Pharmacology of a Ca^{2+} -influx pathway activated by emptying the intracellular Ca^{2+} stores in HL-60 cells: evidence that a cytochrome *P*-450 is not involved

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In HL-60 cells, inhibition of the endoplasmic-reticular Ca^{2+} pump by thapsigargin leads to the emptying of this intracellular Ca^{2+} store and a subsequent activation of plasma-membrane Ca^{2+} influx through a non-voltage-dependent pathway. The elevated intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) produced and maintained by this Ca^{2+} inflow was used to examine the potency of various compounds to inhibit this influx mechanism. As expected, specific blockers of known Ca^{2+} channels, such as nifedipine, ω -conotoxin GVIA and ryanodine were without effect. The less selective inhibitors La^{3+} , SKF-96365 and L-651,582, which are thought to inhibit both voltage-dependent and voltage-independent Ca^{2+} channels, decreased $[\text{Ca}^{2+}]_i$ back to resting levels, with pIC_{50} values of 5.2, 5.9 and 6.2 respectively. It has been proposed that a cytochrome *P*-450 is involved in activating Ca^{2+} -influx pathways in thymocytes, neutrophils and platelets.

Consistent with this idea, the imidazole cytochrome *P*-450 inhibitors miconazole, econazole, clotrimazole and ketoconazole inhibited the thapsigargin-elevated $[\text{Ca}^{2+}]_i$ with pIC_{50} values of 7.1, 7.1, 7.1 and 5.8 respectively. The high affinity of imidazoles for cytochromes *P*-450 is due to co-ordinate binding to the haem. This interaction is greatly decreased in 2-substituted imidazoles. We examined whether the inhibition of Ca^{2+} influx was due to an interaction of the inhibitor imidazole nitrogen with the haem iron of the putative cytochrome *P*-450 by comparing the activity of two compounds, identical except that one was methylated at the imidazole 2-position. They were found to block thapsigargin-activated Ca^{2+} influx with equal potency. These results strongly suggest that a cytochrome *P*-450 is not involved in the activation of the Ca^{2+} influx produced by emptying the intracellular Ca^{2+} stores.

INTRODUCTION

Thapsigargin specifically inhibits the endoplasmic-reticular Ca^{2+} pump [1,2], thereby interrupting the pump-leak cycle and emptying the intracellular Ca^{2+} stores. The depletion of the intracellular Ca^{2+} stores leads to increased Ca^{2+} influx in most types of cells [3,4]. In HL-60 cells, for example, thapsigargin and other pump inhibitors activate the influx of bivalent cations (including Mn^{2+} and Ba^{2+}) and raise the steady-state intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) ([5]; M. V. Kopanitsa, unpublished work). Emptying the stores with $\text{Ins}(1,4,5)\text{P}_3$ also elevates the steady-state $[\text{Ca}^{2+}]_i$ in voltage-clamped cells, an effect which is decreased by depolarization in a manner consistent with the decrease in driving force for Ca^{2+} influx [6]. These findings are consistent with the idea that, in HL-60 cells, emptying the intracellular Ca^{2+} stores leads to increased Ca^{2+} influx through voltage-independent Ca^{2+} channels. One aim of our work was to characterize the pharmacology of this influx pathway.

In a series of papers, Javier Garcia-Sancho and colleagues have suggested that a cytochrome *P*-450 is involved in coupling the emptying of intracellular Ca^{2+} stores to the activation of Ca^{2+} flux into neutrophils, platelets and thymocytes [7–10]. This suggestion is largely based on the ability of various imidazole cytochrome *P*-450 inhibitors to inhibit Mn^{2+} influx activated by emptying the intracellular Ca^{2+} stores. We report here further examination of the involvement of cytochromes *P*-450 in coupling the emptying of Ca^{2+} stores to Ca^{2+} influx in the HL-60 human promyelocytic-leukaemia cell line. Although we found that common imidazole cytochrome *P*-450 inhibitors block Ca^{2+}

influx with reasonable potency, our results suggest that the efficacy of these compounds is not related to their ability to inhibit these oxidases. Some of these results were presented at the Pharmacology '93 Meeting [11].

