ADP evokes biphasic Ca^{2+} influx in fura-2-loaded human platelets

Evidence for Ca^{2+} entry regulated by the intracellular Ca^{2+} store

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Stopped-flow fluorimetric studies at 37 °C have shown that ADP, at optimal concentrations, can evoke Ca^{2+} or Mn^{2+} influx in fura-2-loaded human platelets without measurable delay. In contrast, the release of Ca^{2+} from intracellular stores is delayed in onset by about 200 ms. By working at a lower temperature, 17 °C, we have now shown that the rise in cytosolic calcium concentration ($[\text{Ca}^{2+}]_i$) evoked by ADP in the presence of external Ca^{2+} is biphasic. The use of Mn^{2+} as a tracer for bivalent-cation entry indicates that both phases of the ADP-evoked response are associated with influx. The fast phase of the ADP-evoked rise in $[\text{Ca}^{2+}]_i$, which occurs without measurable delay at both 17 °C and 37 °C, is consistent with Ca^{2+} entry mediated by receptor-operated channels in the plasma membrane. The delayed phase, indicated by Mn^{2+} quench, is coincident with the discharge of the intracellular Ca^{2+} stores. Forskolin did not inhibit the fast phases of ADP-evoked rise in $[\text{Ca}^{2+}]_i$ or Mn^{2+} quench, but completely abolished ADP-evoked discharge of the intracellular stores, the delayed phase of the rise in $[\text{Ca}^{2+}]_i$ observed in the presence of external Ca^{2+} and the second phase of Mn^{2+} quench. The timing of the delayed event appears to be modulated by $[\text{Ca}^{2+}]_i$: the delayed phase of Mn^{2+} quench coincides with discharge of the intracellular stores in the absence of added Ca^{2+} , but with the second phase of the ADP-evoked rise in $[\text{Ca}^{2+}]_i$ in the presence of extracellular Ca^{2+} . Similarly, blockade of the early phase of Ca^{2+} entry by SK&F 96365 further delays the second phase. It is suggested that a pathway for Ca^{2+} entry which is regulated by the intracellular Ca^{2+} store exists in platelets. This pathway operates alongside, and appears to be modulated by the activity of, other routes for Ca^{2+} entry into the cytosol.

INTRODUCTION

We have previously used stopped-flow fluorimetry to show that ADP, at optimal concentrations, evokes Ca^{2+} influx in human platelets without measurable delay [1]. Mn^{2+} entry shows similar kinetics [2]. Recently we have demonstrated ADP-evoked single channels in human platelets, using the patch-clamp technique [3]. ADP evokes single channel activity in cell-attached membrane patches when present in the pipette-filling solution, consistent with the channel being receptor-operated (M. P. Mahaut-Smith, S. O. Sage & T. J. Rink, unpublished work). These channels are permeable to Na^+ and Ba^{2+} and could underly the rapid ADP-evoked bivalent-cation entry.

There is also a contribution to ADP-evoked rises in $[\text{Ca}^{2+}]_i$ arising from the discharge of intracellular Ca^{2+} stores [4]. This intracellular release of Ca^{2+} is slower in onset than ADP-evoked Ca^{2+} influx, being delayed by about 200 ms [1].

In some non-excitable cells, it has been suggested that the intracellular Ca^{2+} stores may regulate a pathway for Ca^{2+} entry [5–9]. Evidence for this influx pathway comes from the finding that stores depleted by agonist stimulation in the absence of extracellular Ca^{2+} may be replenished on exposure to Ca^{2+} , in the absence of agonist, without a rise in $[\text{Ca}^{2+}]_i$. In endothelial cells the rate of Mn^{2+} entry has been shown to correlate with the degree of filling of the store [7].

We have now investigated whether or not the discharge of the intracellular Ca^{2+} stores in platelets is associated with the generation of Ca^{2+} influx. Using Mn^{2+} as a tracer for bivalent-cation entry [2], we have obtained evidence for an influx pathway in platelets which appears to be activated by discharge of the intracellular Ca^{2+} store. This store-regulated pathway is largely obscured by the rapid receptor-operated Ca^{2+} (or Mn^{2+}) entry at 37 °C, but has been clearly resolved at 17 °C.

