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CALCIUM SIGNALING IN HUMAN PLATELETS

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INTRODUCTION

In this chapter we outline our current understanding of the processes by which external Ca^{2+} and internal Ca^{2+} stores are mobilized to generate cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) signals in human platelets, and note briefly what is known of how resting $[Ca^{2+}]_i$ is restored and maintained. Due to limitations of space we will not cover the downstream events of calcium interaction with intracellular targets that evoke functional and structural changes: see Reference 71 for a recent, comprehensive review of platelet activation.

A central role for Ca^{2+} in platelet activation was deduced initially by analogy with other secretory and contractile cells where Ca^{2+} had been established as a key mediator of stimulus-secretion and excitation-contraction coupling. A variety of approaches then supported this notion for platelets. Several groups showed that calcium ionophores could evoke platelet responses similar to those seen with natural ligands acting at surface receptors: ^{45}Ca uptake was found to be increased on stimulation; measurement of chlortetracycline fluorescence (81) was consistent with stimulus-evoked discharge of an internal Ca store; and secretion from electro-permeabilized platelets was stimulated by Ca^{2+} in the range of 0.1 to 10μM (e.g. 21, 52,

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53). The first analyses of agonist-evoked $[Ca^{2+}]_i$ signals awaited the development, in the early 80s, of the fluorescent indicator, quin2, which could be introduced into populations of small cells by cytosolic esterase hydrolysis of the membrane-permeant acetoxymethyl ester (59). This "first-generation" indicator provided considerable insights, which have been summarized in earlier reviews (21, 53). Since 1986 most workers have turned to the "second-generation" dye fura-2. Its greater fluorescence signal permits lower intracellular concentrations of indicator, and hence less cytosolic buffering, and has the advantage of a significant shift of excitation spectrum on calcium binding that allows dual or multiple wavelength ratio techniques e.g. (9). Also, a few groups have made measurements with the photo-protein aequorin (e.g. 26, 84), loaded by reversible cell permeabilization induced by ATP^{4-}, and there have been some further significant data from $^{45}Ca^{2+}$ flux measurements (4, 55). This account will refer mainly to work published between January 1986 and March 1989, but key findings from early work are also referenced. We have focused on studies of human platelets, judged by us to provide important new data and/or insights; we offer the usual apologies for the inevitable omission of some good and significant work.

It is worth noting that the ability to measure and manipulate $[Ca^{2+}]_i$ also allows clearer understanding of the role of other cellular messengers. For instance, it soon became apparent that some functional responses, including secretory exocytosis, shape-change, and myosin phosphorylation, could occur with little or no measured rise in $[Ca^{2+}]_i$ (18, 58, 59). The "Ca-independent" secretory response is probably mediated via protein kinase C (58), which in turn is activated at basal $[Ca^{2+}]_i$ by increased formation of diacylglycerol formed by receptor-mediator activation of phospholipases acting on inositol lipids or on phosphatidylcholine. Ca^{2+}-independent shape-change and myosin phosphorylation seem not to reflect activity of protein kinase C (18, 21) and point to the existence of another excitatory signaling pathway, so far unidentified. Experiments with agents that elevate cAMP and inhibit responses to almost all agonists have shown that cAMP, acting presumably via A-kinase, inhibits both the generation and the actions of intracellular messengers, including elevated $[Ca^{2+}]_i$ (e.g. 1, 39, 41, 57, 64).

IONIC GRADIENTS AND MEMBRANE POTENTIAL

Platelets are in a sense cell fragments, which bud off from megakaryocytes in the bone marrow; they are very small, approximately discoidal, 1 by 3 μm. They lack nuclei and any significant components of protein synthesis, but otherwise appear to have the major components common to larger cell types. The available evidence indicates that platelets share with other cells the usual

gradients of Na^+ and K^+, established and maintained by an ouabain-sensitive Na-K ATPase, and have a plasma membrane selectively permeable to K^+ (23, 31, 43, 69). Mass measurements indicate $[K^+]_i$ to be about 135 mM and $[Na^+]_i$ about 20 mM. There are no measurements of intracellular activities for those ions. Resting pH_i is around 7.0 at 37°C, depending somewhat on the experimental conditions (e.g. 72, 73, 74). We recently found that in the usual bicarbonate-free suspending medium pH_i was 7.02, whereas in the presence of 25 mM HCO_3^- with 5% CO_2, pH_i was 7.15 (25). There is clear evidence for the presence of $Na^+:H^+$ exchange in platelets (72, 89), though it is not clear to what extent this is responsible for the maintainance of pH_i. A recent paper has put free Mg^{2+} in the range of 0.1 to 0.3 mM (85), which is markedly lower than that reported for lymphocytes, and many other cell types. This finding needs independent confirmation.

Resting $[Ca^{2+}]_i$ measured by fluorescence indicators is typically close to 0.1 μM. In more recent work with quin2 or fura-2, values for basal $[Ca^{2+}]_i$ tend to be in the range 70–80 nM. These lower values may reflect closer attention to leaked indicator and perhaps better preparation of the cells. Basal $[Ca^{2+}]_i$ is remarkably insensitive to large changes in external $[Ca^{2+}]$. Estimates of basal $[Ca^{2+}]_i$ in aequorin-loaded platelets have been much higher than measurements with quin2 or fura-2 (26, 84). The reason is not known, although the main proponents of the technique have recently proposed that lower than expected free Mg^{2+} in platelets compared with, for example, lymphocytes (60) may account for part of the discrepancy since the calibration of aequorin signals is markedly influenced by $[Mg^{2+}]$ (85). Other aspects of this aequorin technique are discussed in a previous review (9).

Estimates with fluorescent potential-sensitive dyes, or the distribution of radio-labeled lipophilic cations, indicate a platelet resting potential of -60 to -70 mV (e.g. 31, 43). This potential is dominated by the K^+ gradient and substitution of Na^+ or Cl^- with large organic ions has little detectable effect. Recently, whole cell patch-clamp recordings have been made on rabbit, rat, and human platelets (32, 33). The initial zero-current potential was about -52 mV, and the only detectable conductance was a voltage-dependent K^+ conductance. It is possible that sporadic opening of these channels provides the resting K^+ permeability that supports the resting potential. As in many cells, none of the main cations is at electrochemical equilibrium. Therefore there must be primary (ATP-fueled) or secondary active transporters for them. Clearly one is the Na^+/K^+ ATPase which generates the Na^+ gradient, which in turn can provide the potential energy for secondary active transport e.g. of H^+ by $Na^+:H^+$ exchange.

There is controversy in the literature as to whether $Na^+:Ca^{2+}$ exchange or a Ca^{2+}-ATPase in the plasma membrane provides the Ca^{2+} extrusion needed

for maintainence of, and recovery to, the resting state (e.g. 5, 14, 16, 35, 50, 55, 56, 70). We judge the evidence to favor a more important role for a Ca^{2+}-ATPase.

