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Novel antimigraineur dotarizine releases Ca^{2+} from caffeine-sensitive Ca^{2+} stores of chromaffin cells

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1 The novel antimigraineur, dotarizine (30 μM), increased cytosolic Ca^{2+} concentration, $[Ca^{2+}]_c$, in fura-2-loaded bovine adrenal chromaffin cells. This increase was transient, reached a peak in about 2–5 min (0.53 ± 0.07 μM; $n = 19$) and then declined to basal levels over a further 5 min period.

2 This transient rise of $[Ca^{2+}]_c$ was mimicked by 1 μM thapsigargin and by 30 μM cyclopiazonic acid (CPA), but not by 30 μM flunarizine. Both thapsigargin and CPA occluded the effects of dotarizine and vice versa.

3 All three compounds suppressed the transient $[Ca^{2+}]_c$ rises induced by caffeine (10 mM, 10 s); blockade induced by thapsigargin was irreversible and that induced by CPA and dotarizine was reversible.

4 Of the three compounds, only dotarizine blocked reversibly the $[Ca^{2+}]_c$ spikes induced by short pulses of high K^+ (70 mM, 5 s), suggesting that dotarizine blocks voltage-dependent Ca^{2+} channels but CPA and thapsigargin do not.

5 Dotarizine caused a gradual and reversible depletion of endoplasmic reticulum (ER) Ca^{2+} in chromaffin cells transfected with ER-targeted aequorin. CPA had a similar effect.

6 These data show that dotarizine shares with thapsigargin and CPA the ability to deplete Ca^{2+} in the ER; this novel action of dotarizine could be relevant to its prophylactic effects in migraine. Unlike thapsigargin and CPA, however, dotarizine additionally and reversibly blocks Ca^{2+} entry through voltage-dependent Ca^{2+} channels.

Keywords: Dotarizine; cyclopiazonic acid; thapsigargin; caffeine; intracellular calcium; chromaffin cell

Abbreviations: $[Ca^{2+}]_c$, cytosolic $[Ca^{2+}]$; $[Ca^{2+}]_{ER}$, endoplasmic reticulum $[Ca^{2+}]$; CPA, cyclopiazonic acid; DMEM, Dulbecco's modified Eagle medium; SERCA, sarco-endoplasmic reticulum Ca^{2+} ATPase; ER, endoplasmic reticulum

Introduction

Dotarizine 1-(diphenylmethyl)-4-[3-(2-phenyl-1,3-dioxalan-2-yl-)-propyl]-piperazine, was designed as a prophylactic agent for migraine (Galiano *et al*., 1993; Horga *et al*., 1996). Its antimigraine actions may be associated with its ability to block 5-HT receptors (Brasó *et al*., 1996; Montiel *et al*., 1997) and voltage-dependent Ca^{2+} channels (Tejerina *et al*., 1993; Villarroya *et al*., 1995).

In the course of a study to compare the effects of dotarizine with those of the parent compound, flunarizine, on chromaffin cell viability and cytosolic Ca^{2+} levels, $[Ca^{2+}]_c$, we casually observed that dotarizine (but not flunarizine) caused a transient substantial increase of $[Ca^{2+}]_c$ (Novalbos *et al*., 1999). The present study was designed to investigate the mechanism involved in the elevation of $[Ca^{2+}]_c$ induced by dotarizine. One possible target for the drug is the smooth endoplasmic reticulum Ca^{2+} ATPase (SERCA); thus the effects of dotarizine on $[Ca^{2+}]_c$ were compared with those of thapsigargin, an irreversible blocker of SERCA (Lytton *et al*., 1991; Kijima *et al*., 1991) and cyclopiazonic acid (CPA), a reversible blocker of the enzyme (Demaurex *et al*., 1992). The use of aequorin targeted to the ER (Montero *et al*., 1997; Alonso *et al*., 1999), that directly measures the changes of lumenal Ca^{2+} concentrations in the ER, $[Ca^{2+}]_{ER}$, provided direct, compelling evidence that dotarizine causes the release of ER Ca^{2+} in chromaffin cells.

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Methods

Preparation and culture of bovine chromaffin cells

Bovine adrenal medullary chromaffin cells were isolated as previously described (Livett, 1984) with some modification (Moro *et al*., 1990). After isolation, cells were suspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal calf serum, 10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, 50 IU ml^{-1} penicillin and 50 $\mu g\ ml^{-1}$ streptomycin. Cells were plated on 1 cm diameter glass coverslips pretreated with 0.01 mg ml^{-1} of poly-d-lysine at a density of 5×10^4 cells per coverslip. Cells were used 1–3 days after plating.