EXPERIMENTAL

Preparation of cells

Fura-2-loaded platelets were prepared as previously described [10]. Briefly, platelet-rich plasma was incubated for 45 min with 3 μM -fura-2/AM. The cells were collected by centrifugation and resuspended in a HEPES-buffered saline containing 145 mM-NaCl, 5 mM-KCl, 10 mM-HEPES, 1 mM-MgCl₂, 10 mM-D-glucose, pH 7.4, at 17 °C or 37 °C as appropriate. Apyrase (20 $\mu\text{g}/\text{ml}$) was added to prevent activation or desensitization by residual traces of ADP. To allow the study of primary agonist-evoked effects, without the complication of secondary effects owing to generation of prostaglandin endoperoxides and thromboxanes, the platelet-rich plasma was treated with 100 μM -aspirin before dye loading. Before experimental manipulations, 1 mM- CaCl_2 and/or 50 μM - MnCl_2 were added as appropriate.

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic calcium concentration; IBMX, isobutylmethylxanthine.

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Stopped-flow fluorimetry

The kinetics of fluorescence change from fura-2-loaded platelets were investigated by stopped-flow fluorimetry as previously described [1]. Briefly, a Hi-Tech Scientific SFA-II Rapid Kinetic Accessory was mounted in a Perkin-Elmer MPF-44A spectrophotometer. Dye-loaded cells were injected through one port and the agonist solution through the other. The two solutions were conveyed separately to the mixing chamber, which was mounted in the fluorimeter light path. The tubes carrying the solutions from syringes to the mixing chamber passed through a flexible jacket, the temperature of which could be controlled as appropriate by a water circulator. The syringe pistons were moved when a piston-bar was pushed manually, and 200 μ l of each solution entered the chamber before flow was stopped by the stopping syringe piston meeting a stop bar. The amplified signal from the fluorimeter was recorded directly, by-passing the normal damping circuits to improve response time. The signal was fed to the analogue input port of an Acorn BBC microcomputer, and scans of 500 samples taken at 10 ms intervals were recorded when the stopping syringe piston activated a microswitch mounted on the stop bar. In most cases, each individual experiment consisted of 10 scans taken at 15 s intervals and averaged.

Measurements were made from print-outs of the averaged traces.

Materials

Fura-2/AM was from Molecular Probes, Eugene, OR, U.S.A.; ADP, aspirin, apyrase and IBMX were from Sigma, Poole, Dorset, U.K.; forskolin and Hepes were from Behring Diagnostics, Cambridge Bioscience, Cambridge, U.K. SK&F 96365 (1- β -[3-(*p*-methoxyphenyl)-propyloxy]-*p*-methoxyphenethyl]-1*H*-imidazole hydrochloride) was synthesized by Dr. W. P. Armstrong, Department of Medicinal Chemistry, Smith Kline and French Research Ltd., Welwyn, U.K.

RESULTS AND DISCUSSION

Fig. 1 shows responses evoked in fura-2-loaded platelets by 40 μ M-ADP at 37 °C and 17 °C. In the presence of 1 mM external Ca^{2+} , ADP evoked a fast rise in $[\text{Ca}^{2+}]_i$ without measurable delay (Fig. 1*a*). In some experiments at 37 °C, the rise in $[\text{Ca}^{2+}]_i$ appeared to be biphasic, but two phases were not easily discerned in every case. At 17 °C, the ADP-evoked rise in $[\text{Ca}^{2+}]_i$ was clearly biphasic (Fig. 1*b*). The initial fast rise occurred without measurable delay and was followed by a further rise, delayed by

Fig. 1. Effect of temperature on ADP-evoked responses

Fura-2-loaded platelets were mixed with ADP at a final concentration of 40 μ M at zero time in the presence of 1 mM external Ca^{2+} (*a*, *b*), without added Ca^{2+} (*c*, *d*) or in the presence of 50 μ M- Mn^{2+} (*e*, *f*). The temperature was 37 °C (*a*, *c*, *e*) or 17 °C (*b*, *d*, *f*). Traces show fura-2 fluorescence at an excitation wavelength of 340 nm (*a*–*d*) or 360 nm (*e*, *f*). Emission was recorded at 500 nm in each case. Each trace is an average of 10 scans.

