Relationship between cytoplasmic free calcium and myosin light chain phosphorylation in intact platelets

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1. Human platelets were prepared and loaded with the fluorescent Ca^{2+} indicator quin2. The relation between cytoplasmic free calcium concentration, $[\text{Ca}^{2+}]_i$, and the extent of the phosphorylation of myosin light chains of M_r 20000 could then be examined. 2. When the calcium ionophore ionomycin is used to stimulate platelets, little phosphorylation is seen until $[\text{Ca}^{2+}]_i$ exceeds 400 nM; half-maximal response occurs at 600 nM with a full response at about $1 \mu\text{M}$ - $[\text{Ca}^{2+}]_i$. 3. Under optimal conditions, physiological stimuli such as platelet-activating factor and thrombin can increase $[\text{Ca}^{2+}]_i$ to sufficiently high levels [Rink, Smith & Tsien (1982) FEBS Lett. 148, 21–26; Hallam, Sanchez & Rink (1984) Biochem. J. 218, 819–827] that Ca^{2+} ions could be the trigger for the myosin phosphorylation evoked by these agonists. However, in this paper we show that, in the absence of external calcium, platelet-activating factor and thrombin can stimulate myosin phosphorylation while $[\text{Ca}^{2+}]_i$ remains at levels which are well below those needed when the calcium ionophore is the stimulus. 4. This observation suggests that myosin light chain phosphorylation may be controlled by an additional pathway.

INTRODUCTION

Phosphorylation of the myosin light chains is thought to be one of the primary steps in the activation of contractile events in many types of cells, including blood platelets (Adelstein, 1982; Scholey *et al.*, 1980). Stimulus-dependent phosphorylation of myosin in platelets is correlated with its polymerization and association with actin filaments (Lebowitz & Cooke, 1979; Daniel *et al.*, 1981; Cox *et al.*, 1984). Myosin light-chain kinase is found in platelets and is activated *in vitro* by calcium and calmodulin (Scholey *et al.*, 1980), and in intact platelets, calcium ionophore stimulates the phosphorylation of myosin light chains, M_r 20000 (Kaibuchi *et al.*, 1983). It has therefore been supposed that physiological stimuli promote myosin phosphorylation by elevating cytoplasmic free calcium, $[\text{Ca}^{2+}]_i$ (Daniel *et al.*, 1984a; Adelstein & Eisenberg, 1980). We have examined myosin phosphorylation in human platelets loaded with quin2, the intracellular fluorescent Ca^{2+} indicator, to examine, for the first time in intact cells, the relation between cytoplasmic free calcium and myosin phosphorylation. This relation was examined for cells stimulated by the Ca^{2+} ionophore ionomycin, which transports Ca^{2+} into the cytosol, bypassing receptor-operated transduction systems (Rink *et al.*, 1982a), and for cells stimulated by the natural agonists PAF and thrombin. A preliminary account of some of these results has been published in abstract form (Daniel *et al.*, 1984b).

METHODS

Preparation of cells

Platelets were prepared from freshly drawn human blood anti-coagulated with one-sixth volume of ACD

(2.5 g of sodium citrate, 1.5 g of citric acid and 2.0 g of glucose in 100 ml of water). The citrated blood was centrifuged at 700 g for 5 min and the resulting platelet-rich plasma was incubated at 37 °C for 30 min with 20 μM -quin2 acetoxymethyl ester (Lancaster Synthesis, Morecambe, Lancs., U.K.) added in dimethylsulphoxide (final concn. 0.1%). The cells, now containing approx. 1 mM-quin2, were then centrifuged at 350 g for 20 min and were resuspended in a physiological saline containing 145 mM-NaCl, 5 mM-KCl, 1 mM-MgSO₄, 10 mM-sodium Hepes, 10 mM-glucose, pH 7.4 at 37 °C. Hirudin, 0.05 units/ml (Sigma), was added to prevent activation by residual traces of thrombin and the platelets were incubated with 0.1 mM-aspirin for 10 min before use to inhibit cyclo-oxygenase and block the formation of prostaglandin endoperoxides and thromboxane A₂. Apyrase (10 $\mu\text{g}/\text{ml}$; Sigma grade I), a non-specific adenine nucleotide phosphatase, was also added to the suspension to remove any extracellular ADP. The suspension was then adjusted to pH 6.6 with ACD, centrifuged at 350 g for 20 min and the platelets were resuspended in the Hepes-buffered physiological saline described above containing hirudin and apyrase at pH 7.4. Cell densities were adjusted to approx. $2.0 \times 10^8/\text{ml}$. Before the agonists were added, either 1 mM- Ca^{2+} or 1 mM- $\text{Na}_2\text{H}_2\text{EGTA}$ (to chelate residual traces of Ca^{2+}) was added, and the cells were equilibrated to 37 °C for several minutes.

