Agonist-induced Ca^{2+} influx into human platelets is secondary to the emptying of intracellular Ca^{2+} stores

Maria Teresa ALONSO, Javier ALVAREZ, Mayte MONTERO, Ana SANCHEZ and Javier GARCÍA-SANCHO*
Departamento de Bioquímica, Biología Molecular y Fisiología, Facultad de Medicina, Universidad de Valladolid,
47005-Valladolid, Spain

We have studied the relation between the filling state of the intracellular Ca^{2+} stores and the plasma-membrane permeability to Mn^{2+} , used here as a Ca^{2+} surrogate for Ca^{2+} channels. Emptying of the intracellular Ca^{2+} stores either by incubation in Ca^{2+} -free medium or by treatment with low concentrations of the Ca^{2+} ionophore ionomycin accelerated the influx of Mn^{2+} . Refilling of the Ca^{2+} stores by incubation in Ca^{2+} -containing medium restored low Mn^{2+} permeability. This Ca^{2+} -store-regulated permeability was inhibited by Ni^{2+} and by cytochrome *P*-450 inhibitors. Stimulation of platelets with thrombin produced Ca^{2+} release from the intracellular stores, which was followed, after a temperature-dependent lag (2 s at 37 °C; 5 s at 18 °C), by an acceleration of Mn^{2+} influx. Cytochrome *P*-450 inhibitors prevented the thrombin-induced Mn^{2+} influx, with little effect on the Ca^{2+} mobilization from the intracellular stores. K_i values were similar to those estimated for inhibition of the store-regulated permeability in non-stimulated platelets. Similar results were found in platelets stimulated by platelet-activating factor or by ADP. We propose that agonist-induced Ca^{2+} (Mn^{2+}) influx in platelets is secondary to the emptying of the intracellular Ca^{2+} stores. The activation of the plasma-membrane Ca^{2+} (Mn^{2+}) pathway may take place by a mechanism involving microsomal cytochrome *P*-450, similar to that described previously in thymocytes [Alvarez, Montero & García-Sancho (1991) *Biochem. J.* 274, 193–197] and neutrophils [Montero, Alvarez & García-Sancho (1991) *Biochem. J.* 277, 73–79].

INTRODUCTION

Activation of human platelets is associated with an increase in the cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), which originates both by release of Ca^{2+} from the intracellular Ca^{2+} stores and by entry of Ca^{2+} from the extracellular medium [1–3]. It is generally accepted that the release of Ca^{2+} from the stores is mediated by inositol 1,4,5-trisphosphate (InsP_3) [4,5]. Agonist-induced Ca^{2+} entry through a plasma-membrane pathway, which also allows passage of Ba^{2+} , Mn^{2+} and Co^{2+} [6], has been reported repeatedly [7–11], but the mechanisms have proven harder to elucidate.

Several mechanisms for activation of ‘receptor-operated’ Ca^{2+} channels have been proposed in different tissues. These include either direct coupling to the receptor through G-proteins [12] or indirect coupling via a second messenger such as InsP_3 , inositol 1,3,4,5-tetrakisphosphate (InsP_4) or the concerted action of both [13–16]. It has been recently suggested that the emptying of the intracellular Ca^{2+} stores could cause an increase in plasma-membrane Ca^{2+} (and Mn^{2+}) permeability in several types of cells, including platelets [17–24]. We have characterized such a mechanism in rat thymocytes, where microsomal cytochrome *P*-450 seems to be the link between the Ca^{2+} stores and the plasma membrane [23]. A similar mechanism was responsible for the agonist-induced Ca^{2+} influx in human neutrophils [24].

ADP-activated non-specific cation channels have been identified recently in ‘cell-attached’ platelet membrane patches [25]. Although these channels could be responsible for the entry of Na^+ associated with platelet activation, it is not clear whether they could also explain the Ca^{2+} influx observed in intact cells. Bivalent-cation-specific channels have been incorporated into lipid planar bilayers from plasma membranes isolated from thrombin-stimulated platelets, but activation *in vitro* of in-

corporated channels by thrombin or ADP was not achieved [26]. Using stopped-flow approaches in fura-2-loaded platelets, Rink and co-workers found that all the agonists tested except ADP evoked a Ca^{2+} (and Mn^{2+}) influx which was delayed by at least 0.2 s, the discharge from the internal stores beginning earlier [9,27]. ADP produced a biphasic Mn^{2+} influx, which was best resolved at low temperatures (17 °C), with a fast component showing no measurable delay and a slow one, delayed by 0.7–1.4 s, which coincided with the discharge of the intracellular stores [21]. On these bases, two different pathways for agonist-induced Ca^{2+} entry have been proposed, one directly coupled to the receptor (the fast component of ADP) and the other coupled by a second messenger (slow component of ADP and full response to all the other agonists). Results are consistent with the last pathway being a store-regulated Ca^{2+} influx [3,21].

