

Pharmacological Blockade of ERG K⁺ Channels and Ca²⁺ Influx through Store-Operated Channels Exerts Opposite Effects on Intracellular Ca²⁺ Oscillations in Pituitary GH₃ Cells

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ABSTRACT

In the present study, the effects on intracellular calcium concentration ([Ca²⁺]_i) oscillations of the blockade of ether-a-go-go-related gene (ERG) K⁺ channels and of Ca²⁺ influx through store-operated channels (SOC) activated by [Ca²⁺]_i store depletion have been studied in GH₃ cells by means of a combination of single-cell fura-2 microfluorimetry and whole-cell mode of the patch-clamp technique. Nanomolar concentrations (1–30 nM) of the piperidinic second-generation antihistamines terfenadine and astemizole and of the class III antiarrhythmic methanesulfonanilide dofetilide, by blocking ERG K⁺ channels, increased the frequency and the amplitude of [Ca²⁺]_i oscillations in resting oscillating GH₃ cells. These compounds also induced the appearance of an oscillatory pattern of [Ca²⁺]_i in a subpopulation of nonoscillating GH₃ cells. The effects of ERG K⁺ channel blockade on [Ca²⁺]_i oscillations appeared to be due to the activation of L-type Ca²⁺ channels, because they were prevented by 300 nM nimodipine. By contrast, the piperazinic second-generation antihistamine cetirizine (0.01–30 μM), which served as a negative control, failed to affect ERG K⁺ channels and did not interfere with [Ca²⁺]_i oscillations in GH₃

cells. Interestingly, micromolar concentrations of terfenadine and astemizole (0.3–30 μM), but not of dofetilide (10–100 μM), produced an inhibition of the spontaneous oscillatory pattern of [Ca²⁺]_i changes. This effect was possibly related to an inhibition of SOC, because these compounds inhibited the increase of [Ca²⁺]_i achieved by extracellular calcium reintroduction after intracellular calcium store depletion with the sarcoplasmic or endoplasmic reticulum calcium ATPase pump inhibitor thapsigargin (10 μM) in an extracellular calcium-free medium. The same inhibitory effect on [Ca²⁺]_i oscillations and SOC was observed with the first-generation antihistamine hydroxyzine (1–30 μM), the more hydrophobic metabolic precursor of cetirizine. Collectively, the results of the present study obtained with compounds that interfere in a different concentration range with ERG K⁺ channels or SOC suggest that 1) ERG K⁺ channels play a relevant role in controlling the oscillatory pattern of [Ca²⁺]_i in resting GH₃ cells and 2) the inhibition of SOC might induce an opposite effect, i.e., an inhibition of [Ca²⁺]_i oscillations.

It is widely recognized that pituitary cells display spontaneous changes in intracellular Ca²⁺ (Ca²⁺_i) concentrations ([Ca²⁺]_i), which vary considerably from cell to cell, with a periodic range from 3 to 30 s and a peak amplitude ranging from 40 to 400 nM. These spontaneous fluctuations are defined as [Ca²⁺]_i oscillations (Charles et al., 1999). It has been proposed that frequency and amplitude of these [Ca²⁺]_i oscillations play a role in the regulation of anterior pituitary

hormone secretion and gene expression (Berridge, 1997; Dolmetsch et al., 1998). [Ca²⁺]_i oscillations may be related to changes in plasma membrane potential and action potential frequency. In fact, the sum of sodium, potassium, and chloride currents flowing through the respective plasma membrane channels expressed in pituitary cells at each time determines the cell membrane potential, which forms the basis of the plasma membrane oscillator (Stojilkovic and Catt, 1992; Stojilkovic, 1996). Changes in the plasma membrane oscillator regulate the opening of L-type voltage-dependent Ca²⁺ channels, which are also modulated by tyrosine (Cataldi et al., 1996) and serine-threonine kinases (Cataldi et al., 1999). Furthermore, the participation of a cytoplasmic Ca²⁺ oscillator, composed of intracellular calcium-storing organelles, has also been suggested. This cyto-

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ABBREVIATIONS: ERG, ether-a-go-go-related gene; [Ca²⁺]_i, intracellular calcium concentration; Ca²⁺_e, extracellular calcium; Ca²⁺_i, intracellular calcium; SOC, store-operated channels; GH, growth hormone; DMSO, dimethyl sulfoxide.

plasmic oscillator releases its Ca^{2+} content upon activation of IP_3 -generating mechanisms and is refilled from the extracellular space by specific plasma membrane channels named store-operated channels (SOC) (Stojilkovic, 1996).

K^+ channels play an important role in the regulation of membrane potential in pituitary cells. In the rat growth hormone (GH)- and prolactin-secreting pituitary clonal cell line GH_3 , in particular, Ca^{2+} -dependent, ATP-dependent, outwardly, and classical inwardly rectifying K^+ channels have been described (Barros et al. 1994; Nelson et al., 1996; Jakab et al., 1997). All of these channels contribute to the shaping of the electrophysiological properties of these cells. More recently, a novel type of voltage-dependent K^+ current has been suggested to contribute to the resting membrane potential control in pituitary GH cells (Bauer, 1998; Bauer et al., 1999), as well as in primary lactotrophs (Corette et al., 1996). The molecular basis for this novel K^+ current has recently been identified as the gene product of the ether- α -gogo-related gene (ERG; Warmke and Ganetzky, 1994), which encodes for K^+ channels expressed in several tissues such as the heart, the central nervous system, and tumor cell lines of different histogenesis, including GH_3 cells (Bianchi et al. 1998).

Considering the relevant role played by the membrane potential in the modulation of $[\text{Ca}^{2+}]_i$ oscillations, the present study investigated the possible involvement of ERG K^+ channels and of SOC in $[\text{Ca}^{2+}]_i$ oscillatory pattern in GH_3 cells. $[\text{Ca}^{2+}]_i$ oscillations and the activity of ERG K^+ channels were studied using single-cell fura-2 microfluorimetry and the whole-cell mode of the patch-clamp technique, respectively.

Because it has been shown that the piperidinic second-generation antihistamines terfenadine and astemizole (Roy et al. 1996; Suessbrich et al. 1996; Tagliatalata et al. 1998), as well as the class III antiarrhythmic dofetilide (Kiehn et al., 1996; Snyders and Chaudhary, 1996), block with elevated affinity constitutively and heterologously expressed ERG K^+ channels, we studied the effect of terfenadine and astemizole on I_{ERG} in GH_3 cells; subsequently, the effect of nanomolar concentrations of these second-generation antihistamines and of dofetilide were studied on $[\text{Ca}^{2+}]_i$ oscillations in these cells. Furthermore, because it has been also shown that astemizole may inhibit store-operated Ca^{2+} fluxes when used at micromolar concentrations in rat basophilic leukemia cells (RBL-2H3) (Fischer et al. 1997, 1998a), higher (micromolar) concentrations of this agent were studied on $[\text{Ca}^{2+}]_i$ increase induced by $[\text{Ca}^{2+}]_i$ store depletion and subsequent refilling. In addition, to rule out the possibility that these second-generation antihistamines can interfere with $[\text{Ca}^{2+}]_i$ oscillations by inhibiting Ca^{2+} channels, the effect of astemizole on high-voltage-activated Ca^{2+} channel currents was also investigated. Finally, with the help of selective inhibitors, the role played by other K^+ channel subtypes different from ERG in $[\text{Ca}^{2+}]_i$ oscillations in GH_3 cells was also studied.

The results obtained suggest that the inhibition of ERG K^+ channels achieved by nanomolar concentrations of terfenadine, astemizole, and dofetilide is able to increase the frequency and the amplitude of $[\text{Ca}^{2+}]_i$ oscillations in GH_3 cells. However, when micromolar concentrations of astemizole, terfenadine, and hydroxyzine, but not of dofetilide, were used, an inhibition of the spontaneous oscillatory pattern of $[\text{Ca}^{2+}]_i$ changes was observed. This inhibitory effect seems to be

related to an inhibition of the SOC channels activated upon depletion of $[\text{Ca}^{2+}]_i$ stores. Finally, the piperidinic second-generation antihistamine cetirizine, which is devoid of any inhibitory action on ERG K^+ channels and SOC, did not interfere with $[\text{Ca}^{2+}]_i$ oscillations in GH_3 cells.

Materials and Methods

Cell Culture. GH_3 cells were obtained from Flow Laboratories (Irvine, Scotland) and grown on plastic dishes in Ham's F10 medium (Gibco-BRL, San Giuliano Milanese, Italy) composed of 15% horse serum (Flow), 2.5% fetal calf serum (Hyclone, Logan, UT), 100 I.U. penicillin/ml, and 100 μg streptomycin/ml. The cells were cultured in a humidified 5% CO_2 atmosphere, and the culture medium was changed every 2 days. For microfluorimetric studies, the cells were seeded on glass coverslips (Fisher, Springfield, NJ) coated with poly-L-lysine (30 $\mu\text{g}/\text{ml}$) (Sigma, St. Louis, MO) and used at least 12 h after seeding.

$[\text{Ca}^{2+}]_i$ Measurements and Quantification of $[\text{Ca}^{2+}]_i$ Oscillations. $[\text{Ca}^{2+}]_i$ was measured using a microfluorimetric technique, as previously reported (Cataldi et al., 1996). Briefly, the cells grown on glass coverslips were loaded with 5 μM 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)-ethane- N,N,N',N' -tetraacetic acid pentaacetoxymethyl ester (fura-2 AM) in Krebs-Ringer saline solution (5.5 mM KCl, 160 mM NaCl, 1.2 mM MgCl_2 , 1.5 mM CaCl_2 , 10 mM glucose, and 10 mM HEPES-NaOH, pH 7.4) for 1 h at room temperature. At the end of the fura-2 AM-loading period, the coverslip was introduced into a microscope chamber (Medical System Co., Greenvale, NY) on an inverted Diaphot fluorescence microscope (Nikon, Tokyo, Japan). The cells were kept in Krebs-Ringer saline solution throughout the experiment. All of the drugs tested were introduced into the microscope chamber by fast injection. A 100-W Xenon lamp (Osram, Frankfurt, Germany) with a computer-operated filter wheel bearing two different interference filters (340 and 380 nm) illuminated the microscopic field with UV light, alternating the wavelengths at an interval of 500 ms. The interval between each pair of illuminations ranged from 1 to 3 s, and the interval between filter movements was 1 s. Emitted light was passed through a 400-nm dichroic mirror, filtered at 510 nm, and collected by a charge-coupled device camera (Photonic Science, Robertsbridge, East Sussex, UK) connected to a light amplifier (Applied Imaging Ltd., Dukesway Gateshead, UK). Images were digitized and analyzed with a Magiscan image processor (Applied Imaging Ltd.). Using a calibration curve, the Tardis software (Applied Imaging Ltd.) calculated the $[\text{Ca}^{2+}]_i$ corresponding to each pair of images from the ratio between the intensity of the light emitted when the cells were illuminated at both 340 and 380 nm.

