Inhibition of voltage-gated Ca^{2+} entry into GH_3 and chromaffin cells by imidazole antimycotics and other cytochrome P450 blockers

CARLOS VILLALOBOS, ROSALBA FONTERIZ, MANUELA G. LOPEZ,* ANTONIO G. GARCIA,* AND JAVIER GARCIA-SANCHO[1]

Departamento de Bioquímica y Biología Molecular y Fisiología, Facultad de Medicina, Universidad de Valladolid, Valladolid, Spain and *Departamento de Farmacología y Terapéutica, Facultad de Medicina, Universidad Autónoma de Madrid, Madrid, Spain

ABSTRACT **We have studied the effects of cytochrome P450 inhibitors on the entry of Ca^{2+} and Mn^{2+}, used here as a Ca^{2+} surrogate for Ca^{2+} channels, in fura-2-loaded GH_3 pituitary cells and bovine chromaffin cells depolarized with high-K^+ solutions. Imidazole antimycotics were potent inhibitors (econazole > miconazole > clotrimazole > ketoconazole). α-Naphtoflavone and isosafrole, but not metyrapone, also inhibited the entry of Ca^{2+} and Mn^{2+} induced by depolarization. This inhibitory profile most resembles that reported for IA-type cytochrome P450. However, carbon monoxide (CO), a well-known cytochrome P450 antagonist, had no effect on Ca^{2+} (Mn^{2+}) entry. Given the high selectivity of the imidazole antimycotics for the heme moiety, our results suggest that a hemoprotein closely related to cytochrome P450 (but insensitive to CO) might be involved in the regulation of voltage-gated Ca^{2+} channels. The inhibitory pattern was also similar to that previously reported for agonist-induced Ca^{2+} (Mn^{2+}) influx in neutrophils and platelets, although CO was an efficient inhibitor in this case. These results pose the question of whether similarities in the sensitivity to cytochrome P450 inhibitors exhibited by receptor-operated and voltage-gated channels reflect unknown similarities either in structural features or regulation mechanisms.—Villalobos, C.; Fonteriz, R. Lopez, M. G.; Garcia, A. G.; Garcia-Sancho, J. Inhibition of voltage-gated Ca^{2+} entry into GH_3 and chromaffin cells by imidazole antimycotics and other cytochrome P450 blockers. *FASEB J.* 6: 2742–2747; 1992.**

Key Words: Ca^{2+} channels · cytochrome P-450 · GH_3 pituitary cells · adrenal chromaffin cells

Two different types of Ca^{2+} channels, voltage-gated and receptor-operated,[2] are currently acknowledged with regard to their activation mechanism (1). At least three different subtypes of voltage-gated channels have been described (2). The existence of two of them (L and T) has been documented in GH_3 pituitary cells (3). The presence of L-type Ca^{2+} channels has long been known to exist in adrenal chromaffin cells (4). Recently, two different subtypes of Ca^{2+} channels, one sensitive to and the other resistant to dihydropyridines, have been found in bovine chromaffin cells by using patch-clamp techniques (5, 6).

It has been reported that nordihydroguaiaretic acid (NDGA),[3] a well-known lipoxygenase inhibitor (7) that also antagonizes cytochrome P450 (8), is able to block voltage-activated Ca^{2+} currents in GH_3 cells independently of its effects on arachidonic acid metabolism (9). Recently we have reported that many cytochrome P450 inhibitors, including NDGA, prevent agonist-induced Ca^{2+} and Mn^{2+} entry in human neutrophils (10) and platelets (11) and that they antagonize the plasma membrane Ca^{2+} permeability regulated by Ca^{2+} stores (12). Although no structural similarities between voltage-gated and receptor-operated Ca^{2+} channels are known at present, we thought it was worthwhile to investigate systematically the effects of cytochrome P450 inhibitors on the activity of voltage-gated Ca^{2+} channels.

