

Inhibition by forskolin of cytosolic calcium rise, shape change and aggregation in quin2-loaded human platelets

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The adenylate cyclase stimulator forskolin was used to study the inhibitory effects of elevated cAMP on the activation of washed human platelets loaded with the fluorescent Ca^{2+} indicator quin2. In the presence of 10 μM isobutylmethylxanthine forskolin inhibited rises in $[\text{Ca}^{2+}]_i$ evoked by thrombin and platelet-activating factor (PAF) due to both Ca^{2+} influx and release from internal stores with similar potency. Aggregation evoked by thrombin and PAF was suppressed whilst partial shape-change persisted, even in the absence of a measurable rise in $[\text{Ca}^{2+}]_i$. Forskolin did not affect the rise in $[\text{Ca}^{2+}]_i$ evoked by Ca^{2+} ionophore; aggregation was suppressed but shape-change persisted.

Platelet	Calcium	Fluorescent indicator	Forskolin	Shape-change	Aggregation
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1. INTRODUCTION

Agents which increase cyclic AMP [1,2] inhibit platelet responses to a wide range of agonists. A major effect of cAMP seems to be suppression of the generation of second messengers. The inhibition by cAMP of an agonist-induced rise in $[\text{Ca}^{2+}]_i$ due to both Ca^{2+} influx and release from internal stores has been shown [3,4] as has inhibition of agonist-induced hydrolysis of inositol lipids [5,6].

We have used the diterpene compound forskolin, which elevates platelet cAMP by direct activation of adenylate cyclase [7,8] to study further the effects of cAMP on Ca^{2+} rises induced by thrombin and PAF in the presence and absence of external Ca^{2+} . If the mechanisms or messengers

for Ca^{2+} influx and release from internal stores differ, one might expect cAMP to affect these 2 responses differently. Similar inhibition of the 2 responses could be suggestive of a common mechanism. The effects of cAMP on shape change and aggregation evoked by thrombin and PAF, recorded simultaneously with $[\text{Ca}^{2+}]_i$ (reported by quin2), were also assessed. The effect of cAMP on processes beyond the mobilisation of Ca^{2+} were studied using the Ca^{2+} ionophore ionomycin.

2. EXPERIMENTAL

Human platelets were isolated from freshly drawn blood and loaded with approx. 1 mM quin2 as in [9]. The cells were resuspended in a physiological saline of composition: 145 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 10 mM Hepes, 10 mM glucose, pH 7.4, at 37°C. Hirudin (0.05 units/ml) and apyrase (20 $\mu\text{g}/\text{ml}$) were added to prevent activation by residual traces of thrombin and ADP, respectively. Cells were pretreated with 100 μM aspirin to prevent the generation of prostaglandin

Abbreviations: cAMP, adenosine 3'5'-cyclic monophosphate; IBMX, isobutylmethylxanthine; PAF, platelet activating factor (1-O-alkyl-2-acetyl-sn-glycerol-3-phosphorylcholine); $[\text{Ca}^{2+}]_i$, cytoplasmic free calcium concentration; DMSO, dimethyl sulphoxide