MATERIALS AND METHODS

HL-60 cells (A.T.C.C., Rockville, MD, U.S.A.) were cultured in RPMI 1640 medium plus 10% (v/v) fetal-bovine serum (or Iscove's modification of Dulbecco's modified Eagle's medium plus 20% fetal-bovine serum) and were split to $2 \times 10^5/\text{ml}$ on the day before each experiment. The $[\text{Ca}^{2+}]_i$ of cells in a stirred cuvette (3 ml) at 37 °C was monitored by measuring Fura-2 emission at 510 nm by using a fluorimeter (SPEX SP-CM, Edison, NJ, U.S.A.) which rapidly (100 Hz) alternates the excitation wavelength between 340 and 380 nm. The cells were loaded with Fura-2 by incubating them with Fura-2/AM as described by Demaurex et al. [5], except that the ' Ca^{2+} medium' used in our experiments contained 120 mM NaCl, 5 mM KCl, 1.5 mM MgCl_2 , 1 mM CaCl_2 , 25 mM Na-Hepes (pH 7.4), 10 mM glucose and 250 μM sulfinpyrazone [12]. Control experiments using Mn^{2+} and diethylenetriaminepenta-acetic acid (a membrane-impermeant chelator) showed that over 95% of the Mn^{2+} -quenchable dye was in a compartment which was not immediately accessible to Mn^{2+} and which was inaccessible to the chelator, suggesting that the dye was intracellular.

At the end of each experiment, 10 μM ionomycin was added, followed by 1 mM MnCl_2 . Since Mn^{2+} quenches Fura-2 fluorescence, the proportion of the signal resulting from background

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; L-651,582, 5-amino-[4-chlorobenzoyl]-3,5-dichlorobenzyl]-1,2,3-triazole-4-carboxamide; pIC_{50} , negative log of the IC_{50} ; SKF-96365, 1- β -[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1*H*-imidazole.

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fluorescence and/or incompletely hydrolysed dye could be estimated by this procedure. 'Background' fluorescence ranged from 5 to 20% of the baseline signal at each wavelength. After the appropriate 'background' was subtracted from each trace, the $[Ca^{2+}]_i$ was calculated by the method of Grynkiewicz et al. [13], by using values from dilute solutions of Fura-2 for R_{max} and R_{min} .

The cytochrome *P*-450 oxidase activities associated with CYP17 and progesterone hydroxylation were determined as previously described [14,15].

Fura-2 and Fura-2/AM were from Molecular Probes (Eugene, OR, U.S.A.). Thapsigargin and ionomycin were from CalBiochem (La Jolla, CA, U.S.A.). ω -Conotoxin GVIA was from Bachem California (Torrance, CA, U.S.A.) and ryanodine from Research Biochemicals Inc. (Natick, MA, U.S.A.). Ketoconazole, SKF-96365, L-651,582, RS-14647 and RS-73520 were synthesized by the Institute of Organic Chemistry, Syntex Discovery Research. All other drugs were from Sigma (St. Louis, MO, U.S.A.). For the $[Ca^{2+}]_i$ assay, all drugs were dissolved in ethanol, except ω -conotoxin GVIA and La^{3+} , which were dissolved in buffer. For the cytochrome *P*-450 assays, the compounds were dissolved in methanol (final concn. < 2%).

RESULTS AND DISCUSSION

Thapsigargin is a selective, irreversible, inhibitor of the endoplasmic-reticular Ca^{2+} pump [1,2]. Addition of 10 nM thapsigargin to HL-60 cells resulted in a rapid, transient, rise in $[Ca^{2+}]_i$, which decayed to a new steady-state level (Figure 1). This $[Ca^{2+}]_i$ rise may result from the interruption of an endoplasmic-reticular/cytoplasmic pump-leak cycle by thapsigargin. If, in the absence of thapsigargin, endoplasmic-reticular Ca^{2+} pump activity were at steady-state with a leak out of the endoplasmic reticulum, then inhibition of the Ca^{2+} pump would result in a net efflux of Ca^{2+} from the endoplasmic reticulum. This would be expressed as a rise in $[Ca^{2+}]_i$. Once the Ca^{2+} has leaked out of the endoplasmic reticulum, cellular homeostatic mechanisms, such as the plasma-membrane Ca^{2+} pump, would be expected to return the $[Ca^{2+}]_i$ to resting levels. Instead, an elevated steady-state $[Ca^{2+}]_i$, or 'plateau', was observed (Figure 1), which appears to result from increased Ca^{2+} influx [5]. Although the magnitude of this plateau

