40 Resink, T. J., Bürgisser, E., and Bühler, F. R., Enhanced platelet cyclic AMP response to prostaglandin E_1 in essential hypertension. Hypertension *8* (1986) 662–668.
41 Robinson, B. F., Altered calcium handling as a cause of primary hypertension. J. Hypertens. *2* (1984) 453–460.
42 Takai, Y., Kikkawa, U., Kaibuchi, K., and Nishizuka, Y., Membrane phospholipid metabolism and signal transduction for protein phosphorylation. Adv. cycl. nucl. Res. *18* (1984) 119–158.
43 Vergara, J., Tsien, R. Y., and Delay, M., Inositol 1,4,5-triphosphate: a possible chemical link in excitation-contraction coupling in muscle. Proc. natl Acad. Sci. USA *82* (1985) 6352–6356.
44 Watterson, D. M., and Vincenzi, F. F. (Eds), Calmodulin and cell functions. Ann. N.Y. Acad. Sci. *356* (1980) 1–446.
45 Wei, J.-W., Janis, R. A., and Daniel, E. E., Calcium accumulation and enzymatic activities of subcellular fractions from aortas and ventricles of genetically hypertensive rats. Circ. Res. *39* (1976) 133–140.
46 Zidek, W., Vetter, H., Dorst, K.-G., Zumkley, H., and Losse, H., Intracellular Na^+ and Ca^{2+} activities in essential hypertension. Clin. Sci. *63* (1982) 41 s–43 s.

0014-4754/88/02094-04$1.50 + 0.20/0

Cytosolic calcium in platelet activation

by T. J. Rink

Smith Kline & French Research Ltd, The Frythe, Welwyn, Hertshire AL6 9AR (England)

Summary. Experiments with permeabilised platelets, and with intact platelets loaded with fluorescent Ca^{2+}-indicators, over the past several years have greatly extended our knowledge and understanding of cytosolic Ca^{2+} as a platelet activator and its interactions with other cytosolic regulators. This article outlines insights, gained from the use of the fluorescent dyes, into maintenance and restoration of basal $[Ca^{2+}]_i$, mechanisms of receptor-mediated Ca^{2+}-mobilisation and quantitation of $[Ca^{2+}]_i$/response relations in intact human platelets.
Key words. Calcium; platelet; second messenger.

Introduction

Ca^{2+} was the first identified second messenger, established many years ago as a key trigger for contraction and secretory exocytosis. Subsequently Ca^{2+} was found to influence many other intracellular functions such as membrane transport, glycogenolysis, mitochondrial respiration and non-muscle motility. One of the main cytosolic target proteins, calmodulin, has been found in all eukaryotic cells and is a major protein in platelet cytosol. Furthermore, calmodulin-dependent protein kinase is also present with a wide variety of target proteins in different cell types. These findings point to a cytosolic regulator role for Ca^{2+} in virtually all cells. Evidence for a trigger role of calcium was first found by microinjection mainly into large striated muscle cells, and by finding that some cell responses could be abolished by removal of external Ca^{2+}. This latter manoeuvre suggested a role for Ca-entry, but could have reflected a requirement for Ca^{2+} in surface membrane events of stimulus-response coupling. Also, it became clear from work with skeletal muscle that trigger calcium could come from a rapidly dischargeable internal pool not readily influenced by manipulation of external Ca^{2+}

By the late seventies there were four main pieces of evidence for an important role for calcium in platelet activation. Platelets contain calmodulin, and calmodulin-dependent protein kinase; they undergo cell functions such as secretory exocytosis that were widely thought to be triggered by Ca^{2+}, platelet activation is associated with an increased uptake of $^{45}Ca^{2+}$ (see for example Massini and Lüscher[13]), and perhaps most convincingly the calcium ionophore A 23187 was able to activate platelets[3, 12]. In 1980, Knight and Scrutton[9] demonstrated secretory exocytosis in platelets whose plasma membranes have been rendered selectively permeable by exposure to high voltage discharge. These experiments showed that $[Ca^{2+}]_i$ in the range 1–10 µM was a sufficient stimulus for secretory exocytosis. Further work with this system has allowed analysis of the interaction between Ca^{2+} and other messengers such as cAMP and activators of protein kinase-C in the intracellular control of platelet function and has been the subject of a recent excellent review[10]. Shortly after this came the invention of fluorescent Ca^{2+}-indicators[28] and a way of trapping them in the cytosol of intact cells[22], and the first measurements of $[Ca^{2+}]_i$ in resting and activated platelets. In this article I shall focus on the measurement and manipulation of $[Ca^{2+}]_i$ in intact human platelets loaded with quin 2 and fura-2 and try to summarize the main new data and insights that this work has provided.