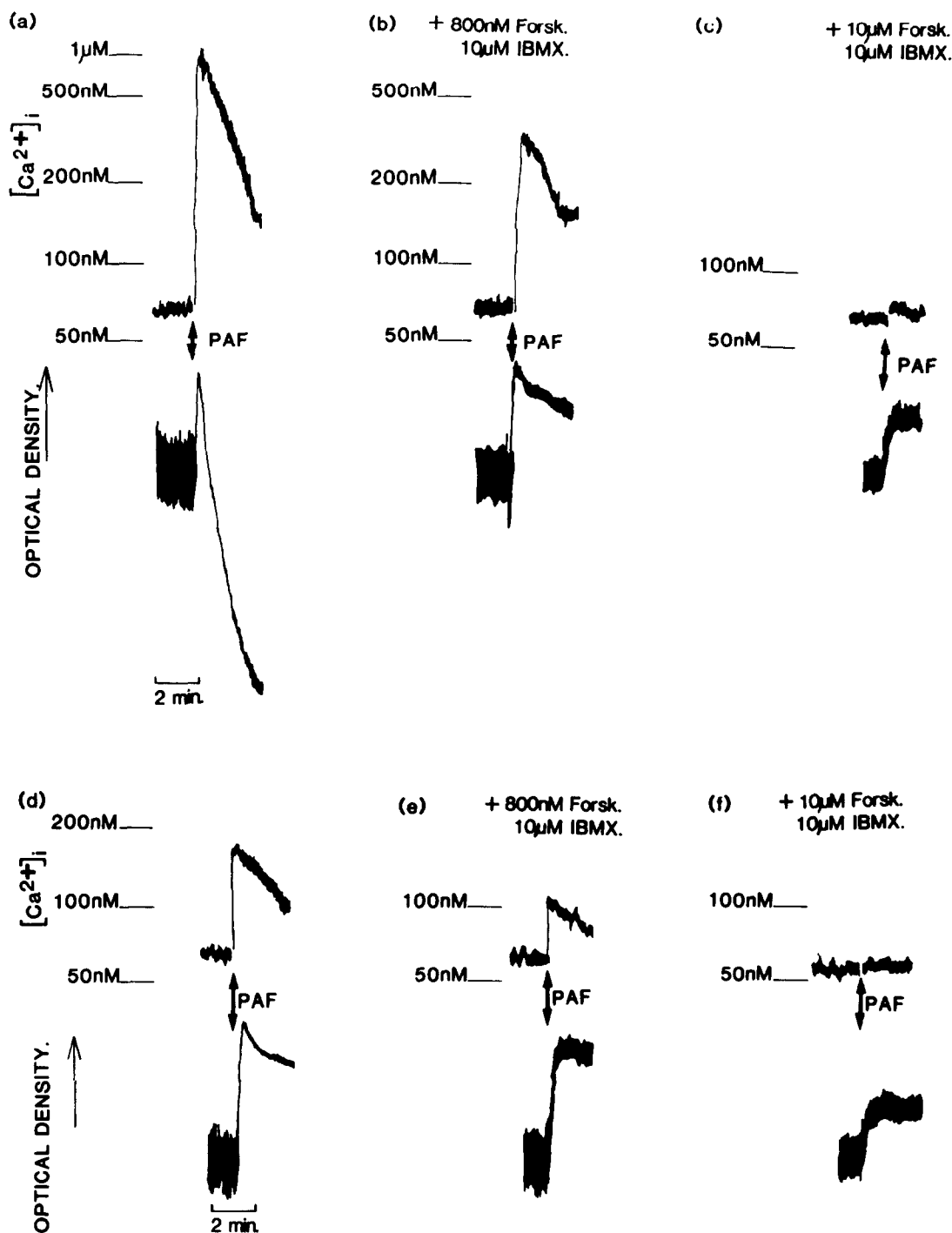


Fig.1. Effect of forskolin and 10 μ M IBMX on responses evoked by 20 ng/ml PAF. Upper traces, quin2 fluorescence; lower traces, absorbance. In (a-c) 1 mM $CaCl_2$ was added, in (d-f) 1 mM EGTA was added. IBMX was added 150 s and forskolin 120 s before the agonist.

endoperoxides and thromboxanes. Prior to experimental procedures the external $[Ca^{2+}]$ was adjusted by the addition of $CaCl_2$ or EGTA (Fluka) as required and the cells were equilibrated at $37^\circ C$ for about 5 min.

Quin2 fluorescence was measured in a stirred, thermostatted cuvette in a Perkin-Elmer MPF44A spectrophotometer with excitation 339 nm and emission 500 nm as previously described [10,11]. The procedure for calibration of the fluorescence signal is detailed elsewhere [11]. The absorbance of the cell suspension was recorded simultaneously with quin2 fluorescence. Shape change and aggregation were assessed from this turbidimetric trace [12]. The presence or absence of an

aggregatory response was verified by single-cell counting and microscopic examination for microaggregates.