Determination of myosin phosphorylation

Aliquots (0.8 ml) of the suspension were stirred during stimulation in a modified Perkin-Elmer MPF 44A fluorescence spectrophotometer. At the end of each incubation 0.6 ml of 0.7 M-HClO₄ was added and the resulting precipitate was then left for a further 10 min at

Abbreviations used: PAF, platelet-activating factor; TPA, phorbol 12-myristate 13-acetate; IP₃, inositol 1,4,5-trisphosphate.

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room temperature. After centrifuging at 1400 *g* for 5 min the pellet was washed twice with 0.5 ml of acetone and then dissolved in 30 μ l of sample buffer containing 8 M-urea, 20 mM-Tris, 122 mM-glycine, 5 mM-dithiothreitol, pH 8.6, with approximately 0.1% Bromophenol Blue dye. Gel electrophoresis was carried out on 10% polyacrylamide slab gels containing 40% (v/v) glycerol with a 3.6% polyacrylamide stacking gel containing 8 M-urea (Cande *et al.*, 1983; Perrie & Perry, 1970). The running buffer used was 20 mM-Tris/122 mM-glycine at pH 8.6 and contained 4 mM-urea. After 2 h at 4 mA/gel, gels were fixed in methanol/acetic acid/water (5:1:4, by vol.) for 1 h to wash out the glycerol, and then stained with Coomassie Brilliant Blue R250. Gels were scanned on a Joyce-Loebl Microdensitometer 3CS and the areas of the peaks for dephosphorylated and phosphorylated 20 kDa myosin light chain were estimated by cutting and weighing. The results were expressed as the percentage of light chains in the phosphorylated form.

Measurement of $[Ca^{2+}]_i$

The cells were continuously stirred with a Teflon-covered magnetic follower in a modified Perkin-Elmer MPF 44A fluorescence spectrophotometer. $[Ca^{2+}]_i$ was reported by quin2 fluorescence (excitation 339 ± 5 nm; emission 500 ± 10 nm) as previously described (Rink *et al.*, 1983; Hallam *et al.*, 1984a). The presence of around 1 mM-quin2 in the cytoplasm of the cells is expected to influence stimulated changes in $[Ca^{2+}]_i$ since it significantly increases the calcium-buffering ability of the cytoplasm as discussed elsewhere (see, e.g., Hallam *et al.*, 1984a). However, the correlation between functional response and measured $[Ca^{2+}]_i$ is applicable for these quin2-loaded cells.

Preparation of permeabilized cells

Platelets were prepared as described above but without loading with quin2, washed once in physiological saline, pH 6.6, before finally resuspending in buffer containing 108 mM-sucrose, 60 mM-potassium gluconate, 20 mM-Mops/NaOH, 20 mM-KCl, 1 mM- KH_2PO_4 , 1.112 mM- $MgSO_4$, 1.112 mM-EGTA and 5 mM- $MgATP$, pH 7.05 at 25 °C. The suspension was then exposed to 15 consecutive high-voltage discharges of 20 kV $\cdot$ cm $^{-1}$ to premeabilize selectively the plasma membrane of the platelets and render it leaky to small molecules and ions (Knight *et al.*, 1982). Aliquots (0.8 ml) were then added to vials containing nothing, 7.5 μ l of 50 mM- or 30 μ l of 500 mM- $CaEGTA$ to adjust the free Ca^{2+} concentration in the suspension and inside the platelets to 0 ($< 10^{-8}$ M), 100 nM or 4 μ M respectively in the presence or absence of 20 μ M-inositol 1,4,5-trisphosphate as indicated. After 1 min at 25 °C the incubations were stopped by the addition of $HClO_4$ and the extent of myosin light chain phosphorylation was determined as previously described. (It should be noted that this protocol permeabilized about 75% of the platelets, as judged from the release of pre-incorporated [^{14}C]adenine nucleotides; 4 μ M- Ca^{2+} caused secretion of about 70% of incorporated 5-hydroxy[3H]tryptamine from the permeabilized cells, but only 20% release from control cells; these values are similar to those reported by Knight *et al.*, 1982).