Features of $Na^+:Ca^{2+}$ exchange are clearly seen in microsomal fractions enriched in plasma membrane (50). Some authors have not found Ca^{2+}-ATPase in plasma membrane fractions, but others have reported two classes of Ca^{2+}-ATPase in platelet membranes and proposed that one, analogous to the real cell Ca-ATPase, serves as a Ca^{2+} pump in the platelet plasma membrane (14, 16). Brass observed a reduced ^{45}Ca efflux and increased uptake into human platelets when N-methyl-D-glucamine replaced external Na^+ (5). However, he observed no measurable change in $[Ca^{2+}]_i$ measured by quin2, a result reflected in other reports. More significant, perhaps, was the limited effect of Na^+ substitution in the recovery of $[Ca^{2+}]_i$ or the efflux of $^{45}Ca^{2+}$ following elevation of $[Ca^{2+}]_i$ by thrombin or ionomycin (55, 56). In a very recent paper it is reported that addition of Na^+ but not K^+, to platelets, gel-filtered, treated with ouabain, and suspended in sucrose solution, promoted a decline in $[Ca^{2+}]_i$ (70). This result is consistent with $Na^+:Ca^{2+}$ exchange, but there were no flux measurements to confirm an efflux rather than sequestration of Ca^{2+}.

INTERNAL CALCIUM RELEASE

The persistence of functional responses, attributed to elevated $[Ca^{2+}]_i$, in the absence of external Ca^{2+} provided indirect evidence for agonist-evoked discharge of intracellular Ca^{2+} stores. This analysis neglected the possibility that non-Ca^{2+} pathways might mediate such responses (as we now believe can happen); but the ability of Ca^{2+} ionophores to activate platelets in the absence of external calcium was more convincing evidence for a pool of Ca^{2+} sequestered in intracellular organelles. The existence of a microsome fraction capable of ATP-dependent accumulation of Ca^{2+}, analagous to sarcoplasmic reticulum (SR) fractions of muscle, was also consistent with this concept (e.g. 7, 39, 40). The demonstration of rapid agonist-evoked elevation of $[Ca^{2+}]_i$ in quin2-loaded platelets suspended in Ca-free medium (59) confirmed the existence of receptor-mediated internal discharge. Subsequent investigation has shown that many platelet receptors including those for platelet activating factor (PAF), vasopressin, thromboxane A_2, 5-hydroxytryptamine, and ADP can mediate internal Ca^{2+} release (13, 21, 53, 81). By contrast, adrenaline and collagen, which are capable of stimulating human platelets to aggregate and secrete, do not appear directly to elevate $[Ca^{2+}]_i$ (at least as measured by quin2 or fura-2) via their own receptors (13, 21, 53). Rather the involvement of Ca^{2+} is indirect: these agents promote aggregation and the liberation of arachidonic acid, probably from phosphatidylcholine, via Ca^{2+}-independent

pathways (46). The arachidonic acid is then converted to thromboxane A_2, which acts on its receptors to cause a delayed $[Ca^{2+}]_i$ response. Activators of protein kinase C, such as oleoylacetylglycerol or phorbol myristate acetate, do not increase platelet $[Ca^{2+}]_i$ (in the presence or absence of external Ca^{2+}) as measured by quin2 (26, 58) or fura-2 (47, 85). These agents do produce a modest increase in light emission from aequorin-loaded platelets, but the implication of such signals in terms of Ca^{2+} mobilization and cell signaling remains unclear (84).

The amount of Ca^{2+} discharged from internal stores can be estimated from the size of the $[Ca^{2+}]_i$ transient in quin2-loaded platelets and the intracellular quin2 concentration (59). Such calculations give values of 2–300 μmol Ca^{2+} per liter cell water. This is a plausible value for Ca^{2+} sequestered in organelles, presumably in the dense tubular system, which occupy a few percent of the cytoplasmic volume. The total Ca^{2+} content in such organelles would then be in the range of 10–20 mM, and the expectation is that much of this Ca^{2+} would be reversibly bound to a calsequestrin-like molecule so that the free $[Ca^{2+}]$ in these organelles might be several hundred μM. This arrangement would be analogous to the sacroplasmic reticulum in muscle and to the specialized calcium-sequestering and discharging organelles, "calciosomes", proposed for nonmuscle cells (34).

The amount of internal Ca^{2+} discharged by thrombin is virtually the same as that discharged by Ca^{2+} ionophores (59), which suggests that all the readily mobilized intracellular Ca^{2+} is accessible to receptor-mediated pathways. The minimal Ca^{2+} release evoked by mitochondrial uncouplers indicates that, as in most other cells, mitochondria are not a significant source of releasable Ca^{2+}. Mass measurements of Ca^{2+} and analysis of the amine-storage dense granules show that the total content of Ca^{2+} in platelets is much higher than the dischargeable store, about 10–20 mmol per liter of cell water. Most of this Ca^{2+} forms an insoluble matrix with biogenic amines and ATP within the secretory granules, thereby permitting a content of secretable product far in excess of that possible if the granule contents were in solution, and thus at osmotic balance with the cytosol.

Experiments with fura-2, loaded at low cell content, have shown that agonist-evoked internal release can increase $[Ca^{2+}]_i$ to at least 1 μM (47) and thus well into the range of $[Ca^{2+}]$, which is expected to influence target proteins. Use of stopped-flow equipment has allowed us to examine the subsecond kinetics of Ca^{2+} mobilization (62, 66, 67). In Ca^{2+}-free solution, thrombin, PAF, vasopressin, U46619 (a thromboxane mimetic), and ADP can elicit a $[Ca^{2+}]_i$ rise after a minimum delay of only some 200 msec, with a peak being reached within one sec. These results show that surface ligands can elicit a greater than tenfold elevation of $[Ca^{2+}]_i$ within a few hundred msecs, just by internal release. The delay and the time to peak response

lengthen as agonist concentration is reduced from the optimal level, or when the cells are treated with forskolin to increase cAMP levels (56, 67).

Inositol Phosphates

The role of inositol 1,4,5-trisphosphate, IP_3, as a link between receptor occupation and Ca^{2+} discharge is as well established for platelets as for many other cell types. Several agonists elicit a prompt formation of IP_3 and diacylglycerol from PIP_2 hydrolysis (10, 13, 17, 71, 80), though it has yet to be shown that IP_3 is formed quickly enough to account for the rapid $[Ca^{2+}]_i$ responses described above. IP_3 can cause Ca^{2+} release from platelet microsomes and from saponin-permeabilized platelets (e.g. 2, 6, 7, 11, 15, 40). The concentration range over which IP_3 is effective, 1–10 μM, is similar to that required in many other preparations and is compatible with the amount of PIP_2 breakdown and with mass measurement of the stimulated formation of inositol phosphates (80). At this point there is no reason to believe that the IP_3 receptor, or the calcium channel it is coupled to, differ from those in other cell types. As in many other systems, IP_3 is subject to a complex array of metabolic conversions starting with dephosphorylation to the 1,4-bisphosphate, or phosphorylation to form the 1,3,4,5-tetrakisphosphate (71). Thus within 10–20 secs much of the trisphosphate is actually in the form of the 1,3,4 isomer (80). Also, a small proportion of 1,2-cyclic 4,5-trisphosphate is formed. The available evidence suggests that the 1,4,5-tris isomer has the major, perhaps exclusive, role in Ca^{2+} release by the inositol lipid pathway; it appears that formation of cyclic IP_3 is too little and too late to be physiologically important (80). The possible role of inositol phosphates in gating Ca^{2+} entry is considered briefly below.

