

Low concentrations of the stable prostaglandin endoperoxide U44069 stimulate shape change in quin2-loaded platelets without a measurable increase in $[Ca^{2+}]_i$

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Received 12 March 1986

Dose-response relationships for raised cytoplasmic free calcium concentration, $[Ca^{2+}]_i$, and shape change were measured simultaneously in quin2-loaded human platelets. With the calcium ionophore ionomycin the threshold $[Ca^{2+}]_i$ for shape change was 300 nM with a maximal response at 800 nM. With 1 mM external Ca^{2+} the U44069 concentrations required to stimulate half-maximal shape change and an increase in $[Ca^{2+}]_i$ were 2 and 41 nM, respectively. For PAF these values were 8.7 and 164 pg/ml, respectively. Low concentrations of U44069 and PAF evoked substantial shape change without any rise in $[Ca^{2+}]_i$. In the absence of external Ca^{2+} , U44069 stimulated half-maximal shape change at 2 nM, and half-maximal elevation of $[Ca^{2+}]_i$ at 69 nM: here, increased $[Ca^{2+}]_i$ never reached the threshold $[Ca^{2+}]_i$ for shape change derived with ionomycin. These results suggest that some transduction mechanism other than elevated $[Ca^{2+}]_i$, as yet unidentified, can cause shape change.

U44069	Ionomycin	Ca^{2+}	Shape change	Platelet	Platelet-activating factor
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1. INTRODUCTION

Within seconds after addition of an agonist such as thrombin, platelet-activating factor (PAF) or prostaglandin endoperoxide, platelets change shape from smooth discs to spiny spheres. Many cellular processes are associated with these changes including repositioning of the equatorial band of microtubules, polymerisation of actin, phosphorylation of myosin light chains, formation of actomyosin, centralisation of secretory granules and formation of filopodia and pseudopodia [1–4]. One link between the binding of an agonist to its receptor and these events appears to be a rise in cytoplasmic free calcium ($[Ca^{2+}]_i$). Indeed, calcium ionophores are effective stimuli for shape

change [5,6]. In quin2-loaded platelets, treated with varying concentrations of the Ca^{2+} ionophore ionomycin, a 3-fold rise in $[Ca^{2+}]_i$ to about 300 nM was found to be the apparent threshold for shape change [7,8]. Maximal shape change occurred when $[Ca^{2+}]_i$ was elevated to between 600 and 800 nM. Physiological agonists that cause shape change also elevate $[Ca^{2+}]_i$ sufficiently for this rise to cause shape change [7–9].

In Ca^{2+} -free media, with 1 mM EGTA, where the cytoplasmic buffering has been increased by quin2, optimal concentrations of agonists or ionophore elevate $[Ca^{2+}]_i$ to no more than 200 nM [8,9]. Under these conditions Ca^{2+} ionophores, as expected, do not cause shape change; yet agonists added alone or after the ionophore do cause shape change and myosin light chain phosphorylation [7–9,11]. We have interpreted such results to indicate that signal transduction systems other than

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elevated $[Ca^{2+}]_i$, available to agonists but not to ionomycin, can trigger shape change. Such studies have been carried out using maximal concentration of agonists in the absence of extracellular Ca^{2+} . Here, we have studied the relationship between the concentration of agonists U44069 and PAF and their ability to evoke shape change and elevate $[Ca^{2+}]_i$ in the presence as well as absence of external Ca^{2+} .

We have shown that under conditions pertinent to those encountered *in vivo*, i.e. 1 mM Ca^{2+} and low agonist concentrations, shape change can be stimulated while $[Ca^{2+}]_i$, as measured by quin2, remains at basal levels and that the EC_{50} for agonist-stimulated shape change is independent of $[Ca^{2+}]_i$.