In the present paper we have further investigated the presence of a store-regulated plasma-membrane Ca^{2+} (Mn^{2+}) pathway in human platelets and its contribution to the Ca^{2+} influx induced by thrombin, ADP and platelet-activating factor (PAF).

METHODS

Fura-2-loaded platelets were prepared as described previously [6]. Briefly, platelets were sedimented (350 g, 20 min) from platelet-rich plasma obtained from fresh blood anticoagulated with acid citrate–dextrose and resuspended at about 10^9 cells/ml in standard incubation medium containing (in mM): NaCl, 145; KCl, 5; MgCl_2 , 1; glucose, 10; sodium Hepes, 10, pH 7.4. Apyrase (20 $\mu\text{g}/\text{ml}$) was added to prevent activation or desensitization by residual traces of ADP. After addition of fura-2/AM (4 μM), the cell suspension was incubated for 45 min at 37 °C. The platelet suspension was then diluted 3-fold with incubation

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; K_i , inhibitor concentration producing 50% inhibition; ETYA, eicosatetra-5,8,11,14-ynoic acid; NDGA, nordihydroguaiaretic acid; PAF, platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine); SK&F 96365, 1- β -[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl-1*H*-imidazole hydrochloride.

* To whom correspondence should be addressed.

medium containing 2% acid citrate-dextrose and 1 mg of BSA/ml, and the cells were sedimented by centrifugation and resuspended at 2×10^8 /ml in standard medium.

Fluorescence measurements were performed, unless otherwise stated, at 37 °C and under magnetic stirring. A fluorescence spectrophotometer constructed by Cairn Research Ltd. (Newnham, Sittingbourne, Kent, U.K.), which allows quick alternation (30–300 Hz) of up to six excitation wavelengths and separate reading of the corresponding emitted fluorescences, was used. Excitation wavelengths were set at 340, 360 and 380 nm and emission was at 530 nm. Readings were integrated at 1 s intervals and stored for later analysis. $[Ca^{2+}]_i$ was calculated from the ratio of the fluorescences excited at 340 and 380 nm ($R_{340/380}$; [28]). The uptake of Mn^{2+} was monitored by the quenching of the fura-2 fluorescence excited at 360 nm. At this excitation wavelength, changes in $[Ca^{2+}]_i$ do not modify fluorescence emission [8,9]. On the other hand, quenching by Mn^{2+} does not modify $R_{340/380}$ as long as the total fura-2 content of the cells is greater than the total content of Mn^{2+} . This procedure has been described in detail elsewhere [10,11].

Fura-2/AM was obtained from Molecular Probes, Eugene, OR, U.S.A. PAF, ionomycin and thrombin were purchased from Calbiochem. Econazole, miconazole, clotrimazole, nordihydroguaiaretic acid (NDGA) and aspirin were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. Eicosatetra-5,8,11,14-ynoic acid (ETYA) and gossypol were from Biomol Research Laboratory, Plymouth Meeting, PA, U.S.A. BW755C {3-amino-1-[*m*-(trifluoromethyl)phenyl]-2-pyrazoline} was generously provided by Dr. S. Moncada, Wellcome Research Laboratories, Beckenham, Kent, U.K. SK&F 96365 was generously provided by Dr. J. E. Merritt, Smith Kline & French Research Ltd., The Frythe, Welwyn, Herts., U.K. Other chemicals were obtained either from Sigma or from E. Merck, Darmstadt, Germany.

RESULTS

In thymocytes, Ehrlich ascites-tumour cells or neutrophils loaded with fura-2, the Ca^{2+} content of the intracellular Ca^{2+} stores can be conveniently monitored by the size of the $[Ca^{2+}]_i$

Fig. 1. Refilling of the intracellular Ca^{2+} stores of human platelets by incubation in Ca^{2+} -containing medium

The size of the $[Ca^{2+}]_i$ peak elicited by thrombin (1 unit/ml) in fura-2-loaded platelets suspended in Ca^{2+} -free medium (Control) or after 1 min incubation with 1 mM- Ca^{2+} (1 min + Ca) is compared. $NiCl_2$ (5 mM) was added just before thrombin in order to avoid entry of extracellular Ca^{2+} . The mean increases in $[Ca^{2+}]_i$ in the 60 s following thrombin addition, estimated by adding up all the values and dividing by 60, were 121 and 176 nM respectively. The experiment shown is representative of nine similar ones (see the text). Each interval on the abscissa represents 1 min.

peak induced by ionomycin in cells suspended in Ca^{2+} -free medium [22–24]. However, this procedure is not suitable for platelets, since most of the stored Ca^{2+} of these cells (> 99% of a total of 20 mmol/l of cells) is contained within the secretory granules [29,30]. Furthermore, this Ca^{2+} is mobilized to the cytoplasm by ionophores, but not by cell agonists. Therefore, in order to measure the size of the agonist-sensitive Ca^{2+} stores, we studied the $[Ca^{2+}]_i$ peak elicited by thrombin.