Basal $[Ca^{2+}]_i$ and restoration of resting levels

For many years before direct measurements were possible it could be deduced that $[Ca^{2+}]_i$ in resting platelets was in the sub-micromolar range, based on the Ca-sensitivities of calmodulin and Ca-activated proteases and phospholipases determined in cell fractions, and analogy with those giant cells in which measurement of $[Ca^{2+}]_i$ by microinjected aequorin or microelectrodes had been possible. Direct measurements with intracellular quin 2 gives values near 100 nM, in line with resting levels determined by various methods in most other cell types examined. In the first quin 2 studies and in much subsequent work, correction has not been made for the error that arises from dye that has leaked into the external medium and which leads to an over-estimate of the value for $[Ca^{2+}]_i$ in calcium-containing medium. More careful recent measurements give values for resting $[Ca^{2+}]_i$ around 70 nM for quin 2-loaded human platelets in physiological saline[24]. Basal $[Ca^{2+}]_i$ is not significantly different over minutes in cells in the presence of physiological levels of extracellular calcium or in medium containing no added calcium and 1 mM EGTA. This implies that the resting membrane is very impermeable and that the basal leak and counter-balancing Ca-pumping are small in the resting state. One consequence of this is that inhibition of Ca-pumping could produce only a very slow elevation of $[Ca^{2+}]_i$ and is not therefore a plausible mechanism for triggering rapid events. The mechanism by which the steady state is maintained in the long term, i.e. by which the inevitable leak of calcium into the cell is counterbalanced by extrusion, is still contentious. Some authors contend that there is no Ca-ATPase in the surface membrane[4, 27]; but a recent report[2] has identified a Ca-ATPase which the authors conclude is likely to be capable of active transport of calcium across the surface membrane. Another

possible mode of Ca-transport across plasma membranes is Ca : Na exchange. We have found that complete replacement of Na by either choline or potassium has little effect of basal $[Ca^{2+}]_i$ over the many tens of minutes[24] suggesting that Na : Ca exchange is not important for Ca-transport in platelets at least over this time period.

Many agonists can produce a rapid rise in $[Ca^{2+}]_i$ into the micromolar range (see below) followed by return towards or to the basal level within minutes. This implies the existence of powerful mechanisms for removing Ca^{2+} from the cytosol into internal pools or by extrusion across the plasma membrane. We have recently started to investigate the mechanisms for this Ca^{2+}-removal and find evidence, as we might have expected, for a component representing resequestration into the internally-dischargeable pool and a component representing extrusion across the plasma membrane[19]. In dye-loaded platelets following application of an agonist it is difficult to disentangle the contributions due to falling calcium influx, falling internal release, internal sequestration, and extrusion. We have not yet found a way to specifically investigate the internal resequestration process; a permeabilised platelet preparation would offer advantages for this type of analysis. We have been able to find a way to examine the extrusion mechanism by looking at cells treated with maximal doses of Ca-ionophore to short circuit the uptake into internal stores[9]. Under these conditions recovery of $[Ca^{2+}]_i$, or loss of $^{45}Ca^{2+}$ into Ca-free medium is indicative of Ca-pumping across the plasma membrane. Following elevation of $[Ca^{2+}]_i$ with ionophore alone, restoration towards the basal level is very slow so that $[Ca^{2+}]_i$ remains well above 200 nM after 10 min at 37 °C. However, addition of either thrombin or phorbol ester following the ionomycin stimulates a more rapid return of $[Ca^{2+}]_i$ to the resting level, and this is paralleled by an enhanced efflux of $^{45}Ca^{2+}$. Therefore we conclude that a natural agonist and a known activator of protein kinase-C can markedly stimulate plasma membrane Ca-pumping. We do not believe that Na : Ca exchange plays an important role in this process since complete removal of sodium and its replacement by *N*-methyl-D-glucamine has rather little effect under these experimental conditions on the fall of $[Ca^{2+}]_i$ or the loss of $^{45}Ca^{2+}$ (Sage and Rink, in preparation).