EXPERIMENTAL PROCEDURES

The strain of GH_3 pituitary cells (13) was kindly provided by Dr. Carlos Dieguez, Departamento de Fisiologia, Universidad de Santiago de Compostela, Spain. They were grown in RPMI 1640 medium supplemented with 15% horse serum and 2.5% fetal calf serum at 37°C and under air-5% CO_2 atmosphere. Before the experiments, the cells were starved in standard medium containing (in mM): NaCl, 145; KCl, 5; Mg_2Cl, 1; $CaCl_2$, 1; glucose, 10; sodium-Hepes, 10, pH, 7.4. The cells were washed twice and resuspended in the same medium at about 10^7 cells/ml.

Bovine adrenal chromaffin cells were isolated as previously described (14). A cell layer enriched in adrenalin-containing cells was taken from the Percoll gradient. Cells were suspended in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum containing 50 IU/ml penicillin and 50 μg/ml streptomycin. Cells were kept in suspension with gentle continuous agitation at 37°C and under air-5% CO_2 atmosphere for 2–3 days before the experiments. At this time, cells were starved, washed twice with standard (see composition above) incubation medium, and resuspended in the same medium at about 10^7 cells/ml.

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[1]To whom correspondence should be addressed, at: Departamento de Fisiología, Facultad de Medicina, 47005-Valladolid, Spain.

[2]The term receptor-operated Ca^{2+} channel is used here to describe a Ca^{2+} entry pathway that activates on interaction of an extracellular messenger with its membrane receptor, either by a direct or a second messenger-mediated action. The channel (as opposed to carrier) nature of this entry pathway has not been extensively documented in all cases.

[3]Abbreviations: NDGA, nordihydroguaiaretic acid; $[Ca^{2+}]_i$, cytosolic free calcium concentration; IC_{50}, inhibitor concentration that produces 50% inhibition.

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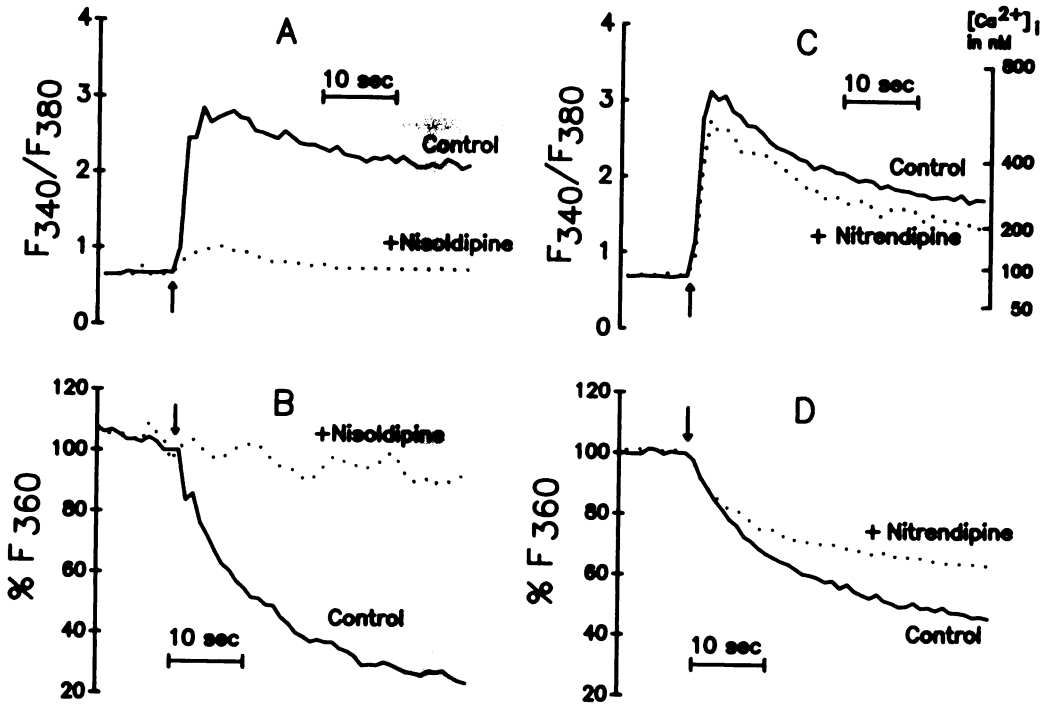