Table 1. ADP-evoked responses at 37 °C and 17 °C

Delays in onset of fura-2 fluorescence rise at an excitation wavelength of 340 nm (in the presence of 1 mM external Ca^{2+} or without added Ca^{2+}) or delay in onset of fluorescence quench at an excitation wavelength of 360 nm (50 μM - Mn^{2+}). Results are means $\pm$ S.E.M. of 6 (37 °C) or 12 (17 °C) experiments. The delayed phase of Mn^{2+} entry at 37 °C could not be resolved (n.r.).

Condition	Delay in onset (ms)			
	37 °C		17 °C	
	Fast phase	Delayed phase	Fast phase	Delayed phase
1 mM- Ca^{2+}	0 ± 0	220 ± 40	0 ± 0	830 ± 50
0 Ca^{2+}	—	280 ± 40	—	1390 ± 40
50 μM - Mn^{2+}	0 ± 0	n.r.	0 ± 0	1390 ± 60

0.83 s (Table 1). In the absence of added Ca^{2+} , ADP evoked a delayed monophasic rise in $[\text{Ca}^{2+}]_i$ at both temperatures (Figs. 1c and 1d). The delay in onset of $[\text{Ca}^{2+}]_i$ rise was 5 times longer at the lower temperature (Table 1).

These results indicate that ADP evokes a rise in $[\text{Ca}^{2+}]_i$ consisting of at least two phases. The initial phase, which occurs without measurable delay, is attributable to Ca^{2+} influx, since it is abolished in the absence of external Ca^{2+} (Figs. 1c and 1d) and is blocked by external Ni^{2+} [1]. The finding that the fast phase of the response continues to be evoked without measurable delay after a decrease in temperature is consistent with the idea that it is mediated by receptor-operated, rather than second-messenger-operated, channels [1,3]. The increased delay in the onset of ADP-evoked $[\text{Ca}^{2+}]_i$ rise in the absence of external Ca^{2+} presumably reflects the slowed production of the second messenger which mediates discharge of the intracellular Ca^{2+} stores. To test whether the delayed phase of the ADP-evoked rise in $[\text{Ca}^{2+}]_i$ observed in the presence of external Ca^{2+} was due to the release of the intracellular stores, or also had a component of additional Ca^{2+} influx, ADP-evoked responses in the presence of external Mn^{2+} were investigated. The entry of Mn^{2+} and consequent quenching of fura-2 fluorescence excited at 360 nm allows bivalent-cation entry to be distinguished from the release of Ca^{2+} from intracellular stores [2,8,11].

Figs. 1(e) and 1(f) show that ADP evoked Mn^{2+} entry without measurable delay at both 37 °C and 17 °C. Mn^{2+} entry was clearly biphasic at the lower temperature (Fig. 1f). The delayed phase of Mn^{2+} entry occurred at the same time after agonist addition as the release of Ca^{2+} from intracellular stores (Fig. 1d). An important point is that both of these events were considerably later than the delayed phase of $[\text{Ca}^{2+}]_i$ rise evoked by ADP in the presence of extracellular Ca^{2+} (Fig. 1b and Table 1).