Figure 1 Effect of thapsigargin on the $[Ca^{2+}]_i$ of HL-60 cells

The $[Ca^{2+}]_i$ was monitored by using Fura-2 as described in the Materials and methods section. At the time indicated by the arrow, 3 μ l of 10 μ M thapsigargin was added to give a final concentration of 10 nM.

varied between experiments, once established it was stably maintained for over 30 min. This suggested a convenient assay for studying the effects of various drugs on the Ca^{2+} influx activated by thapsigargin.

Econazole, an anti-fungal cytochrome *P*-450 oxidase inhibitor, has also been reported to inhibit bivalent-cation flux into thymocytes and neutrophils [7,10]. Addition of econazole during the 'plateau' phase of the $[Ca^{2+}]_i$ response to thapsigargin produced a concentration-dependent inhibition of the $[Ca^{2+}]_i$ (Figure 2a). Quantitative analysis of these data (Figure 2b) indicated that the IC_{50} was approx. 50 nM. Similar experiments were performed with a variety of drugs. Selective blockers of 'classical' Ca^{2+} channels were without effect. Thus nifedipine (30 μ M), a dihydropyridine which blocks L-type voltage-gated Ca^{2+} channels, and ω -conotoxin GVIA (1 μ M), which blocks N-type voltage-gated Ca^{2+} channels, did not decrease the thapsigargin-induced plateau. Ryanodine (100 μ M), which blocks the Ca^{2+} -induced Ca^{2+} -release channel of the sarcoplasmic reticulum, was also without effect.

In contrast, less selective Ca^{2+} -channel blockers inhibited the thapsigargin-induced $[Ca^{2+}]_i$ plateau (Table 1). La^{3+} , SKF-96365 and L-651,582 have been reported to inhibit both L-type voltage-dependent and voltage-independent Ca^{2+} channels [16,17]. All were relatively potent at decreasing the plateau, and, by implication, Ca^{2+} influx. Intriguingly, the potency of SKF-96365 to inhibit the $[Ca^{2+}]_i$ plateau ($pIC_{50} = 5.9$) is very similar to its potency to inhibit a non-selective cation channel (a potential Ca^{2+} -influx pathway) in HL-60 cells that is activated by emptying the intracellular Ca^{2+} stores ($pIC_{50} = 5.7$) [18]. Verapamil, a selective blocker of L-type voltage-dependent Ca^{2+} channels at low concentrations, inhibited the thapsigargin-induced $[Ca^{2+}]_i$ plateau with low potency ($pIC_{50} = 4.7$). In fact, verapamil produces this effect with only slightly higher potency than it inhibits voltage-dependent K^{+} channels ($pIC_{50} = 4.3$) [19] and Na^{+} channels ($pIC_{50} \sim 3.7$ – 4.3) [20]. Since verapamil is thought to bind within the ion-permeation pore [21], this finding may suggest that the channel opened by emptying the intracellular stores shares some structural features with voltage-dependent channels.

Imidazole cytochrome *P*-450 inhibitors greatly slow Mn^{2+} influx activated by emptying the intracellular Ca^{2+} stores of neutrophils, platelets and thymocytes [7–10]. These results have been interpreted to imply that a cytochrome *P*-450 is involved in coupling the emptying of intracellular Ca^{2+} stores to the activation of Ca^{2+} influx [8]. Consistent with this idea, we found that SKF-96365 and L-651,582, respectively an imidazole and a triazole, blocked the thapsigargin-induced $[Ca^{2+}]_i$ plateau in HL-60 cells. To test further whether a similar mechanism was operating in HL-60 cells, we examined the effect of a number of cytochrome *P*-450 inhibitors on the thapsigargin-induced $[Ca^{2+}]_i$ plateau (Table 1). The antifungal imidazole cytochrome *P*-450 inhibitors miconazole, econazole, clotrimazole and ketoconazole all potently blocked this putative Ca^{2+} -influx mechanism. In addition, nordihydroguaiaretic acid, which does not contain a nitrogen atom, but has been reported to inhibit cytochrome *P*-450 activity [22], also inhibited the $[Ca^{2+}]_i$ plateau.