$[\text{Ca}^{2+}]_i$ oscillations were defined as an increase of $[\text{Ca}^{2+}]_i$ above the mean of the basal value ± 2 S.D., occurring with a frequency higher than one peak/3 min. According to Villalobos et al. (1998), two different parameters were used for the quantification of $[\text{Ca}^{2+}]_i$ oscillations: the oscillation index and the mean $[\text{Ca}^{2+}]_i$ value. The oscillation index was calculated by adding all of the absolute differences in $[\text{Ca}^{2+}]_i$ between each $[\text{Ca}^{2+}]_i$ measurement and the previous value; this parameter represents the rate of $[\text{Ca}^{2+}]_i$ changes during the measurements and the frequency and/or amplitude of $[\text{Ca}^{2+}]_i$ oscillations and is independent of the actual $[\text{Ca}^{2+}]_i$ value. Instead, the mean $[\text{Ca}^{2+}]_i$ value was obtained by adding all of the $[\text{Ca}^{2+}]_i$ values measured during the experimental period divided by the number of all of the experimental points measured. This parameter provides a mean value of $[\text{Ca}^{2+}]_i$ over time.

Each experiment was divided into three periods of equal duration (i.e., 100 s when the acquisition time was 1 s and 300 s when the acquisition time was 3 s), and both the oscillation index and the mean $[\text{Ca}^{2+}]_i$ value were calculated for each period. In control conditions (i.e., no pharmacological treatment), no significant change in the oscillation index or the mean $[\text{Ca}^{2+}]_i$ value occurred (data not shown) during these three successive periods. This allowed us to use

the first experimental period of acquisition, in which no experimental maneuver was performed, as a reference control for the following two periods in which the pharmacological treatment was applied. The quantification of the effects of the drugs on [Ca²⁺]_i oscillations was performed by comparing the last period of drug application with the first control period.

Patch-Clamp Recordings. Currents from GH₃ cells were recorded at room temperature using a commercially available amplifier (Axopatch 200A, Axon Instruments, Foster City, CA). The whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) was used with glass micropipettes of 3 to 7 MΩ resistance. No compensation was made for pipette resistance and cell capacitance. For the experiments on ERG K⁺ channels, the relatively small density of the current required the use of a high (100 mM) external K⁺ concentration as a charge carrier. Therefore, GH₃ cells were perfused with an extracellular solution containing (in mM): 100 KCl, 10 EGTA, and 10 HEPES, pH 7.3, with KOH, and the pipettes were filled with (in mM): 110 CsCl, 10 tetraethylammonium-Cl, 2 MgCl₂, 10 EGTA, 8 glucose, 2 Mg-ATP, 0.25 cAMP, and 10 HEPES, pH 7.3. For Ca²⁺ current recordings, the cells were perfused with an extracellular solution containing (in mM): 10 BaCl₂, 125 NaCl, 1 MgCl₂, 10 HEPES, and 300 nM tetrodotoxin, pH 7.3. The pipettes were filled with (in mM): 110 CsCl, 10 tetraethylammonium-Cl, 2 MgCl₂, 10 EGTA, 8 glucose, 2 Mg-ATP, 0.25 cAMP, and 10 HEPES, pH 7.3. The Ba²⁺ current through Ca²⁺ channels was obtained by subtracting the current elicited in the presence of 50 μM CdSO₄.

Drugs and Chemicals. Chemicals were of analytical grade and were purchased from Sigma Italia (Milan, Italy). fura 2-AM was obtained from Calbiochem (La Jolla, CA). Astemizole and dofetilide were kindly provided by Janssen-Cilag (Rome, Italy) and Pfizer, Inc. (Sandwich, UK), respectively. Cetirizine was generously donated by UCB Pharma (Bruxelles, Belgium). All of the drugs were dissolved in 10 mM DMSO, and stock solutions were kept at -20°C. Appropriate dilutions were prepared daily. The maximal DMSO concentration (0.3%) did not affect [Ca²⁺]_i oscillations in GH₃ cells.

Statistical Analysis of the Data. All of the data are expressed as the means ± S.E.M. The statistical analysis was performed using the Student's *t* test for paired or unpaired data, where required. The threshold for statistical significance was set at *P* < .05. The data reported in the present study are the means ± S.E.M. of single-cell determinations obtained by the analysis of all the cells recorded in each of the different experimental sessions. For each pharmacological treatment, at least five cells in at least three experimental sessions were evaluated.

Results

Effect of Second-Generation Antihistamines on ERG K⁺ Currents in GH₃ cells. In GH₃ cells, hyperpolarizing voltage pulses (from 0 mV to -160 mV) delivered after long (10 s) depolarizing pulses to 0 mV to inactivate the delayed rectifier K⁺ current elicited the appearance of inward K⁺ currents with the characteristic slow development and subsequent decay (Fig. 1). These currents, which were first described in GH₃ cells (Bauer et al., 1990) and subsequently shown to also exist in several other cell types, were originally attributed to the voltage-dependent activation of an atypical inwardly rectifying K⁺ channel. More recently, it has been demonstrated that they represent the voltage-dependent closing of delayed rectifier K⁺ currents activated by the previous long depolarization. The molecular basis of these peculiar K⁺ currents has been identified to be the protein product of the ERG. This protein carries a current-denominated *I*_{ERG}.

*I*_{ERG} was completely suppressed in GH₃ cells by the second-generation antihistamine astemizole (Fig. 1A) at 30 nM and 3 μM, a range found to be effective in blocking ERG K⁺

channels heterologously expressed in *Xenopus* oocytes (Suessbrich et al., 1996; Taglialatela et al., 1998) or in mammalian HEK-293 cells (Zhou et al., 1998). Interestingly, when the difference in current (*I*_{control} minus *I*_{drug}) was calculated, no significant difference was observed between the two astemizole concentrations (0.03 and 3 μM), suggesting that maximal *I*_{ERG} inhibition was already occurring at the lowest drug concentration. This is in accordance with the results showing that the IC₅₀ for astemizole blockade of HERG was 0.9 nM (Zhou et al., 1999). In addition, another piperidinic H₁ receptor blocker, terfenadine (3 μM), was able to completely suppress *I*_{ERG} in GH₃ cells (Fig. 1B). By contrast, cetirizine, a piperazinic antihistamine compound shown to lack ERG-blocking capabilities (Taglialatela et al., 1998), did not inhibit the K⁺ current carried by ERG at a concentration of 3 μM in GH₃ cells (Fig. 1C).

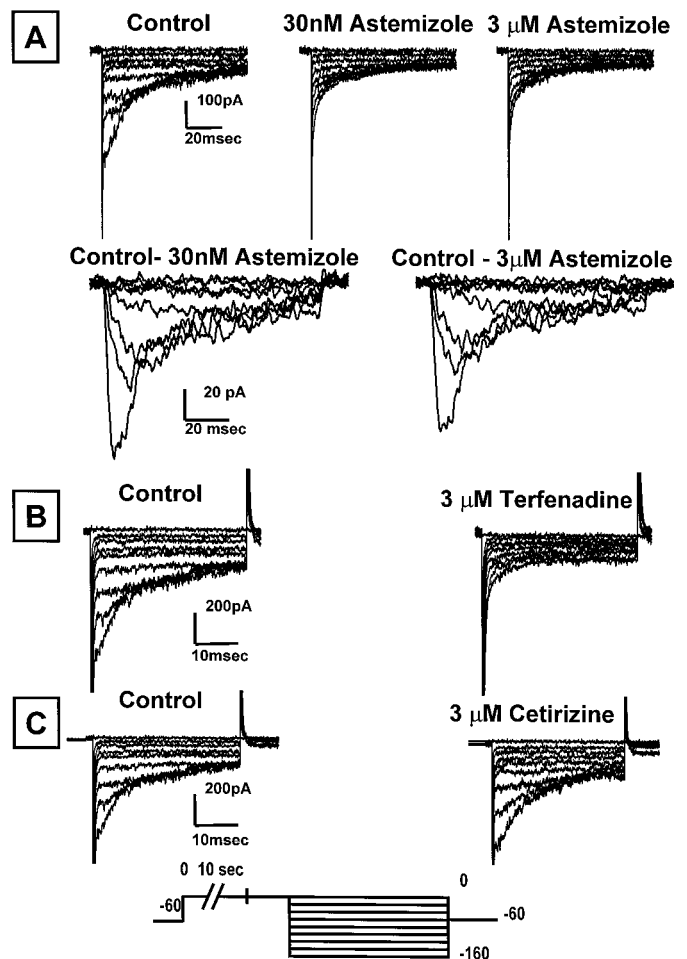


Fig. 1. Effect of astemizole (0.03 and 3 μM), terfenadine (3 μM), and cetirizine (3 μM) on inward ERG K⁺ currents expressed in GH₃ cells. A, representative current traces recorded in GH₃ cells in control conditions (left), after a 5-min perfusion with 0.03 μM (30 nM) astemizole (center), and with 3 μM astemizole (right). The voltage protocol (schematically shown at the bottom of the figure) was the following: holding potential, -60 mV; conditioning pulse, 0 mV (10 s); test pulses, from 0 to -160 mV in 20 mV steps (100 ms); return potential, -60 mV. The two panels at the bottom of A show the subtracted traces for control-astemizole 0.03 μM (30 nM, left) and control-astemizole 3 μM (right). B, representative current traces in GH₃ cells in control conditions (left) and after a 5-min perfusion with terfenadine (3 μM, right panel). The voltage protocol was identical with that described in A. C, representative current traces in GH₃ cells in control conditions (left) and after a 5-min perfusion with cetirizine (3 μM; right). The voltage protocol was identical with that described in A.

