

# Agonists stimulate divalent cation channels in the plasma membrane of human platelets

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Agonists such as thrombin, PAF (platelet-activating factor) and ADP are known to cause a larger elevation in  $[Ca^{2+}]_i$  in quin2-loaded platelets in the presence of extracellular  $Ca^{2+}$  than in its absence. The simplest interpretation of these observations is that in the presence of extracellular calcium there is an influx component across the cell surface. In the presence of  $Mn^{2+}$ , a divalent cation which is known to avidly bind to quin2 and to quench its fluorescence, the agonists produce a small initial rise in quin2 fluorescence followed by a decrease in fluorescence to well below the resting level. The result indicates entry of  $Mn^{2+}$ , presumably through some form of receptor-operated  $Ca^{2+}$  channel.

Manganese      Cation channel      Fluorescence      Platelet      Calcium

## 1. INTRODUCTION

The introduction of the fluorescent  $Ca^{2+}$  indicator quin2 has allowed measurement of the rise in cytoplasmic free calcium concentration,  $[Ca^{2+}]_i$ , in many cells during stimulation by a wide variety of agents [1]. This method cannot directly determine the source of the calcium required for stimulated increases in  $[Ca^{2+}]_i$ , but simple manipulations, for example the removal of extracellular  $Ca^{2+}$ , can give clues. In human platelets incubated in the absence of added  $Ca^{2+}$  and with 1 mM EGTA, the size of the maximal transient rise in  $[Ca^{2+}]_i$  on stimulation with ADP [2], PAF [3], thrombin [4], U46619 [5] or vasopressin [6] is markedly smaller than that seen in the presence of 1 mM external  $Ca^{2+}$ .

Typically optimal concentrations of agonists raise  $[Ca^{2+}]_i$  from the resting level of approx. 100 nM to around 1  $\mu$ M with 1 mM  $Ca^{2+}$  present in the external medium. When the external  $Ca^{2+}$  concentration is reduced to below the cytosolic level agonists stimulated an increase in  $[Ca^{2+}]_i$  in quin2-loaded cells to only 200–300 nM.

The simplest interpretation is that the difference between the two transients is due to an influx of

$Ca^{2+}$  across the plasma membrane, and that the residual-stimulated rise in  $[Ca^{2+}]_i$  observed with 1 mM external EGTA is due to the discharge of  $Ca^{2+}$  from an intracellular store [3]. However, this is not the only possibility. For instance, exposure to low  $[Ca^{2+}]_o$  might deplete the intracellular releasable pool. This is unlikely because the effect of reducing  $[Ca^{2+}]_o$  is seen within 1 min of chelating external  $Ca^{2+}$ , during which time there is little fall in resting  $[Ca^{2+}]_i$ , judged from the quin2 signal. Another possibility is that external  $Ca^{2+}$  is needed for agonists to discharge effectively the internal  $Ca^{2+}$  store.

The site of storage of the dischargeable pool of intracellular calcium is not known but is thought to be a membrane-bound organelle, the dense tubular system [7]. In the presence of 1 mM EGTA, divalent cation ionophores such as ionomycin or A23187 cause the discharge of  $Ca^{2+}$  to increase  $[Ca^{2+}]_i$  in a fashion similar to that produced by agonists [4]. Maximally effective concentrations of agonists added after ionophore usually produce no measurable further increase in quin2 fluorescence [3,4]. Therefore models which do not incorporate influx would have to postulate another site of calcium storage in the platelet which is not bound-

ed by a membrane (or at least that ionophores cannot discharge) and which is released by agonists only in the presence of external calcium.

What is needed is a method that can distinguish the source of  $\text{Ca}^{2+}$ . The most straightforward method should be the use of  $^{45}\text{Ca}^{2+}$ . Previous work has shown an increased uptake of  $^{45}\text{Ca}^{2+}$  in platelets stimulated with thrombin and PAF [8–10]. However, it is difficult to be sure that the cell-associated radioactivity is in the cytosol rather than bound to the cell surface [11]. It seems that the exposure of fibrinogen receptors does result in increased surface  $\text{Ca}^{2+}$  binding [12]

We report here another approach which indicates that thrombin or PAF, can promote  $\text{Mn}^{2+}$  entry across the platelet plasma membrane. The experiments exploit the fact that  $\text{Mn}^{2+}$  binds to quin2 much more avidly than does  $\text{Ca}^{2+}$  and quenches its fluorescence. This ability of  $\text{Mn}^{2+}$  to quench quin2 fluorescence has been used to reveal the autofluorescence of cell suspensions in the calibration of  $[\text{Ca}^{2+}]_i$  measurements [3,6,13]. Any agent which promotes  $\text{Mn}^{2+}$  influx into quin2-loaded cells will therefore result in a reduced signal as has already been shown with the ionophore, ionomycin [13]; finding such a reduction of signal from internal quin2 suggests the uptake of external  $\text{Mn}^{2+}$  due to an increase in  $\text{Mn}^{2+}$  permeability.