Measurements of $[Ca^{2+}]_c$

Chromaffin cells were loaded with fura-2 by incubating them with fura-2/AM (4 μM) for 30 min at room temperature in Krebs-HEPES solution (pH 7.4) containing (in mM): NaCl

145, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2.5, sodium HEPES 10, glucose 10. The loading incubation was terminated by several washes of the coverslip containing the attached cells, using Krebs-HEPES. Then, cells were kept at 37°C in the incubator for 15–30 min. The fluorescence of fura-2 in single cells was measured with a photomultiplier-based system described by Neher (1989), which produces a spatially averaged measure of the $[Ca^{2+}]_c$. Fura-2 was excited with light alternating between 360 and 390 nm, using a Nikon 40× fluorite objective. Emitted light was transmitted through a 425 nm dichroic mirror and 500–545 nm barrier filter before being detected by the photomultiplier. $[Ca^{2+}]_c$ was calculated from the ratios of the light emitted when the dye was excited by the two alternating excitation wavelengths (Grynkiewicz *et al*., 1985).

Measurements of $[Ca^{2+}]_{ER}$ with targeted aequorin

Construction, packaging, and titering of the pHSVerAEQ amplicon vector and expression in chromaffin cells have been recently described (Alonso *et al*., 1999). Chromaffin cells (5×10^5 cells/0.5 ml) were routinely infected with 1.2×10^4 i.v.u. 1 day before measurements. The percentage of cells expressing ER-targeted aequorin was usually around 20%. Aequorin photoluminescence measurements were performed essentially as described (Alonso *et al*., 1999). Cells were depleted of Ca^{2+} by incubation for 5–10 min at 37°C with 10 μM of the SERCA inhibitor 2,5-di-terbutil-benzohydroquinone (BHQ) in standard medium containing (in mM): NaCl 145, KCl 5, $MgCl_2$ 1, glucose 10, HEPES 10, pH 7.4, supplemented with 3 mM EGTA. Cells were then incubated for 1 h at room temperature in standard medium containing 0.5 mM EGTA, 10 μM BHQ, and 1 μM coelenterazine *n*. The coverslip was then placed in the perfusion chamber of a purpose-built thermostatized luminometer and standard medium containing 1 mM Ca^{2+} was perfused to refill the ER with Ca^{2+}. Measurements were performed at 22°C and $[Ca^{2+}]_{ER}$ values were calculated from the luminiscence records using a computer algorithm (Brini *et al*., 1995) which follows the calibration curve reported before (Barrero *et al*., 1997). The total number of counts obtained ranged between 0.3 and 2 million.

Materials and solutions

Dulbecco's modified Eagle medium (DMEM), penicillin, streptomycin and foetal calf serum were obtained from GIBCO, Madrid, Spain. Collagenase from *Clostridium histolyticum* was from Roche Molecular Biochemicals. Dotarizine was obtained from Grupo Ferrer, Barcelona, Spain. Flunarizine was obtained from the Janssen Research Foundation, Belgium. CPA, thapsigargin, BHQ, cytosine arabinoside and fluorodeoxyuridine were obtained from Sigma. Coelenterazine *n* and fura-2-AM were obtained from Molecular Probes.

Dotarizine and flunarizine were dissolved in dimethylsulphoxide (DMSO, Merck) at 10^{-2} M and diluted in Krebs-HEPES solution. At the highest concentration used, 0.3% DMSO had no effect on $[Ca^{2+}]_c$. CPA and thapsigargin were dissolved in an 80% alcoholic solution at 10^{-2} M, and diluted to the desired concentrations. At the highest concentration used (0.24%), ethanol had no effect on $[Ca^{2+}]_c$ changes. All other chemicals were reagent grade.

Statistical analysis

Averaged data are means±s.e.mean. Analysis of variance (ANOVA) was applied to see differences between groups. When significant differences were found, an appropriate multiple comparison test was done (Student-Newman-Kleus). The level of significance was taken as $P<0.05$. Analysis was performed using SPSS software for Windows.

