Cytochrome *P*-450 may link intracellular Ca^{2+} stores with plasma membrane Ca^{2+} influx

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We have studied the mechanism of the regulation of plasma membrane Ca^{2+} permeability by the degree of filling of the intracellular Ca^{2+} stores. Using Mn^{2+} as a Ca^{2+} surrogate for plasma membrane Ca^{2+} channels, we found that Mn^{2+} uptake by rat thymocytes is inversely related to the degree of filling of the intracellular Ca^{2+} stores. This store-dependent plasma membrane permeability is inhibited by oxygen scavenging, CO, imidazole antimycotics and other cytochrome *P*-450 inhibitors. The pattern of inhibition is similar to that reported previously for the inhibition of microsomal cytochrome *P*-450-mediated aryl hydrocarbon hydroxylase activity of lymphocytes. Several calmodulin antagonists, both phenothiazinic (trifluoperazine, fluphenazine and chlorpromazine) and dibenzodiazepinic (clozapine), accelerate Mn^{2+} uptake by cells with Ca^{2+} -filled stores, and this effect is prevented by imidazole antimycotics. Our results suggest that cytochrome *P*-450 may be the link between the stores and the plasma membrane Ca^{2+} pathway. We propose a model in which this cytochrome, sited at the stores, stimulates plasma membrane Ca^{2+} influx. This stimulatory effect is, in turn, prevented by the presence of Ca^{2+} inside the stores, possibly via a calmodulin-dependent mechanism.

INTRODUCTION

Many physiological agonists act by increasing the cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) by release of Ca^{2+} from intracellular stores [1,2]. In several cell lines, emptying of the intracellular Ca^{2+} stores increases the plasma membrane permeability to Ca^{2+} [3–8]. This, via Ca^{2+} influx, reinforces the $[\text{Ca}^{2+}]_i$ signal and secures adequate refilling of the stores. We have shown previously that manoeuvres which empty the intracellular Ca^{2+} stores increase by about 10-fold the plasma membrane permeability to Ca^{2+} in rat thymocytes [9]. These cells are an excellent model for the study of the above interactions, since Ca^{2+} depletion of the stores can be accomplished by incubation in Ca^{2+} -free medium, thus avoiding the use of agonists which could have direct effects on plasma membrane permeability. On the other hand, the use of Mn^{2+} as a Ca^{2+} surrogate for plasma membrane Ca^{2+} channels allows simultaneous monitoring of plasma membrane permeability (Mn^{2+} uptake) and release of Ca^{2+} from the stores (changes of $[\text{Ca}^{2+}]_i$) in fura-2-loaded cells [9–11].

The existence of an unknown mediator which is generated at the Ca^{2+} stores, and which would modulate plasma membrane Ca^{2+} permeability, has been proposed [8]. We have also suggested the involvement of an enzymically produced mediator on the basis of the large temperature dependence [increase in activity caused by a 10 °C increase in temperature (Q_{10}) = 4–5] of the store-regulated Mn^{2+} influx in thymocytes [9]. In this paper we have investigated the nature of the link between plasma membrane permeability to Ca^{2+} and the intracellular Ca^{2+} stores.

MATERIALS AND METHODS

Rat thymocytes [12], Ehrlich ascites tumour cells [13] and human platelets [14] were prepared and handled as described previously. Thymocytes were suspended at 1% cytocrit in nominally Ca^{2+} -free standard incubation medium containing (in mM): NaCl, 150; MgCl_2 , 1; glucose, 10; potassium-Hepes, 10,

pH 7.4. Cells were loaded with fura-2 by incubation with 2–3 μM -fura-2/AM for 30 min at room temperature. This was enough to deplete the Ca^{2+} stores by more than 75% (see Fig. 1a). $[\text{Ca}^{2+}]_i$ was measured in a fluorescence spectrophotometer constructed by Cairn Research Ltd. (Newnham, Sittingbourne, Kent, U.K.), which allows simultaneous excitation of fluorescence at 340, 360 and 380 nm. Fluorescence emission was set at 530 nm. Readings were integrated at 1 s intervals. $[\text{Ca}^{2+}]_i$ was calculated from the ratio of fluorescence excited at 340 nm and at 380 nm [15]. Mn^{2+} uptake was monitored by the quenching of fluorescence excited at 360 nm, which is insensitive to $[\text{Ca}^{2+}]_i$ variations [9–11]. The same experimental protocols were used for the other two cell kinds used, i.e. Ehrlich cells and human platelets.