We have not assumed that IP_3 is the only pathway in nonexcitable cells for discharge of internal Ca^{2+} stores (53, 54, 56, 67). In human platelets ADP is effective in generating $[Ca^{2+}]_i$ signals in the absence of external Ca^{2+} (20). However ADP directly causes little (11) or no (13, 17) PIP_2 hydrolysis and IP_3 formation. These findings could indicate alternative pathways for internal Ca^{2+} discharge.

cAMP and cGMP

The role of cAMP in modulating Ca^{2+} sequestration and IP_3-evoked release remains unclear. Early and more recent reports indicate that cAMP-dependent kinase may act via a 23 kd protein (possibly related to phospholamban) to enhance Ca^{2+}-ATPase and Ca^{2+} uptake by platelet microsomes (1, 39, 71). This has been proposed as a major mechanism for cAMP inhibition of platelet function. However some workers have proposed that cAMP-dependent kinase is facilitatory for IP_3-induced release and reported that "PKI", a peptide

inhibitor of cAMP-dependent kinase catalytic subunit, inhibited IP_3-evoked Ca^{2+} release from a vesicle fraction (15), while another group found PKI to suppress Ca^{2+} uptake (1). O'Rourke et al (39) have re-examined this point and find first, no effect of protein phosphorylation by the catalytic subunit on Ca^{2+} uptake or IP_3-evoked release, and second, no effect of a highly purified PKI on either process. They suggest that previous work may have been confounded by impurities in commercially produced PKI. Our present view is that the main way that cAMP interacts with the inositol phosphate/Ca^{2+} discharge pathway is by inhibiting PIP_2 hydrolysis, but other effects are clearly not excluded (41, 57). A recent paper reports that cAMP-dependent kinase can stimulate Ca^{2+} uptake in a microsome fraction from neurons and also reduce the potency of IP_3 in promoting Ca^{2+} release (76); these findings indicate that interactions between cAMP and IP_3-dischargeable Ca^{2+} pools may be widespread.

Agents such as nitroprusside, which elevate cGMP, also cause inhibition of platelet responses to different agonists, as do permeant forms of cGMP such as dibutyrylcGMP (e.g. 13, 21, 36, 79). Nitroprusside was less effective in reducing responses to Ca^{2+} ionophores or phorbol ester, which suggests that the main site of inhibitory action is in the generation of second messengers (36). Interestingly, cGMP was more effective in inhibiting Ca^{2+} entry evoked by ADP than inhibiting internal release (36), whereas no such differential inhibition is seen with PAF or thrombin stimulation. Because cGMP can increase cAMP, for instance, by effects on cyclic nucleotide phosphodiesterases, it is important to measure both cyclic nucleotides. Morgan & Newby (36) showed that nitroprusside elevated cGMP up to tenfold with no significant effect on cAMP.

C-Kinase

Another important kinase, protein kinase C, can also reduce agonist-evoked PIP_2 hydroylsis (e.g. 90) and internal Ca^{2+} release (13, 30, 45, 90). In a broken cell system diacylglycerol was found to release sequestered Ca^{2+} (7), but we have never seen any elevation of $[Ca^{2+}]_i$ evoked by diacylglycerol or phorbol ester in intact platelets (46, 58). Another report has suggested that phorbol esters can stimulate Ca^{2+} sequestration in permeabilized platelets (87). Thus again we have apparently conflicting mechanisms deduced from broken cell systems and no clear relation to what is observed in intact platelets.

pH_i and Na^+/H^+ Exchange

The role of pH_i and Na^+/H^+ exchange in Ca^{2+} mobilization in platelets has been controversial (25, 51, 68, 72, 73, 89). It is found that thrombin activates Na^+/H^+ exchange and can promote, over many seconds, a 0.1 to 0.2 unit

increase in pH_i (23, 25, 72, 73, 74, 89). Some workers have seen an inhibition in the thrombin-evoked rise in $[Ca^{2+}]_i$ in Ca^{2+}-free medium when Na^+ is replaced by large cations, or in the presence of amiloride or its analogues that inhibit Na^+/H^+ exchange (72, 73). Moreover, IP_3-evoked Ca^{2+} release from permeabilized platelets was found to be enhanced by modest elevations in pH (6). It seems therefore that pH_i can modulate internal Ca^{2+} discharge, at least in some circumstances. For a complex train of biological processes, comprising many polyvalent-charged solutes and macromolecules, this is not too surprising. It has been implied, and even explicitly stated, that elevation of pH_i is a prerequisite of Ca^{2+} mobilization (72). However the available evidence indicates that pH_i elevation is neither sufficient nor necessary for Ca^{2+} mobilization (25, 51, 68, 74, 88). For instance, complete replacement of external Na^+ by N-methyl-D-glucamine or K^+ did not measurably reduce Ca^{2+} release evoked by thrombin, at near maximal effective concentrations, or by PAF (65). [The literature discrepancies in the effects of Na^+ substitution or amiloride and its analogues may partly reflect the different concentrations of agonist as these experimental manipulations seem to shift the thrombin dose-effect curve to the right (72, 77, 78)]. Changes of pH_i imposed by addition of CO_2, nigericin, or NH_4Cl did not alter resting $[Ca^{2+}]_i$ in fura-2-loaded platelets (74). Incidentally, large changes in $[Ca^{2+}]_i$ evoked by ionomycin did not alter pH_i (74). PAF and ADP produced little or no alkalinization under conditions where they produce a substantial discharge of internal Ca^{2+} (25). A particularly critical point is the temporal relation of the observed changes in pH_i and $[Ca^{2+}]_i$, since for alkalinization to play a significant role in Ca^{2+} mobilization, the increase in pH_i must come first (51, 74). In the published experimental records this does not appear to be the case: $[Ca^{2+}]_i$ peaks well ahead of pH_i and typically before measurable increases in pH_i (e.g. 72, 74). We have confirmed these kinetic patterns with stopped-flow measurements (25). Siffert et al have proposed that this may be because shape-changes optically interfere with pH_i records and generate an artifactual fall in pH_i immediately after activation (73). We doubt this interpretation (25) on the basis of our own data wth HCO_3^--containing medium. We find that shape-change is similar to that under HCO_3^--free conditions, but the dip in pH_i is smaller as expected due to the extra buffering of HCO_3^-. A particularly telling result is shown in Figure 4 of Siffert et al (73) in previously shape-changed cells; the thrombin-evoked $[Ca^{2+}]_i$ transient in Ca^{2+}-free medium is clearly much faster than the pH_i rise.

Other Factors

Membrane potential does not appear to influence internal Ca^{2+} discharge in platelets. Depolarization imposed by high K^+ or gramicidin does not elicit Ca^{2+} release, and thrombin, PAF, and ADP evoke their usual $[Ca^{2+}]_i$ rise in K^+-rich Ca^{2+}-free medium (65). In our hands, caffeine, dantroline, and

ryanodine are without obvious effect on basal $[Ca^{2+}]_i$ or on agonist-evoked Ca^{2+} mobilization in human platelets (T. Rink, S. Sage unpublished data), and caffeine was unable to release Ca^{2+} from human platelet internal membrane vesicles (11). Thus agents that have a marked effect on calcium discharge from sarcoplasmic reticulum in striated and smooth muscle appear to have little effect on this process in human platelets. A recent interesting result has been the identification of a monoclonal antibody that binds to platelet membrane vesicles and rather specifically inhibits IP_3-evoked Ca^{2+} release (39). This reagent should prove a valuable tool in elucidating these processes in platelets and most likely in other cell types as well.