Loading of platelets with fura-2 is performed in Ca^{2+} -free medium, in order to avoid cell activation. Therefore, the Ca^{2+} stores of these cells could have been partly depleted of Ca^{2+} during the loading period, as has been previously observed in other cells treated in the same way [22–24]. In thymocytes and neutrophils the stores can be refilled within a few seconds by incubation in Ca^{2+} -containing medium at 37 °C [22–24]. Fig. 1 compares the size of the $[Ca^{2+}]_i$ peaks induced by thrombin in platelets loaded with fura-2 before and after a 1 min incubation with 1 mM- Ca^{2+} . $NiCl_2$ (5 mM) was added to the incubation medium just before thrombin in order to prevent the entry of extracellular Ca^{2+} . Ni^{2+} has been shown before to block agonist-induced Ca^{2+} influx completely [6]. The size of the $[Ca^{2+}]_i$ peak (both height and duration) was increased by the brief pre-incubation with Ca^{2+} . In nine similar experiments performed with platelets isolated from different blood samples, the mean $[Ca^{2+}]_i$ increase in the 60 s following thrombin addition (see Fig. 1 legend) was increased by $54 \pm 8\%$ (mean $\pm$ S.E.M.) by preincubation with Ca^{2+} . This difference was statistically significant ($P < 0.001$, paired *t* test). Note that a larger Ca^{2+} content in the stores would occasion a bigger $[Ca^{2+}]_i$ peak, although the relation between both parameters may be not linear. The Ca^{2+} pump would always dampen the $[Ca^{2+}]_i$ peaks, but much more strongly the larger ones because of the dependence of pump-mediated fluxes on $[Ca^{2+}]_i^2$. In any case, the experiment of Fig. 1 indicates that the Ca^{2+} stores of fura-2-loaded platelets can be refilled by a brief incubation in Ca^{2+} -containing medium.

Fig. 2 compares the influx of Mn^{2+} , evidenced by the quenching of fura-2 fluorescence, in platelets either before (trace A) or after (trace D) refilling of the Ca^{2+} stores. The rate of Mn^{2+} uptake was about 10-fold slower in the cells with refilled stores. It could be argued that this decrease in Mn^{2+} uptake was due to competition with extracellular Ca^{2+} (present in Expt. D but not in Expt. A). However, addition of 1 mM- Ca^{2+} simultaneously with Mn^{2+} (trace B) did not substantially modify the initial rate of Mn^{2+} uptake (compare with trace A). This indicates that the decreased uptake observed in the cells first incubated with Ca^{2+} (trace D) is not due to interactions between Ca^{2+} and Mn^{2+} at the extracellular side of the membrane. The increased Mn^{2+} uptake observed in Ca^{2+} -depleted cells was prevented either by Ni^{2+} (5 mM; results not shown) or by the cytochrome P-450 inhibitor econazole (trace C). Fig. 3 shows that the effect of econazole was concentration-dependent, half-maximal inhibition of Mn^{2+} uptake being achieved at about 2 μ M.

Table 1 shows the K_i values for the effects of several compounds on the acceleration of Mn^{2+} uptake observed in fura-2-loaded non-stimulated platelets. The imidazole antimycotics econazole, miconazole and clotrimazole, considered to be selective antagonists of cytochrome P-450 [31,32], were powerful inhibitors. The lipoxygenase inhibitors NDGA and ETYA [33–35] also inhibited the acceleration of Mn^{2+} uptake. However, these two compounds have also been reported to antagonize cytochrome P-450 [31]. On the other hand, neither the dual lipoxygenase and cyclo-oxygenase inhibitor BW755C [36] nor the cyclo-oxygenase inhibitor aspirin [33] prevented the acceleration of Mn^{2+} uptake. Gossypol, another lipoxygenase inhibitor, also prevented the acceleration of Mn^{2+} uptake. We found, however, that this compound also inhibited cytochrome P-450-mediated

Fig. 2. Comparison of the uptake of Mn^{2+} by platelets with either empty (A) or refilled (D) intracellular Ca^{2+} stores: effect of econazole on the Ca^{2+} -store-regulated Mn^{2+} uptake