Measurement of external Ca^{2+}

Calcium-selective electrodes were constructed by fusing Ca^{2+} -selective poly(vinyl chloride) matrix mem-

Fig. 1. Urea/glycerol polyacrylamide-gel electrophoresis for resolving the unphosphorylated and phosphorylated forms of the 20 kDa myosin light chain in crude protein extracts from quin2-loaded human platelets

Tracks 1, 2 and 3 show the effect in 1 mM- Ca^{2+} medium of no addition, 200 nM-ionomycin or 20 ng of PAF/ml for 30 s respectively. Tracks 4, 5 and 6 show the effect of no addition, 200 nM-ionomycin for 30 s or 200 nM-ionomycin for 1 min followed by 20 ng of PAF/ml for 30 s in the presence of no added Ca^{2+} . P and non P, phosphorylated and non-phosphorylated 20 kDa light chains respectively.

branes (supplied by Pye-Unicam; part no. 1S561CASP) onto 1 mm poly(vinyl chloride) tubing with tetrahydrofuran. The reference electrode was a similar tube filled with 3 M-KCl gelled in agar. The potential difference was measured by a specially constructed high impedance electrometer and the electrode was calibrated in the Ca^{2+} buffers described by Marban *et al.* (1980).

Materials

PAF, human thrombin and ionomycin were all purchased from Calbiochem.

RESULTS AND DISCUSSION

Fig. 1 shows extracts of quin2-loaded platelets in control conditions and after various forms of stimulation run on a glycerol gel. The first track shows that nearly all the myosin light chain from control cells is in the unphosphorylated form. Tracks 2 and 3 show the appearance of bands in the position of the phosphorylated light chain form following stimulation with optimal concentrations of ionomycin and PAF respectively in the presence of 1 mM external Ca^{2+} . (Tracks 4, 5 and 6 show the effects of stimuli applied with very low external Ca^{2+} , and are discussed below.) Fig. 2 shows the data collected from three separate experiments in which $[Ca^{2+}]_i$ was raised to different levels by applying different concentrations of the calcium ionophore, ionomycin. At basal $[Ca^{2+}]_i$, 9% of the light chains were in the phosphorylated form. There is little indication of phosphorylation until $[Ca^{2+}]_i$ exceeds 400 nM and the response appears to saturate at about 1 μ M. Even at the highest $[Ca^{2+}]_i$, only about 70% of the light chains were phosphorylated; this is similar to the maximum extent of phosphorylation

Fig. 2. Ca^{2+} -activation curve for myosin light chain phosphorylation in intact quin2-loaded human platelets

The solid circles show the extent of myosin light chain phosphorylation and the existing $[\text{Ca}^{2+}]_i$ after stimulation with various concentrations of ionomycin for 30 s either in the presence of 1 mM-EGTA or 1 mM- Ca^{2+} . The open circle shows the effect of stimulation with 0.5 unit of thrombin/ml for 30 s (instead of ionomycin) in the presence of 1 mM-EGTA and represents the same data as in Table 1, row 2.

observed with maximal doses of natural agonists (see Daniel *et al.*, 1984a and Table 1). These results demonstrate the first simultaneous measurements of myosin phosphorylation and $[\text{Ca}^{2+}]_i$ in intact cells and show that $[\text{Ca}^{2+}]_i$ in the sub-micromolar range is indeed an effective stimulus for myosin phosphorylation. A somewhat similar relationship between the level of myosin light chain phosphorylation and $[\text{Ca}^{2+}]_i$ is seen

in platelets made permeable to calcium buffers by exposure to high-voltage electric discharge (Haslam & Davidson, 1984), though in those experiments phosphorylation was followed by incorporation of ^{32}P and the relationship looks less steep than that seen in Fig. 2.