2. EXPERIMENTAL

Human platelet-rich plasma (PRP) was isolated from freshly drawn blood and incubated with 15 μ M quin2 AM following the method in [12]. 5 min before the end of the incubation 100 μ M aspirin and 40 μ g/ml apyrase were added to the PRP. The cells were resuspended in a Hepes-buffered physiological saline containing: 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM Hepes, 10 mM dextrose (pH 7.4) at 37°C. The cell suspension also contained 0.05 U/ml hirudin and 20 μ g/ml apyrase. Before measurements were made, aliquots of the cell suspension were equilibrated to 37°C for several minutes and the external calcium concentration adjusted by adding 1 mM $CaCl_2$ or 1 mM Na_2H_2EGTA as required. Quin2 fluorescence was monitored in a continually stirred cell suspension, thermostatted to 37°C in a Perkin Elmer MPF 44A spectrofluorimeter. The instrument was adapted so that the absorbance of the stirred suspension could be monitored producing a turbidometric trace like that produced in a standard aggregometer simultaneously with fluorescence [13]. The quin2 fluorescence signal was calibrated as described [12,14].

Calcium-selective electrodes were constructed by fusing Ca^{2+} -selective PVC matrix membranes supplied by Pye Unicam (part no. 9436 094 75861) onto 1 mm PVC tubing with tetrahydrofuran. The reference electrode consisted of a similar tube filled with 3 M KCl gelled in agar. The potential difference was measured by a specially constructed high independence electrometer and the electrode

calibrated in a series of Ca^{2+} buffers containing free Ca^{2+} in the range 10^{-7} – 10^{-3} M [15].

Shape change was expressed as % maximal deflection in absorbance recorded on the chart recorder. Dose-response curves were fitted to the logistic equation by computer using the ALLFIT program [16]. The points are means \pm SE. Errors quoted for EC_{50} values are approximate SE derived from this fitting procedure.

Apyrase type I and aspirin (acetylsalicylic acid) were obtained from Sigma; EGTA puriss from Fluka; quin2 AM from Lancaster synthesis; U44069 from the Upjohn Co., Kalamazoo and PAF from Calbiochem.

3. RESULTS AND DISCUSSION

Fig.1 shows the relation between increases in $[Ca^{2+}]_i$ and shape change. 200 nM of the Ca^{2+} ionophore ionomycin was added to platelet suspensions with varying external Ca^{2+} concentrations. With low external Ca^{2+} there was a small increase in $[Ca^{2+}]_i$ with no shape change. As the external Ca^{2+} was increased so did the increase in $[Ca^{2+}]_i$ and shape change occurred. The threshold $[Ca^{2+}]_i$ for this response was 300 nM with a maximal response occurring at 0.8–1 μ M. The same relationship between $[Ca^{2+}]_i$ and shape change was observed when external Ca^{2+} was held constant at 1 mM and the ionomycin concentration varied.

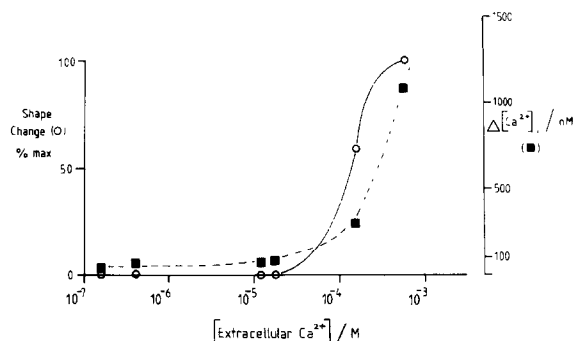


Fig.1. Effect of varying the external Ca^{2+} concentration on ionomycin (200 nM) induced elevation of $[Ca^{2+}]_i$ and shape change. $\Delta[Ca^{2+}]_i$ is the measured increment over the resting level. Shape change is measured as the % of the maximal increment in absorbance. The external Ca^{2+} concentration was varied by adding varying concentrations of EGTA or $CaCl_2$ to the platelet suspension.