The uptake of Mn^{2+} is evidenced by the quenching of the fura-2 fluorescence excited at 360 nm (see the Methods section). Platelets were loaded with fura-2 and stored for 60 min in Ca^{2+} -free medium before starting the experiment. In Expt. A 5 mM- MnCl_2 was added at $t = 0$. In Expt. D 1 mM- CaCl_2 was added, followed 1 min later ($t = 0$ in the Figure) by 5 mM- MnCl_2 . In Expt. B 1 mM- CaCl_2 and 5 mM- MnCl_2 were added simultaneously at $t = 0$. In Expt. C 5 μM -econazole was added, followed 2 min later ($t = 0$ in the Figure) by 5 mM- MnCl_2 . In all the cases fluorescence was normalized to 100% at the time of Mn^{2+} addition. This experiment is representative of four similar ones performed with different cell batches. The uptake of Mn^{2+} was fully prevented by 5 mM- NiCl_2 (not shown in the Figure). The slopes of the lines, estimated from the first 20 s, are (% decrease in F_{360}/s): A, 0.94; B, 0.76; C and D, 0.08.

Fig. 3. Inhibition of the uptake of Mn^{2+} by non-stimulated human platelets by different concentrations (1–8 μM) of econazole

Cells loaded with fura-2 in Ca^{2+} -free medium were incubated with econazole (concn. at the right of each trace, in μM) for 2 min before addition of Mn^{2+} ($t = 0$ in the Figure). Other details were as in Fig. 2.

ethoxyresorufin *O*-de-ethylation by hepatic microsomes (results not shown). The above results are consistent with the involvement of cytochrome *p*-450 in the control of the Ca^{2+} -stores-dependent plasma-membrane Mn^{2+} influx, as was already proposed for other types of cell [23,24].

Table 1. Effects of several drugs on the uptake of Mn^{2+} by non-stimulated platelets with partly empty intracellular Ca^{2+} stores (column A) or on the uptake of Mn^{2+} stimulated by thrombin (column B)

Platelets were loaded with fura-2 in Ca^{2+} -free medium, and the uptake of Mn^{2+} was measured in the resting condition (column A) or after stimulation with thrombin (1 unit/ml; column B). K_i represents the concentration of drug producing 50% inhibition. Other details were as in Figs. 2, 3 and 5.

Inhibitor	K_i value (μM)	
	A (non-stimulated)	B (+ thrombin)
Econazole	2–3	3
Miconazole	1–3	3
Clotrimazole	3–6	6
NDGA	5	6
ETYA	10–20	10–20
Gossypol	6	6
BW755C*	> 200	> 200
Aspirin†	> 100	> 100

* No inhibition was detected at 200 μM .

† No inhibition was detected at 100 μM .

Fig. 4. Effects of 1 nM-ionomycin on the uptake of Mn^{2+} by fura-2-loaded platelets

The experiments were started by addition of 5 mM- MnCl_2 , followed 1 min later ($t = 0$ in the Figure) by 1 nM-ionomycin (Iono). In the two traces either 5 mM- NiCl_2 (labelled + Ni in the Figure) or 5 μM -econazole (labelled + Eco in the Figure) was added to the platelet suspension 1 min before Mn^{2+} . Other details were as in Fig. 2. The slopes of the quenching of fluorescence were (% decrease in F_{360}/s): control, 0.07 before ionomycin and 0.40 after ionomycin; + Ni, 0.22; + Eco, 0.23 (the last two after ionomycin). The experiments shown are representative of three similar ones.

It has been shown above (Fig. 1) that platelets loaded with fura-2 in Ca^{2+} -free medium still contain a considerable amount of Ca^{2+} in their intracellular stores. Further incubation of these cells in Ca^{2+} -free medium for up to 60 min at room temperature did not produce a further decrease in the Ca^{2+} content. Some additional emptying of the Ca^{2+} stores could be achieved by increasing the temperature to 37 °C, but results were irregular and signs of cell deterioration usually appeared. In consequence, this approach was discontinued. Ca^{2+} ionophores are able to release Ca^{2+} from the intracellular stores. We have shown previously in Ehrlich ascites-tumour cells [37], rat thymocytes [22,23] and neutrophils [24] that, by using low concentrations of ionomycin, the intracellular Ca^{2+} stores can be largely emptied of Ca^{2+} with little concomitant effect on the plasma-membrane

Fig. 5. Asynchrony between the increase of $[Ca^{2+}]_i$ and the uptake of Mn^{2+} elicited by thrombin in fura-2-loaded platelets