Several agonists, including PAF and thrombin, are known to stimulate myosin phosphorylation (Kaibuchi *et al.*, 1983; Lyons & Shaw, 1980), and are able to raise $[\text{Ca}^{2+}]_i$ in quin2-loaded platelets towards or above 1 μM (Hallam *et al.*, 1984a; Rink *et al.*, 1982a), so that the increase in myosin phosphorylation they evoke could be due to the elevation of $[\text{Ca}^{2+}]_i$. However, we have found that in certain experimental conditions these agonists can stimulate the platelets to change shape and to secrete the contents of amine storage granules even when $[\text{Ca}^{2+}]_i$ remains at, or close to, basal levels ($< 300 \text{ nM}$), and well below the levels needed to cause shape-change (400 nM) and secretion ($> 1 \mu\text{M}$) when calcium ionophore is used to stimulate the cells (Hallam *et al.*, 1984a; Rink *et al.*, 1982a; Rink & Hallam, 1984). We therefore wondered whether the same might be true for myosin phosphorylation, since this phosphorylation has been proposed to be an important element in the cytoplasmic rearrangements involved in shape-change and secretion in platelets (Daniel *et al.*, 1984a). We tested this idea by measuring myosin phosphorylation after quin2-loaded platelets had been stimulated with PAF and thrombin in a medium containing no added calcium and 1 mM-EGTA. Added alone, these agonists caused a small rise in $[\text{Ca}^{2+}]_i$, which reaches a maximum below 200 nM (see Table 1). Fig. 3(a) shows a typical $[\text{Ca}^{2+}]_i$ transient on stimulation with PAF.

Under these conditions optimal concentrations of ionomycin raised $[\text{Ca}^{2+}]_i$ to only 180 nM, presumably discharging the same internal pool of Ca^{2+} accessible to the natural agonists as shown in Fig. 3(b). Subsequent

Table 1. $[\text{Ca}^{2+}]_i$ and myosin light chain phosphorylation in intact quin2-loaded human platelets upon stimulation

The experimental protocols were similar to those described in Figs. 1 and 2. The extent of myosin phosphorylation shown is that 30 s after the addition of ionophore or agonist when just one agent was added; where ionomycin was followed by an agonist, there was an interval of 1 min between the two and the extent of myosin phosphorylation shown is that recorded 30 s after addition of the agonist. The $[\text{Ca}^{2+}]_i$ values shown are the peak values which are attained within the period of stimulation by the agonist (see Fig. 3). The concentration of agents added was as follows: ionomycin, 200 nM; PAF, 20 ng/ml; thrombin, 0.5 unit/ml; TPA, 20 nM. For most of the experiments 1 mM-EGTA was added to reduce the external Ca^{2+} concentration to $< 10^{-8} \text{ M}$; for the rest 1 mM- CaCl_2 was added. The values shown for $[\text{Ca}^{2+}]_i$ and myosin phosphorylation are the means $\pm$ S.E.M. for the number of determinations shown. In medium containing $< 10^{-7} \text{ M-}\text{Ca}^{2+}$, the stimulated rise in $[\text{Ca}^{2+}]_i$ in response to any of the stimuli were not significantly different from each other. The extent of myosin phosphorylation in response to thrombin, PAF, ionomycin + thrombin, and ionomycin + PAF in the same low- Ca^{2+} medium were all significantly different from that to ionomycin alone ($P < 0.003$).