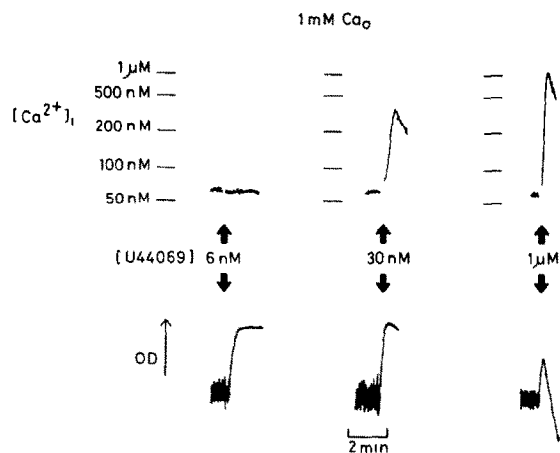


Fig.2. Simultaneous measurements of $[Ca^{2+}]_i$ and absorbance of platelets stimulated with U44069. The figure shows the effect of three concentrations of U44069 on $[Ca^{2+}]_i$ and absorbance in the presence of 1 mM extracellular Ca^{2+} .

Fig.2 shows the effect of adding U44069 at three different concentrations in the presence of 1 mM external Ca^{2+} . 6 nM U44069 produced no increment in $[Ca^{2+}]_i$, but was an effective stimulus for shape change. Increasing the U44069 concentration to 30 nM and 1 μ M produces no further increment in the absorbance signal although $[Ca^{2+}]_i$ is now markedly increased. Scanning electron micrographs confirm that the cells which are normally in a discoid resting state do undergo shape changes as indicated by the increase in absorbance of the suspension. Fig.3A shows the collected data from experiments like those in fig.2. The calculated EC_{50} is 1.8 ± 0.2 nM for shape change and 41 ± 6 nM for elevation of $[Ca^{2+}]_i$; shape change is maximal at the threshold concentration for the $[Ca^{2+}]_i$ rise.

Fig.3B shows the collected data from experiments with U44069 conducted in Ca^{2+} -free solution containing 1 mM EGTA. The EC_{50} for shape change is 2.0 ± 0.1 nM and that for the $[Ca^{2+}]_i$ rise is 69 ± 12 nM. Again the shape change is maximal at the threshold for the $[Ca^{2+}]_i$ rise. One important point is that while the EC_{50} values for the $[Ca^{2+}]_i$ rise are similar in the presence and absence of external Ca^{2+} , the maximal $[Ca^{2+}]_i$ rise in the presence of 1 mM Ca^{2+} , 1500 nM, is very much larger than the rise in the absence of external Ca^{2+} , 100 nM. For shape change both the EC_{50}

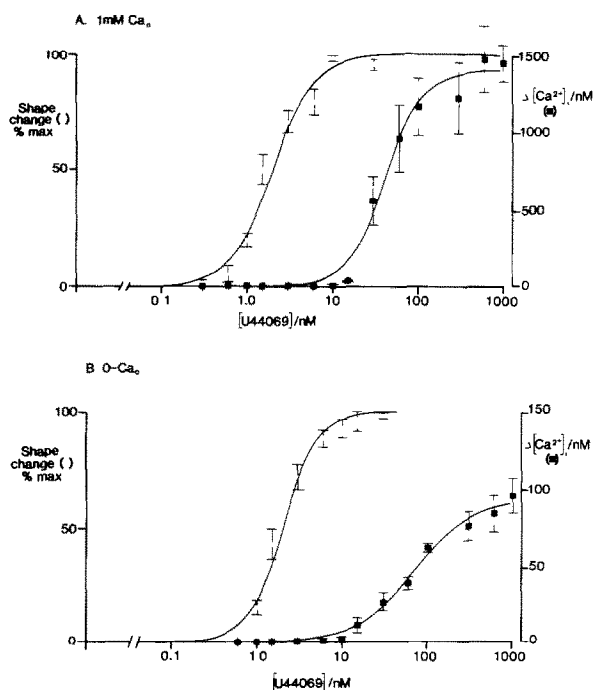


Fig.3. Dose-response relation for U44069-induced shape change and increment in $[Ca^{2+}]_i$ above resting levels. (A) In the presence of 1 mM extracellular Ca^{2+} . (B) In the presence of 1 mM extracellular EGTA with no added Ca^{2+} .

and extent of the response are similar in the presence and absence of calcium judged from the absorbance measurements and from scanning electron microscopy (not shown).