Agonist evoked elevation of $[Ca^{2+}]_i$

In physiological saline containing 1 mM Ca^{2+}, many agonists are able to elevate $[Ca^{2+}]$ reported by either quin 2 or fura-2 to approximately 1 μM[6, 8, 20]. These include platelet-activating factor, vasopressin, stable prostaglandin endoperoxides, and ADP. In our hands, only thrombin is able reliably to raise $[Ca^{2+}]_i$ well above 1 μM. For none of these agonists is the endogenous production of prostaglandin endoperoxides or thromboxane necessary, although the responses are usually a little smaller in cells treated with blockers of cyclooxygenase. With platelets loaded with relatively large, 1–3 mM, amounts of quin 2 there is a very marked diminution in the extent of the $[Ca^{2+}]_i$ rise when the experiments are repeated in the absence of external calcium. This finding implies first, that there is a significant internally dischargeable pool, most probably in the dense tubular system, and second that under normal circumstances a significant component of the elevation of $[Ca^{2+}]_i$ is contributed by a triggered influx of Ca^{2+} across the plasma membrane. When the experiments are repeated with much lower loadings of fura-2, which can be achieved while retaining adequate signal to noise ratio because this dye is some 30 times more fluorescent than is quin 2, one sees much smaller differences between the extent of the $[Ca^{2+}]_i$ rise in the presence and absence of external calcium[17]. However, a statistically significant difference is still present and attributable to an influx component.

We assume along with almost all other workers in this field that at least for the most part the internal realease is attributable to agonist-evoked hydrolysis of phosphatidylinositol bisphosphate with the production of 1,4,5 inositol trisphosphate and the ability of this substance to trigger calcium discharge from the dense tubular system[14]. Because ADP is able to evoke a moderately large internal release of Ca^{2+}, and has been found, at least in aspirin-treated human platelets, to be a very weak activator of phosphatidylinositol bisphosphate hydrolysis we have suggested that perhaps an additional internal messenger for Ca-discharge is at work[6, 25]. This line of reasoning is relatively tenuous and depends critically on detailed quantitative measurements of inositol trisphosphate and $[Ca^{2+}]_i$ release in platelets stimulated by different ligands and it may turn out that inositol trisphosphate is indeed the major or even the only mechanism by which internal discharge is achieved. In recent work we have examined the kinetics of $[Ca^{2+}]_i$ rises in fura-2-loaded human platelets by means of stopped-flow fluorescence[24, 25]. In the absence of external Ca^{2+} all the ligands mentioned are able to cause a rise in measured $[Ca^{2+}]_i$ within 0.25 s but also there appears to be an irreducible delay of about 200 ms before any signal is seen. Clearly this time is quite adequate for the binding of receptor to ligand and all the downstream events that lead to the interaction of inositol trisphosphate with its putative receptor on the dense tubular system. But, the data are also a challenge to the proponents of this generally accepted hypothesis to demonstrate that adequate amounts of inositol trisphosphate are indeed formed within 200 ms of ligand binding to its receptor.