Experimental conditions				
$[\text{Ca}^{2+}]_o(\text{M})$	Stimulus	$[\text{Ca}^{2+}]_i/\text{nM}$	Myosin phosphorylation/%	<i>n</i>
$< 10^{-7}$	Control	78 ± 3	8 ± 1	14
	Thrombin	206 ± 14	39 ± 4	11
	PAF	160 ± 16	22 ± 3	4
	Ionomycin	181 ± 14	14 ± 1	13
	Ionomycin + thrombin	174 ± 16	46 ± 1	5
	Ionomycin + PAF	189 ± 37	32 ± 2	6
	TPA	109 ± 3	9 ± 2	6
Approx. 10^{-3}	Control	105 ± 4	7 ± 1	11
	Ionomycin	> 1000	62 ± 2	9
	Thrombin	> 1000	63 ± 5	5
	TPA	109 ± 3	9 ± 2	6

Fig. 3. Effect of PAF and ionomycin and $[Ca^{2+}]_i$ in the absence of external Ca^{2+}

The quin2 fluorescence traces show the effect of addition of (a) PAF, 20 ng/ml or (b) 200 nM-ionomycin followed by PAF, 20 ng/ml. Prior to stimulation the platelets were equilibrated to 37 °C for several minutes and incubated with 1 mM-EGTA for 2–3 min.

addition of PAF or thrombin then caused no significant rise in $[Ca^{2+}]_i$, as shown in Table 1 and Fig. 3(b), and in our previously published quin2 recordings (Rink *et al.*, 1982a, 1983; Hallam *et al.*, 1984a). Table 1 also shows the extent of myosin phosphorylation measured under these experimental conditions. PAF and thrombin raise $[Ca^{2+}]_i$ to a peak of 160 and 210 nM respectively, and increase the proportion of myosin in the phosphorylated form after 30 s stimulation by 14% and 31%. A similar elevation of $[Ca^{2+}]_i$ by ionomycin caused only a 6% increase in myosin phosphorylation after a similar time period. Subsequent addition of PAF or thrombin (giving no significant increase in $[Ca^{2+}]_i$) produced after a further 30 s a marked increase of 18% and 32% respectively in the proportion of myosin light chains in the phosphorylated form.

These results show that PAF, and more effectively thrombin, can promote myosin phosphorylation when $[Ca^{2+}]_i$ changes either modestly or not at all. This is highlighted in Fig. 2 where the open circle showing the effect of thrombin in Ca^{2+} -free medium lies well to the left of the points representing the responses to ionophore.

We cannot say from this type of experiment whether the phosphorylation seen at low $[Ca^{2+}]_i$ represents a sensitization of a Ca^{2+} -dependent process or an essentially Ca^{2+} -independent mechanism. We should also point out that the extent of phosphorylation seen at low $[Ca^{2+}]_i$, even with thrombin, is less than the maximum seen when $[Ca^{2+}]_i$ exceeds 1 μ M, as shown in Table 1. Maximal myosin phosphorylation may therefore require elevation of $[Ca^{2+}]_i$ to μ M levels. We do not know at present precisely what is the functional role of myosin light chain phosphorylation in platelets. Recent evidence shows a strong correlation between ADP-evoked shape-change and the extent of phosphorylation of myosin chains (Daniel *et al.*, 1984a). It seems possible that phosphorylation plays a major role in the internal reorganization of organelles, sometimes referred to as internal contraction, that accompanies a full shape-change response (Gerrard *et al.*, 1979). In smooth muscle it is generally agreed that the development of tension, if not its maintenance, is correlated with the extent of myosin

light chain phosphorylation (Askoy *et al.*, 1982). In that context an interesting result was reported in smooth muscle loaded with the calcium-indicating photoprotein aequorin (Morgan & Morgan, 1984). Addition of phenylephrine, after the cells had been depolarized with 90 mM- K^+ , caused an increase in tension with no further change in light output (Fig. 8 of Morgan & Morgan, 1984). Could it be that this development of tension without measurable increase in $[Ca^{2+}]_i$ reflects that responsible for the effects we see in platelets at low $[Ca^{2+}]_i$?