Results

Effects of dotarizine, flunarizine, cyclopiazonic acid and thapsigargin on the basal cytosolic concentrations of Ca^{2+}

After a brief delay (seconds), exposure to 30 μM dotarizine of a fura-2-loaded chromaffin cell superfused continuously with normal Krebs-HEPES solution (2.5 mM Ca^{2+}), led to a prompt increase in $[Ca^{2+}]_c$ that reached a peak in about 2–5 min and then gradually declined to basal levels in about 5 min (Figure 1a). In 19 cells the $[Ca^{2+}]_c$ peak reached an

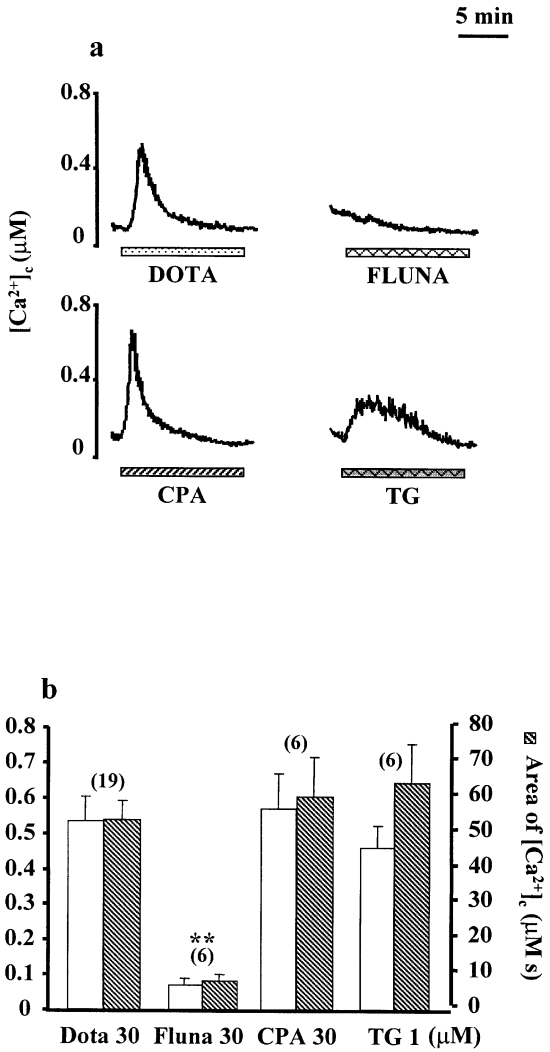

Figure 1 (a) shows the effects of dotarizine (DOTA, 30 μM), flunarizine (FLUNA, 30 μM), cyclopiazonic acid (CPA, 30 μM) and thapsigargin (TG, 1 μM) on cytosolic Ca^{2+} levels, $[Ca^{2+}]_c$. These original traces were obtained in four separate fura-2-loaded chromaffin cells. Ordinates show the $[Ca^{2+}]_c$ in μM; see calibration bar on top right for the time. (b) shows the averaged changes of $[Ca^{2+}]_c$ induced by the compounds in the number of cells shown in parentheses. The left ordinate expresses the magnitude of the $[Ca^{2+}]_c$ signals generated by each compound at the μM concentrations shown in the bottom of each pair of bars. Data are means±s.e.mean. $^{**}P<0.01$ with respect to the other compounds.

average of 0.53 ± 0.07 μM (Figure 1b). In contrast to dotarizine, its parent compound flunarizine (30 μM) did not produce an elevation of $[Ca^{2+}]_c$. CPA (30 μM) increased the $[Ca^{2+}]_c$ with a time course and amplitude similar to dotarizine; thapsigargin (1 μM) also increased the $[Ca^{2+}]_c$ but with a slower time course. Figure 1b shows that the increase of $[Ca^{2+}]_c$ induced by CPA and thapsigargin were similar to those induced by dotarizine; this was true for the magnitude of the peak as well as for the areas of the $[Ca^{2+}]_c$ increments.

Attempts were made to establish a concentration-response relationship for the increase of $[Ca^{2+}]_c$ induced by dotarizine. In the fura-2-loaded cell shown in Figure 2, K^+ challenges were given during superfusion with increasing concentrations of dotarizine. At 10 μM dotarizine did not change the basal $[Ca^{2+}]_c$ but did however reduce the K^+ signal from 1.4 μM to 0.35 μM. At 30 μM dotarizine produced its typical gradual elevation of $[Ca^{2+}]_c$ and fully suppressed the K^+ response. This quickly recovered upon dotarizine washout. At 50 μM dotarizine produced a sharper $[Ca^{2+}]_c$ elevation that reached a peak of 0.45 μM; again, the K^+ response was abolished but recovered partially after dotarizine washout.

Effects of dotarizine and CPA on $[Ca^{2+}]_c$ transients induced by high K^+ and caffeine

In the fura-2-loaded cell shown in Figure 3a the initial basal $[Ca^{2+}]_c$ was 0.1 μM. An initial K^+ pulse (70 K^+/2.5 Ca^{2+}, 5 s) caused a transient elevation of the $[Ca^{2+}]_c$ that peaked at 1.9 μM. The subsequent application of caffeine (10 mM, 10 s) gave rise to a transient $[Ca^{2+}]_c$ peak of similar magnitude (1.6 μM). It is interesting that the time course and shape of both peaks were similar. Thus, it seems that Ca^{2+} entering through voltage-dependent Ca^{2+} channels (the case of K^+ stimulation) and Ca^{2+} released from intracellular stores (the case of caffeine stimulation) can provide global $[Ca^{2+}]_c$ rises quite similar in bovine chromaffin cells (see also Lara *et al*., 1997).