Effect of First- and Second-Generation Antihistamines and of the Antiarrhythmic Methanesulfonamide Dofetilide on $[Ca^{2+}]_i$ Oscillations in GH_3 Cells. In resting conditions, 70% of GH_3 cells ($n = 285/410$ in 51 experiments) displayed spontaneous $[Ca^{2+}]_i$ oscillations. The remaining 30% of the cells ($n = 125/410$ in 51 experiments) that did not display these characteristics were defined as nonoscillating. In oscillating GH_3 cells, astemizole (1–30 nM) and terfenadine (1–10 nM) enhanced in a concentration-dependent fashion the oscillatory pattern of $[Ca^{2+}]_i$ (Fig. 2A; Fig. 3A). This enhancement was quantified as an increase of both the oscillation index and the mean $[Ca^{2+}]_i$ baseline, as

shown in Fig. 2B and Fig. 3B. These two parameters used to quantify $[Ca^{2+}]_i$ oscillations are not necessarily directly correlated; in fact, as shown in the 30 nM terfenadine experiment (Fig. 3B), the oscillation index showed a decrease despite the sustained elevation of the mean $[Ca^{2+}]_i$ baseline. When the two piperidinic second-generation antihistamines were used in the same experimental paradigm at higher micromolar concentrations (1–30 μM astemizole ; 0.3–30 μM terfenadine), a concentration-dependent inhibition of spontaneous $[Ca^{2+}]_i$ oscillations occurred (Fig. 2; Fig. 3). Interestingly, the highest concentrations of both terfenadine and astemizole completely abolished $[Ca^{2+}]_i$ oscillations in GH_3

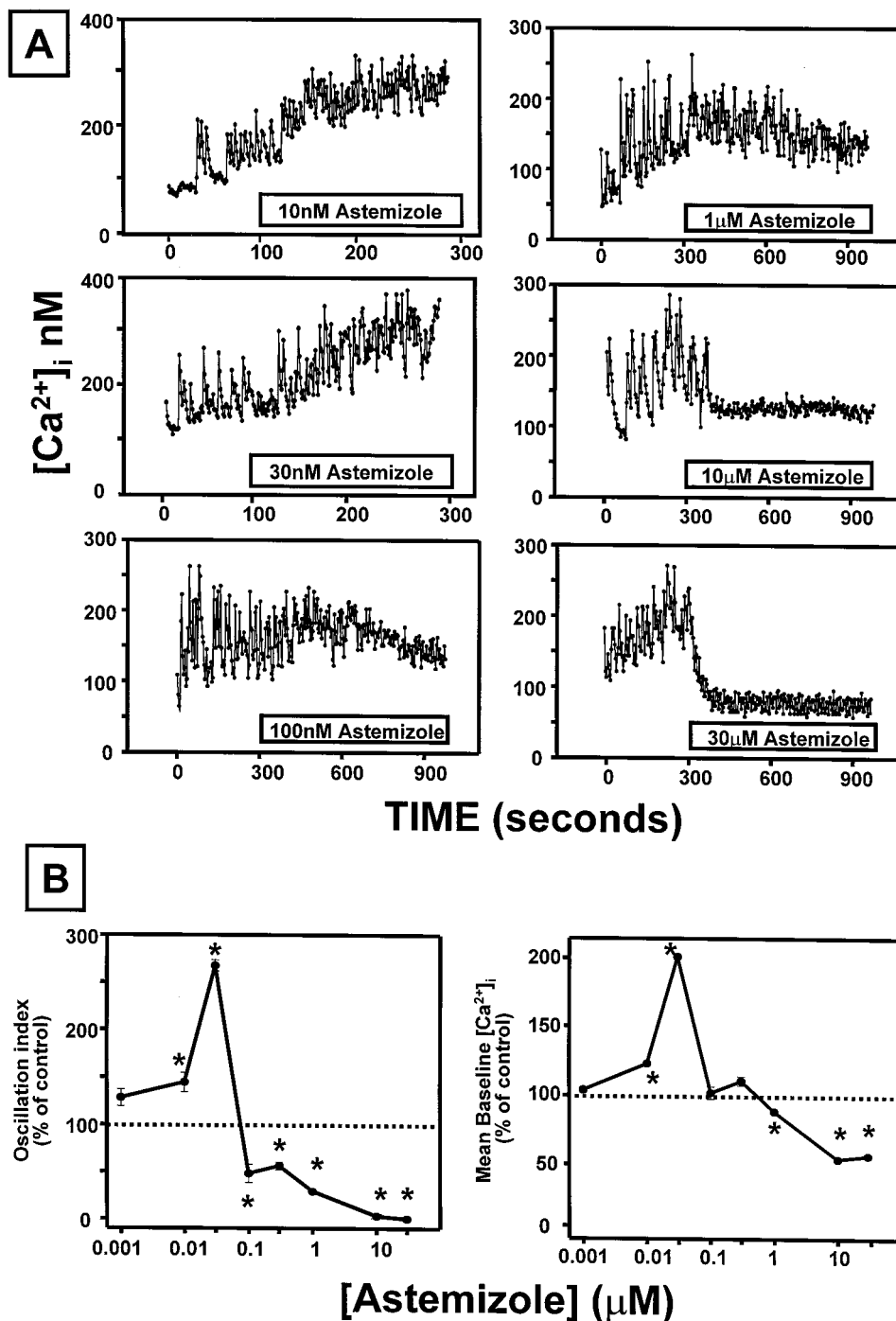


Fig. 2. Effect of astemizole on $[Ca^{2+}]_i$ oscillations in GH_3 cells. A, representative single-cell traces for the effects of astemizole (0.01–30 μM) on $[Ca^{2+}]_i$ oscillations in GH_3 cells. The experiments at lowest concentrations (0.01–0.03 μM) were performed with an acquisition interval of 1 s, whereas those at the highest concentrations (0.1–30 μM) were sampled at 3 s. The drug was added after 100 or 300 s of baseline $[Ca^{2+}]_i$ monitoring and left in the chamber for the remaining period as indicated by the bar. B, the quantification of drug effect on $[Ca^{2+}]_i$ oscillations performed as described under *Materials and Methods*; the drug concentration-effect on the oscillation index (left) and on the baseline $[Ca^{2+}]_i$ (right) are shown. Each point represents the mean \pm S.E. of 15 to 25 cells studied in at least three different experimental sessions.

cells, as revealed by the complete suppression of the oscillation index.

Hydroxyzine, an older first-generation antagonist of the H₁ receptor, dose-dependently (1–30 μ M) inhibited [Ca²⁺]_i oscillations in GH₃ cells (Fig. 4A). The oscillation index was significantly inhibited by this compound at concentrations of 1 to 30 μ M (Fig. 4B, left), whereas a significant inhibition of the mean [Ca²⁺]_i value was observed with hydroxyzine concentrations of 10 and 30 μ M (Fig. 4B, right). By contrast, cetirizine, a second-generation antihistamine that is a more polar *in vivo* metabolite of hydroxyzine and is devoid of any inhibitory action on ERG K⁺ channels (Taglialatela et al., 1998; see also Fig. 1), did not interfere with [Ca²⁺]_i oscillations in a wide range of concentrations from 0.03 to 30 μ M

(Fig. 5A). In fact, both the oscillation index and the mean [Ca²⁺]_i value were unaffected by this range of cetirizine concentration (Fig. 5B).

The class III antiarrhythmic methanesulfonanilide dofetilide, a compound shown to potently inhibit ERG K⁺ channels (Kiehn et al., 1996; Snyders and Chaudhary, 1996), increased [Ca²⁺]_i oscillations in GH₃ cells in concentrations of 10 and 30 nM, whereas higher concentrations of 10 to 100 μ M, in contrast with the results obtained with similar concentrations of terfenadine, astemizole, and hydroxyzine, failed to inhibit [Ca²⁺]_i oscillations (Fig. 6).

Interestingly, concentrations of astemizole (30 nM), terfenadine (10 nM), and dofetilide (30 nM) that were able to increase [Ca²⁺]_i oscillations in oscillating GH₃ cells, also

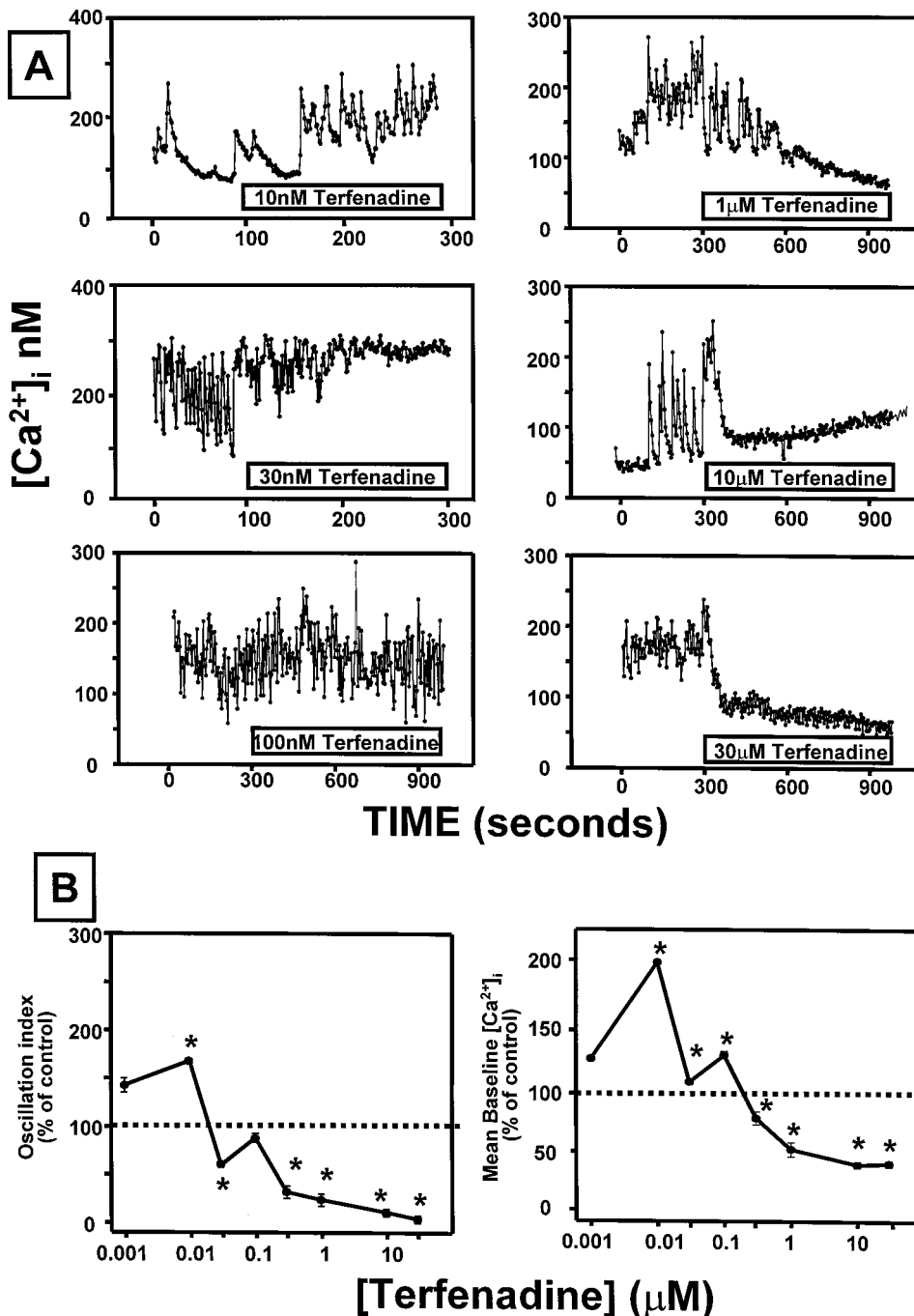


Fig. 3. Effect of terfenadine on [Ca²⁺]_i oscillations in GH₃ cells. **A**, representative single-cell traces for the effects of terfenadine (0.01–30 μ M) on [Ca²⁺]_i oscillations in GH₃ cells. The experiments at lowest concentrations (0.01–0.03 μ M) were performed with an acquisition interval of 1 s, whereas those at the highest concentrations (0.1–30 μ M) were sampled at 3 s. The drug was added after 100 or 300 s of baseline [Ca²⁺]_i monitoring and left in the chamber for the remaining period as indicated by the bar. **B**, the quantification of drug effect on [Ca²⁺]_i oscillations performed as described under *Materials and Methods*; the drug concentration-effect on the oscillation index (left) and on the baseline [Ca²⁺]_i (right) are shown. Each point represents the mean \pm S.E. of 15 to 25 cells studied in at least three different experimental sessions.

induced the appearance of the oscillatory pattern in those GH₃ cells that were quiescent (nonoscillating cells; Fig. 7).