Fura-2/AM was obtained from Molecular Probes, Eugene, OR, U.S.A. Ionomycin was purchased from Calbiochem. Ketoconazole, clotrimazole, miconazole, econazole, metyrapone, α -naphthoflavone, 4-hydroxyandrostenedione, nordihydroguaiaretic acid (NDGA) and aspirin were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Eicosatetra-5,8,11,14-ynoic acid (ETYA) was obtained from Biomol Research Lab., Plymouth Meeting, PA, U.S.A. BW755C was generously provided by Dr. S. Moncada, The Wellcome Research Laboratories, Beckenham, Kent, U.K. Trifluoperazine, clozapine and fluphenazine were gifts from Smith Kline and French, Sandoz and Squibb respectively. Chlorpromazine was obtained from Rhône-Poulenc Farma. Other chemicals were obtained either from Sigma or from E. Merck, Darmstadt, Germany.

RESULTS AND DISCUSSION

Fig. 1(a) shows that a 5 min incubation of fura-2-loaded Ca^{2+} -depleted thymocytes with different Ca^{2+} concentrations resulted in different degrees of store filling, measured as the increase of $[\text{Ca}^{2+}]_i$ on addition of ionomycin after chelation of extracellular Ca^{2+} with EGTA. Quenching of fura-2 fluorescence by Mn^{2+} influx permits estimation of plasma membrane Ca^{2+} permeability

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; ETYA, eicosatetra-5,8,11,14-ynoic acid; NDGA, nordihydroguaiaretic acid.

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Fig. 1. Effect of filling of the intracellular Ca^{2+} stores on Mn^{2+} influx through the plasma membrane

Ca^{2+} -depleted fura-2-loaded thymocytes were incubated in medium containing different Ca^{2+} concentrations for 5 min. Then 1 mM- Mn^{2+} was added and the quenching of fura-2 fluorescence was measured for 3 min. (a) Ca^{2+} contents of the stores. At 5 min after Ca^{2+} addition (concentrations, in mM, are given by the values on the right), 5 mM-EGTA and 15 nM-ionomycin were added ($t = 0$). The relative Ca^{2+} contents of the stores can be estimated from the peak increase in $[Ca^{2+}]_i$ after stimulation with ionomycin. The Ca^{2+} contents of the stores were found to be stable after a 2 min incubation with Ca^{2+} (results not shown; see [9]). (b) Uptake of Mn^{2+} . Fluorescence was normalized to 100% just after the addition of Mn^{2+} . (c) The rate of Mn^{2+} uptake is plotted against either Ca^{2+} refilling level ($\circ$, $\bullet$) or $[Ca^{2+}]_i$ measured just before the addition of Mn^{2+} (Δ , $\blacktriangle$). The refilling level was estimated from the peak increase in $[Ca^{2+}]_i$ over the basal $[Ca^{2+}]_i$ in experiments similar to those of Fig. 1(a). Data from two different experiments are shown. All of the experiments were performed at 25 °C.

[5–11]. Fig. 1(b) shows that cells whose stores were filled to different extents exhibited different uptake rates for Mn^{2+} . The correlation between the extent of store filling and the rate of Mn^{2+} influx is shown in Fig. 1(c). A very similar correlation has been reported recently in human endothelial cells using histamine to empty the Ca^{2+} stores [7]. Fig. 1(c) shows that there is also a good inverse correlation between $[Ca^{2+}]_i$ and Mn^{2+} influx, which

Fig. 2. Effects of 1 nM-ionomycin on Mn^{2+} uptake (a) and $[Ca^{2+}]_i$ (b) in rat thymocytes with refilled Ca^{2+} stores