Similar results were also obtained for PAF-induced shape change and elevation of $[Ca^{2+}]_i$. In the presence of 1 mM extracellular Ca^{2+} the EC_{50} values were 8.7 ± 1.8 and 164 ± 53 pg/ml PAF, respectively. With 1 mM EGTA present the EC_{50} value for shape change was 10.6 ± 1.2 pg/ml and for elevation of $[Ca^{2+}]_i$ was 65 ± 11 pg/ml. As with U44069, the dose-response curve for elevation of $[Ca^{2+}]_i$ lies well to the right of that for shape change and again the EC_{50} values for shape change are the same in the presence and absence of extracellular Ca^{2+} when $[Ca^{2+}]_i$ is quite different.

Our data show that the dose-response relation for U44069 and PAF-induced elevation of $[Ca^{2+}]_i$ is markedly to the right of that for shape change and that these agonists can induce shape change while measured $[Ca^{2+}]_i$ remains unchanged at the

resting level. This suggests that a rise in $[Ca^{2+}]_i$ is not a necessary stimulus, although an imposed rise in $[Ca^{2+}]_i$ can be a sufficient stimulus, for shape change.

Furthermore the similar dose-response relationship for shape change in the presence and absence of external Ca^{2+} , where the change in $[Ca^{2+}]_i$ is very different, suggests that mechanisms other than a rise in $[Ca^{2+}]_i$ can promote the observed changes in shape. An alternate view is that a privileged pool of intracellular Ca^{2+} , not requiring external calcium, inaccessible to calcium ionophore, and immune from the buffering power of quin2, is responsible for the effects.

The finding that a dose-effect curve lies to the left of the agonist binding curve is often considered in terms of spare receptors. This analysis usually assumes that only a small proportion of receptors is needed to generate the second message or that only a small amount of second message is required to produce the observed effect. Clearly only the second possibility applies in the present case if the second messenger is Ca^{2+} , and from the arguments above this seems not to be plausible. Our proposal that another transduction pathway is operating also supposes that only a small fraction of the receptors need be occupied for this pathway to be effective. Whether this is a small proportion of a homogeneous population of receptors or a limited number of high-affinity receptors with special properties cannot be determined from the available data. However, data from binding studies [17] suggest it is likely that less than 5% of the saturable binding sites for U44069 would be occupied at the EC_{50} , 2 nM, for U44069-induced shape change.

Two recent reports [18,19] have suggested that either diacylglycerol or phosphatidylinositol 4,5-bisphosphate may play a part in forming a nucleation site for the polymerisation of actin which forms the microfilaments in pseudopodia. According to existing data [17] there would be little stimulation of inositol lipid turnover at the U44069 concentrations which we find to cause shape change without a measurable rise in $[Ca^{2+}]_i$. It is unclear whether this would be sufficient for the formation of nucleation sites. Changes in cyclic nucleotide levels or in cytoplasmic pH are unlikely to be involved. cAMP and cGMP are inhibitors of platelet function [20,21], and cytoplasmic pH changes less than 0.05 units following stimulation

with U44069, assessed from the fluorescence of trapped biscalboxyethylcarboxyfluorescein (Hallam, Simpson and Rink, unpublished). At present the nature of the putative alternative transduction mechanism remains unclear.

ACKNOWLEDGEMENTS

We thank the SERC and Ciba-Geigy, UK, for financial support.

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