Effect of the Selective Blockade of L-Type Ca²⁺ Channels with Nimodipine on [Ca²⁺]_i Oscillations Induced by ERG K⁺ Channel Blockade in Quiescent and Spontaneously Oscillating GH₃ Cells. To clarify the possible contribution of L-type Ca²⁺ channels in [Ca²⁺]_i oscillations induced by ERG K⁺ channel blockade in quiescent and spontaneously oscillating GH₃ cells, the selective L-type Ca²⁺ channel antagonist nimodipine (300 nM) was used. Panel A of Fig. 8 shows that nimodipine was able to suppress [Ca²⁺]_i oscillations induced by ERG K⁺ channel blockade with 30 nM astemizole in nonoscillating GH₃ cells. Further-

more, the exposure to 300 nM nimodipine prevented the appearance of the oscillatory pattern of [Ca²⁺]_i induced by 30 nM astemizole in nonoscillating GH₃ cells (B). Finally, C shows that the increased frequency of [Ca²⁺]_i oscillations produced by 30 nM astemizole in oscillating GH₃ cells was suppressed by the subsequent exposure to 300 nM nimodipine.

Effect of Selective Blockers of ATP-Dependent, Small-Conductance Ca²⁺-Dependent and Large-Conductance Ca²⁺-Dependent K⁺ Channels on [Ca²⁺]_i Oscillations in GH₃ Cells. Glybenclamide, a specific blocker of ATP-dependent K⁺ channels, in concentrations of 10 μM, a value much higher than the IC₅₀ for blocking these channels (Nelson et al., 1996),

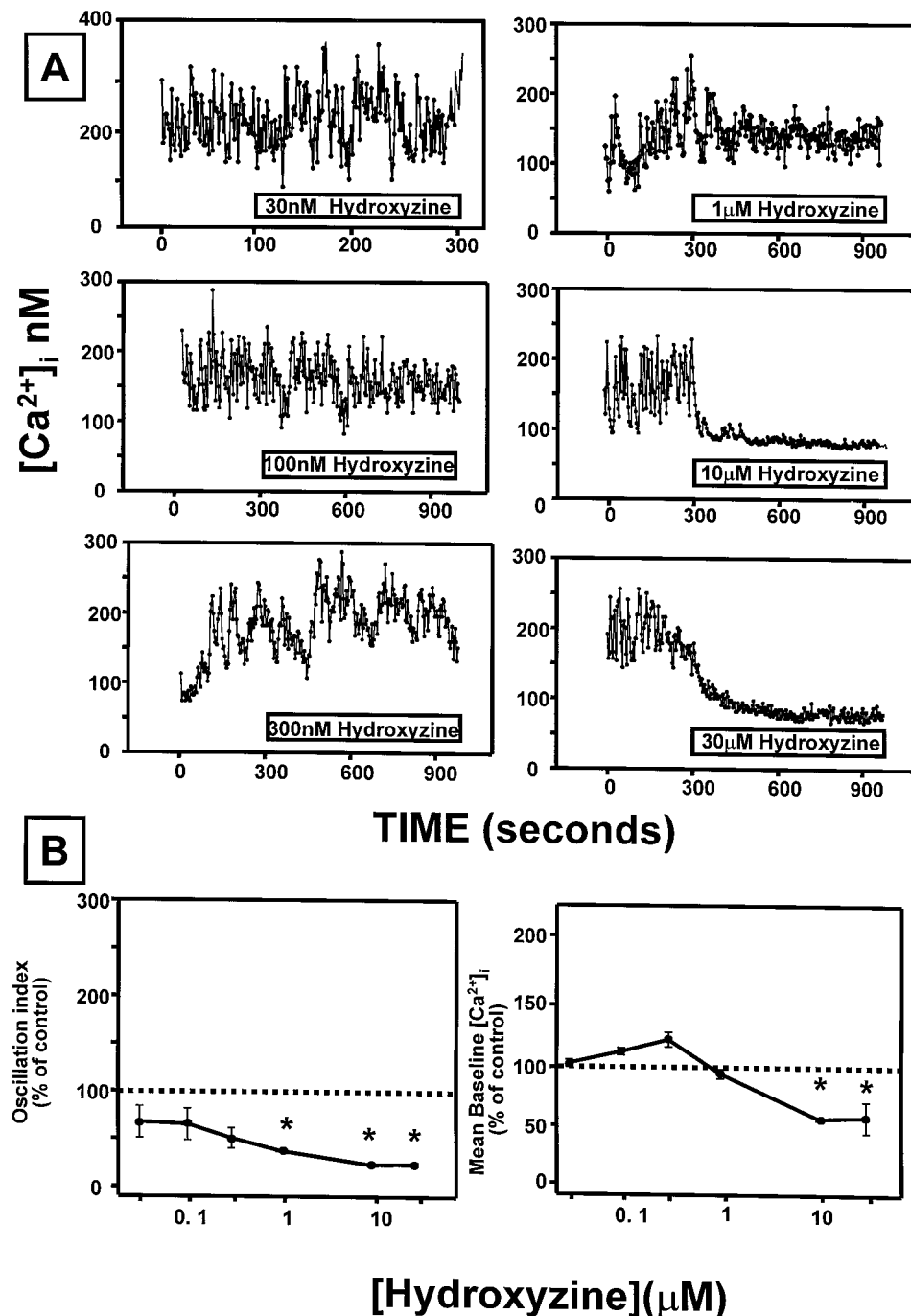


Fig. 4. Effect of hydroxyzine on [Ca²⁺]_i oscillations in GH₃ cells. **A**, representative single-cell traces for the effects of hydroxyzine (0.03–30 μM) on [Ca²⁺]_i oscillations in GH₃ cells. The experiments at the lowest concentration (0.03 μM) were performed with an acquisition interval of 1 s, whereas those at the highest concentrations (0.1–30 μM) were sampled at 3 s. The drug was added after 100 or 300 s of baseline [Ca²⁺]_i monitoring and left in the chamber for the remaining period as indicated by the bar. **B**, the quantification of drug effect on [Ca²⁺]_i oscillations performed as described under *Materials and Methods*; the drug concentration-effect on the oscillation index (left) and on the baseline [Ca²⁺]_i (right) are shown. Each point represents the mean ± S.E. of 15 to 25 cells studied in at least three different experimental sessions.

did not interfere with either the oscillation index or the mean [Ca²⁺]_i value in GH₃ cells (Fig. 9, A and D). Furthermore, apamine (500 nM) and charibdotoxin (200 nM), two blockers of the small-conductance and of the large-conductance Ca²⁺-dependent K⁺ channels, respectively (Jakab et al., 1997), also failed to interfere with [Ca²⁺]_i oscillations (Fig. 9, B and C) as revealed by their ineffectiveness in reducing either the oscillation index or the mean [Ca²⁺]_i value in the same cells (Fig. 9D).

Effect of Micromolar Concentrations of Terfenadine, Astemizole, Hydroxyzine, Dofetilide, and Cetirizine on [Ca²⁺]_i Increase Induced by Depletion of Ca²⁺_i Stores and Subsequent Refilling (SOC) in GH₃ Cells. To identify the possible mechanisms underlying the inhibition of [Ca²⁺]_i oscillations observed with higher micromolar concen-

trations of terfenadine, astemizole, and hydroxyzine, we studied the possible interference of these compounds with Ca²⁺ fluxes induced by the depletion and subsequent refilling of Ca²⁺_i stores (SOC), because this process has been described to play an important role in [Ca²⁺]_i oscillations in GH₃ cells (Stojilkovic, 1996). To this aim, the depletion of Ca²⁺_i stores was achieved by exposing the cells to the sarcoplasmic or endoplasmic reticulum calcium ATPase pump inhibitor thapsigargin (10 μM) in the absence of extracellular calcium (Ca²⁺_e; Fatatis et al., 1994). This depletion is known to activate the plasma membrane SOC (Stojilkovic, 1996). Under this experimental condition, the subsequent reintroduction of 3 mM Ca²⁺_e allows the detection of the possible inhibitory effects of compounds acting on SOC (Fatatis et al.,