The mechanisms of agonist-evoked calcium entry have been more difficult to analyse, and there has even been some doubt as to the existence or importance of this process. Although the difference between the signals seen with quin 2 in the presence and absence of external calcium are most easily explained by the existence of activated Ca-entry, a possible alternative explanation has always been that external Ca^{2+} is necessary for the adequate coupling of the receptor to the internal discharge mechanism. Hallam and I recently used the ability of Mn^{2+} to quench the fluorescence of quin 2 to demonstrate that thrombin, platelet-activating factor and ADP all promoted an influx of Mn^{2+} into quin 2-loaded human platelets and proposed that this was strong evidence for ligand-evoked entry of a divalent cation[7]; we assume that Mn^{2+} was subverting routes more physiologically used by Ca^{2+} ions. Mechanisms of agonist-evoked Ca^{2+}-entry remain somewhat mysterious. We are now reasonably, though not absolutely, convinced that changes of platelet membrane potential play no role in the triggering in Ca^{2+}-entry. Perhaps the best evidence for this conclusion is that complete substitution of sodium by choline causes ligands such as thrombin and ADP to slightly hyperpolarise platelets, as assessed by the fluorescent probe dis-C_3-(5), as opposed to the usual small depolarisation that is seen in normal saline[15]. Nonetheless, the elevation of $[Ca^{2+}]_i$ evoked by these ligands whether in the absence or the presence of external Ca^{2+} is not significantly diminished. We have not found that the organic 'Ca-antagonists' such as nifedipine, diltiazem or verapamil have any significant effect on agonist-evoked elevation of $[Ca^{2+}]_i$ at the concentrations at which they severely suppress calcium currents through L-type voltage sensitive calcium channels[7]. We did find, however, that certain transition metals e.g. Co^{2+} and Li^{2+} are relatively effective at blocking the rise in quin 2 fluorescence in the presence of external calcium, suggesting that these transition metals are able to block receptor-operated Ca-entry[7]. Our recent kinetic analysis has shown that the rise in fura-2 fluorescence examined by stopped-flow meth-

ods occurs some 40–50 ms earlier in the presence than in the absence of external Ca^{2+} [25] suggesting that Ca-entry precedes internal discharge and eliminating a variety of proposed mechanisms in which entry is in some way consequent upon the mechanisms that cause discharge or the internal discharge itself. With all agonists so far tested, other than ADP, there is always an irreducible lag of some 150 ms prior to the measureable elevation of fura-2 fluorescence providing time for quite complex coupling mechanisms between the receptor and the Ca-entry process. With high concentrations of ADP the lag is reduced to less than the 20 ms dead time of our apparatus suggesting although not compellingly, more direct coupling of Ca-entry to this particular receptor. As yet there is no evidence linking inositol lipid hydrolysis to receptor-mediated Ca-entry in platelets. A finding that may implicate a G-Protein in this process is the slow elevation of fura-2 fluorescence evoked by a combination of 10 μM Al^{3+} and 10 mM F^- [1].

The ability of PGI_2 to inhibit platelet responses and by activation of adenylate cyclase and elevation of cAMP is well documented. At least one major part of the inhibitory action of cAMP appears to be a suppression of both Ca-discharge from internal stores and Ca-entry across the plasma membrane. Elevation of cAMP by PGI_2 or forskolin inhibits the increase in quin 2 fluorescence evoked by a variety of agonists. Where the cAMP-dependent phosphorylation is acting is not clear but one site is probably phospholipase-C [23]. Another interesting recent observation is that activation of protein kinase-C can lead to a somewhat slower but marked inhibition of calcium mobilisation in human platelets [11]. This protein kinase is therefore both activating itself and synergistic with activator calcium, and also acts as a negative feedback regulator by this slower inhibitory effect.