After the initial challenges with K^+ and caffeine the cell shown in Figure 3a was superfused with 30 μM dotarizine. This caused a gradual $[Ca^{2+}]_c$ rise which peaked at 0.4 μM and then started to decline. During this decline, the application of a K^+ pulse did not increase the $[Ca^{2+}]_c$; this was likely due to blockade by dotarizine of Ca^{2+} entry through voltage-dependent Ca^{2+} channels (Villarroya *et al*., 1995). The subsequent application of caffeine produced only a tiny elevation of $[Ca^{2+}]_c$. The simplest explanation for the suppression of the caffeine response is that dotarizine elevated the $[Ca^{2+}]_c$ by liberating Ca^{2+} from intracellular stores, thereby causing their depletion. This seems to be the case, since the subsequent application of CPA (30 μM) did not cause an elevation of $[Ca^{2+}]_c$. It is interesting that the K^+ response remained during superfusion with CPA, indicating that contrary to dotarizine, this compound did not block Ca^{2+} channels. The caffeine response, however, remained suppressed, suggesting that the Ca^{2+} stores continued depleted. A partial recovery of the caffeine response was seen after washout of CPA.

The cell shown in Figure 3b produced initial $[Ca^{2+}]_c$ peaks to K^+ and caffeine similar to those found in Figure 3a (near 2 μM). In this case, CPA was given first and then, dotarizine. CPA produced a slow $[Ca^{2+}]_c$ rise that reached a plateau at around 0.25 μM (Figure 3b). In three additional cells the averaged $[Ca^{2+}]_c$ amounted to 0.57 ± 0.1 μM. The K^+ response was preserved in the presence of CPA but the caffeine signal was largely suppressed. The substitution of CPA for dotarizine (30 μM) did not cause an increase in $[Ca^{2+}]_c$. This time, both the K^+ and the caffeine responses were cancelled; these responses recovered gradually and fully upon washout of dotarizine.

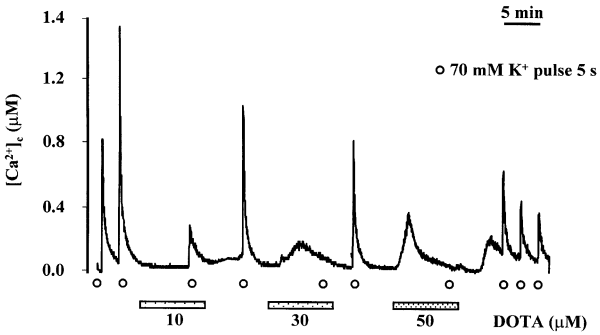

Figure 2 Effects of increasing concentrations of dotarizine on basal and K^+-induced increases of $[Ca^{2+}]_c$. This experiment was performed in a fura-2-loaded cell continuously superfused with Krebs-HEPES solution containing 2 mM Ca^{2+}. K^+ pulses (70 mM K^+ during 5 s) were applied as indicated by white circles. Dotarizine (DOTA) was given at the μM concentrations shown by the numbers at the bottom. Similar results were obtained in three additional cells.