In inhibitory experiments the phosphodiesterase inhibitor IBMX ($10 \mu M$) was added (from methanol stock) to enhance the effect of forskolin in elevating cAMP and to minimise the known effects of thrombin and PAF in inhibiting adenylate cyclase [13,14] over the period of activation. Cells were incubated with IBMX for 30 s before the addition of forskolin (Calbiochem, from DMSO stock) and left for a further 120 s before addition of agonist. Experiments were performed alternately in the presence of $1 \text{ mM } Ca^{2+}$ and 1 mM EGTA . Thrombin (Sigma) and PAF (Calbiochem) were added

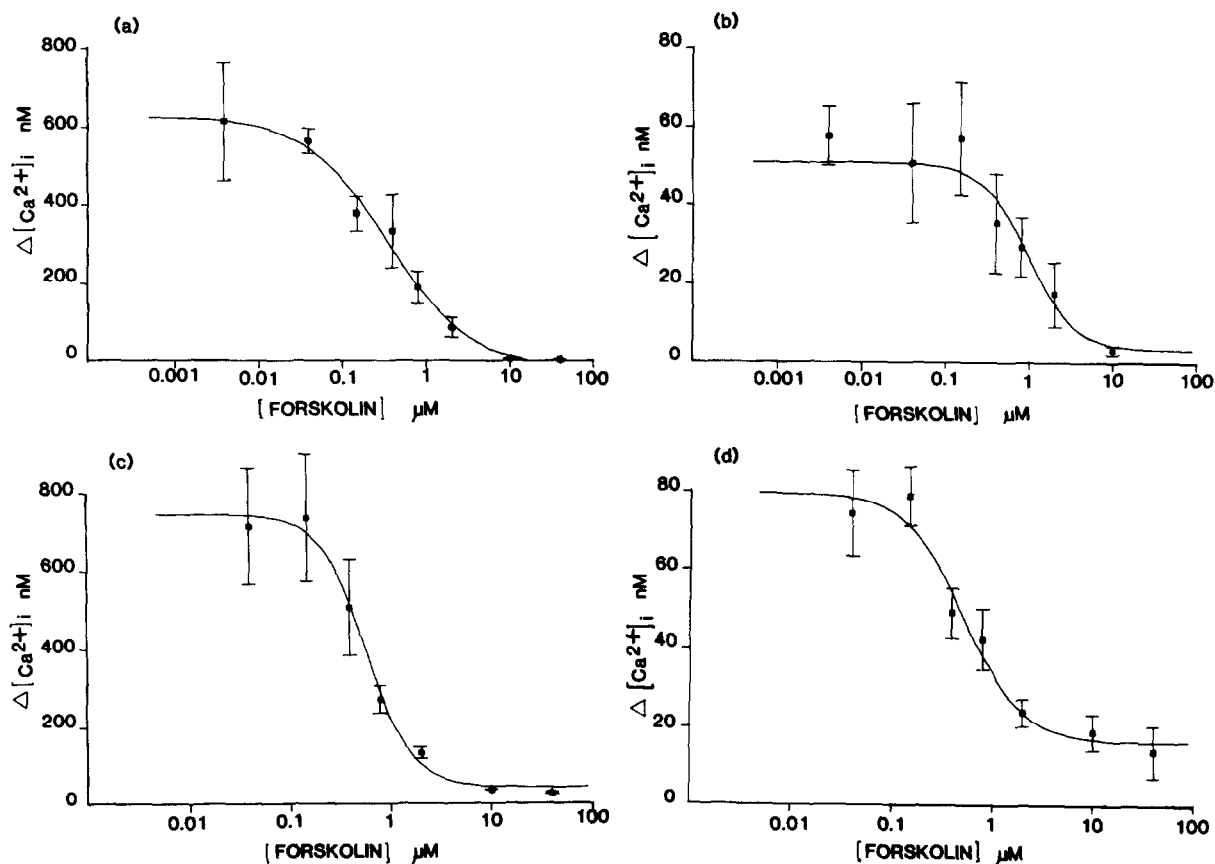


Fig.2. Forskolin dose-inhibition curves in the presence of $10 \mu M$ IBMX. On response evoked by (a) 0.5 units/ml thrombin in the presence of 1 mM external Ca^{2+} ; (b) 0.5 units/ml thrombin in the presence of 1 mM EGTA ; (c) 20 ng/ml PAF in the presence of $1 \text{ mM } Ca^{2+}$; (d) 20 ng/ml PAF in the presence of 1 mM EGTA . Bars show SE of 3-5 determinations. Curves generated by application of Allfit and Ploteasy programmes.