The finding that ADP evokes biphasic Mn^{2+} entry suggests that Ca^{2+} influx is similarly biphasic, since we assume that both ions enter the cell through common pathways [2,11,12]. The temporal coincidence of the delayed phase of Mn^{2+} entry with discharge of the intracellular Ca^{2+} stores suggests that the two events may be causally linked, and that store-regulated Ca^{2+} entry may occur in platelets as has been proposed in other cell types [5–9]. (We usually assume that Mn^{2+} quench of fura-2 fluorescence reflects the direct entry of Mn^{2+} into the cytosol from the extracellular medium, although we cannot be sure that it does not pass via the releasable Ca^{2+} pool. One should consider the possibility that Mn^{2+}

Fig. 2. Effects of forskolin on ADP-evoked responses at 17 °C

Fura-2-loaded platelets were mixed with ADP at a final concentration of 40 μM at zero time in the presence of 1 mM external Ca^{2+} (a), without added Ca^{2+} (b) or in the presence of 50 μM - Mn^{2+} (c). Traces show responses under control conditions (—) superimposed on those obtained after treatment of cells with forskolin (20 mM) and IBMX (10 μM) for 5 min before stimulation (---). Fura-2 fluorescence was recorded at an excitation wavelength of 340 nm (a and b) or 360 nm (c), with emission at 500 nm in each case. Each trace is the average of 10 scans.

Fig. 3. Effect of external Ca^{2+} on ADP-evoked Mn^{2+} quench of fura-2 fluorescence at 17 °C

Fura-2-loaded platelets were mixed with ADP at a final concentration of $40 \mu\text{M}$ at zero time in the presence of 1 mM-Ca^{2+} without (—) or with (----) $50 \mu\text{M-Mn}^{2+}$. Traces show fura-2 fluorescence at an excitation wavelength of 340 nm (—) or 360 nm (----), with emission recorded at 500 nm in each case. Each trace is the average of 10 scans.

Table 2. Timing of the delayed phase of Mn^{2+} influx in the presence and absence of added Ca^{2+}

Delays in onset of fura-2 fluorescence rise at an excitation wavelength of 340 nm (in the presence of 1 mM external Ca^{2+} or without added Ca^{2+}) or delay in onset of fluorescence quench at an excitation wavelength of 360 nm (1 mM-Ca^{2+} and/or $50 \mu\text{M-Mn}^{2+}$). Results are means $\pm$ S.E.M. of 11–15 experiments conducted at 17 °C.

Condition	Delay in onset (ms)	
	Fast phase	Delayed phase
1 mM-Ca^{2+}	0 ± 0	660 ± 20
0 Ca^{2+}	—	1350 ± 30
$50 \mu\text{M-Mn}^{2+}$	0 ± 0	1340 ± 60
$1 \text{ mM-Ca}^{2+} + 50 \mu\text{M-Mn}^{2+}$	0 ± 0	700 ± 60

might somehow accumulate in the store without passing via the cytosol, and thus escape chelation by the cytosolic fura-2. The intra-store Mn^{2+} might then be discharged into the cytosol by InsP_3 -operated channels in the store membrane.)

Further evidence for a link between the discharge of the intracellular Ca^{2+} stores and the generation of a delayed phase of influx was obtained from observations of the inhibitory effects of cyclic AMP on ADP-evoked responses. We have previously reported that, at 37 °C, the elevation of platelet cyclic AMP by high concentrations of forskolin [13,14], in the presence of the phosphodiesterase inhibitor IBMX, can completely block ADP-evoked release of Ca^{2+} from intracellular stores. Under the same conditions, the fast rise in $[\text{Ca}^{2+}]_i$ evoked in the presence of external Ca^{2+} persists [1]. Fig. 2 shows the effects of forskolin ($20 \mu\text{M}$) in the presence of $10 \mu\text{M-IBMX}$ at 17 °C. The elevation of cyclic AMP completely inhibited the release of the intracellular Ca^{2+} stores (Fig. 2b). The delayed phases of Ca^{2+} or Mn^{2+} influx were similarly abolished, whereas the fast phases persisted (Figs. 2a and 2c). These results are consistent with the

Fig. 4. Effects of SK&F 96365 on ADP-evoked responses at 17 °C

Fura-2-loaded platelets were mixed with ADP at a final concentration of $40 \mu\text{M}$ at zero time in the presence of 1 mM external Ca^{2+} (a), without added Ca^{2+} (b) or in the presence of $50 \mu\text{M-Mn}^{2+}$ and 1 mM-Ca^{2+} (c). Traces show responses under control conditions (—) superimposed on those obtained after SK&F 96365 ($20 \mu\text{M}$) was added to the cells 1 min before stimulation (----). Traces show fura-2 fluorescence at an excitation wavelength of 340 nm (a and b) or 360 nm (c). Emission was recorded at 500 nm in each case. Each trace is the average of 8–10 scans.

idea that the release of Ca^{2+} from the intracellular store is linked to the generation of the delayed phase of bivalent-cation entry.