$[Ca^{2+}]_i$ is represented here by the ratio between the fluorescences excited at 340 and at 380 nm (see the Methods section; $\circ$, scale at left). The increase in $[Ca^{2+}]_i$ reflects release from the intracellular Ca^{2+} stores, since the incubation medium contained no Ca^{2+} . The uptake of Mn^{2+} is represented by the quenching of the fura-2 fluorescence excited at 360 nm (see the Methods section; $\bullet$, scale at right). (a) Experiment performed at 37 °C; 0.2 mM- $MnCl_2$ was added to the cell suspension, followed 1 min later by 1 unit of thrombin/ml (vertical dotted line, $t = 0$ in the Figure); each value is the mean of three individual data obtained with the same batch of cells; vertical bars represent S.D. (b) Details as in (a) except that the experiment was performed at 18 °C to slow down the effects of thrombin.

permeability. In platelets selective permeabilization of the stores by ionomycin could not be achieved. Fig. 4 shows that addition of 1 nM-ionomycin to non-stimulated platelets incubated with 5 mM-Mn increased 5-fold the rate of quenching of fura-2. The increase in Mn^{2+} permeability seemed to be delayed by 5–10 s with respect to the addition of ionomycin. When either 5 mM- Ni^{2+} or 5 μ M-econazole was also present, the increase in Mn^{2+} permeability was inhibited by about 50%. Since neither Ni^{2+} nor econazole interferes with ionomycin-mediated transport, the results of Fig. 4 indicate that the treatment with ionomycin triggers a Mn^{2+} entry which is not mediated through the ionophore. This action of ionomycin is very similar to that reported previously for other cell types [22–24].

Fig. 5 compares the time courses of the increase in $[Ca^{2+}]_i$ (F_{340}/F_{380} ; $\circ$) and of the uptake of Mn^{2+} (quenching of F_{360} ; $\bullet$) in platelets stimulated by thrombin. Both measurements were performed simultaneously in the same cells (see the Methods section). The increase in $[Ca^{2+}]_i$ reflects only release of Ca^{2+} from the intracellular stores, since the incubation medium contained no Ca^{2+} . In the experiment shown in Fig. 5(a), performed at 37 °C, it can be observed that the acceleration of Mn^{2+} uptake begins at least 2 s later than the increase in $[Ca^{2+}]_i$, at a time when a large amount of Ca^{2+} had already been released from the

Fig. 6. Effects of econazole (5 μ M) on the uptake of Mn^{2+} (a) and on the release of Ca^{2+} from the intracellular stores (b) induced by thrombin (Thr; 1 unit/ml) in fura-2-loaded platelets

Experimental details were as in Fig. 5. Each interval on the abscissa represents 1 min.

Fig. 7. Effects of CO on the uptake of Mn^{2+} induced by thrombin (Thr; 1 unit/ml) in fura-2-loaded platelets

The cell suspension was gassed with CO in the spectrofluorimeter cuvette and under magnetic stirring for 10 min before addition of Mn^{2+} and thrombin. Controls were treated similarly but without gassing with CO. Each value is the mean of four independent data; vertical bars represent S.D. Other details were as in Fig. 5.

intracellular stores. In the experiment shown in Fig. 5(b), the temperature was decreased to 18 °C in order to slow down the effect of thrombin. In this case the time lag between the increase in $[Ca^{2+}]_i$ and the acceleration of Mn^{2+} uptake was even more conspicuous. These findings are consistent with previous results obtained by using stopped-flow procedures, where a lag of 1–2 s between the Ca^{2+} release from the internal stores and the influx of Mn^{2+} had been found in platelets stimulated with 4 units of thrombin/ml at 37 °C [9]. The existence of this time lag between the release of Ca^{2+} from the intracellular stores and the entry of

Fig. 8. Effects of econazole ($5 \mu\text{M}$) on the uptake of Mn^{2+} (upper panels) and on the release of Ca^{2+} from the intracellular stores (lower panels) induced by PAF (20 ng/ml ; left-hand panels) and by ADP ($20 \mu\text{M}$; right-hand panels)

Experimental details were as in Fig. 5. Each interval on the abscissa represents 1 min.

Mn^{2+} is consistent with a cause-and-effect relation between both processes.

Fig. 6 illustrates the effects of econazole on the acceleration of Mn^{2+} influx (quenching of F_{360} ; Fig. 6a) and on the mobilization of Ca^{2+} from the intracellular stores ($[\text{Ca}^{2+}]_i$; Fig. 6b) induced by thrombin. Econazole did not significantly modify Ca^{2+} mobilization, but prevented the acceleration of Mn^{2+} uptake. The last column of Table 1 shows the K_i values estimated for several compounds for inhibition of the thrombin-induced Mn^{2+} uptake. These values agreed with those obtained for inhibition of the increased Mn^{2+} uptake observed in cells with partly emptied Ca^{2+} stores (first column in Table 1).