## 2. EXPERIMENTAL

Platelet-rich plasma (PRP) was prepared from whole blood anti-coagulated with 1/6 vol. of ACD (2.5 g trisodium citrate, 1.5 g citric acid, 2.0 g glucose in 100 ml  $\text{H}_2\text{O}$ ) by centrifugation at  $700 \times g$  for 5 min at room temperature. The PRP was then incubated in the presence of  $10 \mu\text{M}$  quin2 acetoxymethyl ester at  $37^\circ\text{C}$  for 30 min to give a loading of between 0.5 and 1.0 mM quin2 trapped in the platelet cytoplasm. The PRP was then centrifuged at  $350 \times g$  for 20 min to pellet the platelets which were then resuspended in a medium containing 145 mM NaCl, 5 mM KCl, 1 mM  $\text{MgSO}_4$ , 10 mM glucose and buffered to pH 7.4 with 10 mM Hepes 1 mM  $\text{CaCl}_2$  or 1 mM  $\text{K}_2\text{H}_2\text{EGTA}$  were usually added as required. In experiments which examined the influx of  $\text{Mn}^{2+}$  no  $\text{CaCl}_2$  was added to the medium. However, EGTA could not be used since it avidly binds manganese ions. The

fluorescence of quin2 was measured in a Perkin Elmer MPF 44A spectrofluorimeter as described previously [3,14,15]. Excitation and emission wavelengths were 339 and 500 nm, respectively, with 10 and 20 nm band widths

To remove free  $\text{Mn}^{2+}$  from the suspension at the end of an experiment, a chelator was used. Diethylenetriaminepentaacetic acid, DTPA, is a membrane-impermeant heavy metal chelator and was therefore used to chelate manganese ions in the suspending medium but not inside the cells [3]

The results shown are typical of at least 4 other experiments.

## 3 RESULTS AND DISCUSSION

Fig.1. shows the effects of  $\text{Mn}^{2+}$  entry mediated by the divalent ionophore ionomycin. In fig.1A, ionomycin was added in the presence of 0.5 mM  $\text{Ca}^{2+}$ . The increased  $\text{Ca}^{2+}$  influx across the plasma membrane increases  $[\text{Ca}^{2+}]_i$  until the intracellular quin2 is saturated. The subsequent addition of 1 mM  $\text{Mn}^{2+}$  causes a small sharp drop followed by a decline in fluorescence. The sharp drop is interpreted as the immediate quenching of leaked extracellular quin2 while the slow decline presumably results from  $\text{Mn}^{2+}$  entering the cells and displacing  $\text{Ca}^{2+}$  from the intracellular quin2 and quenching

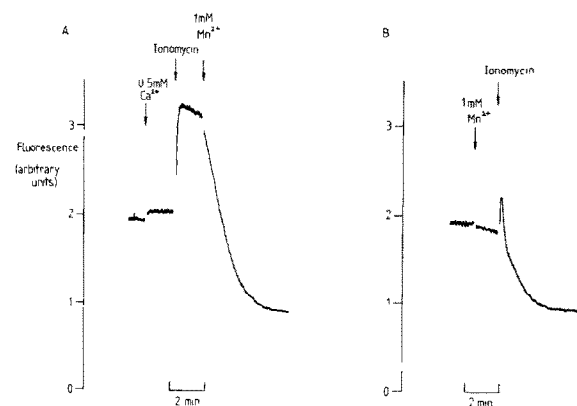


Fig 1 Effect of  $\text{Mn}^{2+}$  on quin2 fluorescence of platelets stimulated with ionomycin. Platelets were stimulated with ionomycin, 400 nM, in the presence (A) or absence (B) of 0.5 mM  $\text{Ca}^{2+}$ . 1 mM  $\text{MnCl}_2$  was added as indicated. After lysing the platelets with  $50 \mu\text{M}$  digitonin at the end of the experiment the resting level of  $[\text{Ca}^{2+}]_i$  was found to be  $\sim 90 \text{ nM}$ .