Although the delayed phase of Mn^{2+} entry coincides with the release of Ca^{2+} from the intracellular stores when observed in the absence of external Ca^{2+} , these events are considerably later in onset than the delayed phase of the rise in $[\text{Ca}^{2+}]_i$ evoked by ADP in the presence of external Ca^{2+} (Fig. 1 and Table 1). There are at least three possible explanations of this phenomenon. Firstly, there could be an additional route for Ca^{2+} entry through channels in the plasma membrane which are

Table 3. Effects of SK&F 96365 on ADP-evoked responses at 17 °C

Delays in onset of fura-2 fluorescence rise at an excitation wavelength of 340 nm (in the presence of 1 mM external Ca^{2+} or without added Ca^{2+}) or delay in onset of fluorescence quench at an excitation wavelength of 360 nm (1 mM- Ca^{2+} + 50 μM - Mn^{2+}). Results are means $\pm$ S.E.M. of 9–12 experiments.

Condition	Delay in onset (ms)			
	Control		20 μM -SK&F 96365	
	Fast phase	Delayed phase	Fast phase	Delayed phase
1 mM- Ca^{2+}	0 $\pm$ 0	650 $\pm$ 20	–	1200 $\pm$ 80
0 Ca^{2+}	–	1270 $\pm$ 90	–	1240 $\pm$ 90
1 mM- Ca^{2+} + 50 μM - Mn^{2+}	0 $\pm$ 0	660 $\pm$ 50	–	1240 $\pm$ 70

poorly permeable to Mn^{2+} . The opening of such channels might be mediated by a second messenger, and hence show a lag [2]. Secondly, the absence of external Ca^{2+} might artifactually delay the release of Ca^{2+} from the intracellular stores and so the opening of a store-regulated entry pathway, either by interfering with agonist-receptor binding or by affecting some subsequent stage of the transduction process. Thirdly, the release of Ca^{2+} from the intracellular stores might be modulated by $[\text{Ca}^{2+}]_i$, such that release occurs at an earlier time if $[\text{Ca}^{2+}]_i$ is already elevated by the fast phase of ADP-evoked Ca^{2+} influx. In an attempt to differentiate between these hypotheses, cells were stimulated by ADP at 17 °C in the presence of 50 μM - Mn^{2+} with or without 1 mM- Ca^{2+} .

Fig. 3 shows that in the presence of 1 mM- Ca^{2+} the delayed phase of Mn^{2+} entry occurred at a much earlier time than in the absence of added Ca^{2+} (Fig. 1f) and that this coincided with the delayed phase of the ADP-evoked rise in $[\text{Ca}^{2+}]_i$ observed in the presence of 1 mM Ca^{2+} alone (Fig. 3 and Table 2). In the same cell preparations, the delayed phase of Mn^{2+} quench in the absence of added Ca^{2+} coincided with the release of Ca^{2+} from the intracellular stores (Table 2). These findings suggest that only two influx pathways are evoked by ADP, but do not distinguish between a modulating role for $[\text{Ca}^{2+}]_i$ in the timing of the release of Ca^{2+} from the intracellular store and an artifact caused by the removal of external Ca^{2+} affecting the release. To differentiate between these possibilities, the actions of SK&F 96365, a newly identified compound which can selectively inhibit receptor-mediated Ca^{2+} entry [15], were investigated.