Refilling Internal Stores

Following stimulation, the dischargeable store can be refilled. Probably a significant resequestration occurs because the elevation $[Ca^{2+}]_i$ causes enhanced Ca^{2+} pumping into the dense tubular system. However it is also likely that a significant part of the discharged Ca^{2+} is pumped out across the plasma membrane (55, 56). Indeed, in Ca^{2+}-free medium, stimulation by thrombin promotes a loss of approximately half of the ^{45}Ca taken up over a previous 30 min loading period (55). In normal Ca^{2+}-containing medium, the dense tubular system could regain lost Ca^{2+} in two ways. A receptor-mediated Ca^{2+} entry into the cytosol could provide extra Ca^{2+} for subsequent pumping by the Ca-ATPase, or the depletion of Ca^{2+} from the store might induce filling directly from the external medium, as has been suggested for other cell types (see e.g. 22, 54). The available evidence, as discussed below and in Reference 62, does not preclude this second class of mechanism in human platelets.

CALCIUM INFLUX

Four main types of evidence indicate the presence of a significant Ca^{2+} entry into stimulated platelets (3): (*a*) increased uptake of ^{45}Ca (29, 52); (*b*) a much larger $[Ca^{2+}]_i$ response in quin2-loaded platelets in the presence than in the absence of external Ca^{2+} (59); (*c*) stimulated entry of Mn^{2+} detected by the ability of this ion to quench quin2 or fura-2 fluorescence (19, 62); (*d*) stopped-flow fluorescence measurements showing an earlier agonist-evoked $[Ca^{2+}]_i$ signal in the presence rather than in the absence of external Ca^{2+} (65, 66, 67). As mentioned above, if one loads platelets with low concentrations of fura-2 to minimize cytosolic Ca^{2+} buffering, the peak $[Ca^{2+}]_i$ evoked by thrombin is little reduced in Ca^{2+}-free medium (46), but the declining phase of the response is prolonged in the presence of external Ca^{2+}, as in many other cell types. With stimulation by ADP, we also find, as detailed below, a very fast early phase of Ca^{2+} entry (66, 67). Thus Ca^{2+} entry can provide a faster and more prolonged signal than that provided by internal release only.

Lack of Voltage-Gated Ca^{2+} Channels

Voltage-operated Ca^{2+} channels do not appear to mediate agonist-evoked Ca^{2+} entry in platelets (12, 52, 56, 65). Although a number of agonists have been demonstrated to evoke Na^+-dependent depolarization of the platelet membrane (23, 31, 43), these are only 5–10 mV in magnitude, less than typically required to promote voltage-gated Ca^{2+} entry. Replacement of external Na^+ with impermeant organic cations such as choline or N-methyl-D-glucamine converts the small depolarization into a small hyperpolarization (43), but it does not reduce the elevations in $[Ca^{2+}]_i$ evoked by thrombin or PAF (65). Imposed depolarization using high K^+ does not elevate $[Ca^{2+}]_i$ (65), although agonist-evoked Ca^{2+} influx is substantially reduced in high K^+ (56, 65). A similar reduction in Ca^{2+} influx in high K^+ has been reported in other nonexcitable cells. This effect may be attributed to a reduced driving force for Ca^{2+} entry following depolarization, an idea supported by the finding that depolarization of the platelet membrane using gramicidin also reduces agonist-evoked Ca^{2+} entry. (S. Sage & T. Rink unpublished observations). Organic Ca^{2+} antagonists are relatively ineffective in blocking the agonist-evoked rise in $[Ca^{2+}]_i$ in platelets at concentrations that are effective in excitable cells, although these compounds are reported to have inhibitory effects at high concentrations (e.g. 3, 20). Also, there are no detectable binding sites for verapamil and nitrendipine on human platelets (38), while such sites are readily found on cells such as smooth or cardiac muscle with well documented voltage-dependent Ca^{2+} channels. Interestingly, the platelet plasma membrane has voltage-gated K^+ channels (33). The threshold for activation of these channels lies close to the resting membrane potential, such that they would oppose membrane depolarization during cation entry and maintain the gradient for influx.

Receptor-Mediated Calcium Entry

Receptor-mediated calcium entry may be divided into at least three classes (22): (*a*) receptor-operated calcium channels, which open as a direct consequence of agonist-receptor binding; (*b*) Ca^{2+} entry coupled to activated receptors via G-proteins; and (*c*) second messenger-operated calcium channels, which are opened by a messenger produced in response to agonist-receptor interaction.

Receptor-Operated Channels

Analysis of the subsecond kinetics of rises in $[Ca^{2+}]_i$ in fura-2-loaded platelets by stopped-flow fluorimetry has revealed that, in the presence of extracellular Ca^{2+}, optimal concentrations of ADP evoke a response without measurable delay, i.e. within 10 to 20 msec (66, 67). This early event is due

to Ca^{2+} influx. ADP evokes Mn^{2+} entry with similar kinetics (61, 62), and, in the absence of external Ca^{2+}, the onset of the rise in $[Ca^{2+}]_i$ due to discharge of the intracellular stores is delayed by at least 200 msec (66). Rapid ADP-evoked responses have also been observed in indo-1-loaded cells using a continuous flow system (27), although temporal resolution was not at the 10 msec level achieved in our stopped-flow system. The rapidity of ADP-evoked influx suggests close coupling between receptor occupation and the Ca^{2+} channel. A delay of less than 20 msec may point to direct ligand gating, but the finding that there is a delay in ADP-evoked influx at sub-optimal ligand concentrations might be more easily explained by coupling of the receptor to channel opening by a G-protein, (56, 67).

Recently, we obtained direct electrophysiologic evidence from cell-attached patch recordings that ADP can stimulate the opening of channels that carry inward current in intact human platelets (32). Single channel inward currents are observed in physiologic saline solutions containing 1 mM Ca^{2+}. The currents are very similar when Cl^- is replaced with gluconate, which indicates cation entry rather than Cl^- efflux. The slope conductance of the channel in physiologic saline is approximately 11pS at a pipette potential of 0mV (the resting potential), and the open probability is not obviously dependent on potential (32). Similar channels, approximately 10 pS, are evoked when the pipette contains 110 mM $BaCl_2$, thus indicating that divalent cations can permeate. Therefore it appears that ADP opens channels in the plasma membrane through which Ca^{2+} could enter. We suspect that these channels provide the pathway for the early Ca^{2+} entry, but need further data to support this idea.

Channels selective for Ba^{2+} over Na^+ have been demonstrated by the incorporation of platelet membrane vesicles into artificial bilayers (91). Channel activity was present only in membranes from thrombin-stimulated platelets, but not from resting platelets. The channel gating was not influenced by the potential across the bilayer and the activity was blocked by Ni^{2+}, which is known to block thrombin-stimulated Ca^{2+} and Mn^{2+} entry in intact platelets (19, 67). The rather surprising finding that the thrombin-evoked channels survive the cell-fractionation procedure (91) suggests that there may be some covalent modification of the channel, for example by phosphorylation. The prolonged survival of this channel is consistent with the finding that thrombin responses, unlike those evoked by other agonists, do not rapidly desensitize (e.g. 62). Although these reconstitution studies provide direct evidence for a thrombin-evoked calcium channel, it is at present unclear whether the origin of the channel was the plasma membrane or contaminating membrane from intracellular organelles and how it may be coupled to the receptor. From its Ba^{2+}/Na^+ selectivity, this channel seems to be different from the one we have detected in ADP-stimulated intact platelets.

Second Messenger-Operated Channels

Stopped-flow kinetic studies indicate that, of the agonists we have tested, only ADP evokes Ca^{2+} influx without measurable delay. Responses evoked by other agonists, including thrombin, PAF, vasopressin, and U46619, show an irreducible delay of at least 200 msec (56, 67). These results suggest that, with the exception of the ADP response, one or more biochemical steps lie between receptor occupation and the generation of Ca^{2+} influx, which could therefore be explained by the action of a diffusible second messenger.