the fluorescence signal. This effect resembles that first reported in lymphocytes by Hesketh et al. [13]. Fig.1B shows the effect of adding ionomycin in the presence of 1 mM  $Mn^{2+}$ , and without added  $Ca^{2+}$  (free  $[Ca^{2+}]_o$  measured by a  $Ca^{2+}$ -selective electrode to be  $\sim 40 \mu M$ ). Under these conditions there is a brief rise in signal followed by a decline to well below the resting level. We interpret this to reflect the discharge of  $Ca^{2+}$  from internal stores which causes a rapid increase in the fluorescence followed by a steady influx of  $Mn^{2+}$ , progressively quenching it.

Fig.2 compares the responses to thrombin, PAF and ADP in nominally  $Ca^{2+}$ -free medium ( $[Ca^{2+}]_o \sim 40 \mu M$ ) without and with 1 mM extracellular  $Mn^{2+}$ . In the presence of  $Mn^{2+}$ , the signal first rises rapidly, presumably mainly reflecting the discharge of intracellular  $Ca^{2+}$ , and then

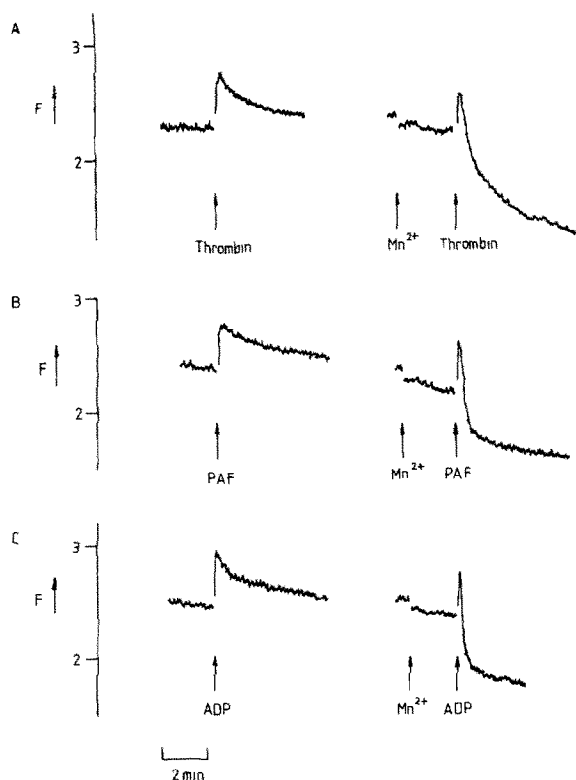


Fig 2 Effect of  $Mn^{2+}$  on fluorescence of quin2-loaded platelets during stimulation with thrombin, PAF or ADP. The figure shows the effect of addition of 1 mM  $MnCl_2$  on the stimulated quin2 fluorescence responses to (A) 0.5 U/ml thrombin, (B) 20 ng/ml PAF or (C) 20  $\mu M$  ADP.

declines to below the resting level. This decline is due to either (a) an increase in the permeability of the plasma membrane to  $Mn^{2+}$  which enters and quenches the fluorescence of intracellular quin2, or (b) a non-selective increase in the permeability of the plasma membrane so that there is an increased leakage of quin2 into the extracellular medium. Such an effect would predictably cause an overall drop in the quin2 fluorescence because an increasing proportion of the dye would be quenched by extracellular  $Mn^{2+}$ .

Experiments like that shown in Fig.3 suggest that the quenching is largely due to  $Mn^{2+}$  entry. Here, excess DTPA was added after PAF. There was a small abrupt rise in signal as  $Mn^{2+}$  was stripped off the leaked extracellular dye. However, the increase that followed addition of DTPA was similar to the decrease seen when the  $Mn^{2+}$  was originally added. As both changes give an estimate of the proportion of extracellular leaked dye it is apparent that little extra leakage has occurred during the period of stimulation. Addition of 50  $\mu M$  digitonin which lyses the cells now causes a rapid increase in quin2 fluorescence since the quin2 inside the cells is now exposed to excess DTPA which binds the  $Mn^{2+}$  and thus relieves the quenching effect leaving the dye to be saturated by  $Ca^{2+}$ . This effect provides strong evidence that the PAF had caused substantial entry of  $Mn^{2+}$ .