Fig. 4(a) shows the characteristic biphasic response evoked by ADP in the presence of external Ca^{2+} . SK&F 96365 (20 μM), in the presence of 1 mM external Ca^{2+} , blocked the fast phase of the ADP-evoked $[\text{Ca}^{2+}]_i$ rise; the delayed phase persisted. The time of onset of this persistent phase was later than the time of onset of the delayed phase observed in control responses; the timing in fact coincided with that of the rise in $[\text{Ca}^{2+}]_i$ observed in the absence of external Ca^{2+} (Fig. 4b; Table 3). SK&F 96365 (20 μM) did not measurably affect the ADP-evoked response in the absence of external Ca^{2+} (Fig. 4b). When cells were stimulated in the presence of 1 mM external Ca^{2+} and 50 μM - Mn^{2+} , the delayed phase of Mn^{2+} quench under control conditions again coincided with the delayed phase of the ADP-evoked rise in $[\text{Ca}^{2+}]_i$ (Figs. 4a and 4c; Table 3). In the presence of SK&F 96365, the fast phase of Mn^{2+} quench was abolished and

the delayed phase occurred at a later time than in controls (Fig. 4c). The persistent phase of Mn^{2+} quench coincided with the delayed rise in $[\text{Ca}^{2+}]_i$ observed in the presence of extracellular Ca^{2+} and the compound (Fig. 4a), and also with the response observed in the absence of added Ca^{2+} (Fig. 4b and Table 3). These results suggest that the greater interval before the onset of the delayed phase of ADP-evoked bivalent-cation influx, and so, presumably, release of Ca^{2+} from intracellular stores, is the result of the absence of the early rise in $[\text{Ca}^{2+}]_i$, rather than the lack of external Ca^{2+} itself. In agreement with this conclusion are findings in cells which had partially desensitized to ADP; the initial phase of the ADP-evoked rise in $[\text{Ca}^{2+}]_i$ was absent, but the delayed phase persisted and occurred at a similar time to the response in the absence of external Ca^{2+} (results not shown). We can think of several explanations for the apparent modulating role of $[\text{Ca}^{2+}]_i$. It may be that the earlier discharge of the intracellular stores reflects a more rapid production of $\text{Ins}(1,4,5)\text{P}_3$ resulting from the stimulation of phospholipase C by the increased $[\text{Ca}^{2+}]_i$. Alternatively, the elevation in $[\text{Ca}^{2+}]_i$ might accelerate the production of $\text{Ins}(1,3,4,5)\text{P}_4$, which has been proposed to regulate the passage of Ca^{2+} between $\text{Ins}(1,4,5)\text{P}_3$ -sensitive and -insensitive regions of the intracellular Ca^{2+} store and also between the store and the extracellular space [16]. The results obtained with SK&F 96365 also indicate that the pathways for bivalent-cation entry evoked by ADP are distinct, since the initial phase of influx is blocked by the compound at concentrations which leave the second phase intact. (Interestingly, in cuvette experiments, this concentration of SK&F 96365 blocks about half of the measured Mn^{2+} quench [16]).

In summary, we have shown that ADP evokes a biphasic rise in $[\text{Ca}^{2+}]_i$ which may be easily resolved at 17 °C. Both phases of the rise in $[\text{Ca}^{2+}]_i$ appear to be associated with influx, since stimulation of cells in the presence of extracellular Mn^{2+} results in a biphasic quenching of fura-2 fluorescence. The delayed phase of the ADP-evoked Mn^{2+} quench is temporally coincident with, and could be the result of, the release of Ca^{2+} from intracellular stores. This idea is supported by the finding that the elevation of platelet cyclic AMP by forskolin similarly abolishes the release of Ca^{2+} from intracellular stores, the second phase of the ADP-evoked rise in $[\text{Ca}^{2+}]_i$ observed in the presence of external Ca^{2+} and the second phase of ADP-evoked Mn^{2+} quench. The timing of the delayed ADP-evoked event is modulated by $[\text{Ca}^{2+}]_i$,

being more rapid in onset when $[Ca^{2+}]_i$ is elevated. It is suggested that a pathway for Ca^{2+} influx which is regulated by the depletion of the intracellular store exists in platelets. This pathway may contribute, to a greater or lesser extent, to the Ca^{2+} influx generated in response to all platelet agonists which evoke the release of Ca^{2+} from intracellular stores.

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