The effects of CO, another well-known cytochrome *P*-450 inhibitor, on the acceleration of Mn^{2+} uptake induced by thrombin were also studied. Fig. 7 shows that equilibration of the platelet suspension with CO before addition of thrombin largely prevented the acceleration of Mn^{2+} uptake (60–70% inhibition of the initial rate of quenching). In similar experiments performed with GH_3 pituitary cells, treatment with CO did not modify the uptake of Mn^{2+} through L-type Ca^{2+} channels elicited by depolarization with high- K^+ solutions (C. Villalobos, unpublished work).

Fig. 8 illustrates the effects of econazole on the acceleration of Mn^{2+} influx (upper panels) and on the mobilization of Ca^{2+} from the intracellular stores (lower panels) induced by ADP (left panels) and by PAF (right panels). As shown above for thrombin, econazole prevented the agonist-induced Mn^{2+} uptake, with minor effects on Ca^{2+} mobilization.

DISCUSSION

This paper analyses the effects of the degree of filling of the intracellular Ca^{2+} stores on the plasma-membrane permeability to Mn^{2+} in human platelets and the possible involvement of this mechanism in the Mn^{2+} influx induced by several agonists. Mn^{2+} is used here as a Ca^{2+} surrogate for plasma-membrane Ca^{2+} channels. Mn^{2+} permeates through agonist-induced plasma-membrane Ca^{2+} channels in platelets [6–11], and it is more suitable than Ca^{2+} itself for influx measurements, since, not being a substrate for the Ca^{2+} pump, the interfering back-flux through this transport system is avoided. On the other hand, the use of Mn^{2+} as the tracer for extracellular Ca^{2+} allows, by taking full advantage of the spectral properties of fura-2, simultaneous and independent monitoring of Ca^{2+} release from the intracellular stores by measuring changes in $[\text{Ca}^{2+}]_i$ [10,11]. The experiments shown here with platelets were guided by previous findings in rat thymocytes and in human neutrophils, where the control of plasma-membrane permeability by the degree of filling of the intracellular stores and the participation of this mechanism in agonist-induced Ca^{2+} influx has been documented previously [22–24].

We show here that the partial emptying of the intracellular stores that takes place during fura-2 loading in Ca^{2+} -free medium increases the plasma-membrane permeability to Mn^{2+} and that refilling of the stores by incubation in Ca^{2+} -containing medium restores a low plasma-membrane permeability (Figs. 1 and 2). When the Ca^{2+} stores were further emptied by ionomycin (Fig. 4)

or by several platelet agonists (thrombin, PAF or ADP; Figs. 5–8), the plasma-membrane permeability to Mn^{2+} was further increased. Finally, the acceleration of Mn^{2+} influx induced by any of the store-emptying manoeuvres, including agonists, was prevented by CO, econazole and other cytochrome *P*-450 inhibitors (Figs. 3, 4, 6, 7 and 8). These results are very similar to those reported previously in other cell types [23,24], suggesting that the mechanisms involved may be the same.

We have proposed that microsomal cytochrome *P*-450 could be the link between the plasma-membrane Ca^{2+} (Mn^{2+}) pathway and the Ca^{2+} stores in rat thymocytes [23] and in human neutrophils [24]. Imidazole antimycotics are considered to be specific inhibitors of cytochrome *P*-450 [31,32]. We show here that econazole, miconazole and clotrimazole inhibited the acceleration of Mn^{2+} uptake induced by any of the store-emptying manoeuvres. The K_i values obtained were the same whether store depletion was achieved either by prolonged incubation in Ca^{2+} -free medium or by treatment with thrombin (Table 1), and were very similar to the values reported previously for the store-dependent Mn^{2+} permeability in other cell types [23,24]. Inhibition by CO (Fig. 7) is also consistent with the involvement of cytochrome *P*-450. The lipoxygenase inhibitors NDGA, ETYA and gossypol also antagonized the acceleration of Mn^{2+} influx. However, other lipoxygenase inhibitors such as BW755C had no effect (Table 1). Since NDGA and ETYA have also been reported to inhibit cytochrome *P*-450 [31], and we find here the same effect for gossypol, we conclude that the inhibition of Mn^{2+} influx must be related to this action. Similar conclusions were drawn in rat thymocytes and human neutrophils [23,24]. It has been reported recently that compound SK&F 96365 inhibits agonist-induced Mn^{2+} influx in platelets without affecting Ca^{2+} release from the intracellular stores [38]. This compound contains an *N*-substituted imidazole group, and such a group is the key factor for the inhibitory interaction of imidazole antimycotics with the haem group of cytochrome *P*-450 [32,39,40]. On the other hand, inhibition of ethoxyresorufin *O*-de-ethylase activity of hepatic microsomes by SK&F 96365 has now been documented in our laboratory (C. Villalobos, unpublished work). On these bases, we suggest that SK&F 96365 may also act on Mn^{2+} influx by inhibiting cytochrome *P*-450.