Imposed elevations of $[Ca^{2+}]_i$

I have indicated above the value of permeabilised platelets in allowing the investigator to set $[Ca^{2+}]_i$ by means of known external buffered $[Ca^{2+}]$ and to vary other smaller molecular weight constituents that can equilibrate with the internal millieu of the permeabilised cells. However, there are a number of responses that are not so easily measured or worked with in permeabilised cells and one cannot be sure that vital components of the small molecular weight composition of the cytosol have not been unknowingly changed during the process. We have used graded concentrations of calcium ionophore to impose essentially step changes in $[Ca^{2+}]_i$ with quin 2- and fura-2-loaded platelets and then constructed $[Ca^{2+}]_i$/response curves for various monitored platelet responses, with $[Ca^{2+}]_i$ as the quasi-independent variable. The great advantage of this approach over simply applying calcium ionophores and observing the responses is that one gets some quantitation of the level of the $[Ca^{2+}]_i$ which one must achieve in order to elicit the desired response. One sees a gradation of responsiveness for various end-points with shape-change [26] and myosin phosphorylation [5] being the most sensitive, having apparent $[Ca^{2+}]_i$ thresholds of 300 nM and complete activation by 800 nM to 1 μM. Aggregation appears to require $[Ca^{2+}]_i$ in the range 700 to 900 nM [20] and secretory exocytosis [20], liberation of arachidonate [18] and TxA_2 production need $[Ca^{2+}]_i$ [16] in the micromolar range. These values are those we have obtained in platelets not exposed to any activator other than Ca^{2+}, so far as we could determine. It rapidly became clear that $[Ca^{2+}]_i$ could synergise with other excitatory intracellular messengers such as diacylglycerol, so that one got substantial responses at levels of $[Ca^{2+}]_i$ that were themselves quite ineffective. We first detected this with stimulus-secretion coupling where diacylglycerol, or phorbol ester as a direct activator of protein kinase-C, could elicit a substantial, if somewhat delayed and sluggish, secretory exocytosis while $[Ca^{2+}]_i$ remains at or below resting levels [21]. Elevation of $[Ca^{2+}]_i$ to 500 nM by Ca ionophore elicited shape-change but no secretion. Subsequent addition of diacylglycerol or phorbol ester produced no further rise in $[Ca^{2+}]_i$, but there was a brisk and secretory response more like that seen with natural agonists, which can be presumed to produce both a rise in $[Ca^{2+}]_i$ and a supply of diacylglycerol to activate protein kinase-C. We also found other responses that natural agonists could evoke with $[Ca^{2+}]_i$ levels well below those needed when Ca ionophore, by-passing receptor mediated mechanisms and therefore presumably by-passing other signal pathways, was the stimulus. Shape-change and the phosphorylation of myosin light-chains could be evoked by thrombin or PAF while $[Ca^{2+}]_i$ remained below 160 nM [5, 22]. We have not yet identified the additional pathway that acts with or alongside Ca^{2+} to produce these responses.

More recently we have found that collagen is able to elicit a substantial liberation of arachidonate, mainly from phosphatidylcholine, while measured $[Ca^{2+}]_i$ remains between 100 and 115 nM [18]. We have not yet elucidated this apparently Ca-independent mechanism for the liberation of arachidonate but this finding fits in with our earlier conclusions on the sequence of excitatory events following the binding of collagen to platelets. The initial event is stimulation of phospholipid hydrolysis, both phosphatidylinositol and phosphatidylcholine, to form diacylglycerol and arachidonate. The diacylglycerol can promote a modest 'Ca-independent secretion'. In drug-free cells, the arachidonate is converted to TxA_2 which then acts at surface receptors to mobilise Ca^{2+} and evoke a delayed rise of $[Ca^{2+}]_i$ [20].

This kind of analysis has exploited one of the major advantages of new technology, namely the ability to quantify the levels of calcium required for different responses under different conditions. One then begins to see, not only what Ca^{2+} *can* do when applied at elevated concentrations to broken systems, permeabilised cells or even to intact cells by means of ionophore, but also what $[Ca^{2+}]_i$ *does* do when it is part of a complex activation cascade, evoked by the binding of a natural ligand to its receptor, in concert with the many other excitatory messengers produced, for example by the hydrolysis of phosphoinositides.