Figure 1. Entry of Ca^{2+} (upper panels) and Mn^{2+} (lower panels) induced by high K^+ in GH_3 cells (left) and in bovine chromaffin cells (right). Inhibition by dihydropyridines. Cells were loaded with fura-2 as described in Methods. Aliquots of 0.5 ml of the cell suspension were placed in the spectrofluorimeter cuvette and allowed to equilibrate to temperature for 3 min. At the arrow, 45 mM KCl_2 was added from a concentrated (3 M) stock solution. In the experiments shown in the lower panels 0.2 mM $MnCl_2$ was added 1 min before K^+. Crude records of F_{340}/F_{380} (as an index of $[Ca^{2+}]_i$) and F_{360} (as an index of Mn^{2+} entry) are shown. Scale for estimated $[Ca^{2+}]_i$ is shown at right. F_{360} is expressed as percent of the value at $t = 0$. In the traces labeled, either 10^{-7} nisoldipine (GH_3 cells) or 10^{-6} M nitrendipine (chromaffin cells) was added 2 min before K^+. The experiment with GH_3 cells is representative of three similar ones. With chromaffin cells eight similar experiments were performed using several dihydropyridines (see footnote 4).

Cells were loaded with fura-2 by incubation with 4 μM fura-2/AM for about 60 min at room temperature. The loading incubation was terminated by dilution with 3 volumes of fresh standard medium followed by centrifugation. The sedimented cells were then suspended at 1-3 x 10^6 cells/ml in standard medium.

Fluorescence measurements of cell suspensions were performed under magnetic stirring in a fluorescence spectrophotometer that allowed rapid (30-300 Hz) alternation of up to six different excitation wavelengths (Cairn Research Ltd., Newnham, Sittingbourne, Kent, U.K.). Temperature was 37°C. Emitted fluorescence was measured at 530 ± 10 nm and integrated at every 1-s period. The cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was estimated from the ratio of the fluorescence values excited at 340 and at 380 nm (F_{340}/F_{380}; see ref 15). In some experiments Mn^{2+}, used here as a Ca^{2+} surrogate for Ca^{2+} channels (see Results), was added to the cell suspension. The entry of Mn^{2+} into the cells was evidenced by the quenching of the fura-2 fluorescence excited at 360 nm. This wavelength is insensitive to changes in $[Ca^{2+}]_i$, but it is quenched by manganese (16). On the other hand, F_{340}/F_{380} is not modified by manganese provided the total cell content of fura-2 exceeds that of manganese. Thus, simultaneous and independent measurement of the changes of $[Ca^{2+}]_i$ and the uptake of Mn^{2+} was possible. This procedure has been described in detail elsewhere (17-19).

For single-cell fluorescence measurements, GH_3 cells were allowed to attach to glass coverslips and grown for 24-48 h. Then the coverslips were transferred to wells containing 1 ml of standard medium and 5 μM fura-2/AM and incubated with gentle shaking for 1 h at room temperature. The coverslips were then mounted under the microscope in a chamber thermostated at 36°C and epiilluminated alternately at 340 and 380 nm. Light emitted above 520 nm was recorded by an extended ISIS-M camera (Photonic Science, Robertbridge, East Sussex, U.K.) and analyzed using a Joyce-Loebl Magical image processor (Newcastle, U.K.) with 32 Mbyte video RAM. Four video frames of each wavelength were averaged by hardware, with an overall time resolution of about 3 s for each pair of images at alternate wavelengths. Consecutive frames obtained at 340 and 380 nm excitation were ratioed pixel by pixel and $[Ca^{2+}]_i$ was estimated from F_{340}/F_{380} by comparison with fura-2 standards.