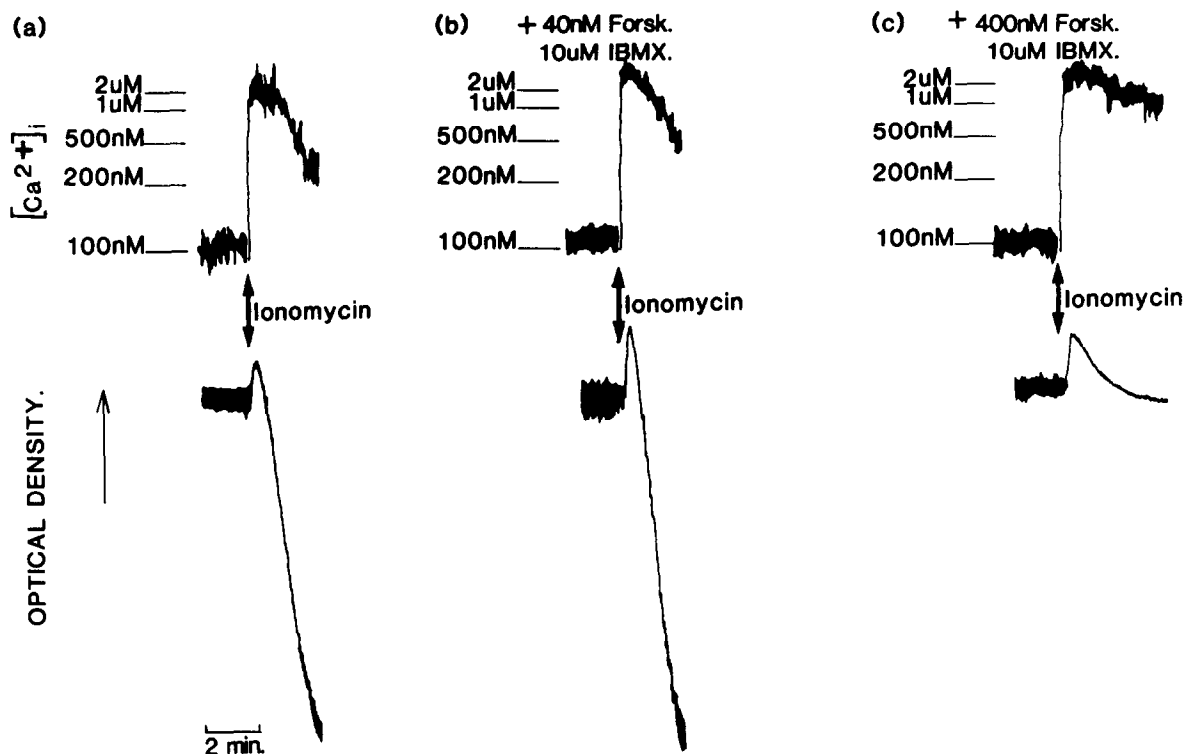


Fig.3. Effect of forskolin and 10 μ M IBMX on responses evoked by 200 nM ionomycin in the presence of 1 mM external Ca^{2+} . Other conditions as for fig.1.

from aqueous stock, ionomycin (Squibb) was added from DMSO stock. The maximum concentration of DMSO was 0.1% (v/v) which was itself without effect.

3. RESULTS AND DISCUSSION

3.1. Effect of forskolin on changes in internal calcium produced by PAF and thrombin

The upper traces of fig.1 show that PAF (20 ng/ml) produces a rapid rise in $[\text{Ca}^{2+}]_i$ to around 600 nM in the presence of 1 mM external Ca^{2+} (fig.1a). IBMX (10 μ M) has only a negligible effect on this response ($98 \pm 2\%$ control; SE, $n = 5$), which is inhibited by rising concentrations of forskolin in the presence of 10 μ M IBMX (figs 1b,c and 2a).

