigma. The major intracellular organelle implicated in Ca^{2+} sequestration is the dense tubular system, and cAMP is known to promote accumulation of Ca^{2+} by this organelle [8]. The qualitatively similar effects elicited by NaNP and PMA would suggest that cGMP and DAG also promote Ca^{2+} sequestration in the dense tubular system. The role of cGMP in Ca^{2+} sequestration within platelets has not been examined. In other systems, e.g. vascular smooth muscle, although reported to promote Ca^{2+} sequestration within a microsomal fraction [22], the effects of cGMP on Ca^{2+} homeostasis are controversial [23]. Although phenothiazines inhibit Ca^{2+} uptake by the dense tubular system, such effects could not be attributed to an interaction with calmodulin [9]. However, phenothiazines are known to inhibit protein kinase C in platelets [10]. Such a role for protein kinase C in Ca^{2+} sequestration would be consistent with the effects of PMA observed in the present study using platelets, and in other tissues [24].

As platelet stimulatory agonists do not elicit cAMP formation, it is unlikely that, unless exposed to an adenylate cyclase stimulant, endogenous cAMP would subserve a physiological role in modulating $[Ca_f]$ in platelets. Certain platelet stimulatory agonists have been reported to cause cGMP formation [13]: this occurs relatively late after addition of agonist and thus could be of importance in limiting or terminating platelet responsiveness. Our failure to detect cGMP formation in response to PAF may indicate some agonist specificity in platelet cGMP formation, or may reflect that aggregation (which did not occur under our experimental conditions) is a prerequisite for cGMP formation [13]. Irrespective of their physiological importance, cAMP or cGMP may be of pharmacological or therapeutic importance as endogenous regulators of $[Ca_f]$ in platelets. In contrast, phosphoinositide hydrolysis, DAG formation and activation of protein kinase C are also sequelae of receptor occupancy that accompany agonist-induced elevation of $[Ca_f]$. Thus DAG, via activation of protein kinase C, may serve as an endogenous negative feedback regulator of elevated platelet $[Ca_f]$. In view of the role of DAG in mediating the so-called 'calcium-independent' pathway of platelet activation, this would indicate that DAG can function as a bi-directional regulator of platelet reactivity. Indeed, evidence for such a function is now emerging in numerous cell types [15,25].

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