Ca^{2+} -depleted cells were first incubated for 5 min at 25 °C with 1 mM- Ca^{2+} in order to refill the stores. Then 1 nM-ionomycin was added ($t = 0$) in either the absence (solid lines) or the presence (broken lines) of 5 mM- Ni^{2+} . Other details were as in the legend to Fig. 1.

might suggest that plasma membrane Ca^{2+} permeability is inhibited by an elevated $[Ca^{2+}]_i$. However, we have shown previously that $[Ca^{2+}]_i$ can be dissociated from Mn^{2+} influx under several experimental conditions [9]. The experiment shown in Fig. 2 reinforces this point. Low concentrations of ionomycin effectively released Ca^{2+} from the intracellular stores, with little effect on Mn^{2+} influx. After about 30 s, once the intracellular stores were empty and $[Ca^{2+}]_i$ was high, the influx of Mn^{2+} increased abruptly. The acceleration of Mn^{2+} influx was blocked by Ni^{2+} (Fig. 2), which does not antagonize the Mn^{2+} influx induced directly by ionomycin (results not shown). It is therefore shown that an accelerated Mn^{2+} influx can be associated with either a low (Fig. 1) or a high (Fig. 2) $[Ca^{2+}]_i$, thus excluding a regulatory role for $[Ca^{2+}]_i$ on plasma membrane Ca^{2+} permeability. The data are consistent, however, with plasma membrane Ca^{2+} permeability being controlled by the extent of filling of the intracellular Ca^{2+} stores. The results in Fig. 2 also suggest that some physiological agonists which induce fast Ca^{2+} release from the stores but result in a delayed Ca^{2+} influx might be acting like ionomycin, i.e. the effect on the plasma membrane may be not direct, but a consequence of the emptying of the stores.

The nature of the transport mechanism responsible for store-regulated Ca^{2+} influx was investigated next. Depolarization by increasing the extracellular K^+ concentration to 35 mM neither modified $[Ca^{2+}]_i$ nor accelerated Mn^{2+} influx in thymocytes with filled Ca^{2+} stores. On the other hand, the stimulated Mn^{2+} influx found in Ca^{2+} -depleted cells was not inhibited by 1 μ M-nisoldipine (results not shown). These results suggest that voltage-dependent

Table 1. Inhibition of the Mn^{2+} uptake in Ca^{2+} -depleted cells by several cytochrome *P*-450, lipoxygenase and cyclo-oxygenase inhibitors

Inhibition by CO was obtained by gassing the cell suspension with 100 % CO for 1–3 min before the addition of Mn^{2+} . The oxygen scavenging system used was as follows. Catalase (10000 units/ml), glucose oxidase (0.1 mg/ml) and glucose (20 mM) were added, and then the cell suspension was gassed with 100 % N_2 for 5 min before the addition of Mn^{2+} . Values in parentheses indicate percentage inhibition.

Inhibitor	K_i for Mn^{2+} influx (μM) (or % inhibition)
CO	(77)
Oxygen-scavenging system	(82)
Econazole	0.2
Miconazole	0.5
Clotrimazole	2
Ketoconazole	15
α -Naphthoflavone	10
Metirapone (2 mM)	(30)
4-Hydroxyandrostenedione (40 μM)	(0)
NDGA	12
ETYA	4
BW755C (1 mM)	(30)
Aspirin (1 mM)	(0)

Ca^{2+} channels are not involved. Extracellular Na^+ removal with or without prior Na^+ depletion of the cells did not modify store-regulated Mn^{2+} influx either. These results suggest that Na^+ -linked Ca^{2+} transport mechanisms are not involved.

Fig. 3. Effects of different concentrations of econazole on Mn^{2+} uptake by Ca^{2+} -depleted thymocytes

Trace a is the control with no econazole; trace b corresponds to another control without econazole which was incubated with 1 mM- Ca^{2+} for 3–4 min before the addition of Mn^{2+} . Mn^{2+} was added at $t = 0$; econazole was added about 2 min before the addition of Mn^{2+} . Econazole concentrations in μM are given on the right.