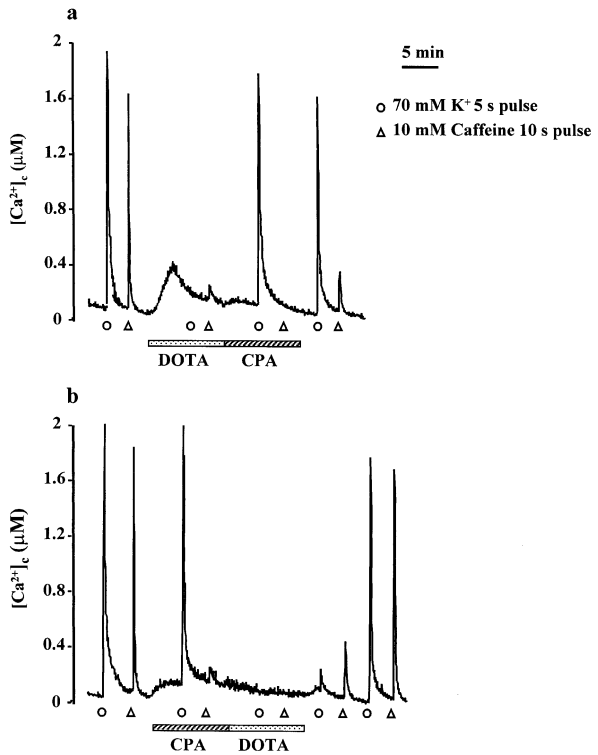

Figure 3 Effects of dotarizine (DOTA) and cyclopiazonic acid (CPA) on the $[Ca^{2+}]_c$ transients induced by high K^+ and caffeine in fura-2-loaded chromaffin cells. Cells were continuously superfused with Krebs-HEPES containing 2.5 mM Ca^{2+}. K^+ pulses (70 mM, 5 s) and caffeine pulses (10 mM, 10 s) were applied as indicated by their respective symbols at the bottom of the traces. DOTA (30 μM) and CPA (30 μM) were sequentially superfused during the time periods shown by the horizontal bars (a). (b) shows a cell first treated with CPA and then with DOTA. The $[Ca^{2+}]_c$ changes are expressed in μM (ordinate); time can be calculated using the calibration bar on top right. This experiment was repeated in five additional cells from different cultures with similar results.

Effects of dotarizine and thapsigargin on $[Ca^{2+}]_c$ transients induced by high K^+ and caffeine

Experiments similar to those described above were performed to compare dotarizine with thapsigargin, a compound that causes irreversible blockade of SERCA (Lytton *et al*., 1991). The cell shown in Figure 4a was initially challenged with K^+ and caffeine. This produced initial $[Ca^{2+}]_c$ peaks of 1.8 μM and 0.85 μM, respectively. Dotarizine caused a slow elevation of $[Ca^{2+}]_c$ that peaked at 0.8 μM, and then declined to near basal levels in about 5 min. Added immediately after removal of dotarizine, thapsigargin (1 μM) did not modify the $[Ca^{2+}]_c$. A K^+ challenge applied while thapsigargin was being superfused, induced a sharp $[Ca^{2+}]_c$ peak of 1.7 μM; this indicates that thapsigargin does not block Ca^{2+} channels. After washout of thapsigargin the K^+ response remained, but the caffeine response was nearly fully suppressed.

In the cell shown in Figure 4b, thapsigargin was applied first and then this compound was substituted by dotarizine. A few seconds after adding thapsigargin, the basal $[Ca^{2+}]_c$ tended to increase slowly. In the presence of thapsigargin, an initial caffeine pulse produced a rise in $[Ca^{2+}]_c$ to 0.6 μM, likely due to an incomplete depletion of the Ca^{2+} store because enough time was not allowed before adding caffeine, a subsequent caffeine challenge failed to produce a $[Ca^{2+}]_c$ peak, while the K^+ pulse produced a peak of 1.9 μM. The subsequent application of 30 μM dotarizine did not modify the basal $[Ca^{2+}]_c$; in the presence of the compound both caffeine and K^+ pulses failed to evoke $[Ca^{2+}]_c$ spikes. Washout of dotarizine allowed the recovery of the K^+ response but not that of caffeine; this was surely due to the previous treatment with thapsigargin which causes an irreversible blockade of SERCA thus preventing the refilling of the caffeine-sensitive Ca^{2+} stores.

Quantitative analysis of the results obtained in various separate experiments using these protocols provided the following results. Control peak $[Ca^{2+}]_c$ induced by K^+ pulses were 1.8 ± 0.11 μM ($n = 20$ pulses). In the presence of CPA the peaks amounted to 2 ± 0.16 μM ($n = 6$ pulses); in contrast, in the presence of dotarizine the K^+-induced $[Ca^{2+}]_c$ peaks were drastically reduced to near basal values (0.12 ± 0.01 μM; $n = 6$ pulses; $P < 0.001$). In the presence of thapsigargin the K^+ peaks were 1.6 ± 0.18 μM ($n = 6$ pulses). Control caffeine-induced $[Ca^{2+}]_c$ peaks amounted to 1.2 ± 0.11 μM ($n = 16$ caffeine pulses). In the presence of dotarizine the caffeine pulses were reduced to 0.14 ± 0.03 μM ($n = 8$ pulses; $P < 0.001$). In the presence of CPA the caffeine peaks were reduced to

