Econazole inhibits thapsigargin-induced platelet calcium influx by mechanisms other than cytochrome *P*-450 inhibition

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Cytochrome *P*-450 has been suggested as a mediator of the signal between depleted platelet calcium stores and an increase in plasma membrane permeability to calcium which follows depletion of the stores. This hypothesis is based on the observations that inhibitors of cytochrome *P*-450, such as the imidazole antifungal agents, also inhibit influx of a calcium surrogate (manganese) into calcium-depleted platelets. We tested the effects of econazole and of a cytochrome *P*-450 inhibitor, carbon monoxide (CO), on thapsigargin (TG)-induced platelet $^{45}Ca^{2+}$ influx. TG specifically depletes internal calcium stores and activates store-regulated calcium influx. Econazole blocked $^{45}Ca^{2+}$ influx when it was added before TG (IC_{50} 11 μM). Econazole at a concentration (20 μM) that inhibited 83 % of TG-induced calcium influx was not inhibitory to TG-induced calcium efflux from $^{45}Ca^{2+}$-loaded platelets, and did not affect calcium fluxes in resting platelets. This econazole concentration was also inhibitory to calcium influx even when it was added after the stores had been calcium-depleted by EGTA and TG for 15 min and the signal to increase calcium influx had already been generated. Inhibition of cytochrome *P*-450 with CO bubbled through platelet suspensions did not change calcium influx in resting cells and potentiated TG-induced calcium influx (160 % of control calcium accumulation at 20 min). This effect appeared to be concentration-dependent, such that a 5 min exposure to CO produced a greater influx potentiation than a 3 min exposure. These observations indicate that (1) cytochrome *P*-450 does not mediate store-regulated calcium influx, and (2) econazole probably inhibits store-regulated calcium influx by an alternative mechanism, such as interaction with plasma membrane calcium channels.

INTRODUCTION

Calcium influx into platelets that have been activated with agonists capable of elevating inositol phosphates has been shown to be regulated, in part, by the level of calcium remaining in internal calcium stores and releasable by inositol 1,4,5-trisphosphate (IP_3) [1,2]. Depletion of calcium stores generates a signal leading to opening of plasma membrane calcium channels and an increased influx of extracellular calcium [3,4]. These channels remain open until stores have been refilled by the action of a Ca^{2+}-ATPase. Evidence for store-regulated calcium influx, also known as capacitative calcium influx [3], has been strengthened by the discovery of a non-phorbol ester tumour promoter, thapsigargin (TG) [5]. This drug depletes calcium stores by inhibiting the Ca^{2+}-ATPase dedicated to refilling them [5,6], without elevation of inositol phosphates [7]. In platelets, TG elevates cytosolic calcium and increases calcium influx [8].

The signal that relates levels of calcium in the stores to an increase in plasma membrane permeability to calcium has not been elucidated, but a number of mechanisms have been proposed [9–12]. One theory suggests that, upon depletion of the calcium stores, a store-associated cytochrome *P*-450 produces a signal which then regulates plasma membrane calcium channels [11]. The potential role of cytochrome *P*-450 has been based on the ability of imidazole antimycotic compounds, which inhibit cytochrome *P*-450 [13,14], to decrease the influx of calcium into a variety of calcium-depleted cells [15,16], including platelets [11,17]. Of these compounds, econazole has been reported to be the most effective inhibitor of calcium influx [11]. Econazole has a chemical structure that is almost identical to that of another compound, SK&F 96365, which has been shown to be a calcium channel blocker that inhibits capacitative calcium influx (IC_{50} 10 μM) in platelets, neutrophils and monocytes [18]. Because of these two possible sites for the action of econazole, we devised experiments aimed at identifying whether this compound could block the signal between the calcium stores and plasma membrane, or whether its inhibitory action could be localized elsewhere, such as at a calcium channel. We tested the effects of econazole, and of a structurally distinct cytochrome *P*-450 inhibitor, carbon monoxide (CO), on TG-induced $^{45}Ca^{2+}$ influx into platelets. Econazole inhibited calcium influx but its mechanism was independent of the signal between stores and plasma membrane and appeared to be localized to the extracellular platelet surface, possibly at a calcium channel. CO potentiated TG-induced calcium influx, indicating that cytochrome *P*-450 is an unlikely generator of the signal between stores and plasma membrane.