Possible intermediaries in channel gating include Ca^{2+} and inositol phosphates (8, 22, 28, 83). Calcium-sensitive Ca^{2+} channels in the plasma membrane, which would be opened following the elevation of $[Ca^{2+}]_i$ by discharge of the intracellular store, have been proposed to mediate Ca^{2+} influx in neutrophils (83). Stopped-flow studies suggest that a similar pathway does not operate in platelets (62, 67). The onset of $[Ca^{2+}]_i$ rise in the presence of external Ca^{2+} precedes that observed in the absence of external Ca^{2+} or when Ca^{2+} influx is blocked using Ni^{2+}, which indicates that Ca^{2+} influx precedes the discharge of the intracellular stores.

There is currently little evidence concerning the possible role of inositol phosphates in mediating calcium influx directly across the platelet plasma membrane (as opposed to entry via the intracellular store, discussed below). A model in which Ins(1,4,5)P_3 opened Ca^{2+} channels in both the plasma and intracellular store membranes would be compatible with the observed temporal separation of Ca^{2+} influx and store discharge: since the messenger is generated at the plasma membrane, it might be expected to take longer to reach the receptors of the store (67). The only evidence supporting a direct role for Ins(1,4,5)P_3 in the generation of Ca^{2+} influx in platelets at present is the finding that this ligand released Ca^{2+} from membrane vesicles enriched in plasma membrane (49). Inward currents evoked by Ins(1,4,5)P_3 has been reported in human T-lymphocytes (28) and in rat mast cells (42). There has yet to be a similar direct electrophysiologic investigation of this point in platelets. Agonist-evoked phosphatidyl-Ins(4,5)P_2 breakdown in many cells results in the accumulation of Ins(1,3,4)P_3 formed from the initial hydrolysis product, Ins(1,4,5)P_3. This conversion is particularly rapid in platelets (80). There has been considerable speculation over the possible signaling roles of Ins(1,3,4,5)P_4, the intermediate in the conversion of Ins(1,4,5)P_3 to Ins-(1,3,4)$_3$. In particular, the possible role of Ins(1,3,4,5)P_3 in the generation of Ca^{2+} influx has been considered (24, 37). Evidence that Ins(1,3,4,5)P_4, acting in concert with Ins(1,4,5)P_3, may be involved in mediating Ca^{2+} influx has been obtained in lacrimal gland cells (8).

Store-Regulated Calcium Influx

In recent years evidence has been presented in a number of cell types suggestive of a pathway by which Ca^{2+} enters the cytosol via some fraction of

the intracellular Ca^{2+} store (see e.g. 22, 54). The evidence from refilling experiments, where the stores are discharged in the absence of extracellular Ca^{2+} and are then shown to be recharged after brief exposure to Ca^{2+}, indicates some kind of private pathway between the extracellular space and the store, since $[Ca^{2+}]_i$ is not elevated during the refilling. The simplest explanation of these phenomena is that some form of channel, perhaps like a gap junction, links the lumen of intracellular store to the extracellular space and that the opening of this channel is controlled by the $[Ca^{2+}]$ inside the store. It has been considered that Ca^{2+} entering the store by such a route might gain entry into the cytosol only after passage into an Ins(1,4,5)P_3-sensitive fraction of the store, which might require another messenger such as Ins(1,3,4,5,)P_4 (8). Recently results consistent with a store-regulated Ca^{2+} influx have been seen in platelets (62, 63). ADP evokes a biphasic rise in $[Ca^{2+}]$, which is poorly resolved at 37°C, but is clearly separated into two events at 17°C. At this temperature an initial rise in $[Ca^{2+}]_i$ occurs without measurable delay and is followed by a further elevation approximately 700 msec later. When the cells are stimulated in the presence of both Ca^{2+} and Mn^{2+}, the second phase of $[Ca^{2+}]_i$ rise coincides wth Mn^{2+} entry. In the absence of external Ca^{2+} there is a single ADP-evoked rise in $[Ca^{2+}]_i$ delayed in onset by approximately 1400 msec. When the cells are stimulated in the presence of Mn^{2+} alone, an early Mn^{2+} entry occurs without measurable delay, followed by a second phase of entry, also delayed by approximately 1400 msec. Hence the second phase of divalent-cation entry coincides with discharge of the intracellular Ca^{2+} stores, which could reflect regulation of influx by the Ca^{2+} in the store. These experiments also indicate that the timing of the second ADP-evoked event is modulated by the presence of external Ca^{2+}, since the second phase of Mn^{2+} entry occurs at a markedly earlier time if both ions are present in the external medium, perhaps because the key event in eliciting an earlier second phase of entry is the elevation of $[Ca^{2+}]_i$ during the initial phase. One can only speculate on the way in which $[Ca^{2+}]_i$ modulates store discharge. For example, it could be that Ca^{2+} stimulation of phospholipase C results in an earlier generation of Ins-(1,4,5)P_3.

Modulation of Calcium Entry

Activation of platelet protein kinase C inhibits Ca^{2+} influx (82). This may in part reflect the inhibition of PIP_2 hydrolysis (90) with consequently reduced Ca^{2+} entry through second messenger-operated Ca^{2+} channels and by the store-dependent pathway, since intracellular release is also diminished. Inhibition by protein kinase C may involve a change in receptor affinity, but there also appears to be inhibition at the level of the G-protein, which stimulates the phosphoinositidase, or at a subsequent stage of transduction, since fluoride-stimulated Ca^{2+} entry is reduced (44). The channels responsi-

ble for gating Ca^{2+} influx may also be modulated, but as yet there has been no direct electrophysiologic test. Influx, assessed by Mn^{2+} entry, does not appear to be as sensitive to protein kinase C-mediated inhibition as is intracellular release (82), although influx is slower to recover from prolonged exposure to diacylglycerol (DAG).

Cyclic AMP also inhibits Ca^{2+} influx (e.g. 41, 57). As with protein kinase C, the cAMP-dependent kinase probably has several sites of action. Inositol lipid hydrolysis is inhibited (see 71), which could reduce Ca^{2+} influx through putative inositol phosphate-operated channels as well as through the putative store-dependent pathway. There may also be phosphorylation of the Ca^{2+} channels that conduct the influx, although the early phase of ADP-evoked entry is surprisingly insensitive; elevation of cAMP using forskolin can completely inhibit ADP-evoked release of the intracellular stores while Ca^{2+} influx continues to be evoked without measurable delay (67). Resolution of the ADP-evoked influx into its constituent phases at low temperature shows that the second phase of Ca^{2+} or Mn^{2+} entry can be completely abolished while the initial phase, generated without measurable delay, persists (63). In contrast, influx and intracellular release evoked by thrombin or PAF shows similar sensitivity to inhibition by cAMP (64, 67).

Agents that elevate cGMP also inhibit Ca^{2+} influx in platelets (21, 36, 56). As with cAMP, the mechanism probably involves, at least in part, a reduction in PIP_2 hydrolysis (79). But recent results (36) show that Ca^{2+} or Mn^{2+} entry evoked by ADP are preferentially inhibited by nitroprusside. This contrasts with the effect of forskolin mentioned above, which indicates that cAMP preferentially spares the rapid phase of ADP-evoked Ca^{2+} or Mn^{2+} entry.