The activation mechanism of 'receptor-operated' Ca^{2+} channels in platelets is poorly understood. It has been suggested recently that a store-regulated plasma-membrane Ca^{2+} pathway could be involved in agonist-induced Ca^{2+} influx (see [3]). This proposal was based on the delay, observed by stop-flow procedures, of the Ca^{2+} (or Mn^{2+}) influx with regard to Ca^{2+} discharge from the intracellular stores [9,21,27]. This conclusion was criticized because of possible contribution of shear stress generated during the stop-flow experiments (see [41,42]). We now confirm a delay of the thrombin-induced Mn^{2+} influx with regard to the release of Ca^{2+} from the internal stores under circumstances in which shear stress could not be a disturbing factor (Fig. 5). Our temporal resolution (1 s) was not enough to evidence such a delay when PAF or ADP was used as the agonist. Additionally, our results show that the agonist-induced Mn^{2+} influx can be uncoupled from Ca^{2+} release from the intracellular stores by cytochrome *P*-450 inhibitors with the same affinity pattern as in the cells not treated with agonists, suggesting the identity of both mechanisms. We must conclude that activation of 'receptor-operated' channels by agonists (with the exception, perhaps, of a part of the ADP effect; see the Introduction) is achieved by a rather indirect mechanism, involving neither direct interaction with the channel nor even direct generation of a channel-activating second messenger. This could contribute to explain previous failures to obtain evidence for receptor-operated channels in excised membrane patches, where the regulating

mechanisms (the Ca^{2+} stores) are absent. The operation of this mechanism has been documented previously for the agonist-induced Ca^{2+} (Mn^{2+}) influx in human neutrophils [24]. Previous reports with pancreatic [17] and parotid [18] acinar cells, smooth-muscle cells [20], endothelial cells [19], chromaffin cells [43] and even with platelets [21] had suggested the existence of a pathway for Ca^{2+} entry which was regulated by the filling state of the intracellular Ca^{2+} stores, although the possible role of a cytochrome *P*-450 had not been assessed.

This work was supported by a grant from DGICYT (PB89-0359). We thank Mr. Jesús Fernández for excellent technical assistance.