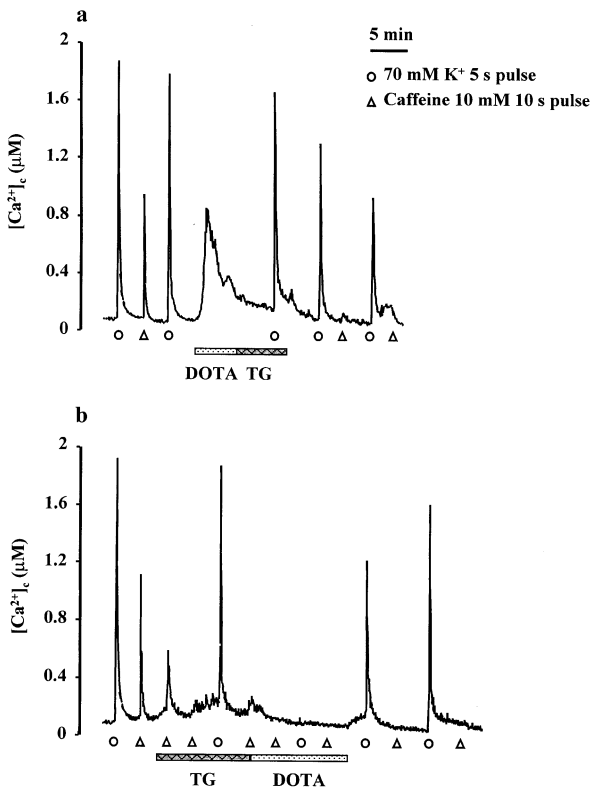

Figure 4 Effects of dotarizine (DOTA) and thapsigargin (TG) on the $[Ca^{2+}]_c$ transients induced by high K^+ and caffeine. Cells were continuously superfused with Krebs-HEPES containing 2.5 mM Ca^{2+}. K^+ pulses (70 mM, 5 s) and caffeine pulses (10 mM), 10 s) were applied as indicated by their respective symbols at the bottom of the traces. DOTA (30 μM) and thapsigargin (1 μM) were sequentially superfused during the time periods shown by the horizontal bars. (b) shows a cell superfused firstly with thapsigargin and then dotarizine. The $[Ca^{2+}]_c$ changes are expressed in μM (ordinate); time can be calculated using the calibration bar. This experiment was repeated in six additional cells from different cultures with similar results.

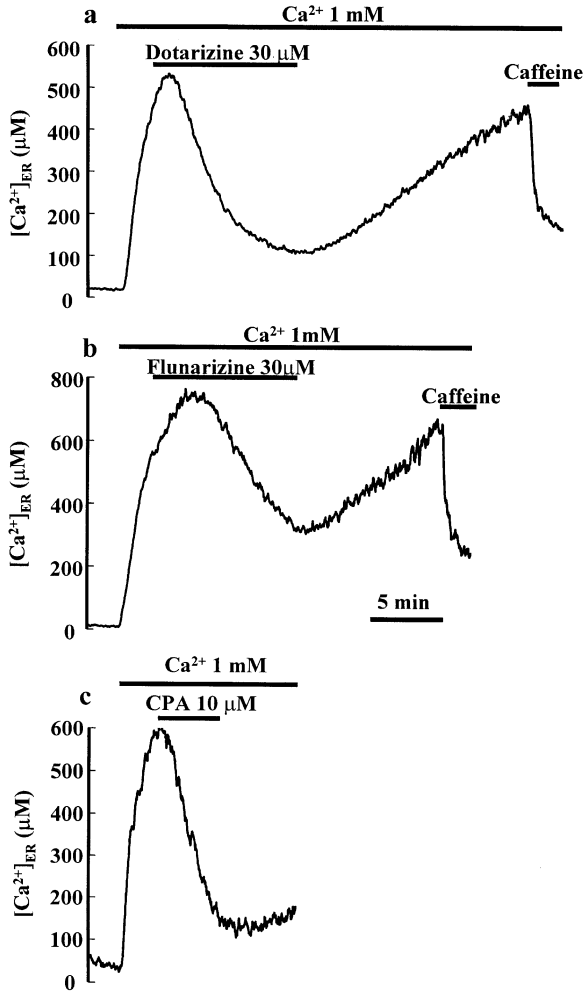

Figure 5 Effects of dotarizine, flunarizine and cyclopiazonic acid (CPA) on endoplasmic reticulum Ca^{2+} concentrations, $[Ca^{2+}]_{ER}$ (ordinates), in chromaffin cells transfected with ER-targeted aequorin (see Methods). Ca^{2+} (1 mM) was given as shown by top horizontal bars. After store Ca^{2+} refilling, the compounds were given at the concentrations shown in (a) (dotarizine), (b) (flunarizine) and (c) (CPA). Caffeine (10 mM) was given at the end of experiments (a and b). Similar results were obtained in three (dotarizine and flunarizine) and five additional experiments (CPA).