The enhanced Mn^{2+} uptake found in Ca^{2+} -depleted cells was inhibited by oxygen scavengers or by CO (Table 1). This is consistent with the involvement of a cytochrome. Econazole, one of a series of imidazole antimycotics which are specific inhibitors of cytochrome *P*-450 [16,17], also inhibited Mn^{2+} uptake ($K_i = 0.2 \mu\text{M}$; Fig. 3). Table 1 lists the K_i values for other imidazole

Fig. 4. Effects of fluphenazine (a), clozapine (b), trifluoperazine (c) and chlorpromazine (d) on Mn^{2+} uptake by rat thymocytes

The cells were first incubated for 2 min with 1 mM- Ca^{2+} in order to refill the Ca^{2+} stores. Mn^{2+} (1 mM) was added at $t = 0$. Trace B is the control with no drug added. Anti-calmodulin drugs were added 2 min before Mn^{2+} addition at the concentrations (μM) indicated in the Figure. When indicated (+E), 5 μM -econazole was added together with the anti-calmodulin drug. A control with Ca^{2+} -depleted cells whose stores were not refilled by incubation with 1 mM- Ca^{2+} is also shown for comparison (trace A). The treatment with these drugs did not modify the Ca^{2+} contents of the intracellular stores (results not shown).

antimycotics (miconazole, clotrimazole and ketoconazole). Several lipoxygenase inhibitors such as NDGA and ETYA, which have also been reported previously to inhibit cytochrome *P*-450 [18], were also effective inhibitors of store-regulated Mn^{2+} influx (Table 1). However, neither the dual lipoxygenase/cyclooxygenase inhibitor BW755C [18] nor aspirin, a cyclo-oxygenase inhibitor [19], antagonized Mn^{2+} influx. These results suggest that store-regulated Ca^{2+} influx is not related to arachidonic acid metabolism. Direct comparison of the levels of arachidonic acid metabolites [20] in thymocytes with either empty or filled Ca^{2+} stores showed no significant differences (results not shown). We found extremely slow arachidonic acid metabolism under both sets of conditions, which is consistent with previous observations in this cell type [21].

Within the cytochrome *P*-450 family, inhibition by α -naphthoflavone, but not by metyrapone or 4-hydroxyandrostenedione, suggests the involvement of cytochrome *P*₁-450 or *P*-448 [22–24]. These cytochromes have been found in microsomes of human lymphocytes, where they are responsible for enzymic activities such as aryl hydrocarbon hydroxylase [22,25–27]. Our results therefore suggest that microsomal cytochrome *P*-450 is involved in the increase in the plasma membrane permeability to Ca^{2+} observed when the Ca^{2+} stores are empty. Cytochrome *P*-450 has been proposed previously to modulate other membrane fluxes such as those associated with gap-junctional channels [28] and the microsomal ATP-dependent Ca^{2+} pump [29].

If emptying of the Ca^{2+} stores increases Ca^{2+} (Mn^{2+}) entry via a cytochrome *P*-450, then this cytochrome should be somehow inhibited by the refilling of the Ca^{2+} stores. It has been reported that aldosterone production by cytochrome *P*-450 in bovine adrenocortical mitochondria is inhibited in a calmodulin-dependent fashion when the intramitochondrial $[Ca^{2+}]$ rises to the micromolar range [30]. Histochemical studies have shown that the endoplasmic reticulum contains significant amounts of calmodulin [31]. On the other hand, it has been shown in platelets that low concentrations (10 μM) of the calmodulin antagonist trifluoperazine facilitate thrombin-induced elevation of cytosolic Ca^{2+} [32].

We have tested the effects of several calmodulin antagonists on Mn^{2+} uptake by thymocytes. Fig. 4 shows that the phenothiazines trifluoperazine, fluphenazine and chlorpromazine, and the dibenzodiazepine clozapine, increased the uptake of Mn^{2+} by thymocytes whose Ca^{2+} stores were filled. This increase of Mn^{2+} influx was inhibited by econazole. In contrast, the uptake of Mn^{2+} by Ca^{2+} -depleted cells was not affected by the calmodulin antagonists (results not shown). The concentration needed to increase the Mn^{2+} uptake correlated well with the K_i of these drugs for inhibition of calmodulin-dependent enzymes (trifluoperazine, 9–10 μM ; fluphenazine, 10 μM ; chlorpromazine, 22–42 μM ; clozapine, 80 μM [33,34]).