MATERIALS AND METHODS

Gel filtration

Human platelets obtained by cytapheresis from normal donors were incubated with 10 μM indomethacin for 15 min at room temperature prior to gel filtration on Sepharose 2B equilibrated with Tyrode's/Hepes solution (concentrations in mM: NaCl 138, KCl 2.9, NaH_2CO_3 12, NaH_2PO_4 0.4, dextrose 5.0, Hepes 5.0; plus 0.35 % BSA, pH 7.4). Collected platelets were adjusted to a count of 750000 cells/μl and bivalent cations (0.2 mM $CaCl_2$, 0.5 mM $MgCl_2$) were added. Platelets were equilibrated for 30 min at room temperature prior to the start of experiments.

Abbreviations used: IP_3, inositol 1,4,5-trisphosphate; TG, thapsigargin.

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Platelet counts were determined on a Baker Instruments System 9000 (Winchester, VA, U.S.A.).

Calcium influx

$^{45}Ca^{2+}$ (0.5 μCi) was added to 0.5 ml of gel-filtered platelet mixture equilibrated at room temperature in 0.2 mM $CaCl_2$ immediately prior to adding TG. After specific time intervals, 220 μl was removed from the mixture, added to 8 μl of EGTA (final concentration of 3.5 mM) and incubated for 2 min. Duplicate 100 μl aliquots of the mixture were layered on 250 μl of phthalate oil mixture (1.5 parts dibutyl phthalate, 1 part dioctyl phthalate) in 0.4 ml Microfuge tubes and centrifuged at 8000 *g* for 30 s. Tubes were then quick-frozen, and the tips containing platelet pellets were cut off and dissolved overnight in 5 ml of scintillation cocktail (Aquasol; Amersham) prior to determination of radioactivity. Econazole was added 5 min before addition of $^{45}Ca^{2+}$ tracer and TG. We used platelets treated with indomethacin (10 μM) to prevent activation during isolation. In previous studies indomethacin did not alter resting platelet calcium influx [19]. However, indomethacin (10 μM) inhibits 20% of the TG-induced calcium influx (results not shown), which can be attributed to generation of thromboxane B_2 and the further interaction of thromboxane B_2 with its receptor [20].

Calcium efflux

Gel-filtered platelets in Tyrode's/Hepes buffer (0.5 mM $MgCl_2$ and 0.2 mM $CaCl_2$) were loaded with $^{45}Ca^{2+}$ (20 μCi/10 ml) for 2 h at room temperature and then refiltered into fresh buffer. Econazole was incubated with the calcium-loaded and refiltered platelets for 5 min prior to addition of TG and sample collection at the first time point. Platelet mixtures were sampled at the indicated time points and processed as described above. Efflux data were interpreted in terms of a two-compartment model [19]. Half-lives for the two phases of efflux were estimated from plots of log(% of total platelet calcium content) versus time.

Depletion of calcium stores

One set of gel-filtered platelets in Tyrode's/Hepes buffer without calcium (0.5 mM EGTA) was incubated with econazole (20 μM) for 5 min. $CaCl_2$ (1 mM), with 5 μM $^{45}Ca^{2+}$ as tracer, was then added, the mixture was sampled for determination of radioactivity at time zero and the cells were activated with TG (1 μM). The second set of platelets in Tyrode's/Hepes buffer (0.5 mM EGTA) was initially incubated with TG (1 μM) only. After 10 min econazole was added to the mixture and at 15 min 1 mM $CaCl_2$ with 5 μM $^{45}Ca^{2+}$ tracer was added, and the time zero sample was taken. The effects of econazole on TG-induced calcium uptake were determined under each condition by comparison with uptake in the absence of drugs.

Exposure of platelets to CO

Gel-filtered platelets (3 ml) in Tyrode's/Hepes buffer (0.5 mM $MgCl_2$, 0.2 mM $CaCl_2$) were bubbled with CO for 3, 5 or 10 min, or with N_2 for 5 min. Gases were passed through round microcapillary pipette tips (PGC Scientifics, Gaithersburg, MD, U.S.A.) at a rate which generated a cluster of 3–4 bubbles, each of 1 mm diam., every 2 s. Control platelets were not bubbled with CO or N_2, but were otherwise treated in the same way. After bubbling, $^{45}Ca^{2+}$ was added as tracer followed by TG (1 μM) and the mixture was sampled as described above.