Fura-2/AM was obtained from Molecular Probes, Eugene, Oreg., USA. Nisoldipine was a generous gift from Bayer A.G., Germany. BW755C was generously provided by Dr. S. Moncada, The Wellcome Research Laboratories, Beckenham, Kent, U.K. Gossypol was from Biomol Res. Lab., Plymouth Meeting, Pa., USA. Other chemicals were obtained either from Sigma (London) or from E. Merck, Darmstandt, Germany.

RESULTS

Depolarization of GH_3 (**Fig. 1**, left) or chromaffin cells (Fig. 1, right) with high $[K^+]$ (50 mM) resulted in an increase of $[Ca^{2+}]_i$, evidenced by an increase of F_{340}/F_{380} (upper panels). Depolarization also accelerated the uptake of Mn^{2+}, as evidenced by the quenching of F_{360} (lower panels). These results suggest the opening by depolarization of a plasma membrane pathway that allows the passage of Ca^{2+} and

Mn^{2+}. The increase of $[Ca^{2+}]_i$ and the acceleration of Mn^{2+} entry induced by high K^+ in GH_3 cells were both almost completely prevented by nisoldipine, a dihydropyridine inhibitor of L-type Ca^{2+} channels (Fig. 1*A* and Fig. 1*B*). In chromaffin cells, the increase of $[Ca^{2+}]_i$ induced by high K^+ was only partly prevented by dihydropyridines (Fig. 1*C*). Similar results were found when the uptake of Mn^{2+} was used as the index of Ca^{2+} channels activation (Fig. 1*D*).[4]

Figure 2 shows the effects of econazole, an imidazole antimycotic able to block cytochrome P450 activity (8, 21), on the increase of $[Ca^{2+}]_i$ (upper panel) and the uptake of Mn^{2+} (lower panel) induced by high K^+ in GH_3 cells. The blocking effect of econazole increased in a concentration-dependent manner, half-maximal effect being achieved between 0.2 and 0.5 μM. The effects on the increase of $[Ca^{2+}]_i$ (upper panel) and on the acceleration of Mn^{2+} uptake (lower panel) were similar.

Figure 3 shows the effects of the imidazole antimycotic miconazole on the increase of $[Ca^{2+}]_i$ (upper panel) and on the uptake of Mn^{2+} (lower panel) induced by high K^+ in bovine chromaffin cells. Note that in contrast with the results found with dihydropyridines (Fig. 1), inhibition by miconazole was complete at 5 μM. IC_{50} ranged between 0.5 and 2 μM in different experiments.

Table 1 lists the IC_{50} values estimated in experiments similar to those of Fig. 2 and Fig. 3 for several drugs known to be cytochrome P450 inhibitors. Among imidazole antimycotics, econazole and miconazole were the most potent inhibitors, followed closely by clotrimazole. Ketoconazole was less efficient. Polar imidazole derivatives such as 1-methyl-imidazole or metronidazole had no effect at concentrations of up to 100 μM (results not shown). Among well-known nonimidazolic cytochrome P450 inhibitors, metyrapone had no effect at 200 μM whereas α-naphtoflavone had an IC_{50} value similar to that reported for inhibition of aryl hydrocarbon hydroxylase activity (22). Isosafrole (23) was also an efficient inhibitor. The lipoxygenase inhibitors gossypol and nordihydroguaiaretic acid also inhibited K^+-induced Mn^{2+} uptake, but these drugs are also known to inhibit cytochrome P450 activity (8, 11, 12). Another well-known lipoxygenase inhibitor, BW755C (24), had no effect at 200 μM.