Our data, and those of others who have looked at the phosphorylation of myosin light chains in intact cells, do not identify the enzymes responsible; we cannot be sure that the phosphorylation observed in our experiments was entirely caused by stimulation of calcium-calmodulin-dependent myosin light chain kinase. It has recently been reported that phorbol ester, TPA, albeit at extremely high concentrations, can promote a slow and partial phosphorylation of myosin light chains (Naka *et al.*, 1983), an effect attributable to intense stimulation of protein kinase C (Castagna *et al.*, 1982). Since we have shown that TPA exerts its effect on platelets without raising $[Ca^{2+}]_i$ (Rink *et al.*, 1983), one must consider whether myosin phosphorylation evoked by PAF and thrombin at low $[Ca^{2+}]_i$ is similar to that produced by TPA. This seems unlikely since: (1) the effect of TPA reported by Naka *et al.* (1983) was slow, taking several minutes to reach 20% of the level of phosphorylation produced by thrombin, whereas our measurements were made at 30 s, at which point TPA causes no increase in light chain phosphorylation, as seen in Table 1; (2) TPA stimulated phosphorylation at a site distinct from that phosphorylated after stimulation by thrombin (Naka *et al.*, 1983); and (3) more modest concentrations of TPA than those used by Naka *et al.* (1983) produce optimal activation of protein kinase-C, but do not cause significant phosphorylation of myosin light chains (Table 1 and Castagna *et al.*, 1982).

Next, one has to consider what the alternative intracellular signal for myosin phosphorylation evoked by thrombin and PAF at low $[Ca^{2+}]_i$ might be.

Diacylglycerol, the natural activator of C-kinase (Nishizuka, 1984), is one candidate, but if it does control myosin phosphorylation it is unlikely to be through the activation of C-kinase for reasons discussed above in connection with TPA. Another possible candidate is the other product of stimulated hydrolysis of inositol lipids, namely IP_3 (Berridge & Irvine, 1984). At present there is no method for introducing IP_3 into intact platelets and so we resorted to a permeabilized preparation produced by exposing the cells to a high voltage discharge (Knight *et al.*, 1982). Using this preparation $[Ca^{2+}]$ was fixed at either very low levels with 1.1 mM-EGTA with no added Ca^{2+} , or at 100 nM- $[Ca^{2+}]$. At very low $[Ca^{2+}]$ or at 100 nM, the extent of myosin phosphorylation was $9 \pm 2\%$ ($n = 3$) and $11 \pm 2\%$ ($n = 6$) respectively, values which are not significantly different from intact cell controls, $10 \pm 1\%$ ($n = 4$). Raising $[Ca^{2+}]$ to 4 μM as expected produced a large stimulation of myosin phosphorylation to $50 \pm 3\%$ ($n = 6$). This result, and the fact that the cells secreted previously incorporated $[^3H]$ serotonin under these conditions, confirmed the functional $[Ca^{2+}]$ competence of the preparation. After addition of 20 μM - IP_3 for 1 min at very low $[Ca^{2+}]$ $12 \pm 1\%$ ($n = 3$) of myosin was phosphorylated and at 100 nM- $[Ca^{2+}]$ only $9 \pm 2\%$ ($n = 7$) of the myosin light chains were phosphorylated after 1 min exposure to 20 μM - IP_3 . This negative result does not support the suggestion that IP_3 might stimulate the phosphorylation of myosin light chains observed in intact cells. It does not of course rule out this possibility, since the conditions chosen might not have been suitable. While this manuscript was in preparation Lapetina *et al.* (1984) reported that IP_3 does cause phosphorylation of human platelet M_r 20000 protein. However, in their studies no inhibitors of prostanoid synthesis were used. Activation of phospholipase A_2 result in the release of activating prostaglandins, and through surface receptors promote phosphorylation secondarily. In our studies such effects of endogenous prostanoids were avoided by prior incubation of the platelets with aspirin.

It is unlikely that cyclic nucleotides could mediate these effects, since in platelets these are inhibitory messengers (Haslam *et al.*, 1980; Feinstein *et al.*, 1981; Hallam *et al.*, 1984b). In some cells H^+ ions can have important controlling effects (Rink *et al.*, 1982b) but we find that PAF and thrombin evoke minimal (less than 0.05 unit) changes in cytoplasmic pH in quin2-loaded platelets (T. J. Hallam, A. W. M. Simpson & T. J. Rink, unpublished work). One is therefore left to suggest the existence of a so far unidentified second messenger pathway which our future efforts will need to track down.