RESULTS

Thapsigargin-induced $^{45}Ca^{2+}$ influx

Calcium influx in resting and TG-activated platelets was measured with trace quantities of $^{45}Ca^{2+}$ added to 0.2 mM $CaCl_2$ in the medium. The $^{45}Ca^{2+}$ associated with platelet pellets represents internalized platelet calcium, since surface-bound calcium was stripped off with EGTA prior to separation of platelets from media [19]. Figure 1 compares the calcium uptake by resting and TG-treated platelets over 30 min. In resting platelets the rate of calcium uptake was 0.54 pmol/min per 10^8 cells, while in TG-activated platelets the rate of influx was 3.0 pmol/min per 10^8 cells. On subtracting the resting calcium, the TG-specific platelet accumulation was 76 ± 10 pmol of calcium/10^8 cells at 30 min (mean $\pm$ S.E.M., $n = 9$).

Inhibition of TG-induced calcium influx by econazole

The effects of econazole on TG-specific calcium accumulation were tested by comparing platelet $^{45}Ca^{2+}$ content following a 30 min incubation with or without the drug. TG-induced calcium influx was inhibited by econazole with an IC_{50} of 11 μM (Figure 2), while resting calcium influx was not affected. The inhibitory effect of econazole on TG-induced calcium influx was dependent on the extracellular calcium concentration. Econazole (20 μM) decreased TG-induced calcium influx to 17 ± 10% of control (mean $\pm$ S.E.M., $n = 3$) at 0.2 mM $CaCl_2$, but the same concentration of econazole decreased this influx to only 72.5 ± 6.4% of control (mean $\pm$ S.E.M., $n = 3$) at 2 mM $CaCl_2$.

Effects of econazole on TG-induced calcium efflux

To explore further the site of inhibition by econazole, we examined the effect of the drug on calcium efflux from platelets. Gel-filtered platelets were loaded with $^{45}Ca^{2+}$ for 2 h, refiltered into Tyrode's/Hepes buffer containing 0.2 mM unlabelled calcium, and the decrease in internalized platelet $^{45}Ca^{2+}$ in control and TG-treated cells was then determined over time. In control platelets calcium efflux was biphasic, with the first phase having a half-life of 75 min and the second phase having a half-life of

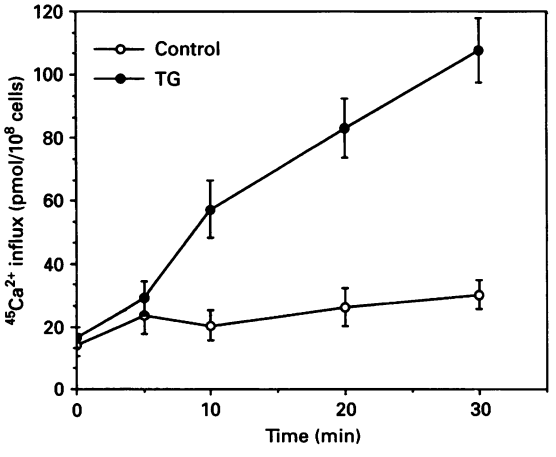

Figure 1 TG-induced calcium influx

$^{45}Ca^{2+}$ uptake by gel-filtered resting platelets (○) and by TG (1 μM)-activated platelets (●) is shown. Results are means $\pm$ S.E.M., $n = 9$, where n indicates the number of donors whose platelets were tested. Within individual experiments, data points were always collected in duplicate and averaged.

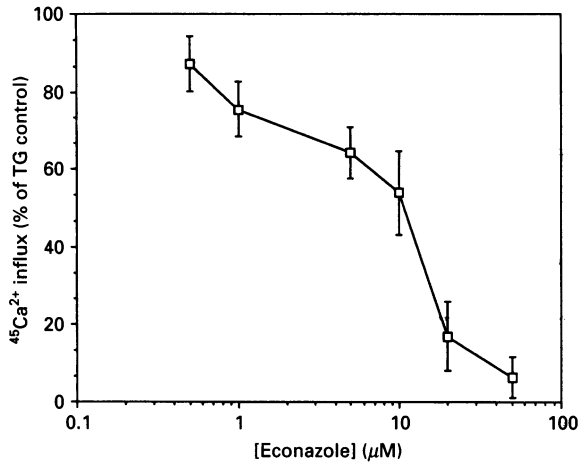

Figure 2 Inhibition of TG-induced calcium uptake by various concentrations of econazole