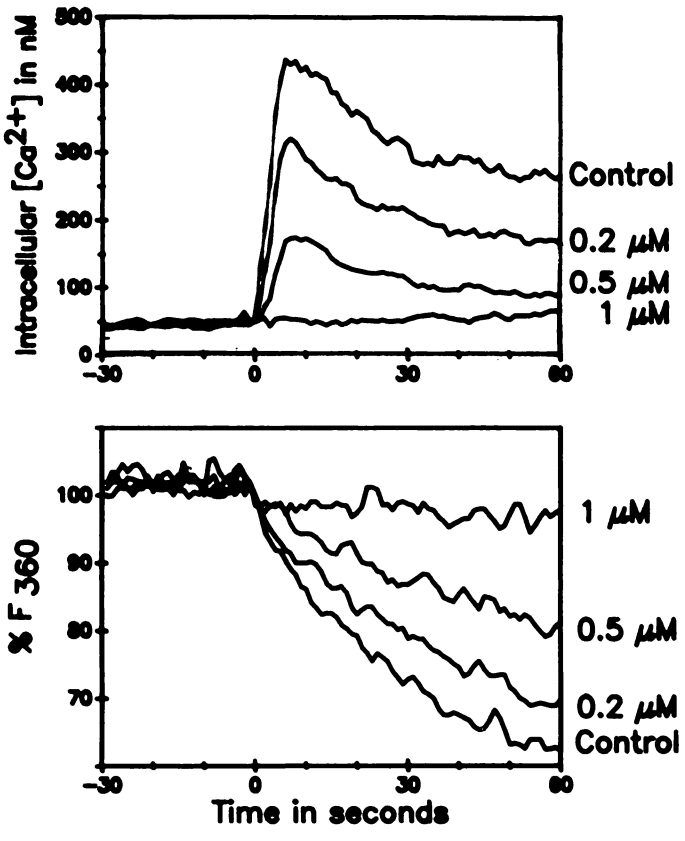

Figure 2. Inhibition by econazole of the entry of Ca^{2+} (upper panel) or Mn^{2+} (lower panel) induced by high K^+ in pituitary GH_3 cells. Different concentrations of econazole, as shown at the right-hand side of the traces, were added 2 min before high K^+. *T'* 0 corresponds to the addition of 45 mM KCl. $[Ca^{2+}]_i$ was estimated from F_{340}/F_{380} (see Methods). Other details are as in Fig. 1. These experiments are representative of three similar ones. For estimates of IC_{50} see Table 1.

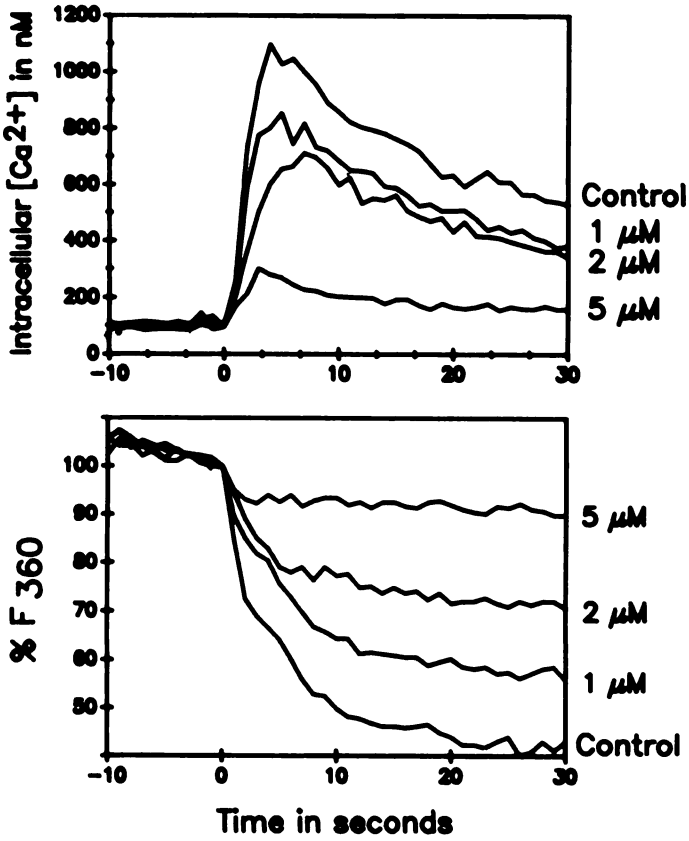

Figure 3. Inhibition by miconazole of the entry of Ca^{2+} (upper panel) or Mn^{2+} (lower panel) induced by high K^+ in bovine chromaffin cells. Details as in Fig. 2. These experiments are representative of three similar ones. For estimates of IC_{50} see Table 1.