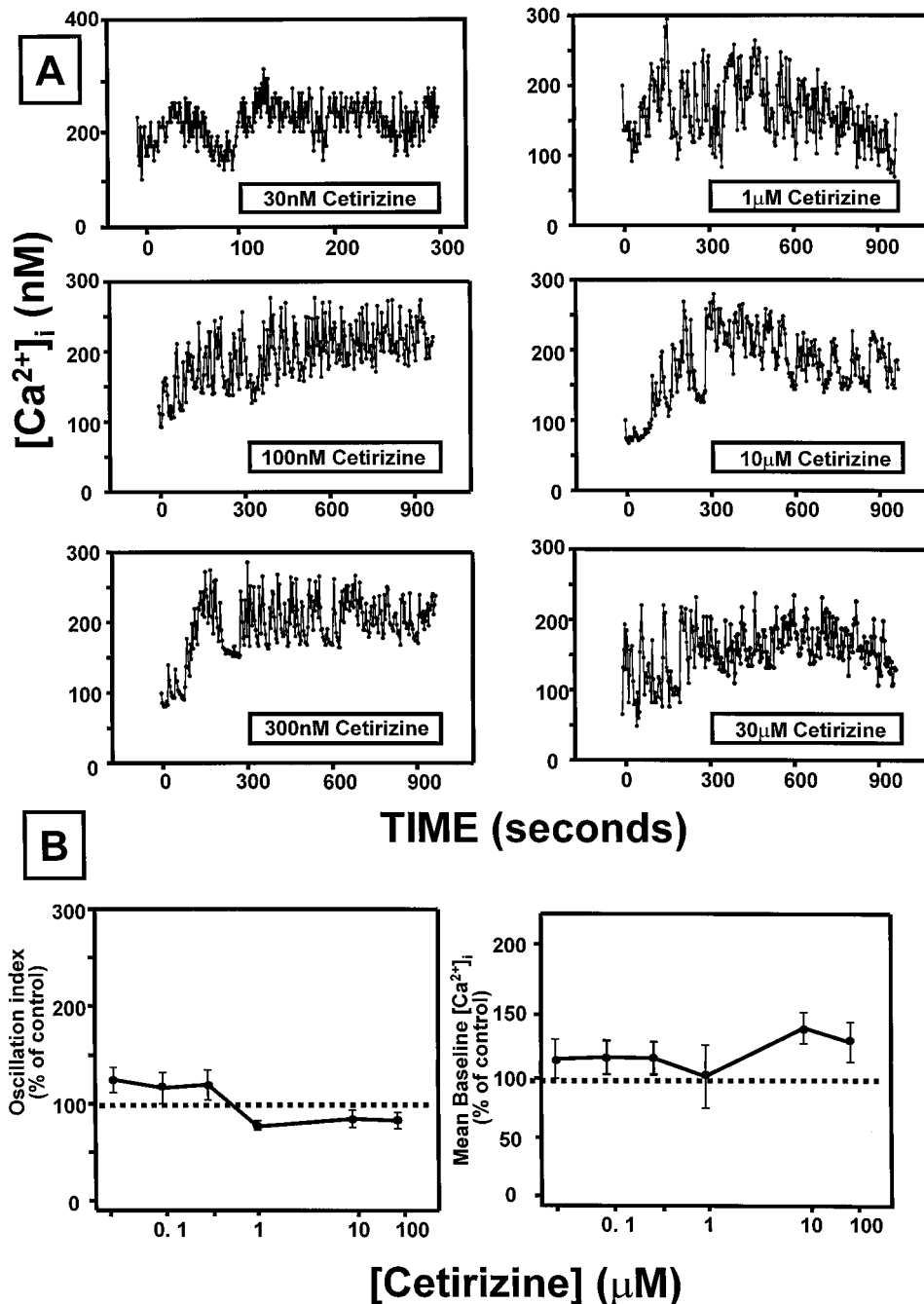


Fig. 5. Effect of cetirizine on [Ca²⁺]_i oscillations in GH₃ cells. A, representative single-cell traces for the effects of cetirizine (0.03–30 μM) on [Ca²⁺]_i oscillations in GH₃ cells. The experiments at the lowest concentration (0.03 μM) was performed with an acquisition interval of 1 s, whereas those at the highest concentrations (0.1–30 μM) were sampled at 3 s. The drug was added after 100 or 300 s of baseline [Ca²⁺]_i monitoring and left in the chamber for the remaining period as indicated by the bar. B, the quantification of drug effect on [Ca²⁺]_i oscillations performed as described under *Materials and Methods*; the drug concentration-effect on the oscillation index (left) and on the baseline [Ca²⁺]_i (right) are shown. Each point represents the mean ± S.E. of 15 to 25 cells studied in at least three different experimental sessions.

1994). In controls, the reintroduction of 3 mM Ca^{2+}_e induced an increase in $[\text{Ca}^{2+}]_i$ that did not decline over a 5- to 6-min period (Fig. 10, left top). By contrast, astemizole (Fig. 10, top right), terfenadine (Fig. 10, middle left), and hydroxyzine (Fig. 10, middle right) caused a time- and concentration-dependent (1–30 μM) decline of $[\text{Ca}^{2+}]_i$ after the reintroduction of 3 mM $[\text{Ca}^{2+}]_e$, with IC_{50} values of 11.3, 5.7 and 19.2 μM , respectively (Fig. 11). On the other hand, both dofetilide (10 μM ; Fig. 10, bottom right) and cetirizine (10 μM) (Fig. 10, bottom left) proved to be ineffective in the same experimental model (Fig. 11).

Effect of Cetirizine and Astemizole on Voltage-Dependent Ca^{2+} Channels in GH_3 Cells. Because $[\text{Ca}^{2+}]_i$ oscillations have been shown to be critically dependent on the

opening of the L-subtype of voltage-dependent Ca^{2+} channels and blockers of these channels suppress $[\text{Ca}^{2+}]_i$ oscillations in GH_3 cells (Charles et al., 1999), there was a possibility that the inhibition of the $[\text{Ca}^{2+}]_i$ oscillatory pattern observed with micromolar concentrations of first- and second-generation antihistamines could be attributed to a blocking action exerted by these compounds at the level of high-voltage-activated Ca^{2+} channels. Figure 12A shows that 3 μM astemizole, a concentration that effectively inhibited $[\text{Ca}^{2+}]_i$ oscillations, failed to prevent inward Ca^{2+} currents elicited by depolarizing pulses to 0 mV in GH_3 cells. On the other hand, cetirizine, which failed to interfere with $[\text{Ca}^{2+}]_i$ oscillations, was also ineffective in blocking high-voltage-activated Ca^{2+} channels in these cells (Fig. 12B). The peak in-

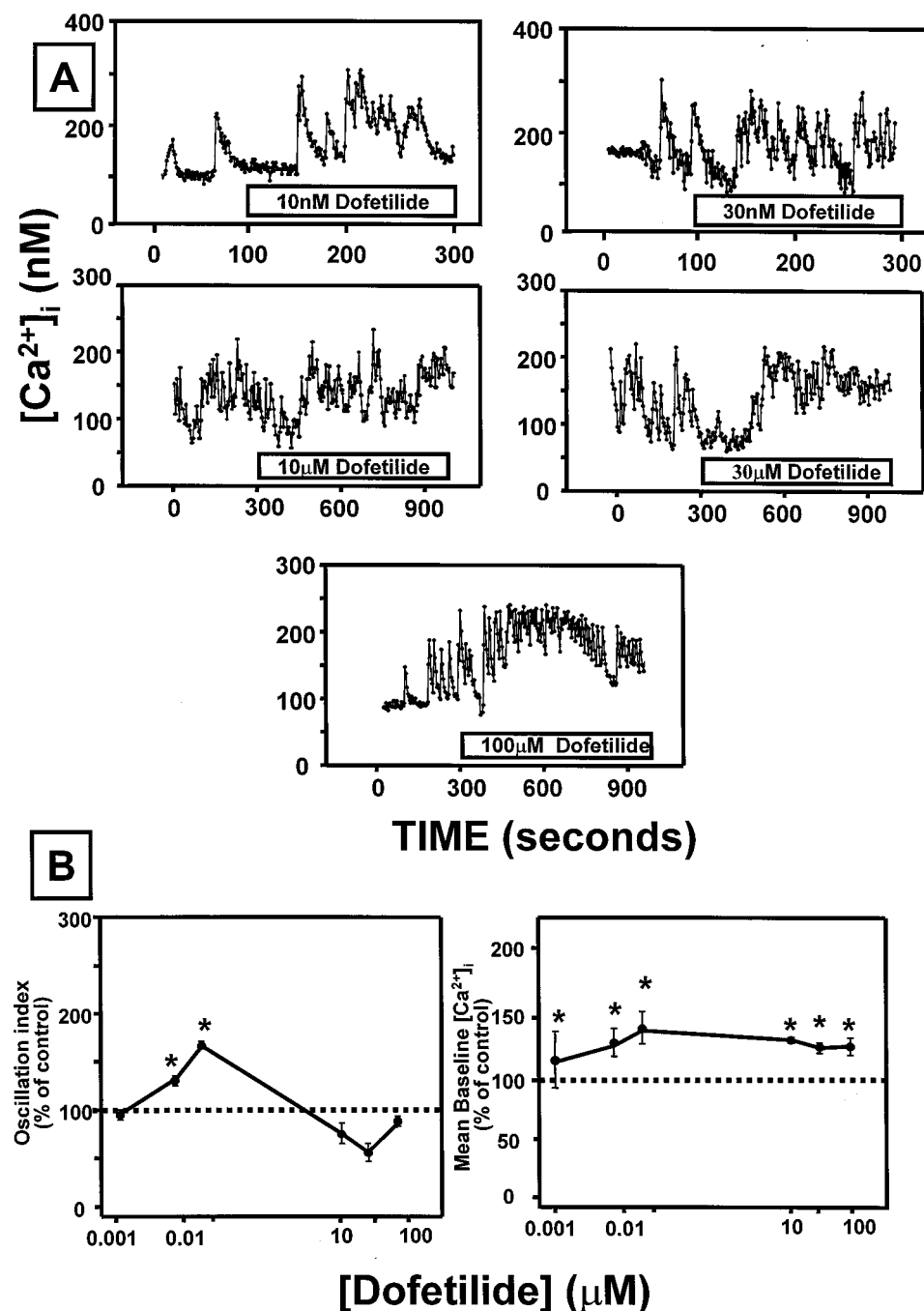


Fig. 6. Effect of dofetilide on $[\text{Ca}^{2+}]_i$ oscillations in GH_3 cells. **A**, representative single-cell traces for the effects of dofetilide (0.001–100 μM) on $[\text{Ca}^{2+}]_i$ oscillations in GH_3 cells. The experiments at lowest concentrations (0.001–0.03 μM) were performed with an acquisition interval of 1 s, whereas those at the highest concentrations (0.1–100 μM) were sampled at 3 s. The drug was added after 100 or 300 s of baseline $[\text{Ca}^{2+}]_i$ monitoring and left in the chamber for the remaining period as indicated by the bar. **B**, the quantification of drug effect on $[\text{Ca}^{2+}]_i$ oscillations performed as described under *Materials and Methods*; the drug concentration-effect on the oscillation index (left) and on the baseline $[\text{Ca}^{2+}]_i$ (right) are shown. Each point represents the mean \pm S.E. of 15 to 25 cells studied in at least three different experimental sessions.

ward Ba²⁺ current recorded after 3 min of superfusion with vehicle (0.1% DMSO), astemizole (3 μ M), or cetirizine (3 μ M) was not significantly different from the respective controls recorded before drug application. In fact, these values were $87.5 \pm 3.2\%$ ($n = 3$), $86.2 \pm 2.6\%$ ($n = 9$), and $89.4 \pm 2.9\%$ ($n = 5$), respectively, of the value recorded before vehicle or drug perfusion. This reduction was attributed to spontaneous rundown of channel activity. In addition, the amplitudes of the inward Ba²⁺ currents at 0 mV were indistinguishable in the three experimental groups: -159 ± 23 , -166 ± 40 , and -170 ± 46 pA in vehicle, 3 μ M astemizole, and 3 μ M cetirizine groups, respectively. By contrast, the inorganic Ca²⁺ channel blocker Cd²⁺ (50 μ M) completely suppressed high-voltage-activated Ca²⁺ channels (Fig. 12, A and B). C shows a time course of Ca²⁺ currents in a single GH₃ cell subsequently exposed to control solution, 3 μ M astemizole, 50 μ M Cd²⁺, washout, and 3 μ M cetirizine. The results obtained confirm the ineffectiveness of the second-generation antihis-

tamines astemizole and cetirizine in interfering with L-type Ca²⁺ channels in GH₃ cells.