REFERENCES

1. Rink, T. J., Tsien, R. Y., Sanchez, A. & Hallam, T. J. (1985) in *Calcium in Biological Systems* (Robin, R. P., Weiss, G. B. & Putney, J. W., eds.), pp. 115–164. Plenum Publishing Co., New York.
2. Siess, W. (1989) *Physiol. Rev.* **69**, 58–178.
3. Rink, T. J. & Sage, S. O. (1990) *Annu. Rev. Physiol.* **52**, 431–449.
4. Berridge, M. J. & Irvine, R. F. (1989) *Nature (London)* **341**, 197–205.
5. Putney, J. W., Jr., Takemura, H., Hughes, A. R., Horstman, D. A. & Thastrup, O. (1989) *FASEB J.* **3**, 1899–1905.
6. Alonso, M. T., Sanchez, A. & García-Sancho, J. (1990) *Biochem. J.* **272**, 435–443.
7. Hallam, T. J. & Rink, T. J. (1985) *FEBS Lett.* **186**, 175–179.
8. Merritt, J. E. & Hallam, T. J. (1988) *J. Biol. Chem.* **263**, 6161–6164.
9. Sage, S. O., Merritt, J. E., Hallam, T. J. & Rink, T. J. (1989) *Biochem. J.* **258**, 923–926.
10. Alonso, M. T., Sanchez, A. & García-Sancho, J. (1989) *Biochem. Biophys. Res. Commun.* **162**, 24–29.
11. García-Sancho, J., Alonso, M. T. & Sanchez, A. (1989) *Biochem. Soc. Trans.* **17**, 980–982.
12. Brown, A. M. & Birnbauer, L. (1988) *Am. J. Physiol.* **254**, H401–H410.
13. Irvine, R. F. & Moor, R. M. (1986) *Biochem. J.* **240**, 917–920.
14. Irvine, R. F. & Moor, R. M. (1987) *Biochem. Biophys. Res. Commun.* **146**, 284–290.
15. Pittet, D., Lew, D. P., Mayr, G. W., Monod, A. & Schlegel, W. (1989) *J. Biol. Chem.* **264**, 7251–7261.
16. Kuno, M. & Gardner, P. (1987) *Nature (London)* **326**, 301–304.
17. Pandolf, S. J., Schoeffield, M. S., Fimmel, C. J. & Muallem, S. (1987) *J. Biol. Chem.* **262**, 16963–16968.
18. Takemura, H., Hughes, A. R., Thastrup, O. & Putney, J. W., Jr. (1989) *J. Biol. Chem.* **264**, 12266–12271.
19. Jacob, R. (1990) *J. Physiol. (London)* **421**, 55–77.
20. Missiaen, L., Declercq, I., Droogmans, G., Plessers, L., De Smedt, H., Raeymaekers, L. & Casteels, R. (1990) *J. Physiol. (London)* **427**, 171–186.
21. Sage, S. O., Reast, R. & Rink, T. J. (1990) *Biochem. J.* **265**, 675–680.
22. Montero, M., Alvarez, J. & García-Sancho, J. (1990) *Biochem. J.* **271**, 535–540.
23. Alvarez, J., Montero, M. & García-Sancho, J. (1991) *Biochem. J.* **274**, 193–197.
24. Montero, M., Alvarez, J. & García-Sancho, J. (1991) *Biochem. J.* **277**, 73–79.
25. Mahaut-Smith, M. P., Sage, S. O. & Rink, T. J. (1990) *J. Biol. Chem.* **265**, 10479–10483.
26. Zschauer, A., van Breemen, C., Bühler, F. R. & Nelson, M. T. (1988) *Nature (London)* **334**, 703–705.
27. Sage, S. O. & Rink, T. J. (1987) *J. Biol. Chem.* **262**, 16364–16369.
28. Grynkiewicz, G., Poenie, M. & Tsien, R. (1985) *J. Biol. Chem.* **260**, 3440–3450.
29. Gerrard, J. M., Peterson, D. A. & White, J. G. (1981) in *Platelets in Biology and Pathology*, vol. 2 (Gordon, J. L., ed.), pp. 407–436. Elsevier/North-Holland, Amsterdam.
30. Brass, L. F. (1984) *J. Biol. Chem.* **259**, 12563–12570.
31. Capdevila, J., Gil, L., Orellana, M., Marnett, L. J., Mason, J. I., Yadagiri, P. & Falk, J. R. (1988) *Arch. Biochem. Biophys.* **261**, 257–263.
32. Rodrigues, A. D., Gibson, G. G., Ioannides, C. & Parke, D. V. (1987) *Biochem. Pharmacol.* **36**, 4277–4281.
33. Rittenhouse-Simmons, S. & Deykin, D. (1981) in *Platelets in Biology and Pathology*, vol. 2 (Gordon, J. L., ed.), pp. 349–373. Elsevier/North-Holland, Amsterdam.

34. Piomelli, D., Volterra, A., Dale, N., Siegelbaum, S. A., Kandel, E. R., Schwartz, J. H. & Belardetti, F. (1987) *Nature (London)* **328**, 38–43
35. Kurachi, Y., Ito, H., Sugimoto, T., Shimizu, T., Miki, I. & Ui, M. (1989) *Nature (London)* **337**, 555–557
36. Higgs, G. A., Flower, R. J. & Vane, J. R. (1979) *Biochem. Pharmacol.* **28**, 1959–1961
37. Artalejo, A. R. & García-Sancho, J. (1988) *Biochim. Biophys. Acta* **941**, 48–54
38. Merritt, J. E., Armstrong, P., Benham, C. D., Hallam, T. J., Jacob, R., Jaxa-Chamiec, A., Leigh, B. K., McCarthy, S. A., Moores, K. E. & Rink, T. J. (1990) *Biochem. J.* **271**, 515–522
39. Ballard, S. A., Lodola, A. & Tarbit, M. H. (1988) *Biochem. Pharmacol.* **37**, 4643–4651
40. Yoshida, Y. & Aoyama, Y. (1987) *Biochem. Pharmacol.* **36**, 229–235
41. Jones, G. D. & Gear, A. R. (1990) *Biochem. J.* **265**, 305–306
42. Sage, S. O. & Rink, T. J. (1990) *Biochem. J.* **265**, 306–307
43. Liu, P.-S., Lin, Y.-J. & Kao, L.-S. (1991) *J. Neurochem.* **56**, 172–177

Received 27 February 1991/8 July 1991; accepted 18 July 1991