Cytochrome P450 is known to bind CO. **Figure 4** shows that treatment with CO had no significant effect on K^+-induced $[Ca^{2+}]_i$ increase (upper panel) or on Mn^{2+} uptake (lower panel) in GH_3 cells.

Figure 5 shows the effects of econazole on the K^+-induced $[Ca^{2+}]_i$ increase measured in a single GH_3 cell. The increase of $[Ca^{2+}]_i$ produced by successive high K^+ pulses (two of which are shown in Fig. 5) was very reproducible. Perfusion with econazole for 1 min before high K^+ depolarization

[4]Inhibition by dihydropyridines (in addition to nitrendipine, nisoldipine, and nimodipine were tested at 1 μM) was variable with cell batches, but always incomplete (range, 10–70% in eight experiments). This relative insensitivity of bovine chromaffin cell Ca^{2+} channels to dihydropyridines agrees with previous findings (20).

TABLE 1. *Values of IC_{50} for inhibition of Ca^{2+} (Mn^{2+}) channels by several cytochrome P450 inhibitors*[a]

Inhibitor	Voltage-gated channels		Receptor-operated channels[b]
	GH_3	Chromaffin	
Econazole	0.2-0.5	0.7-2	0.2-3
Miconazole	0.2-0.5	0.5-2	0.5-3
Clotrimazole	0.6-1	1-2	2-6
Ketoconazole	6-10	5-10	15-25
α-Naphtoflavone	4	15	10
Isosafrole	25-50	—	10-50[c]
Gossypol	10-20	—	6
Nordihydroguaiaretic acid	6	10-20	6-12

[a]The values given for IC_{50} correspond to the ranges observed in different experiments with a design similar to those shown in Fig. 2 and Fig. 3. Three experiments with different cell batches were performed in all cases except for α-naphtoflavone (two experiments in GH_3 and one experiment in chromaffin cells), isosafrole, gossypol, and nordihydroguaiaretic acid (one experiment). The values were similar for inhibition of the increase of $[Ca^{2+}]_i$ and of the entry of Mn^{2+} in GH_3 cells, and usually somewhat larger for inhibition of the $[Ca_2^+]_i$ increase in chromaffin cells. [b]Values obtained for agonist-induced Ca^{2+} (Mn^{2+}) entry in human neutrophils and platelets (10, 11). [c]Unpublished results in neutrophils and platelets.

produced a concentration-dependent inhibition of the $[Ca^{2+}]_i$ increase. This inhibition was slowly reversed on washing of the inhibitor by perfusion with standard medium. In the experiment depicted by Fig. 5, the response was 80% restored after a 25-min washing. Reversal was much faster (completed within 5 min) when 0.5% albumin was added to the washing solution. This suggests that the slowness of reversal on washing with standard medium was due to slow extraction of the highly lipid-soluble drug from the tissue.

The inhibitory effects of econazole were very homogeneous among different cells. Thus in 16 different GH_3 cells analyzed, inhibition of the increase of $[Ca^{2+}]_i$ by 1, 2, and 4 μM of this drug was (mean ± SE) 45.2 ± 1.9, 73.8 ± 1.6, and 81.7 ± 3.0, respectively. The IC_{50} values were larger than those estimated in experiments with cell suspensions (about 1.2 μM for econazole; compare with Table 1). Similar results were found with miconazole (not shown). Perfusion with 50 μM NDGA produced nearly 90% inhibition of the K^+-induced $[Ca^{2+}]_i$ increase. This inhibition was fully reversed by a 5-min washing with standard medium (results not shown).