Discussion

The results of the present study demonstrate that depolarization-activated, inwardly rectifying ERG K⁺ channels, constitutively expressed in GH₃ cells (Barros et al., 1994, 1997; Bianchi et al., 1998), where they participate in the regulation of the resting membrane potential, are involved in the oscillatory pattern of [Ca²⁺]_i fluctuations in this clonal cell line. In particular, nanomolar concentrations of the second-generation H₁ receptor blockers astemizole and terfenadine, which have ERG K⁺ channel-blocking ability (Roy et al., 1996; Suessbrich et al., 1996; Taglialatela et al., 1998), caused an increase of spontaneous [Ca²⁺]_i oscillations. In agreement with this hypothesis, the class III antiarrhythmic dofetilide,

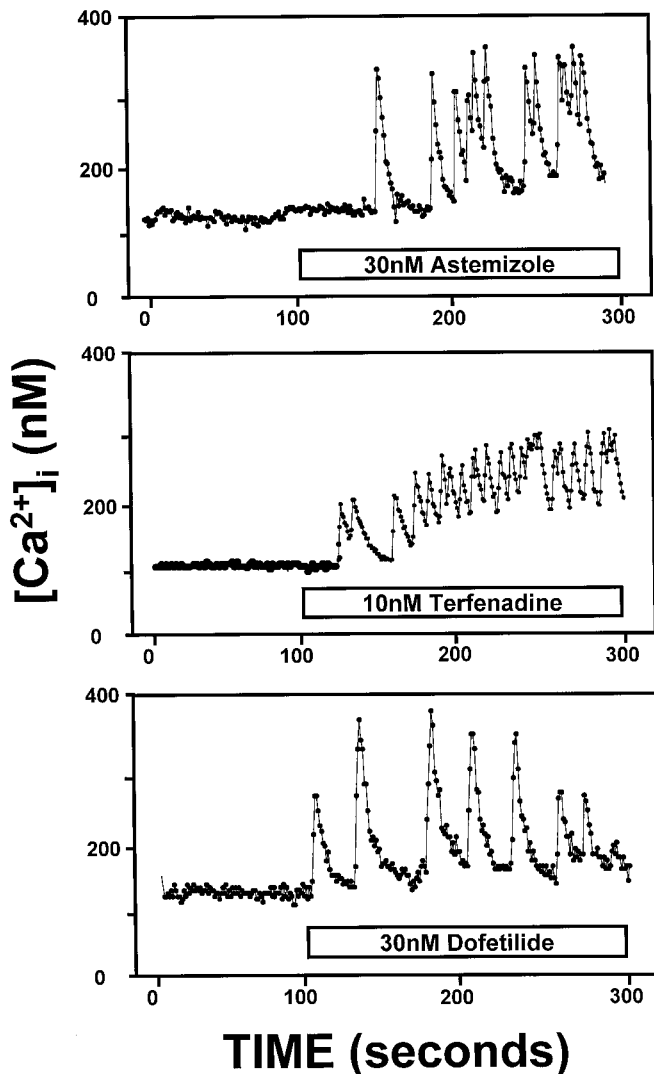


Fig. 7. Effect of nanomolar concentrations of astemizole, terfenadine, and dofetilide on [Ca²⁺]_i in nonoscillating GH₃ cells. Representative single-cell traces for the effects of astemizole (0.03 μ M), terfenadine (0.01 μ M), and dofetilide (0.03 μ M) on [Ca²⁺]_i in GH₃ cells. The experiments were performed with an acquisition interval of 1 s. The traces are representative of six to 10 cells for each experimental group studied in at least three different experimental sessions.

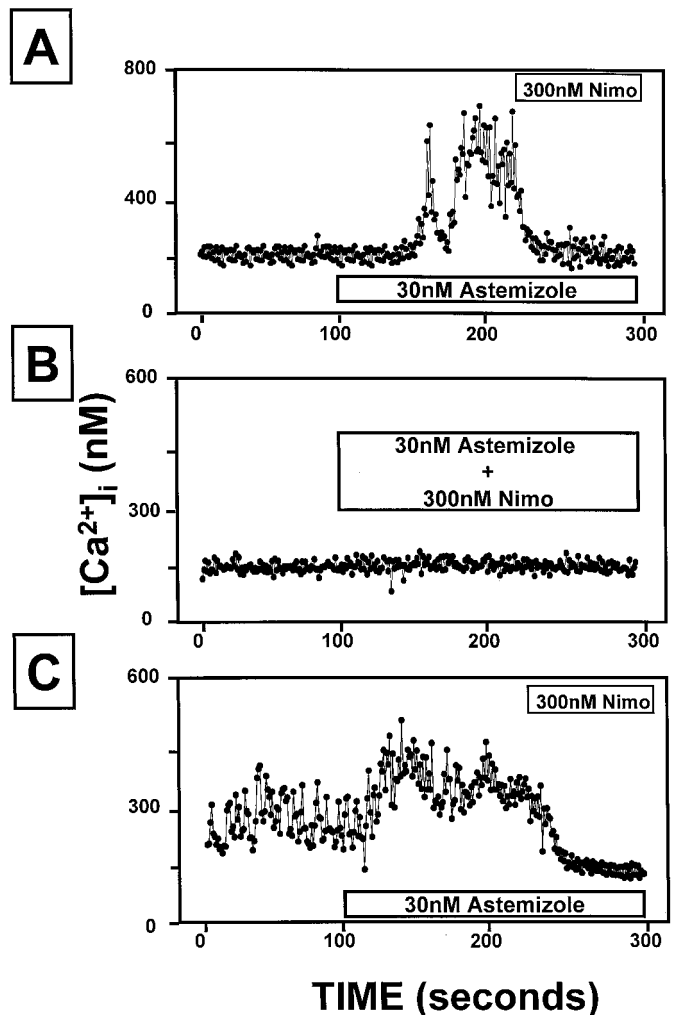


Fig. 8. Effect of the selective L-type Ca²⁺ channel blocker nimodipine on [Ca²⁺]_i oscillations induced by the ERG K⁺ channel blocker astemizole in GH₃ cells. A, representative single-cell trace for the effects of nimodipine (Nimo, 300 nM) added after astemizole (0.03 μ M) in a quiescent GH₃ cell. B, representative single-cell trace for the effects of nimodipine (300 nM) added simultaneously to astemizole (0.03 μ M) in a quiescent GH₃ cell. C, Representative single-cell trace for the effects of nimodipine (300 nM) added after astemizole (0.03 μ M) in an oscillating GH₃ cell. The experiments were performed with an acquisition interval of 1 s. The traces are representative of six to 10 cells for each experimental group studied in at least three different experimental sessions.

a methanesulfonanilide compound structurally unrelated to the above-mentioned H_1 receptor blockers and that has been widely used as a blocker of ERG K^+ channels (Kiehn et al., 1996; Snyders and Chaudhary, 1996), also enhanced spontaneous $[Ca^{2+}]_i$ oscillations. In contrast, cetirizine, another second-generation H_1 receptor blocker that is completely devoid of ERG-inhibitory properties (Taglialatela et al., 1998), failed to interfere with $[Ca^{2+}]_i$ oscillations. A further support to the hypothesis that ERG K^+ channels play a crucial role in controlling the resting membrane potential that underlies spontaneous $[Ca^{2+}]_i$ oscillations in GH_3 cells, came from the observation that those cells that were quiescent under resting condition (nonoscillating cells) were shifted toward an oscillatory pattern by ERG K^+ channel blockade induced by low nanomolar concentrations (1–30 nM) of astemizole, terfenadine, or dofetilide. Collectively, the results of this set of experiments suggest that the inhibition of ERG K^+ channels that participate in the maintenance of the resting membrane potential leads to a depolarization of the membrane of GH_3 cells, thus causing the activation of voltage-dependent L-type Ca^{2+} channels and an increase in $[Ca^{2+}]_i$ oscillations frequency. This view seems to be supported by the ability of nimodipine, a dihydropyridinic L-type Ca^{2+} channel blocker, to inhibit the following effects induced by astemizole: 1) the appearance of $[Ca^{2+}]_i$ oscillations in nonoscillating cells and 2) the increase of $[Ca^{2+}]_i$ oscillations frequency in spontaneously oscillating GH_3 cells.

The hypothesis, supported by the present results, that ERG K^+ channels play a pivotal role in $[Ca^{2+}]_i$ oscillations in resting GH_3 cells, is also suggested by recent data showing that they can also participate in the ability of thyrotropin-releasing hormone, via a yet unidentified intracellular path-

way, to depolarize the resting membrane potential, increase action potential frequency, and enhance $[Ca^{2+}]_i$ oscillations in pituitary clonal cells (Barros et al., 1994; Bauer, 1998). Furthermore, an effect similar to that described for thyrotropin-releasing hormone has also been recently found with astemizole in GH_3 cells, because this compound, used at nanomolar concentrations, by suppressing ERG K^+ channels, caused an increase in action potential frequency (Barros et al., 1997). In addition, the antiarrhythmic methanesulfonanilide E-4031 has recently been shown to also inhibit ERG K^+ channels and cause a moderate depolarization of rat primary lactotrophs, determining an increase in prolactin release (Bauer et al., 1999). The involvement of ERG K^+ channels in the spontaneous oscillatory behavior of $[Ca^{2+}]_i$ in GH_3 cells seems to also be suggested by the recent observation that Cs^+ increased the frequency of Ca^{2+} oscillations in these cells (Charles et al., 1999), an effect possibly related to its ability to block inwardly rectifying K^+ channels (underlined by ERG K^+ channels), although the possible interference of this monovalent cation with other cationic channels could not be excluded in the study of Charles et al. (1999) (Hille, 1997).