0.18 ± 0.04 μM ($n=6$ pulses; $P<0.001$). Finally, in the presence of thapsigargin the caffeine peaks were reduced to 0.16 ± 0.05 μM ($n=6$ pulses; $P<0.001$).

Direct measurements of $[Ca^{2+}]_{ER}$ using targeted aequorin: effects of dotarizine, flunarizine and CPA

A more direct approach to study the effects of dotarizine on $[Ca^{2+}]_{ER}$ is the measurement of changes in lumenal ER Ca^{2+} concentrations by using ER-targeted aequorin (Alonso *et al*., 1999). In the experiments shown in Figure 5, cells were superfused first with a $0Ca^{2+}$/EGTA solution. To refill the ER with Ca^{2+}, 1 mM Ca^{2+} was reintroduced, as shown by the top horizontal bars. The $[Ca^{2+}]_{ER}$ increased gradually to reach a peak in the range of 500–800 μM. Dotarizine (30 μM) caused a gradual decrease in $[Ca^{2+}]_{ER}$ that, in the case of the experiment shown in Figure 5a, caused above 80% of ER Ca^{2+} depletion. Upon washout of dotarizine, $[Ca^{2+}]_{ER}$ recovered slowly to reach near 90% of the initial refilling value. Caffeine (10 mM) then caused a fast Ca^{2+} release from the ER. Figure 5b shows that although slower, flunarizine (30 μM) also caused a gradual $[Ca^{2+}]_{ER}$ depletion; the extent of depletion was smaller (about 55%). The effect of flunarizine was also slowly reversible and caffeine also induced fast Ca^{2+} release. The third experiment was performed with 10 μM CPA, that caused a faster $[Ca^{2+}]_{ER}$ release of over 80% (Figure 5c). The $t_{1/2}$ for the rate of $[Ca^{2+}]_{ER}$ depletion was 115 ± 30 s for dotarizine ($n=4$), 110 ± 10 s for CPA ($n=6$), and 8 ± 3 min for flunarizine ($n=4$). The difference of the rate of ER Ca^{2+} release between dotarizine and flunarizine were significant at $P<0.007$. Therefore, dotarizine releases Ca^{2+} at the same rate as CPA, but 4 fold faster than flunarizine.

In the experiments shown in Figure 6, the extracellular Ca^{2+} and the compounds were simultaneously added to the solution superfusing the cells. Dotarizine (30 μM) decreased the extent of store refilling to about 400 μM and then it caused a drastic store emptying (Figure 5a). Upon dotarizine washout, the store was slowly refilled to values higher than the initial. Flunarizine (Figure 5b) prevented store refilling to a smaller extent than dotarizine and caused a subsequent milder store depletion. Recovery upon washout was slow and reached values that also were above the initial values of store refilling.

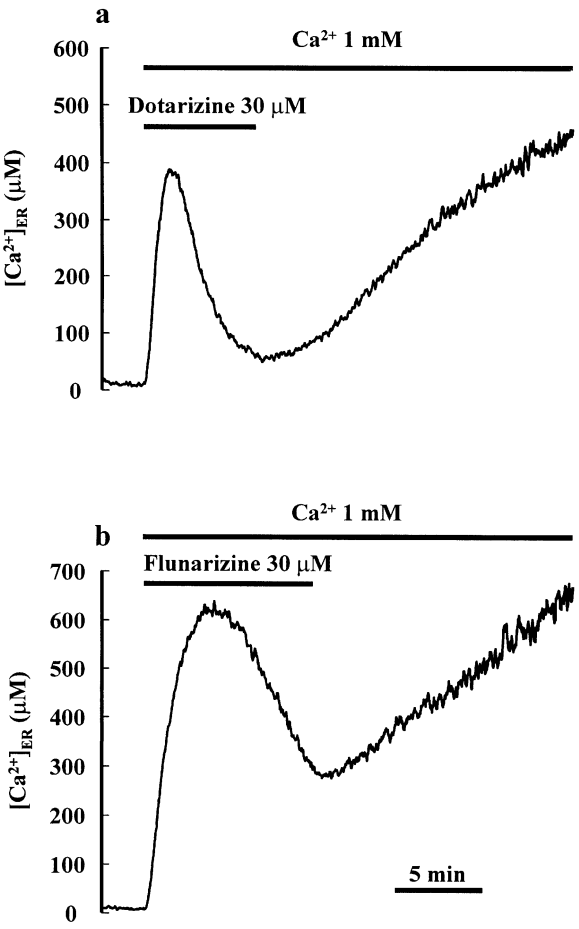

Figure 6 Effects of dotarizine and flunarizine on the refilling and emptying of ER Ca^{2+} stores in chromaffin cells transfected with ER-targeted aequorin. The compounds (30 μM) each were given simultaneously with Ca^{2+} reintroduction, as shown by top horizontal bars.