Gel-filtered platelets in Tyrode's/Hepes buffer (0.2 mM $CaCl_2$) were incubated with econazole for 5 min prior to addition of $^{45}Ca^{2+}$ and TG. The platelet $^{45}Ca^{2+}$ content was determined after 30 min. The resting platelet calcium content was subtracted from that of TG-activated cells; results are expressed as percentages of the TG-specific calcium uptake observed in the absence of econazole. Results are means ± S.E.M., $n = 3$.

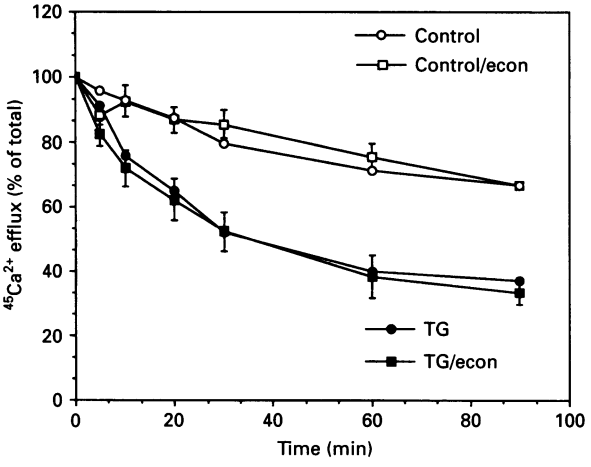

Figure 3 TG-induced calcium efflux

Gel-filtered platelets were loaded with $^{45}Ca^{2+}$ and refiltered into Tyrode's/Hepes buffer (0.2 mM $CaCl_2$). Econazole (econ; 20 μM) was incubated with calcium-loaded platelets for 5 min before addition of TG (1 μM) and sample collection at the first time point. Results are means, $n = 4$. S.E.M.s (error bars) displayed for control curves are representative of the S.E.M.s for the other curves.

170 min. TG-treated cells showed calcium efflux with a first phase half-life of 45 min followed by a second phase half-life of 160 min. Econazole (20 μM) did not change efflux from resting cells or from TG-activated cells (Figure 3).

Effects of econazole on generation of the calcium store-regulated signal

To determine whether econazole influences the generation of the signal to open plasma membrane calcium channels, we tested its