There is little evidence that pH_i mediates agonist-evoked influx. Abolition of Na^+-dependent alkalinization by ionic substitution has no effect on the magnitude of agonist-evoked rises in $[Ca^{2+}]_i$ (65), while kinetic studies indicate that peak $[Ca^{2+}]_i$ is attained before pH_i rises above basal levels (25).

At present little is known of the structural elements in the plasma membrane that conduct the entry of Ca^{2+} into the cell. The IIb.IIIa membrane glycoprotein complex may be involved in Ca^{2+} transport across the plasma membrane in resting (4) and stimulated cells (48, 86). Monoclonal antibodies or smaller peptide fragments, which bind to the IIb.IIIa complex, reduce agonist-evoked influx (48, but see also 20), although at present it is not clear if these proteins are associated with any of the putative pathways for Ca^{2+} entry.

SUMMARY

The past three years have seen significant advances in our knowledge and understanding of Ca^{2+} mobilization in platelets. Some of the data has shown

that systems demonstrated in other cell types operate in platelets, while in certain respects platelet studies have provided the lead with new insights and approaches. An increasing body of evidence supports a key role for Ins1,4,5-trisphosphate in mediating internal release, but it has yet to be experimentally demonstrated that this messenger is formed fast enough to account for the observed kinetics of internal release that can reach its maximum rate within 250 msec. There also remains a question as to the presence of an alternative or additional pathway linking at least ADP receptors to internal Ca^{2+} release. The controversy over the role of pH_i in Ca^{2+} mobilization appears to be resolved; changes in pH_i are neither sufficient nor necessary but can modulate the process in some instances. Elevated cAMP and protein kinase C inhibit Ca mobilization, but the sites and mechanisms of action are not worked out. Analysis of receptor-mediated Ca^{2+} entry by stopped-flow fluorescence has increasingly revealed a complex array of mechanisms, but there is no evidence for voltage-gated Ca^{2+} entry. There are indications of at least three pathways: a fast entry closely coupled to the ADP receptor; a process that may be generated by a diffusible second messenger, possibly an inositol phosphate; and an entry regulated by the state of filling of the discharged Ca^{2+} store. A recent advance has been the successful application of the patch-clamp to these tiny cells, with evidence for voltage-gated K^+ channels and ADP-stimulated single channels that could be the pathway for the fast phase of ADP-evoked $[Ca^{2+}]_i$ elevation.

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Literature Cited

1. Adunyah, S. E., Dean, W. L. 1987. Regulation of human platelets membrane Ca^{2+} transport by cAMP and calmodulin-dependent phosphorylation. *Biochim. Biophys. Acta* 930:401–9
2. Authi, K. S., Crawford, N. 1985. Inositol 1,4,5-trisphosphate release of sequestered Ca^{2+} from highly purified human platelet intracellular membranes. *Biochem. J.* 230:247–53
3. Avodin, P. V., Menshikor, M. Y., Svitina-Ulitana, I. V., Tkachuk, V. A. 1988. Blocking of the receptor-stimulated calcium entry into human platelets by verapamil and nicardipine *Thromb. Res.* 52:587–97
4. Brass, L. F. 1985. Ca^{2+} Transport across the platelet plasma membrane. A role for membrane glycoproteins IIB & IIIA. *J. Biol. Chem.* 260:2231–6
5. Brass, L. F. (1984). The effect of Na^+ on Ca^+ homeostasis in unstimulated platelets. *J. Biol. Chem.* 259:12571–5
6. Brass, L. F., Joseph, S. K. 1985. A role for inositol trisphosphate in intracellular Ca^{2+} mobilization and granule secretion in platelets. *J. Biol. Chem.* 260:15172–79
7. Grass, L. F., Laporta, M. 1987. Diacylglycerol causes Ca release from the platelet dense tubular system: comparison with Ca release caused by inositol tris 1,4,5-trisphosphate. *Biochem. Biophys. Res. Commun.* 142:7–14
8. Changya, L., Gallacher, D. V., Irvine, R. F., Potter, B. V. L., Peterson, O. H.

1989. Inositol 1,3,4,5 tetratasphosphate is essential for substained activation of the Ca^{2+}-dependent K^+ current in single internally perfused mouse lacrimal acinar cells. *J. Membrane. Biol.* 109:85–93
9. Cobbold, P. J., Rink, T. J. 1987. Fluorescent and bioluminescent measurement of cytosolic free calcium. *Biochem. J.* 248:1–16
10. Daniel, J. L., Dangelmaier, C. A., Selak, M., Smith, J. B. 1986. ADP stimulates IP_3 formation in human platelets. *FEBS Letts.* 206:299–303
11. Dean, W. C., Adunyah, S. E. 1986. Effects of sulfhydryl reagents and other inhibitors on Ca^{2+} transport and inositol trisphosphate-induced Ca^{2+} release from human platelet membranes. *J. Biol. Chem.* 261:13071–75
12. Doyle, V. M., Ruegg, U. T. 1985. Lack of evidence for voltage dependent calcium channels on platelets. *Biochem. Biophys. Res. Comm.* 127:161–67
13. Drummond, A. H., MacIntyre, D. E. 1987. Inositol lipid metabolism and calcium flux. *In Platelets in Biology and Pathology III*, eds. D. E. MacIntyre, J. L. Gordon, pp. 373–431. Amsterdam: Elsevier. 589 pp.
14. Enouf, J., Bredeux, R., Bourdean, N., Levy-Toledano, S. 1987. Two different Ca^{2+} transport systems are associated with plasma and intracellular human platelet membranes. *J. Biol. Chem.* 262:9293–97
15. Enouf, J., Girand, F., Bredoux, R., Bourdean, N., Levy-Toledano, S. 1987. Possible role of a cAMP-dependent phosphorylation in the calcium release mediated by inositol 1,4,5-trisphosphate in human platelet membrane vesicles. *Biochim. Biophys. Acta* 928:76–82
16. Enyedi, A., Sakardi, B., Folder-Papp, Z., Monostory, J., Gardos, G. 1986. Demonstration of two distinct calcium pumps in human platelet membrane vesicles. *J. Biol. Chem.* 261:9558–563
17. Fisher, G. J., Bakshian, S., Baldassare, J. J. 1985. Activation of human platelets by ADP causes a rapid rise in cytosolic free Ca^{2+} without hydrolysis of phosphatidylinositol-4,5-bisphosphate. *Biochem. Biophys. Res. Commun.* 129:958–64
18. Hallam, T. J., Daniel, J. L., Kendrick-Jones, J., Rink, T. J. 1985. Relationship between cytoplasmic free calcium and myosin light chain phosphorylation in intact platelets. *Biochem. J. 232*:373–77
19. Hallam, T. J., Rink, T. J. 1985. Agonists stimulate divalent cation channels in the plasma membrane of human platelets. *FEBS Lett.* 186:175–79
20. Hallam, T. J., Rink, T. J. 1985. Responses to adenosine diphosphate in human platelets loaded with the fluorescent calcium indicator quin2. *J. Physiol.* 368: 131–46
21. Hallam, T. J., Rink, T. J. 1987. Insights into platelet function gained with fluorescent Ca^{2+} indicators. In: *Platelets in Biology and Pathology III*. eds. D. F. MacIntyre, J. L. Gordon, pp. 353–372. Amsterdam:Elsevier. 589 pp.
22. Hallam, T. J., Rink, T. J. 1989. Receptor-mediated Ca^{2+} entry: diversity of mechanism and function. *Trends Pharm. Sci.* 10:8–10
23. Horne, W. C., Norman, N. E., Schwartz, D. B., Simons, E. R. 1981. Changes in cytoplasmic pH and in membrane potential in thrombin-stimulated human platelets. *Eur. J. Biochem.* 120: 295–302
24. Irvine, R. F., Moor, R. M. 1986. Micro-injection of inositol 1,3,4,5-tetrakisphosphate activates sea urchin eggs by a mechanism dependent on external Ca^{2+}. *Biochem. J.* 240:917–20
25. Jobson, T. M., Rink, T. J., Sage, S. O. 1990. Agonist-evoked changes in cytosolic pH and calcium concentration in human platelets: Studies in physiological bicarbonate. *J. Physiol.* In press
26. Johnson, P. C., Ware, J. A., Cliveden, P. B., Smith, M., Dvorak, A. M., Salazman, E. W. 1985. Measurement of ionized calcium in blood platelets with the photoprotein aequorin. *J. Biol. Chem.* 260(4):2069–76
27. Jones, G. D., Gear, A. R. L. 1988. Subsecond calcium dynamics in ADP- and thrombin-stimulated platelets: A continuous-flow approach using Indo-1. *Blood* 71:1539–43
28. Kuno, M., Gardner, P. 1987. Ion channels activated by inositol 1,4,5-trisphosphate in plasma membrane of human T-lymphocytes. *Nature* 326:301–4
29. Lee, T., Malone, B., Blank, M. L., Synder, F. 1981. PAF stimulates Ca influx into rabbit platelets. *Biochem. Biophys. Res. Commun.* 102:1262
30. MacIntyre, D. E., McNicol, A., Drummond, A. H. 1985. Tumor-promoting phorbol esters inhibit agonist-induced phosphate formation and CA^{2+} flux in human platelets. *FEBS Lett.* 180:160–64
31. MacIntyre, D. E., Rink, T. J. 1982. The role of platelet membrane potential in the initiation of platelet aggregation. *Thromb. Haemost.* 47:22–26
32. Mahaut-Smith, M. P., Rink, T. J., Sage, S. O. 1989. Single channels in