Discussion

The results of this study show a new pharmacological target for the novel antimigraine drug dotarizine, i.e. a Ca^{2+}-releasing effect from internal ER stores of excitable cells. Being a lipophilic compound (log $P=6$; Novalbos *et al*., 1999), dotarizine can certainly cross the plasma membrane and target ER endomembranes. It is interesting that the blocking actions of dotarizine on Ca^{2+} entry and $[Ca^{2+}]_c$ increase in cells stimulated with K^+, quickly reversed upon washout (Figures 2, 3 and 4). In contrast, the ER previously depleted of Ca^{2+} by dotarizine recovered its normal $[Ca^{2+}]_{ER}$ very slowly. This may be explained by the fact that the dotarizine, that binds to Ca^{2+} channels in the plasmalemma, diffuses away rapidly because this superficial membrane is in contact with the superfusion medium bathing the cells. This was not the case for dotarizine that targeted endomembranes, which are more abundant than plasmalemmal membranes and additionally, are sited away from the cell superfusion solutions.

The use of ER-targeted aequorin allowed direct measurements of the effect of dotarizine and flunarizine on $[Ca^{2+}]_{ER}$ (Montero *et al*., 1997). Both dotarizine and CPA produced a rapid and reversible decrease in $[Ca^{2+}]_{ER}$ with half-times around 2 min. It is puzzling that flunarizine also caused some $[Ca^{2+}]_{ER}$ depletion (Figures 5 and 6), but did not change $[Ca^{2+}]_c$. This may be explained by its slower Ca^{2+}-depleting effects (half-time of 8 min) in comparison with dotarizine or CPA. This slow $[Ca^{2+}]_{ER}$ depletion might not be able to produce a net $[Ca^{2+}]_c$ increase because both the Na^+/Ca^{2+} exchanger and the Ca^{2+} pump of the plasma membrane might be extruding Ca^{2+} at the same rate as it is being released. In the case of dotarizine and CPA, the rate of release was faster and thus a clear increase of $[Ca^{2+}]_c$ was produced. This may also explain why 10 μM dotarizine did not produce an increase of $[Ca^{2+}]_c$, likely because of a slow release of Ca^{2+} from the ER.

The depletion of Ca^{2+} from the ER which is induced by dotarizine could, in principle, be explained by two possible mechanisms: inhibition of ER-Ca^{2+} uptake through SERCA or activation of Ca^{2+} release from the ER. However, the kinetics of the effects of dotarizine are more compatible with blockade of SERCA. Both the $[Ca^{2+}]_c$ elevation and the $[Ca^{2+}]_{ER}$ decrease induced by dotarizine were relatively slow, with kinetics similar to those of the effects of CPA or thapsigargin, two established inhibitors of SERCA. Instead, activation of Ca^{2+} release through ryanodine receptors or inositol trisphosphate receptors produces a much faster (half times below 5 s) $[Ca^{2+}]_c$ increase or $[Ca^{2+}]_{ER}$ decrease (Alonso *et al*., 1999; see also Figure 5).

Whatever the ultimate mechanism involved, we feel that dotarizine emerges as a new and interesting tool to cause reversible depletion of endoplasmic reticulum Ca^{2+} stores. Such depletion was readily reversible and thus is closer to that produced by CPA than to thapsigargin. Dotarizine also blocks, in a reversible manner, the neuronal voltage-dependent Ca^{2+} channels (Villarroya *et al*., 1995, and Figures 2, 3 and 4); this property is not shared by CPA nor thapsigargin, and thus dotarizine might become a useful tool to deplete intracellular Ca^{2+} stores, particularly if we do not wish the intracellular Ca^{2+} signal to be 'contaminated' with Ca^{2+} entry through those channels.

Dotarizine is actually under clinical development for the prophylaxis of migraine (Galiano *et al*., 1993; Horga *et al*., 1996). Its prophylactic effects might be related to 5-HT receptor blockade and/or Ca^{2+} channel blockade (see Introduction). It might be interesting if the Ca^{2+} mobilizing actions here described for dotarizine were also relevant to its antimigraine effects. However, the plasma concentrations of dotarizine after oral administration are around 0.23 μM, a figure much lower than the concentration needed to acutely deplete ER Ca^{2+}. However, chronic administration of dotarizine, which is a highly lipophilic drug, could lead to accumulation in endomembranes and thus cause the release of ER Ca^{2+}.

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