Conclusions

The use of permeabilised platelet preparations, and particularly the exploitation of fluorescent calcium-indicator dyes that can be loaded into intact functioning platelets, have confirmed and substantially extended the earlier deductions of the importance of $[Ca^{2+}]_i$ as a platelet regulator. Basal and $[Ca^{2+}]_i$ levels have been measured, mechanisms for the maintenance for the resting state have begun to be investigated. The processes by which the resting state is restored following stimulation have been shown to have both a component of calcium extrusion and re-uptake into internal stores; the Ca-extrusion seems not to have an important component of Na : Ca exchange. A variety of agonists are able to elevate $[Ca^{2+}]_i$ by both stimulated discharge of internal stores and Ca-entry across the plasma membrane. Recent experiments with relatively small amounts of the indicator dye fura-2, and hence relatively little added buffering capacity to the cytosol, have shown that internal discharge can be sufficient to raise $[Ca^{2+}]_i$ into the micromolar level. This might raise the question why bother with Ca-influx at all. The reason seems to be that if one has powerful extrusion systems in the membrane to guard against potentially unwanted or dangerous elevations in $[Ca^{2+}]_i$ then any elevation of $[Ca^{2+}]_i$ even that evoked by internal discharge will have an automatic consequence of a substantial efflux of Ca^{2+} from the cell. To

avoid Ca^{2+} depletion in repeatedly stimulated cells, one therefore needs an extra influx to accompany each elevation of $[Ca^{2+}]_i$. Internal discharge of Ca^{2+} is very likely, mainly, if not entirely, triggered by 1,4,5 inositol trisphospate. Stimulated Ca-entry seems not to require changes in membrane potential and may involve complex coupling processes including linkage through G-proteins. $[Ca^{2+}]_i$/response curves can be constructed by producing step elevations $[Ca^{2+}]_i$ with graded doses of Ca-ionophores and show that some responses occur as calcium rises above 300 nM, shape-change, while others e.g. secretory exocytosis require $[Ca^{2+}]_i$ in the micromolar range. Comparison of responses evoked by ionophores which by-pass recepted mediated signal transduction and those evoked by natural ligands acting through the receptors, show that other messenger pathways can synergise with or act alternatively to elevated $[Ca^{2+}]_i$, turning what might have seemed like a sub-threshold elevation of $[Ca^{2+}]_i$ into a very significant component of the excitatory cascade.

Acknowledgment. I thank Ms Maureen Bowden and Ms Beatrice Leigh for their help in preparing this manuscript.