Fig.4 shows the effects of  $Ni^{2+}$ . Like  $Mn^{2+}$  the divalent metal also strongly quenches quin2. On addition of  $Ni^{2+}$  there is a small drop in signal

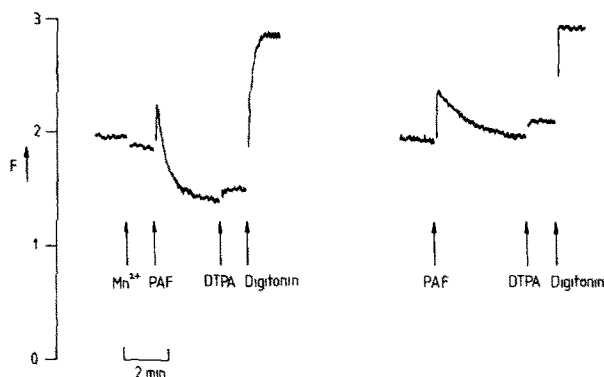


Fig.3. Effect of DTPA on the quenched quin2 signal caused by PAF in the presence of  $Mn^{2+}$ . 1 mM  $MnCl_2$ , 20 ng/ml PAF, 1.5 mM Ca DTPA, and 50  $\mu M$  digitonin (from 50 mM stock in DMSO) were added as indicated.