The above results should be interpreted with great caution, since the calmodulin antagonists are fairly unspecific [35]. The results are consistent, however, with the idea that calmodulin could be required for the inhibition of cytochrome *P*-450 produced by refilling of the Ca^{2+} stores. Note that the calmodulin involved would have to be located inside the Ca^{2+} stores since, as discussed above, Mn^{2+} influx through the plasma membrane depends on the Ca^{2+} content of the stores and not on the $[Ca^{2+}]_i$. A relevant question, therefore, is what is the actual free Ca^{2+} concentration within the stores? Although the total Ca^{2+} content in the endoplasmic reticulum is in the millimolar range, the only available estimate of the free Ca^{2+} concentration in this pool is about 0.5 μM [36]. Even if this value were an underestimate, it is reasonable to assume that, after store emptying, the Ca^{2+} concentration within the stores may well fall to the range of the K_d for the binding of Ca^{2+} by calmodulin.

Fig. 5. Effects of refilling the intracellular Ca^{2+} stores on the uptake of Mn^{2+} by Ehrlich ascites tumour cells (a) and by human platelets (b)

Trace C corresponds to Ca^{2+} -depleted cells (loaded with fura-2 and stored in nominally Ca^{2+} -free medium). Trace A corresponds to Ca^{2+} -depleted cells preincubated with 1 mM- Ca^{2+} for either 15 min at room temperature (Ehrlich cells) or 2 min at 37 °C (platelets). Trace B is same as C, except that econazole (1 μM) was added 2 min before Mn^{2+} . Trace D (platelets only) corresponds to cells in which Mn^{2+} and Ca^{2+} (1 mM each) were added simultaneously at $t = 0$. The experiments with Ehrlich cells were performed at 20 °C and those with platelets at 37 °C. Other details were as in the legend to Fig. 1.

In order to investigate whether cytochrome *P*-450 inhibitors affect store-dependent Ca^{2+} (Mn^{2+}) permeability in other cell types, experiments were performed in Ehrlich ascites tumour cells and human platelets. We have shown before that preincubation in Ca^{2+} -free medium decreases the Ca^{2+} content of the intracellular stores in Ehrlich cells [9]. In human platelets the Ca^{2+} content of the intracellular stores, measured as the $[Ca^{2+}]_i$ peak after addition of thrombin in EGTA-containing medium, was also decreased by incubation in Ca^{2+} -free medium (results not shown). Fig. 5 shows that refilling the intracellular stores decreased the uptake of Mn^{2+} by both Ehrlich cells and platelets (compare curves A and C). The inhibition of Mn^{2+} uptake cannot be attributed to competition with the extracellular Ca^{2+} , since the uptake of Mn^{2+} was not decreased by the simultaneous addition of Ca^{2+} (curve D). On the other hand, econazole inhibited the uptake of Mn^{2+} in Ca^{2+} -depleted cells (compare curves B and C). These results suggest that plasma membrane permeability to Ca^{2+} is controlled by the degree of filling of the intracellular Ca^{2+} stores in the Ehrlich cells and in human platelets also and that a cytochrome *P*-450 is involved in such control.

On the basis of our results we propose the following model for control of plasma membrane Ca^{2+} permeability by the intracellular Ca^{2+} stores in rat thymocytes. When the stores are empty, calmodulin within them is not bound to Ca^{2+} . This permits disinhibition of microsomal cytochrome *P*-450 activity

which, either directly or indirectly (perhaps by means of a metabolite travelling from the stores to the plasma membrane), opens a plasma membrane Ca²⁺ pathway. The entry of Ca²⁺ from the extracellular medium facilitates refilling of the Ca²⁺ stores. Then calmodulin binds Ca²⁺ and inhibits cytochrome P-450 activity, so that plasma membrane Ca²⁺ permeability returns to resting levels. Experiments with Ehrlich ascites tumour cells and with human platelets indicate that the model described here may also be operative in these cells. It is tempting, therefore, to speculate that this regulatory mechanism of Ca²⁺ influx plays a general role in intracellular calcium homeostasis.

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