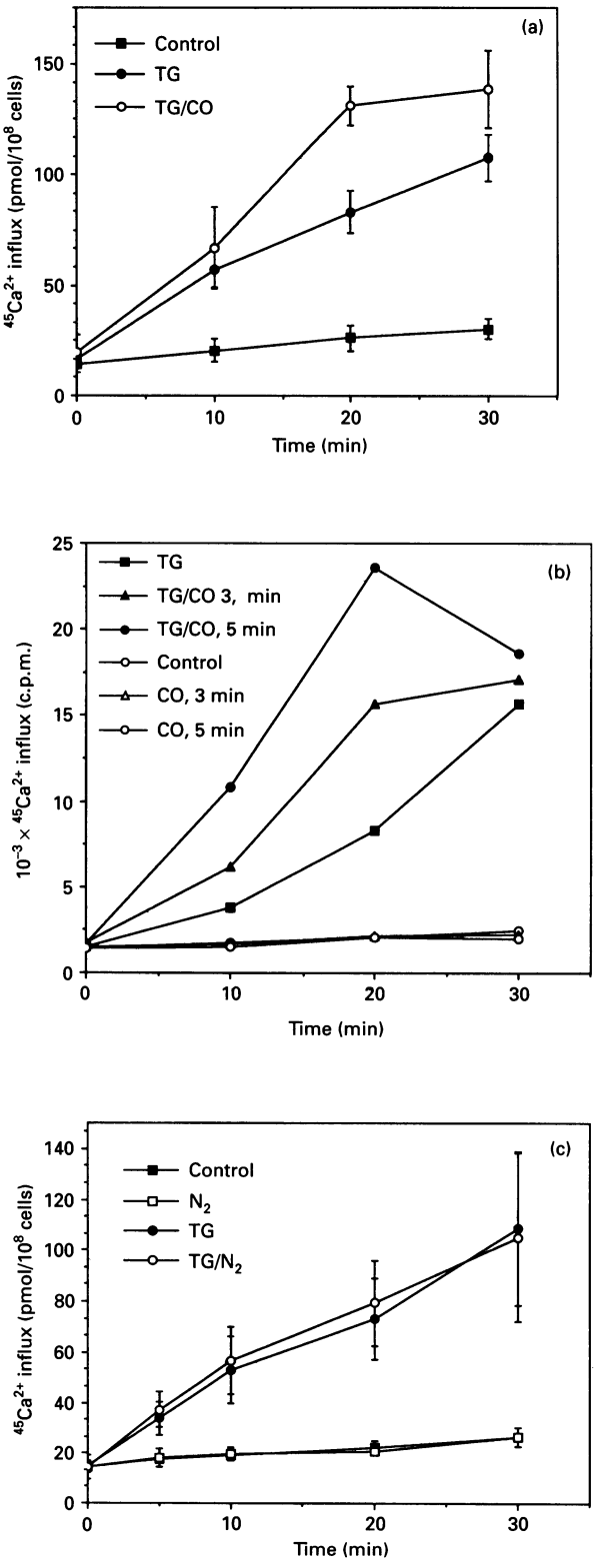

Figure 4 Effect of CO on TG-induced calcium influx

(**a**) Gel-filtered platelets in Tyrode's/Hepes buffer (0.2 mM $CaCl_2$, 0.5 mM $MgCl_2$) were bubbled with CO for 5 min, $^{45}Ca^{2+}$ was added and platelets were activated with TG (1 μM). Platelets were sampled for calcium content as described above. Data points are means ± S.E.M. TG/CO curve (○), $n = 4$; TG curve (●), $n = 9$. CO did not enhance calcium uptake in unactivated platelets. (**b**) A single experiment showing the effects of 3 and 5 min of CO exposure on TG-induced calcium influx. (**c**) Effect of bubbling N_2 at the same rate as with CO on TG-induced calcium influx. Platelets were bubbled with N_2 for 5 min and otherwise treated as for cells bubbled with CO. Data points are means ± S.E.M., $n = 5$.

effect in platelets which had already had their calcium stores depleted. Such cells could have already generated the signal from the stores to the plasma membrane, and any inhibitory effects of the drug on calcium influx would then be localized to other sites. The calcium stores were emptied by adding TG to cells in a calcium-free medium. When extracellular calcium was replaced after 15 min, the extent of calcium influx in these cells was equivalent to the influx in cells where the extracellular calcium was replaced before addition of TG. This observation indicated that the store-regulated channels remained open while the stores were depleted, as has been previously reported [3]. Addition of econazole (20 μM) prior to TG-induced store depletion decreased influx to 53 % of the control, and similar inhibition (to 39 %) was present even when TG was added after depletion of the stores ($n = 2$).

Effect of CO on TG-induced calcium influx

Since econazole appeared to be blocking store-regulated calcium influx by a mechanism other than inhibition of a signal from the stores, we tested the effects of a well-known cytochrome *P*-450 inhibitor on this influx. CO inhibits the activity of purified platelet cytochrome *P*-450 [21] and cytochrome *P*-450 activity in crude platelet microsomes [22]. Gel-filtered platelets were exposed to CO by bubbling the gas into the platelet mixture for 3 or 5 min. A 5 min exposure to CO potentiated the TG-induced calcium accumulation to 160 % of that in control TG-activated platelets at 20 min, and to 130 % of control at 30 min (Figure 4a). The CO effect on activated platelets was dose-dependent (Figure 4b), but CO did not affect calcium influx in resting platelets even with a 10 min exposure. The sensitivity of platelets from different donors varied greatly, with some platelets showing potentiation after a 3 min exposure, whereas in others potentiation was evident only after a 5 or 10 min exposure to CO. The CO effect on TG-induced calcium influx was not due to the physical effects of bubbling, since bubbling chemically inert N_2 gas into the platelet sample did not potentiate calcium influx in control or TG-activated platelets (Figure 4c).