The present data also suggest that the role played by ERG K^+ channels in the control of the membrane potential in resting GH_3 cells seems to be dominant when compared to that of other K^+ channel subtypes; in fact, concentrations higher than the IC_{50} values of blockers of the small- and large-conductance Ca^{2+} -dependent K^+ channels, such as apamine (500 nM) and charibdotoxin (200 nM), respectively, as well as of ATP-dependent K^+ channels, such as glibenclamide (10 μ M), failed to interfere with $[Ca^{2+}]_i$ oscillations. The functional role of ATP-sensitive K^+ channels in endo-

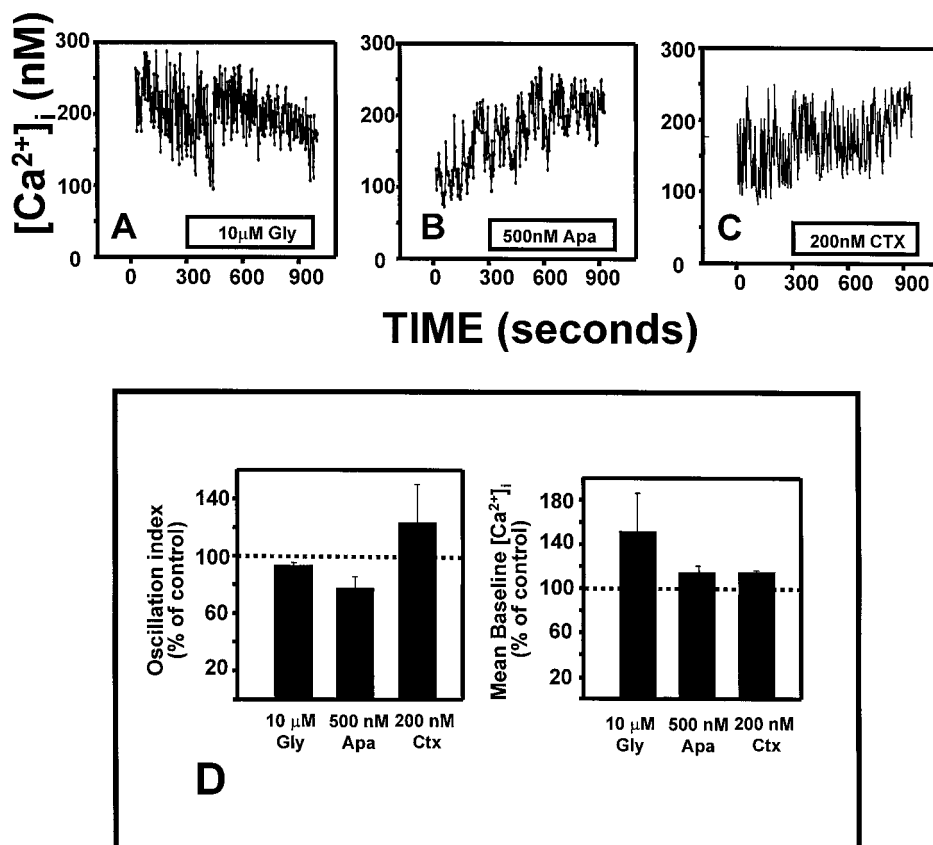


Fig. 9. Effect of glybenclamide (Gly), apamine (Apa), and charibdotoxin (CTX) on $[Ca^{2+}]_i$ oscillations in GH_3 cells. A, B, and C, representative single-cell traces for the effects of glybenclamide (10 μ M), apamine (0.5 μ M), and charibdotoxin (0.2 μ M), respectively, on $[Ca^{2+}]_i$ oscillations in GH_3 cells. The experiments were performed with an acquisition interval of 3 s. D, the quantification of drug effect on $[Ca^{2+}]_i$ oscillations performed as described under *Materials and Methods*; the drug concentration-effect on the oscillation index (left) and on the baseline $[Ca^{2+}]_i$ (right) are shown. Each point represents the mean \pm S.E. of 15 to 25 cells studied in at least three different experimental sessions.

crine cells has been investigated by Bernardi et al. (1993), who showed that the inhibition of ATP-dependent K⁺ channels expressed in adenohypophyseal cells may depolarize the cell membrane and enhance the release of GH. However, it should be noted that in the study of Bernardi et al. (1993) the functional contribution of ATP-sensitive K⁺ channels was investigated under conditions of metabolic exhaustion or previous channel activation by diazoxide rather than under resting conditions as in the present study. Thus, the functional role of this channel subtype may be different under physiological or pathological conditions. On the other hand, in GC cells, a rat pituitary subclone, which in contrast to GH₃ only releases GH, charibdotxin was able to enhance spike amplitude and duration, whereas apamine reduced after-spike hyperpolarization and increased spike duration, suggesting that these two K⁺ channel subtypes contribute to endogenous pacemaker activity (Kwiecien et al., 1998). However, in another study, Bauer et al. (1999) found that, in primary rat lactotrophs, apamine and charibdotxin elicited depolarizing responses in only about 50% of the cells and in this subpopulation of cells the extent of the depolarizing response was

about half of that observed with ERG K⁺ channel blockers (4.1 versus 7.2 mV). Therefore, it seems possible to conclude that a heterogeneous set of K⁺ channels may differentially shape the electrophysiological properties of distinct hormone-secreting pituitary cells.

Surprisingly, when higher concentrations of the second-generation antihistamines astemizole and terfenadine were used, an opposite effect to that occurring when these drugs were used in the nanomolar range was observed. In fact, micromolar concentrations of both these compounds suppressed the spontaneous oscillatory pattern of [Ca²⁺]_i in GH₃ cells. The molecular basis for this inhibitory effect seems to rely on the ability of astemizole and terfenadine to inhibit the flux of extracellular Ca²⁺ occurring upon SOC activation. In fact, these compounds were able to block, in a concentration-related fashion, [Ca²⁺]_i increase activated by Ca²⁺_i store depletion induced by sarcoplasmic or endoplasmic reticulum calcium ATPase pump inhibition with thapsigargin followed by the reintroduction of Ca²⁺_e. The effect of the inhibition of SOC by micromolar concentrations of the second-generation antihistamines astemizole and terfenadine on [Ca²⁺]_i oscil-

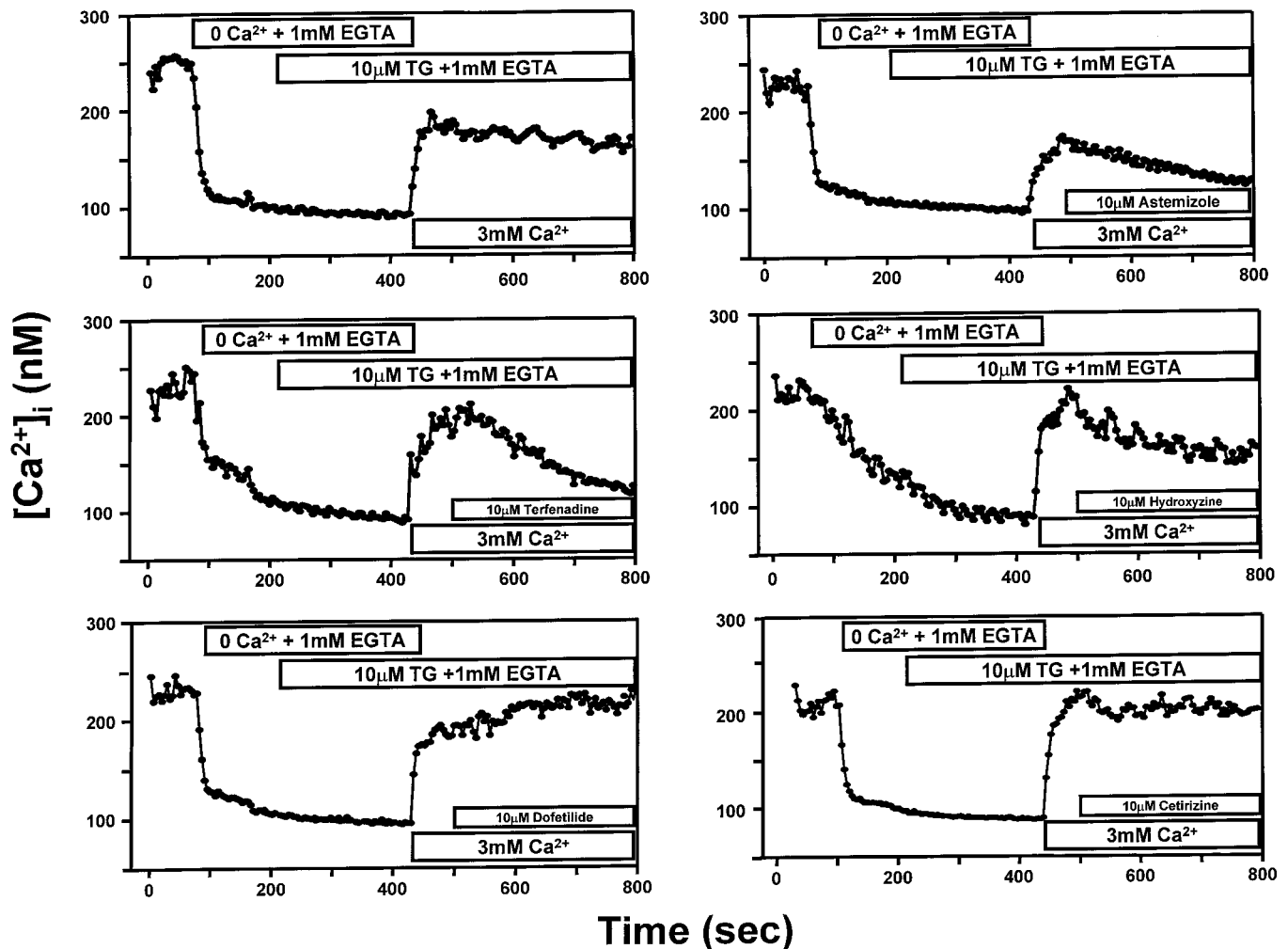


Fig. 10. Effect of astemizole, terfenadine, hydroxyzine, cetirizine, and dofetilide on Ca²⁺ influx activated by Ca²⁺_i store depletion SOC in GH₃ cells. Top left, the model of SOC activation: after removal of extracellular Ca²⁺ with 1 mM EGTA in a Ca²⁺_e-free solution, the cells were treated with 10 μM thapsigargin (TG); subsequently, 3 mM Ca²⁺_e was reintroduced, producing an increase in [Ca²⁺]_i that remained constantly elevated for the following 6 min. After 100 s from Ca²⁺_e reintroduction, 10 μM astemizole (top right), 10 μM terfenadine (middle left), 10 μM hydroxyzine (middle right), 10 μM cetirizine (bottom right), or 10 μM dofetilide (bottom left) was introduced, as indicated by the respective bar. Each trace is representative of 15 to 30 cells for each experimental group studied in at least three different experimental sessions.