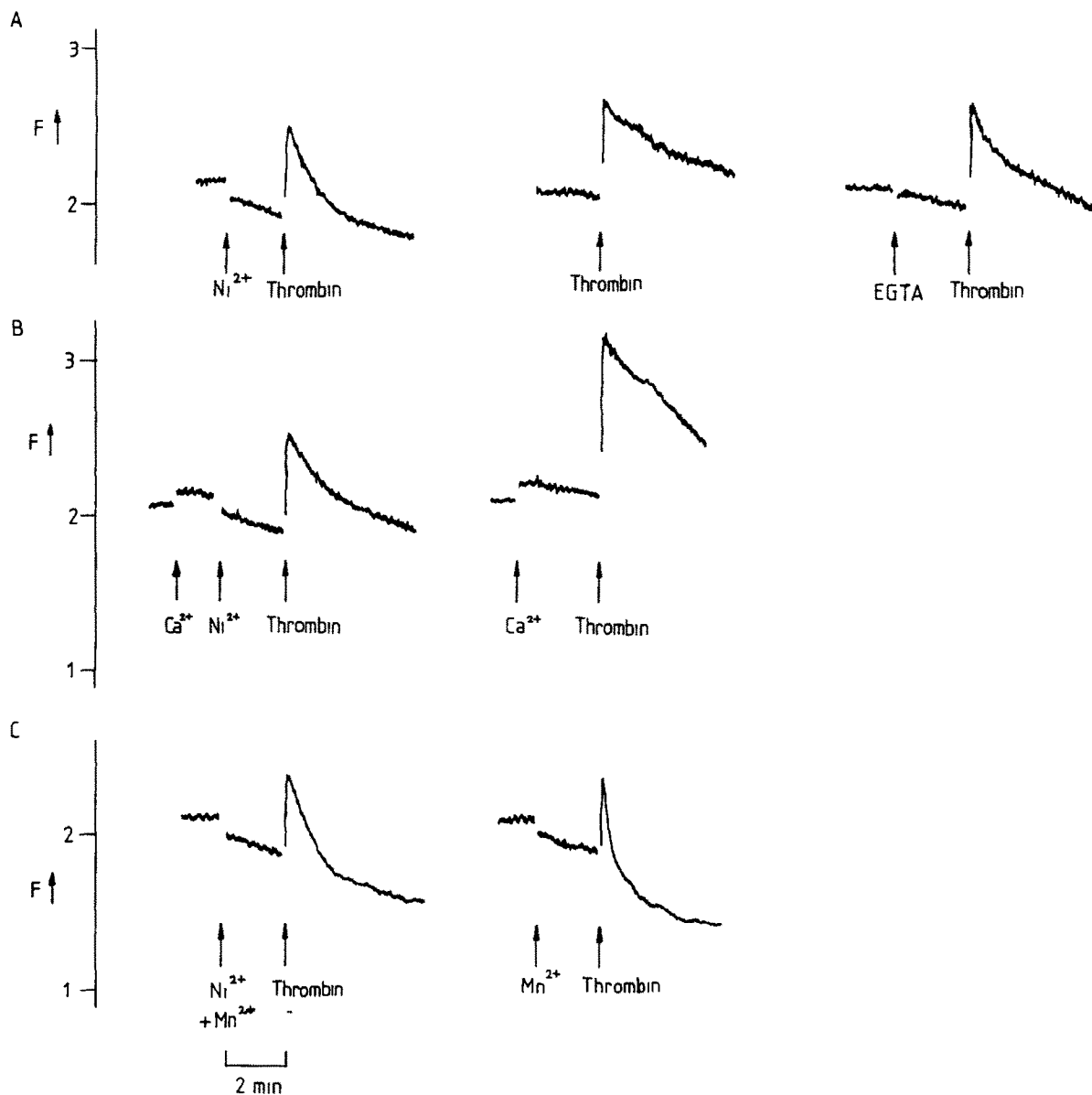


Fig.4 Effect of 2 mM  $\text{Ni}^{2+}$  added as indicated on the quin2 responses to 0.5 U/ml thrombin. (A) In the presence of no added  $\text{Ca}^{2+}$  (1 mM EGTA was added where indicated). (B) In the presence of 0.5 mM  $\text{Ca}^{2+}$ . (C) In the presence of 1 mM  $\text{Mn}^{2+}$ .

presumably due to quenching of leaked dye. The effects of  $\text{Ni}^{2+}$  on the quin2 responses to thrombin are however different from those of  $\text{Mn}^{2+}$ . Fig.4A shows that without added  $\text{Ca}^{2+}$  and with 2 mM  $\text{Ni}^{2+}$  there is a transient rise in signal followed by a return towards, but not below, the baseline. This pattern contrasts with that seen in fig.2A and in-

dicates that the cation entry process excludes  $\text{Ni}^{2+}$ . Fig.4B compares the rise in quin2 fluorescence evoked by thrombin in the presence of 0.5 mM extracellular  $\text{Ca}^{2+}$  with and without 2 mM extracellular  $\text{Ni}^{2+}$ . In the presence of  $\text{Ni}^{2+}$  the rise in quin2 fluorescence is much smaller than in the control. This effect is interpreted to be caused by the

$\text{Ni}^{2+}$  inhibiting the influx of  $\text{Ca}^{2+}$ , possibly by blocking the plasma membrane divalent cation channel. This seems likely since the rise in quin2 fluorescence in the presence of 1 mM extracellular  $\text{Ca}^{2+}$  with 2 mM  $\text{Ni}^{2+}$  is similar in size to that produced by thrombin in the presence of extracellular EGTA (fig.4A) suggesting that the remaining transient is due to the discharge of  $\text{Ca}^{2+}$  from intracellular stores with very little  $\text{Ca}^{2+}$  influx component. Fig.4C shows the effect of stimulation with thrombin in the presence of both extracellular  $\text{Ni}^{2+}$  and  $\text{Mn}^{2+}$  but with no added  $\text{Ca}^{2+}$ . Here, the same concentration of  $\text{Ni}^{2+}$  which inhibits the  $\text{Ca}^{2+}$  influx component of the quin2 transient (fig.4B), also markedly inhibits the slow decline in quin2 fluorescence normally observed in the presence of  $\text{Mn}^{2+}$  on stimulation with thrombin and suggests that  $\text{Mn}^{2+}$  and  $\text{Ca}^{2+}$  may be translocated across the plasma membrane through the same divalent cation channel.

The available evidence argues against the presence of voltage-gated calcium channels in platelet membranes: organic calcium channel blockers are ineffective at blocking  $[\text{Ca}^{2+}]_i$  rises evoked by ADP and thrombin in quin2-loaded platelets [2,17] (various reports that diltiazem and verapamil inhibit aggregation and secretion induced by PAF have been confirmed (Hallam and Rink, unpublished), but found not to inhibit the elevation in  $[\text{Ca}^{2+}]_i$  in the same cells; depolarisation of the membrane by gramicidin (E. Pipili and Rink, unpublished) or  $\text{K}^+$ -rich solutions [2,17] does not increase  $[\text{Ca}^{2+}]_i$ ; agonist-evoked rises in  $[\text{Ca}^{2+}]_i$  are not prevented by additions of valinomycin which markedly hyperpolarise the platelet membrane; and, finally, thrombin, ADP, and PAF produce only very small 5–10 mV depolarisations of the platelet membrane judged from the fluorescence of the potential sensitive dye di-S-C<sub>3</sub>-(5) (E. Pipili, in preparation). It therefore seems that these agonists promote divalent cation influx via receptor-operated channels, the detailed mechanism of which awaits elucidation.

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