DISCUSSION

Our results demonstrate that activation of Ca^{2+} channels by depolarization with high K^+ solutions can be detected in fura-2-loaded cells by the increase of $[Ca^{2+}]_i$ and by the entry of Mn^{2+}. This implicitly indicates that Mn^{2+} is able to permeate voltage-gated channels in the cells used here. In agreement with previous findings (6, 7, 20, 25), dihydropyridines blocked most of the depolarization-induced Ca^{2+} (Mn^{2+}) entry in GH_3 cells, but only a part in bovine chromaffin cells (Fig. 1).

Several cytochrome P450 inhibitors were able to prevent the entry of Ca^{2+} and Mn^{2+} through voltage-gated channels. In bovine chromaffin cells the inhibition was larger than with dihydropyridines, suggesting that in addition to L-type channels, other voltage-gated Ca^{2+} entry pathways may be blocked by cytochrome P450 inhibitors. Inhibitors tested included several N_1-substituted imidazole antimycotics, which are considered to be quite selective inhibitors of cytochrome P450 (8, 21, 26, 27). Polar N_1 imidazole derivatives, which have been shown before to be scarcely efficient in inhibiting microsomal cytochrome P450 activity (26, 28), had no effect on voltage-gated Ca^{2+} entry.

Among the other inhibitors tested, α-naphtoflavone had an IC_{50} value in the same order of magnitude as that reported previously for inhibition of aryl hydrocarbon hydroxylase, a cytochrome P450-mediated activity (22, 23, 29). As with aryl hydrocarbon hydroxylase (22), metyrapone, another well-known cytochrome P450 inhibitor, had no effect. This inhibitory profile most resembles that reported previously for IA-type cytochrome P450 (23). However, CO had no effect, suggesting that the structure involved in the control of voltage-gated Ca^{2+} entry is not a cytochrome P450 but a closely related molecule. Our results provide no information on whether it is a part of the ion channel protein or a separate regulatory mechanism. Nordihydroguaiaretic acid has been reported to prevent Ca^{2+} currents in excised membrane patches (9; see below), suggesting that it interacts either directly with the channel or with a membrane-bound regulator.

It has been reported recently that compounds SK&F 96365 and SC 38249 block voltage-gated Ca^{2+} entry in several cell types (30-32). Both compounds are nonpolar N_1-substituted imidazole derivatives, similar in structure to the imidazole antimycotics used here. Also, we have found that SK&F 96365 inhibits cytochrome P450 activity in liver

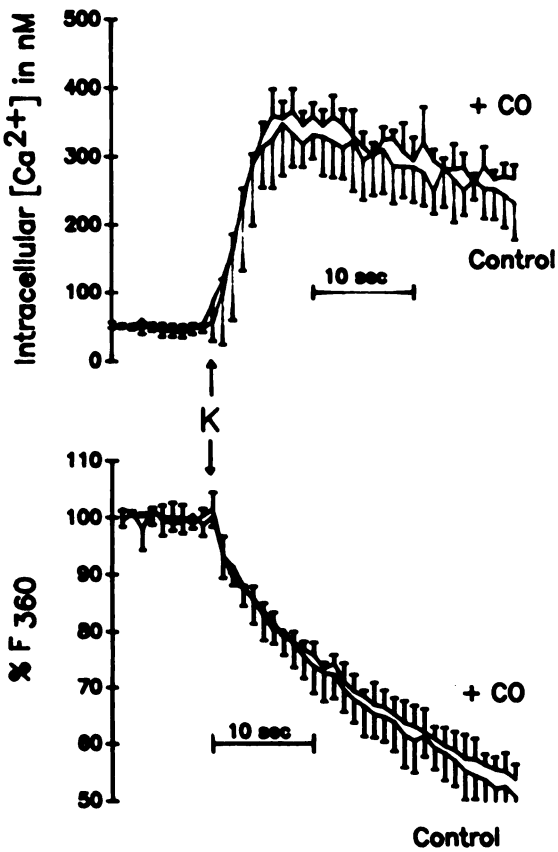

Figure 4. Effects of CO on the increase of $[Ca^{2+}]_i$ (upper panel) and on the entry of Mn^{2+} (lower panel) induced by high K^+ in pituitary GH_3 cells. The cell suspension was equilibrated with CO for 10 min before the addition of Mn^{2+} and K^+ (see Methods). Each value is the mean of four independent data. Vertical bars represent SD. Other details as in Fig. 1.