Imidazoles inhibit cytochrome *P*-450 oxidase activity via interaction of the non-bonded electrons of the nitrogen at the 3-position of the imidazole ring with the haem group of the cytochrome *P*-450 [23]. Substitutions at the 2-position of the imidazole ring decrease the activity of such compounds by 10–100-fold or more, owing to steric interactions with the haem porphyrin [23]. To test whether such an interaction was involved in the imidazole inhibition of Ca^{2+} influx, we examined the activities of two compounds which differ only at the 2-position

Figure 2 Effect of various concentrations of econazole on the elevated $[\text{Ca}^{2+}]_i$ produced by thapsigargin

(a) The $[\text{Ca}^{2+}]_i$ of HL-60 cells was monitored with Fura-2 as described in the Materials and methods section. At the times indicated by the arrows 30 nM thapsigargin (TG), ethanol (E), or the indicated concentration of econazole was added. After each addition, the $[\text{Ca}^{2+}]_i$ was allowed to reach a new steady state before the subsequent addition was performed. The rapid down-stroke coinciding with each addition is an artifact of opening the sample compartment. The apparent rise in $[\text{Ca}^{2+}]_i$ after addition of 3 μM econazole is a fluorescence artifact, since only the 340 nm excitation signal was affected. (b) The steady-state $[\text{Ca}^{2+}]_i$ after each drug addition in (a) was measured. The data were normalized to the post-thapsigargin 'plateau' (100% response) and the pre-thapsigargin baseline (0% response). The line represents the fit of the data to the equation:

$$\text{Response} = \text{MAX} - \{(\text{MAX} - \text{MIN})[\text{drug}]/([\text{drug}] + \text{IC}_{50})\}$$

where MAX is the (fitted) maximum thapsigargin response and MIN is the (fitted) lowest $[\text{Ca}^{2+}]_i$ attained.

Table 1 Potency of compounds to inhibit the thapsigargin-induced $[\text{Ca}^{2+}]_i$ plateau

The potency of various compounds to inhibit the elevated $[\text{Ca}^{2+}]_i$ produced by thapsigargin was determined as described in the Materials and methods section. The negative log of the IC_{50} (pIC_{50}) was calculated for each data set (see e.g., Figure 2b). The mean $\pm$ S.E.M. of the pIC_{50} values are shown for each compound, with the number of individual experiments in parentheses. At the concentrations used, these compounds had little or no effect on the $[\text{Ca}^{2+}]_i$ in the absence of thapsigargin.

Compound	pIC_{50}
LaCl_3	5.2 ± 0.30 (3)
SKF-96365	5.9 ± 0.10 (4)
L-651,582	6.2 ± 0.31 (3)
Verapamil	4.7 ± 0.18 (4)
Clotrimazole	7.1 ± 0.14 (7)
Econazole	7.1 ± 0.12 (6)
Ketoconazole	5.8 ± 0.11 (3)
Miconazole	7.1 ± 0.14 (7)
Nordihydroguaiaretic acid	6.0 ± 0.14 (3)

Figure 3 Structures of RS-14647 and RS-73520

Table 2 Potency of RS-14647 and RS-73520 to inhibit cytochrome P-450 and the thapsigargin-induced $[\text{Ca}^{2+}]_i$ plateau

The closely related compounds RS-14647 and RS-73520 differ by a methyl group at the imidazole 2-position (Figure 3). The potencies of these compounds to inhibit various cytochrome P-450 oxidases and the elevated $[\text{Ca}^{2+}]_i$ produced by thapsigargin were determined as described in the Materials and methods section. The activity of the cytochrome P-450 CYP17 was measured in pig testis microsomes, and the progesterone hydroxylase activities were measured in rat liver microsomes. The pIC_{50} was calculated for each data set. Where applicable, the mean $\pm$ S.E.M. of the pIC_{50} values are shown, with the numbers of individual experiments in parentheses.