1 Brass, L. F., Laposata, M., Banga, H. S., and Rittenhouse, S. E., Regulation of the phosphoinositide hydrolysis pathway in thrombin-stimulated platelets by a pertussis toxin-sensitive guanine nucleotide-binding protein. J. biol. Chem. *261* (1986) 16838–16847.
2 Enyedi, A., Sarkadi, B., Foldes-Papp, Z., Monostory, S., and Gardos, G., Demonstration of two distinct calcium pumps in human platelet membrane vesicles. J. biol. Chem. *261* (1986) 9558–9563.
3 Feinstein, M. B., and Fraser, C., Human platelet secretion and aggregation induced by calcium ionophores. J. gen. Physiol. *66* (1975) 561–581.
4 Hack, N., Croset, M., and Crawford, N., Studies on the bivalent-cation activated ATPase activities of highly purified human platelet surface and intracellular membranes. Biochem. J. *233* (1986) 661–668.
5 Hallam, T. J., Daniel, J. L., Kendrick-Jones, J., and Rink, T. J., Relationship between cytoplasmic free calcium and myosin light chain phosphorylation in intact platelets. Biochem. J. *232* (1985) 373–377.
6 Hallam, T. J., and Rink, T. J., Response to adenosine diphosphate in human platelets loaded with the fluorescent Ca^{2+} indicator quin 2. J. Physiol. *368* (1985) 131–146.
7 Hallam, T. J., and Rink, T. J., Agonists stimulate divalent cation channels in the plasma membrane of human platelets. FEBS Lett. *186* (1985) 175–179.
8 Hallam, T. J., Sanchez, A., and Rink, T. J., Stimulus-response coupling in human platelets: changes evoked by platelet-activation factor in cytoplasmic free calcium monitored with the fluorescent Ca indicator, quin 2. Biochem. J. *218* (1984) 819–827.
9 Knight, D. E., and Scrutton, M. C., Direct evidence for a role for Ca^{2+} in amine storage granule secretion by human platelets. Thrombosis Res. *20* (1980) 437–446.
10 Knight, D. E., and Scrutton, M. C., Gaining access to the cytosol: the technique and applications of electropermeabilization. Biochem. J. *234* (1986) 497–506.
11 MacIntyre, D. E., Buchfield, M., and Shaw, A. M., Regulation of platelet cytosolic free calcium by cyclic nucleotides and protein kinase C. FEBS Lett. *188* (1985) 383–388.
12 Massini, P., and Luscher, E. F., Some effects of ionophores for divalent cations on blood platelets. Comparison with the effects of thrombin. Biochim. biophys. Acta *372* (1974) 109–121.
13 Massini, P., and Luscher, E. F., On the significance of the influx of calcium ions into stimulated human blood platelets. Biochim. biophys. Acta *416* (1976) 652–663.
14 O'Rourke, F. A., Halenda, S. P., Zavoico, G. B., and Feinstein, M. B., Inositol trisphosphate-induced Ca^{2+} release from human platelet membranes. J. biol. Chem. *260* (1985) 956–962.
15 Pipili, E., Platelet membrane potential: simultaneous measurement of dis-C_3-(5) fluorescence and optical density. Thromb. Haemostasis *54* (1985) 645–651.
16 Pollock, W. K., Irvine, R. F., and Rink, T. J., Free Ca^{2+} requirements of agonist-induced thromboxane-A_2 synthesis in human platelets. Eur. J. Pharmac. *132* (1986) 309–313.
17 Pollock, W. K., and Rink, T. J., Thrombin and ionomycin can raise platelet cytosolic Ca^{2+} to micromolar levels by discharge of internal Ca^{2+} stores: studies using fura-2. Biochem. biophys. Res. Commun. *139* (1986) 308–314.
18 Pollock, W. K., Rink, T. J., and Irvine, R. F., Liberation of $[^3H]$ arachidonic acid and changes in cytosolic free calcium in fura-2-loaded human platelets stimulated by ionomycin and collagen. Biochem. J. *235* (1986) 869–877.
19 Pollock, W. K., Sage, S. O., and Rink, T. J., Stimulation of Ca^{2+} efflux from fura-2-loaded platelets activated by thrombin or phorbol myristate acetate. FEBS Lett. *210* (1987)132–136.
20 Rink, T. J., and Hallam, T. J., What turns platelets on? Trends biochem. Sci. *9* (1984) 215–219.
21 Rink, T. J., Sanchez, A., and Hallam, T. J., Diacylglycerol and phorbol ester stimulate secretion without raising cytoplasmic free calcium in human platelets. Nature *305* (1983) 317–319.
22 Rink, T. J., Smith, S. W., and Tsien, R. Y., Cytoplasmic free Ca^{2+} in human platelets: Ca^{2+} thresholds and Ca-independent activation for shape-change and secretion. FEBS Lett. *148* (1982) 21–26.
23 Sage, S. O., and Rink, T. J., Inhibition by forskolin of cytosolic calcium rise, shape change and aggregation in quin 2-loaded human platelets. FEBS Lett. *188* (1985) 135–140.
24 Sage, S. O., and Rink, T. J., Effects of ionic substitution on $[Ca^{2+}]_i$ rises evoked by thrombin and PAF in human platelets. Eur. J. Pharmac. *128* (1986) 99–107.
25 Sage, S. O., and Rink, T. J., Kinetic differences between thrombin-induced and ADP-induced calcium influx and release from internal stores in fura-2-loaded human platelets. Biochem. biophys. Res. Commun. *136* (1986) 1124–1129.
26 Simpson, A. W. M., Hallam, T. J., and Rink, T. J., Low concentrations of the stable prostaglandin endoperoxide U 44 069 stimulate shape change in quin2-loaded platelets without a measurable increase in $[Ca^{2+}]_i$. FEBS Lett. *201* (1986) 301–305.
27 Steiner, B., and Luscher, E. F., Evidence that the platelet plasma membrane does not contain a $[Ca^{2+} + Mg^{2+}]$-dependent ATPase. Biochem. biophys. Acta *818* (1985) 299–309.
28 Tsien, R. Y., Pozzan, T., and Rink, T. J., Calcium homeostasis intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator. J. Cell Biol. *94* (1982) 335–340.

0014-4754/88/020097-04$1.50 + 0.20/0