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REFERENCES

Adelstein, R. S. (1982) *Cell* **30**, 349–350

- Adelstein, R. S. & Eisenberg, E. (1980) *Annu. Rev. Biochem.* **49**, 921–956
- Askoy, M. O., Murphy, R. A. & Kamm, K. E. (1982) *Am. J. Physiol.* **242**, C109–C116
- Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312**, 315–321
- Cande, W. Z., Tooth, P. J. & Kendrick-Jones, J. (1983) *J. Cell Biol.* **97**, 1062–1071
- Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. & Nishizuka, Y. (1982) *J. Biol. Chem.* **257**, 7847–7851
- Cox, A. C., Carroll, R. C., White, J. G. & Rao, G. H. R. (1984) *J. Cell Biol.* **98**, 8–15
- Daniel, J. L., Molish, I. R. & Holmsen, H. (1981) *J. Biol. Chem.* **256**, 7510–7514
- Daniel, J. L., Molish, I. R., Rigmaiden, M. & Stewart, G. (1984a) *J. Biol. Chem.* **259**, 9826–9831
- Daniel, J. L., Hallam, T. J. & Rink, T. J. (1984b) *J. Physiol. (London)* **357**, 108P
- Feinstein, M. B., Rodan, G. A. & Cutter, L. S. (1981) in *Platelets in Biology and Pathology* (Gordon, J. L., ed.), pp. 437–472, Elsevier/North-Holland, Amsterdam
- Gerrard, J. M., Schollmeyer, J. V., Phillips, D. R. & White, J. G. (1979) *Am. J. Pathol.* **94**, 509–528
- Hallam, T. J., Sanchez, A. & Rink, T. J. (1984a) *Biochem. J.* **218**, 819–827
- Hallam, T. J., Sanchez, A. & Rink, T. J. (1984b) in *Prostaglandins and Membrane Ion Transport* (Braquet, P. *et al.*, eds.), pp. 157–163, Raven Press, New York
- Haslam, R. J. & Davidson, M. M. L. (1984) *Biochem. J.* **222**, 351–361
- Haslam, R. J., Salama, S. E., Fox, J. E. B., Lynham, J. A. & Davidson, M. M. L. (1980) in *Platelets* (Rotman, A. *et al.*, eds.), pp. 213–231, John Wiley, Chichester
- Kaibuchi, K., Takai, Y., Sawamura, M., Hoshijima, M., Fukijura, T. & Nishizuka, Y. (1983) *J. Biol. Chem.* **258**, 6701–6704
- Knight, D. E., Hallam, T. J. & Scrutton, M. C. (1982) *Nature (London)* **296**, 256–257
- Lapetina, E. G., Watson, S. P. & Cuatrecasas, P. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7431–7435
- Lebowitz, E. A. & Cooke, R. (1979) *J. Biol. Chem.* **253**, 5443–5447
- Lyons, R. M. & Shaw, J. O. (1980) *J. Clin. Invest.* **65**, 242–255
- Marban, E., Rink, T. J., Tsien, R. W. & Tsien, R. Y. (1980) *Nature (London)* **286**, 845–850
- Morgan, J. P. & Morgan, K. G. (1984) *J. Physiol. (London)* **351**, 155–167
- Naka, M., Nishikawa, M., Adelstein, R. S. & Hidaka, H. (1983) *Nature (London)* **306**, 490–492
- Nishizuka, Y. (1984) *Nature (London)* **308**, 693–698
- Perrie, N. T. & Perry, S. V. (1970) *Biochem. J.* **119**, 31–38
- Rink, T. J. & Hallam, T. J. (1984) *Trends Biochem. Sci.* **9**, 29–34
- Rink, T. J., Smith, S. W. & Tsien, R. Y. (1982a) *FEBS Lett.* **148**, 21–26
- Rink, T. J., Tsien, R. Y. & Pozzan, T. (1982b) *J. Cell Biol.* **95**, 189–196
- Rink, T. J., Sanchez, A. & Hallam, T. J. (1983) *Nature (London)* **305**, 317–319
- Scholey, J. M., Taylor, K. A. & Kendrick-Jones, J. (1980) *Nature (London)* **287**, 233–235
- Steer, M. & Salzman, E. (1980) *Adv. Cyclic Nucleotide Res.* **12**, 71–94

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