lations in GH₃ cells is in line with the results of Fischer et al. (1997, 1998a), obtained in rat basophilic leukemia RBL-2H3 cells, showing that astemizole inhibited SOC-mediated Ca²⁺ fluxes and β -hexoseaminidase release. Subsequently, the same group of investigators (Fischer et al., 1998b), by studying the inhibitory effects of a long series of astemizole derivatives, concluded that SOC inhibition was strictly correlated with the degree of lipophilicity of their chemical structure. In accordance with these findings, in addition to astemizole, an inhibition of SOC and of [Ca²⁺]_i oscillations was also observed in the present study with micromolar concentrations of terfenadine and of the first-generation H₁ receptor antagonist hydroxyzine, two molecules displaying elevated lipophilicity (Timmerman, 1999). Interestingly, cetirizine, which is the main in vivo metabolite of hydroxyzine and is much more

hydrophilic than its metabolic precursor because of the presence of an ionizable carboxyl group, failed to affect SOC channels and [Ca²⁺]_i oscillations. The fact that hydroxyzine, although provided of a certain degree of inhibitory action on ERG K⁺ channels (Taglialatela et al., 2000), was unable to enhance the frequency and amplitude of [Ca²⁺]_i oscillations in GH₃ cells when used at low concentrations (30–300 nM) could be explained by the much weaker affinity of this compound for ERG K⁺ channels when compared with astemizole, terfenadine, and dofetilide and that these hydroxyzine concentrations were not sufficient to significantly interfere with ERG K⁺ channel activity. In fact, the IC₅₀ for hydroxyzine inhibition of ERG K⁺ channels heterologously expressed in *Xenopus* oocytes was at least 100-fold higher when compared to those of the second-generation antihistamines or the an-

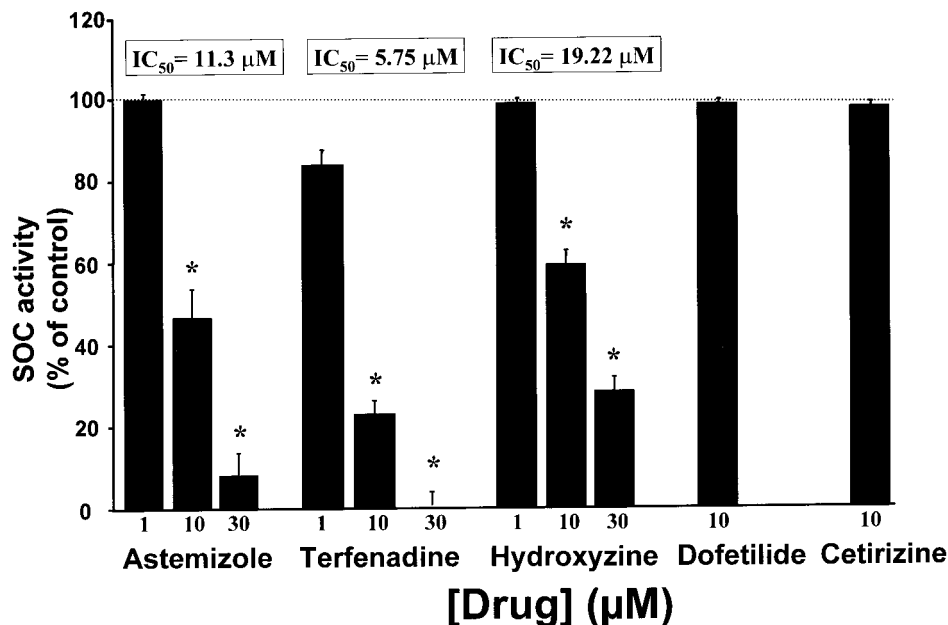


Fig. 11. Quantification of the effect of astemizole, terfenadine, hydroxyzine, cetirizine, and dofetilide on Ca²⁺ influx activated by Ca²⁺ store depletion SOC in GH₃ cells. Each column shows the percentage of inhibition of [Ca²⁺]_i increase upon [Ca²⁺]_e reintroduction by each pharmacological treatment compared with its respective control. The data were calculated by dividing the mean of the last 10 points of each experimental trace obtained in the presence of the drug (≈1 min) by the mean of the last 10 points (≈1 min) acquired before drug introduction. To estimate the IC₅₀ of drug effect on SOC activity, the data were fitted by the equation: SOC activity: MAX · [drug]/([drug] + 9.66), where MAX is the maximum SOC activity. *, denotes values statistically different (*P* < .05) versus the control group. Each bar is the mean ± S.E. of 15 to 30 cells studied in at least three different experimental sessions.

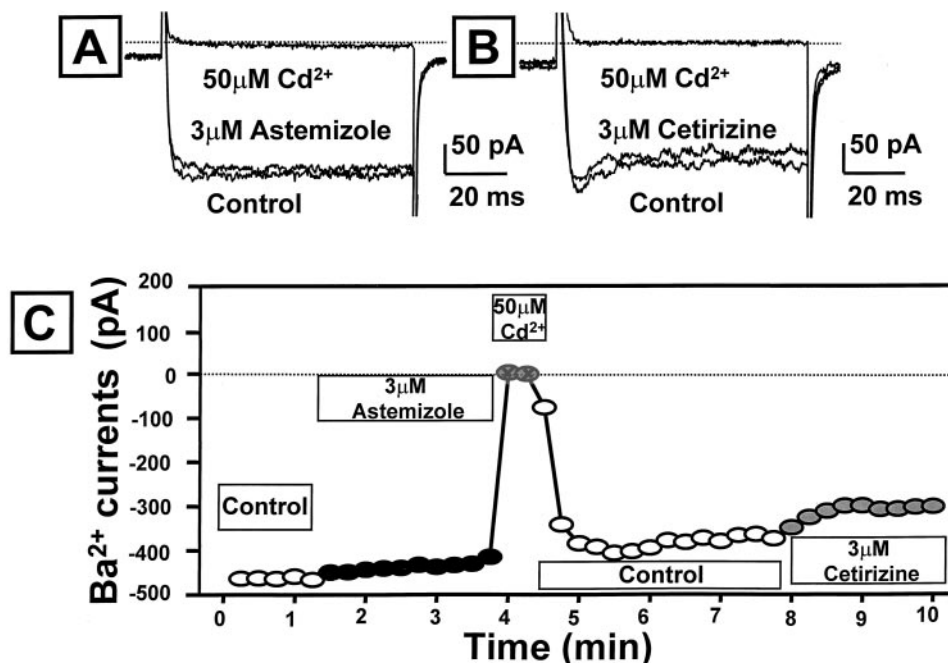


Fig. 12. Effect of astemizole (3 μM) and cetirizine (3 μM) on high-voltage-activated L-type Ca²⁺ currents in GH₃ cells. A, the effects of astemizole (3 μM) and cetirizine (3 μM) on high-voltage-activated Ca²⁺ channels in two different GH₃ cells. Ba²⁺ currents flowing through VGCC were activated by test pulses from -60 mV to 0 mV (100 ms duration) elicited at 0.066 Hz frequency (1 pulse every 15 s). The traces shown in A and B represent control and drug effect after 3 min of perfusion with each drug. For comparison, the Ba²⁺ current trace obtained after complete blockade of Ca²⁺ channels by 50 μM Cd²⁺ is also shown. In C, a time course in a single GH₃ cell of Ba²⁺ currents flowing through Ca²⁺ channels exposed to the conditions indicated is shown. The amplitude of the Ba²⁺ currents was measured at the end of each depolarizing pulse. A quantification of the data shown can be found under *Results*.

tiarrhythmic compound (Taglialatela et al., 2000). This evidence gives further support to the hypothesis of lipophilicity as a major determinant for drug effect on these refilling Ca²⁺ channels activated by Ca²⁺_i stores depletion. Another aspect that emerges from the present study is that the inhibition of SOC observed with terfenadine and astemizole is not a mandatory property of all ERG-blocking drugs; in fact, dofetilide, although displaying high affinity for ERG K⁺ channel inhibition, failed to affect SOC in GH₃ cells, even if used in concentrations up to 100 μM. Overall, these experiments suggest that, in spontaneously oscillating GH₃ cells, the inhibition of SOC can prevent the oscillatory pattern of [Ca²⁺]_i, thus reinforcing the hypothesis that, in addition to a plasma membrane oscillator, a cytoplasmic [Ca²⁺]_i oscillator may also play a certain role in such physiological phenomena and that SOC participates in the depletion-refilling cycle of such an oscillator (Stojilkovic, 1996; Parekh and Penner, 1997). A hypothetical model that accounts for the participation of SOC in [Ca²⁺]_i oscillation in GH₃ cells may involve the spontaneous and rhythmic phospholipase C-induced generation of IP₃ prompted by the plasma membrane oscillator-dependent L-type Ca²⁺ channel activation (Meyer and Stryer, 1991). In addition, the observation that, when both ERG K⁺ channels and SOC are simultaneously inhibited, [Ca²⁺]_i oscillations are completely abolished suggests that the activity of the two oscillators are tightly coordinated and that SOC plays a pivotal role in such coordination.

Because it has been shown that L-type voltage-gated Ca²⁺ channels play a crucial role in [Ca²⁺]_i oscillations in GH₃ cells, as demonstrated by the ability of the L-type Ca²⁺ channel inhibitor nifedipine to reduce the frequency of [Ca²⁺]_i oscillations (Schleger et al., 1987), the possibility existed that the inhibitory action on [Ca²⁺]_i oscillations displayed by micromolar concentrations of the antihistamines evaluated in the present study could be due to their inhibition of L-type voltage-gated Ca²⁺ channels (Ming and Nordin, 1995; Liu et al., 1997). However, the present observation that concentrations of astemizole that effectively suppressed Ca²⁺ oscillations failed to inhibit high-voltage-activated Ca²⁺ currents (mainly of the L-subtype) recorded with direct electrophysiological measurements in GH₃ cells does not support this hypothesis. In addition, it should be underlined that the possible contribution of voltage-dependent Ca²⁺ channels in the model of SOC activation presently utilized should be minimal, because the concentration of thapsigargin used (10 μM) has been reported to completely suppress L-type Ca²⁺ currents in GH₃ cells (Nelson et al., 1994).

In conclusion, the results of the present study seem to suggest that ERG K⁺ channels play a prominent role in controlling the oscillatory pattern of [Ca²⁺]_i in resting GH₃ cells, possibly by modulating the membrane potential variations that are crucial for the opening of voltage-dependent Ca²⁺ channels underlying the oscillatory behavior. On the other hand, the refilling of cytoplasmic Ca²⁺ stores also plays an important role, as demonstrated by the blockade of [Ca²⁺]_i oscillations achieved by micromolar concentrations of astemizole, terfenadine, and hydroxyzine but not of dofetilide or cetirizine. Furthermore, because the blockade of ERG K⁺ channels and of SOC channels induced opposite effects on [Ca²⁺]_i oscillations in GH₃ cells, this clone might represent a valuable model to evaluate the potential activity of drugs interfering with these two membrane channels.

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