human platelets activated by ADP. *J. Physiol.* 415:24P
33. Maruyama, Y. 1987. A patch-clamp study of mammalian platelets and their voltage-gated potassium current. *J. Physiol.* 391:467–85
34. Meldolesi, J., Volpe, P., Pozzan, T. 1988. The intracellular distribution of calcium. *Trends Neurosci.* 11:449–52
35. Menashi, S., Authi, K. S., Carey, F., Crawford, N., 1984. Characterization of the calcium-sequestering process associated with human platelet intracellular membranes isolated by free-flow electrophoresis. *Biochem. J.* 222:413–17
36. Morgan, R. O., Newby, A. C. 1989. Nitroprusside differentially inhibits ADP-stimulated calcium influx and mobilization in human platelets. *Biochem. J.* 258:447–54
37. Morris, A. P., Gallacher, D. V., Irvine, R. F., Petersen, O. H. 1987. Synergism of inositol trisphosphate and tetrakisphosphate in activating Ca^{2+}-dependent K^+ channels. *Nature* 330:653–56
38. Motulsky, H. J., Snavely, M. D., Hughes, P. J., Insel, P. A., 1983. Interaction of verapamil and other calcium channel blockers with α- and β-adrenergic receptors. *Cir. Res.* 52:226–31
39. O'Rourke, F., Zavoico, G. B., Feinstein, M. B. 1989. Release of Ca^{2+} by inositol 1,4,5-trisphosphate in platelet membrane vesicles is not dependent on cyclic AMP-dependent protein kinase. *Biochem. J.* 257: In press
40. O'Rourke, F., Zavoico, G. B., Smith, L. H., Feinstein, M. B. 1987. Stimulus response-coupling in a cell-free platelet membrane system. *FEBS Lett.* 214:176–80
41. Pannocchia, A., Hardisty, R. M. 1985. Cyclic AMP inhibits platelet activation independently of its effect on cytosolic free calcium. *Biochem. Biophysical. Res. Commun.* 127:339–45
42. Penner, R., Matthew, G., Neher, E. 1988. Regulation of calcium influx by second messengers in rat mast cell. *Nature* 334:499–504
43. Pipili, E. 1985. Platelet membrane potential: Simultaneous measurements of dis-C_3-(5) fluorescence and optical density. *Thromb. Haemost.* 54:645–49
44. Poll, C., Kryle, P., Westwick, J. 1986. Activation of protein kinase-C inhibits sodium fluoride-induced elevation of human platelet cytosolic free calcium and thromboxane B_2 generation. *Biochem. Biophys. Res. Commun.* 136:381–89
45. Poll, C., Westwick, J. 1987. Phorbol esters modulate thrombin-operated calcium mobilization and dense granule release in human platelets. *Biochim. Biophys. Acta* 886:434–40
46. Pollock, W. K., Rink, T. J. 1986. 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.*
47. Pollock, W. K., Rink, T. J., Irvine, R. F. 1986. Liberation of [^{3}H] arachidonic acid and changes in cytosolic free calcium in fura-2-loaded human platelets stimulated by ionomycin and collagen. *Biochem. J.* 235:869–77
48. Powling, M. J., Hardisty, R. M. 1985. Glycoprotein IIb-IIIa complex and Ca^{2+} influx in stimulated platelets. *Blood* 66:731–33
49. Rengasamy, A., Feinberg, H. 1988. Inositol 1,4,5-trisphosphate-induced calcium release from platelet plasma membrane vesicles. *Biochem. Biophys. Res. Commun.* 150:1021–26
50. Rengasamy, A., Soura, S., Feinberg H., 1987. Platelet Ca^{2+} hemostasis: Na^+-Ca^{2+} exchange in plasma membrane vesicles. *Thromb. Haemost.* 57:337–40
51. Rink, T. J. 1987. Intracellular pH and cytoplasmic free Ca^{2+}. *Nature* 327:375–76
52. Rink, T. J. 1988. Cytosolic calcium in platelet activation. *Experientia* 44:97–100
53. Rink, T. J., Hallam, T. J. 1984. What turns platelets on? *Trends Biochem. Sci.* 9:215–19
54. Rink, T. J., Hallam, T. J. 1989. Calcium signaling in non-excitable cells: Notes on oscillations and store refilling. *Cell Calcium* In press
55. Rink, T. J., Sage, S. O. 1987. Stimulated calcium efflux from fura-2-loaded human platelets. *J. Physiol.* 393:513–24
56. Rink, T. J., Sage, S. O. 1988. Initiation and termination of Ca^{2+} signals. Studies in human platelets. In *Cell Physiology of Blood.* eds. R. B. Gunn, J. C. Parker. *Soc. Gen. Physiol. 41st Symp.* pp. 381–394. New York: Rockefeller Univ. Press. 402 pp.
57. Rink, T. J., Sanchez, A. 1984. Effects of prostaglandin I_2 and forskolin on the secretion from platelets evoked at basal concentrations of cytoplasmic free calcium by thrombin, collagen, phorbol ester, and exogenous diacylglycerol. *Biochem. J.* 222:833–36
58. Rink, T. J., Sanchez, A., Hallam, T. J. 1983. Diacylglycerol and phorbol ester stimulate secretion without raising cytoplasmic free calcium in human platelets. *Nature* 305:317–19