CO is also known to activate guanylate cyclase and to increase platelet cyclic GMP [23]. Inhibition of the enzyme phosphodiesterase with isobutylmethylxanthine (0.5 mM) decreases breakdown of cyclic GMP, but addition of isobutylmethylxanthine did not change the CO effect on TG-induced calcium influx (results not shown).

DISCUSSION

An increase in calcium influx into cells induced by TG defines the presence of capacitative calcium influx [7,24]. TG has high affinity for the microsomal Ca^{2+}-ATPase, with a dissociation constant in the nanomolar range [6,25] and, once introduced into the cell, it permanently blocks refilling of the calcium stores. Thus a signal to increase the plasma membrane permeability to calcium generated by depletion of the calcium stores cannot be reversed by influx of calcium, since uptake into the stores is blocked. The increase in plasma membrane permeability will therefore persist, and in our system the TG-induced calcium flux is evident even 2 h after initiation. Under these conditions the signal from the stores to the plasma membrane can be considered to be a single event induced by a rise in cytosolic calcium or by loss of calcium from the stores. An equally plausible hypothesis is that the signal is a continuously generated metabolite, the production of which is required for continued elevation of calcium influx.

We were able to show that econazole blocked TG-induced platelet capacitative influx. The effect of econazole was not secondary to preventing release of stored calcium by TG, since the concentration of econazole which blocked 83 % of this influx did not affect TG-induced calcium efflux. This discrepancy of effects on influx and efflux suggests that exiting calcium may traverse the plasma membrane by another pathway, such as through the action of a plasma membrane Ca^{2+}-ATPase, or that the channel inhibition by econazole is unidirectional. The latter possibility is supported by the observation that elevated extracellular calcium levels decrease the inhibitory effectiveness of econazole, suggesting that econazole and calcium may compete for the same extracellular binding site.

The inhibitory effect of econazole was also demonstrable when calcium stores had been depleted by TG prior to applying econazole. Under such conditions a signal from the stores had already been generated and the plasma membrane calcium channels were open. If econazole were a specific inhibitor of a single event signal, it should have been ineffective once the signal had been generated. Inhibition of calcium influx by econazole even after depletion of the stores thus indicates either that econazole can inhibit a continuously generated signal or that it inhibits at a site different from that where the signal is produced. Such a signal is not generated by cytochrome *P*-450, since CO, a well-known inhibitor of cytochrome *P*-450, potentiated rather than inhibited the TG-induced calcium influx. Recently it has been demonstrated that cytochrome *P*-450-generated metabolites of arachidonic acid inhibit TG-induced calcium influx in platelets [26]. Arachidonic acid is released secondarily to activation of phospholipase A_2 by elevated cytosolic calcium, as occurs during TG activation [27,28]. A likely explanation of our observation is that when CO inhibits cytochrome *P*-450 it prevents generation of the inhibitory arachidonic metabolites, which then results in increased calcium influx.

In previous studies CO was shown to inhibit thrombin-induced calcium influx, and the mechanism of inhibition was concluded to be inhibition of cytochrome *P*-450 [11]. It is now appreciated that CO can have a variety of effects on living cells [29]. In particular, it has been demonstrated that CO activates guanylate cyclase and elevates cellular cyclic GMP [23]. In platelets it has been shown that CO elevates cyclic GMP [30] and prevents agonist-induced platelet activation [31,32]. The site of inhibition has been localized to prevention of phospholipase C activation by cyclic GMP, thus leading to decreased inositol phosphate production and decreased release of internal calcium stores by IP_3 [30]. It is therefore very likely that previous experiments looking at the effects of CO on thrombin-activated platelets which demonstrated a decrease in thrombin-induced calcium influx were only showing the CO-mediated inhibition of phospholipase C. In TG-activated platelets phospholipase C is not required to deplete the stores [7], and CO was found not to be inhibitory to TG-induced calcium influx.

Since functional cytochrome *P*-450 is not required for capacitative calcium influx, the inhibition of a continuous cytochrome *P*-450-generated signal by econazole is also excluded. Based on the structural similarity of econazole to the calcium channel blocker SK&F 96365, the reversal of econazole-induced inhibition of influx by high extracellular calcium concentrations and the inhibition by econazole of calcium influx but not efflux, we conclude that the localization of the econazole interaction appears to be at a plasma membrane calcium channel.

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