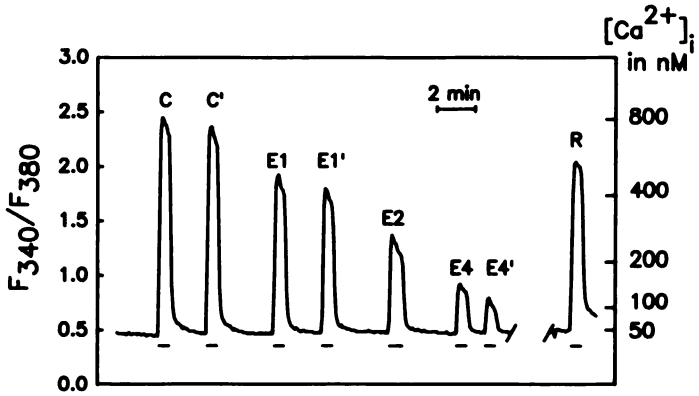

Figure 5. Effects of econazole on the increase of $[Ca^{2+}]_i$ induced by high-K^+ depolarization in a single GH_3 cell. A coverslip with attached fura-2-loaded GH_3 cells (see Methods) was perfused at about 2 ml/min with standard medium followed by a 30-s pulse with high K^+ medium (50 mM KCl replacing the same amount of NaCl, horizontal lines under the traces) and returned to standard medium. Two successive K^+ pulses (C and C') are shown. Then the cells were perfused for 1 min with standard medium containing 1 μM econazole, followed by a 30-s pulse with high K^+ medium. Two of such pulses are shown (E1 and E1'). E2, E4 and E4' correspond to K^+ pulses in cells pretreated for 1 min with 2 and 4 μM econazole, respectively. R corresponds to a 30-s pulse with high K^+ solution after 25 min washing of the cells with standard solution. The values of F_{340}/F_{380}, obtained by dividing pixel by pixel two consecutive frames and averaging those corresponding to the selected cell, are shown. Values of $[Ca^{2+}]_i$, estimated from calibration of a fura-2 solution with the same microscope objective, are shown at right.

microsomes with an IC_{50} value of 10 μM, similar to that reported for inhibition of Ca^{2+} entry (33). These findings suggest that compounds SK&F 96365 and SC 38249 act on voltage-gated Ca^{2+} channels by the same mechanisms as imidazole antimycotics. It has also been reported recently that NDGA is able to inhibit voltage-activated Ca^{2+} currents in GH_3 cells. Both transient and sustained Ca^{2+} currents were inhibited to a similar degree with an IC_{50} of 19 μM (9). This value is close to the one reported here for inhibition of the entry of Ca^{2+} and Mn^{2+} induced by depolarization (Table 1). The effect on Ca^{2+} currents seemed to be independent of the actions on arachidonic acid metabolism, because as was described for BW755C, other lipoxygenase inhibitors had no effect (9).

The inhibitory profile reported here for voltage-gated Ca^{2+} entry is similar to that reported before for inhibition of the agonist-activated Ca^{2+} entry in neutrophils and platelets (10-12) except that the last was also blocked by CO. Cytochrome P450 has also been claimed to reduce the activity of the microsomal ATP-dependent Ca^{2+} pump (34, 35). Overall, these results suggest that the different pathways for Ca^{2+} transport might share either similar structural features or similar regulatory mechanisms. FJ

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