59. Rink, T. J., Smith, S. W., Tsien, R. 1982. Cytoplasmic free Ca^{2+} in human platelets: Ca^{2+} thresholds and Ca-independent activation for shape change and secretion. *FEBS. Lett.* 148:21–26
60. Rink, T. J., Tsien, R. Y., Pozzan, T. 1982. Cytoplasmic pH and free Mg^{2+} in lymphocytes. *J. Cell. Biol.* 95:189–96
61. Sage, S. O. 1987. Kinetics of ADP-evoked and thrombin-evoked rises in cytosolic calcium in human platelets studies with manganese and nickel. *J. Physiol.* 396:43P
62. Sage, S. O., Merritt, J. E., Hallam, T. J., Rink, T. J. 1989. Receptor-mediated calcium entry in fura-2-loaded human platelets stimulated with ADP and thrombin: Dual wavelength studies with Mn^{+}. *Biochem. J.* 258:923–26
63. Sage, S. O., Reast, R., Rink, T. J. 1989. ADP evokes a biphasic Ca^{2+} influx in human platelets: Evidence for store-dependent Ca^{2+} entry. *Biochem. J.* In press
64. Sage, S. O., Rink, T. J. 1985. Inhibition by forskolin of cytosolic calcium rise, shape change and aggregation in quin2-loaded human platelets. *FEBS Lett.* 188:135–40
65. Sage, S. O., Rink, T. J. 1986. Effects of ionic substitution on $[Ca^{2+}]_i$ rises evoked by thrombin and PAF in human platelets. *Eur. J. Pharm.* 128:99–107
66. Sage, S. O., Rink, T. J. 1986. 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.* 136:1124–29
67. Sage, S. O., Rink, T. J. 1987. The kinetics of changes in intracellular calcium concentrations in fura-2-loaded human platelets. *J. Biol. Chem* 262:16364–69
68. Sanchez, A., Alouso, M. T., Collazos, J. M. 1988. Thrombin-induced changes of intracellular $[Ca^{2+}]$ and pH_i in human platelets. *Biochim. Biophys. Acta* 938: 497–500
69. Sandler, W. C., Le Breton, G. C., Feinberg H. 1980. Movement of sodium into human platelets. *Biochim. Biophys. Acta* 600:448–55
70. Schaeffer, J., Blaustein, M. P. 1989. Platelet free calcium concentrations measured with fura-2 are influenced by the transmembrane sodium gradient. *Cell Calcium* 10:101–13
71. Siess, W. 1989. Molecular mechanisms of platelet activation. *Physiol. Rev.* 69:58–178
72. Siffert, W., Akkerman, J. W. N. 1987. Activation of sodium-proton exchange is a prerequisite for Ca^{2+} mobilization in human platelets. *Nature* 325:456–58
73. Siffert, W., Siffert, G., Scheid, P., Akkerman, J. W. N. 1989. Activation of Na^{+}/H^{+} exchange and Ca^{2+} mobilization start simultaneously in thrombin-stimulated platelets. Evidence that platelet shape change disturbs early rises of BCECF fluorescence which causes an underestimation of actual cytosolic alkalinization. *Biochem. J.* 258:521–27
74. Simpson, A. W. M., Rink, T. J. 1987. Elevation of pH_i is not an essential step in calcium mobilization in fura-2-loaded human platelets. *FEBS Lett.* 22:144–48
75. Sinigaglia, F., Bisio, A., Torti, M., Balduini, C.-L., Balduini, C. 1988. Effects of GPIIb-IIIa complex ligands on calcium ion movement and cytoskeleton organization in activated platelets. *Biochem. Biophys. Res. Commun.* 154: 258–64
76. Supattapone, S., Danoff, S. K., Theibert, A., Joseph, S. K., Sieiner, J., Snyder, S. H. 1988. Cyclic AMP-dependent phosphorylation of a brain inositol trisphosphate receptor decreases its release of calcium. *Proc. Natl. Acad. Sci. USA* 85:8747–50
77. Sweatt, J. D., Blair, I. A., Cragoe, E. J., Limbird, L. E. 1986. Inhibitors of Na^{+}/H^{+} exchange block epinephrine- and ADP-induced stimulation of human platelets phospholipase C by blockade of arachidonic acid release at a prior step. *J. Biol. Chem.*261:8660–66
78. Sweatt, J. D., Connolly, T. M., Cragoe, E. J., Limbird, L. E. 1986. Evidence that Na^{+}/H^{+} exchange regulates receptor-mediated phospholipase A_2 activation in human platelets. *J. Biol. Chem.*261:8667–73
79. Takai, Y., Kaibuchi, K., Samo, K., Nishizuta, Y. 1982. Counteraction of calcium activated phospholipid-dependent protein kinase activation by adenosine 3'5' monophosphate and guanosine 3',5'-monophosphate in platelets. *J. Biochem.* 91:403–6
80. Tarver, A. P., King, W. G., Rittenhouse, S. E. 1987. Inositol 1,4,5-trisphosphate and inositol 1,2-cyclic 4,5-trisphosphate are minor components of total mass of inositol trisphosphate in thrombin-stimulated platelets. *J. Biol. Chem.* 262:17268–71
81. Thompson, N. T., Scrutton, M. C. 1985. Intracellular calcium fluxes in human platelets. *Eur. J. Biochem.* 147(2): 421–27
82. Valone, F. H., Johnson, B. 1987. Mod-

ulation of platelet-activating-factor-induced calcium influx and intracellular calcium release in platelets by phorbol esters. *Biochem. J.* 247:669–74
83. von Tscharner, V., Prod'hom, B., Baggiolini, M., Reuter, H. 1986. Ion channels in human neutrophils activated by a rise in free cytosolic calcium concentration. *Nature* 324:369–72
84. Ware, J. A., Saitah, M., Smith, M., Johnson, P. C., Slazman, E. W. 1989. Response of aequorin-loaded platelets to activators of protein kinase-C. *Am. J. Physiol.* 256:C35-C43
85. Ware, J. A., Smith, M., Fossel, E. T., Slazman, E. W. 1988. Cytoplasmic Mg^{2+} concentration in platelets: implications for determination of Ca^{2+} with aequorin. *Am. J. Physiol.* 255: H855–59
86. Yamaguchi, A., Yamamoto, N., Kitagawa, H., Tamone, K., Yamazaki, H. 1987. Ca^{2+} influx mediated through the GPIIb/IIIa complex during platelet activation. *FEBS Lett.* 225:228–32
87. Yoshida, K., Nachmias, V. T. 1987. Phorbol ester stimulates calcium sequestration in saponinized human platelets. *J. Biol. Chem.* 262:16048–54
88. Zavoico, G. B., Cragoe, E. J. 1988. Ca^{2+} mobilization can occur independently of acceleration of Na^+/H^+ exchange in thrombin-stimulated human platelets. *J. Biol. Chem.* 263:9635–39
89. Zavoico, G. B., Cragoe, E. J., Feinstein, M. B. 1986. Regulation of intracellular pH in human platelets. Effects of thrombin, A23187, and ionomycin and evidence for activation of Na^+/H^+ exchange and its inhibition by amiloride analogs. *J. Biol. Chem.* 261: 13160–67
90. Zavoico, G. B., Halenda, S. P., Sha'afi, R. I., Feinstein, M. B. 1985. Phorbol myristate acetate inhibits thrombin-stimulated Ca^{2+} mobilization and phosphatidylinositol 4,5-bisphosphate hydroysis in human platelets. *Proc. Natl. Acad. Sci. USA* 82:3859–62
91. Zschauer, A., Van Breemen, C., Buhler, F. R., Nelson, M. T. 1988. Calcium channels in thrombin-activated human platelet membrane. *Nature* 334: 703–5