Assay	RS-14647	RS-73520
Androgen synthase		
CYP17	6.5 (1)	< 4.0 (1)
Progesterone hydroxylases		
CYP2C11	5.2 (1)	4.3 (1)
CYP3A	5.3 (1)	< 4.0 (1)
CYP2C6	5.5 (1)	4.5 (1)
Thapsigargin-elevated $[\text{Ca}^{2+}]_i$	6.5 ± 0.18 (4)	6.7 ± 0.12 (4)

(Figure 3). As expected, the compound with a hydrogen atom at the 2 position, RS-14647, inhibited the tested cytochrome P-450 oxidase activities with approximately micromolar potency, whereas the compound with a methyl group at the 2-position, RS-73520, is at least 8-fold less potent (Table 2). However, when these compounds were tested for their ability to decrease the thapsigargin-induced $[\text{Ca}^{2+}]_i$ plateau, they had very similar potencies, which were not significantly different ($P > 0.44$; t test). These results suggest that imidazoles inhibit thapsigargin-stimulated Ca^{2+} influx by an unknown mechanism, not by inhibiting a cytochrome P-450 oxidase. Consistent with this idea, structure-activity studies of SC-38249 demonstrate that the imidazole moiety is unnecessary for inhibition of platelet Ca^{2+} influx [24]. In addition, Randriamampita and Tsien [25] found that the release of a Ca^{2+} -influx factor in response to emptying intracellular Ca^{2+} stores was not inhibited by econazole, whereas the activation of Ca^{2+} influx by the factor was blocked. Thus it may be only coincidental that these cytochrome P-450 inhibitors block Ca^{2+} influx.

Very recently, Vostal and Fratanoni [26] reported that CO, an irreversible inhibitor of cytochrome P-450 oxidases, does not block thapsigargin-activated $^{45}\text{Ca}^{2+}$ influx. Although these findings support the conclusion that a cytochrome P-450 is not involved, they appear to contradict the finding by Alonso et al. [9] that CO pretreatment greatly slowed thrombin-stimulated Mn^{2+} influx. Perhaps the different results reflect the different techniques used to activate (thapsigargin versus thrombin) and to measure influx ($^{45}\text{Ca}^{2+}$ versus Mn^{2+} and Fura-2).

How do imidazoles inhibit thapsigargin-activated Ca^{2+} influx? SKF-96365, L-651,582 and the imidazole cytochrome P-450 inhibitors all inhibit various voltage-dependent ion channels [16,17,27,28]. The inhibition of L-type voltage-dependent Ca^{2+}

channels appears to result from a direct interaction, since these compounds decrease binding of labelled dihydropyridine (L. Kunysz, personal communication). Although the influx pathway (presumably a channel) activated by emptying the intracellular Ca^{2+} stores is not blocked by nifedipine, and appears to be voltage-independent, it may share some properties with voltage-dependent channels. Thus, by extension, the simplest explanation is that these compounds block the influx channel.

One curious observation arising from studying the effect of imidazoles on Ca^{2+} influx is that both the relative and absolute potencies of the inhibitors appear to vary between cell types. Thus García-Sancho and co-workers [7,9] report that econazole is 10-fold more potent than clotrimazole in thymocytes, but equipotent in platelets. We found that econazole and clotrimazole were equipotent in HL-60 cells, but with 10-fold higher potencies than those reported in platelets. We also found that SKF-96365 was about 10-fold more potent in HL-60 cells than has been reported in platelets [16]. Although these results may simply reflect differences in experimental technique and conditions, a more appealing explanation is that there are multiple channels, with the differences in potency reflecting the different properties of the channels or mixtures of channels. Consistent with this idea, emptying the intracellular Ca^{2+} stores of various cell types has been observed to activate ion channels which differ in their single-channel conductance and/or ion selectivity [18,29,30].

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