In the absence of external Ca^{2+} PAF produces a small rise in $[\text{Ca}^{2+}]_i$ (fig.1d). This response is slightly reduced by IBMX (10 μ M) alone (to $83 \pm 6\%$ control; SE, $n = 5$) and is inhibited by in-

creasing concentrations of forskolin in the presence of 10 μ M IBMX (figs 1e,f and 2b). Dose-response curves for the effect of forskolin on the PAF-induced $[\text{Ca}^{2+}]_i$ rise in the presence and absence of external Ca^{2+} (fig.2a,b) show the similar potency of forskolin in inhibiting both types of response. The IC_{50} values were 354 and 953 nM, respectively, which were not significantly different at the 0.05 level ($P = 0.17$).

Fig.2c and d shows dose-response curves for the effect of forskolin on the thrombin-induced Ca^{2+} rise in the presence and absence of external Ca^{2+} . The potency of this compound was similar in inhibiting both types of response; IC_{50} values were 580 and 530 nM, respectively.

The small rise in $[\text{Ca}^{2+}]_i$ which occurs in the presence of very low external Ca^{2+} is attributed to release from internal stores, whilst the larger rise occurring on stimulation in the presence of a high (1 mM) external concentration is attributed largely to Ca^{2+} influx [4,11].

The mechanisms by which agonists induce Ca^{2+} influx and release from internal stores are uncertain. It is unlikely that influx occurs through voltage-sensitive channels and ligand-operated channels have been proposed [15,16]. Inositol 1,4,5-trisphosphate (from agonist-induced hydrolysis of phosphatidylinositol biphosphate by phospholipase C) has been proposed as the mediator of internal release [17]. The same molecule could act at ligand-operated Ca^{2+} channels in the plasma membrane to cause influx.

The forskolin dose-response curves reported here show similar inhibition of both Ca^{2+} influx and release from internal stores by thrombin and PAF. These results thus do not support (though, of course, cannot exclude) the idea of any difference between the mechanisms of agonist-induced Ca^{2+} influx and release from internal stores.

3.2. *Effect of forskolin on shape change and aggregation*

The lower trace in fig.1a shows that PAF induces aggregation in the presence of 1 mM external Ca^{2+} . This response is unaffected by 10 μM IBMX. Concentrations of forskolin that (in the presence of 10 μM IBMX) inhibit PAF-induced rises in $[\text{Ca}^{2+}]_i$ also inhibited aggregation, as assessed from absorbance and shown in fig.1b,c. Single platelet counts and microscopic examination of the suspension confirmed that no microaggregates had been formed. Similar results were obtained with thrombin (not shown).

PAF induces shape change in the presence and absence of external Ca^{2+} (fig.1a,d). Concentrations of forskolin which (in the presence of 10 μM IBMX) completely inhibit aggregation fail to abolish completely shape change induced by PAF (fig.1e,f). A partial response persists even in the absence of a measurable rise in $[\text{Ca}^{2+}]_i$ (fig.1f). Similar data were obtained with thrombin (not shown). These results suggest that a Ca^{2+} -independent pathway for shape change exists, as proposed previously from other experimental protocols in which shape change occurs without a measurable rise in $[\text{Ca}^{2+}]_i$, and that this stimulus-response coupling mechanism is relatively resistant to the effects of cAMP.

Substantial rises in $[\text{Ca}^{2+}]_i$ evoked by the Ca^{2+} ionophore ionomycin (which by-pass the normal

receptor process) are an effective stimulus, producing shape change and aggregation (fig.3a). As expected, IBMX and forskolin have no effect on ionomycin-induced Ca^{2+} transients (fig.3b,c) [3,18]. Concentrations of forskolin which (in the presence of 10 μM IBMX) suppress aggregation evoked by PAF and thrombin also inhibit aggregation induced by ionomycin (fig.3c). This shows that cAMP not only suppresses the generation of the Ca^{2+} signal by natural agonists but also reduces the effectiveness of Ca^{2+} in producing the aggregatory response. Shape change was found to persist when ionomycin-induced aggregation was suppressed, demonstrating that cAMP is ineffective in interfering with the ability of an adequate Ca^{2+} stimulus to produce shape change.

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