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Sarco-Endoplasmic ATPase Blocker 2,5-Di(*tert*-butyl)-1,4-benzohydroquinone Inhibits N-, P-, and Q- but Not T-, L-, or R-Type Calcium Currents in Central and Peripheral Neurons

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ABSTRACT

The effects of 2,5-di(*tert*-butyl)-1,4-benzohydroquinone (tBHQ), a synthetic phenolic antioxidant and a blocker of the sarco-endoplasmic ATPase, were evaluated on low and high voltage-activated Ca^{2+} currents (ICas) with rodent dorsal root ganglion, hippocampal, and motor neurons. In all cell types tested, tBHQ (IC_{50} = 35 μM) blocked ICa at concentrations used to inhibit sarco-endoplasmic ATPase. This effect was specific to tBHQ because the other sarco-endoplasmic reticulum calcium ATPase pump inhibitors (thapsigargin and cyclopiazonic acid) had no effect. Selective blockade of the N-type current with ω-conotoxin GVIA and of P- (motoneuron) or Q-type currents (hippocampal neuron) with ω-agatoxin IVA indicated that tBHQ inhibited N, P, and Q types of ICa. tBHQ had no effect on nitrendipine-sensitive (L-type) and residual drug-resistant (R-type) ICa, nor on the low voltage-activated T-type ICa. Contrary to neuronal cells, the L-type ICa was inhibited by tBHQ in a differentiated mouse neuroblastoma and rat glioma hybrid cell line. Injection of cDNAs encoding the α1A, α1B, α1C, and α1E subunits into oocytes showed that tBHQ blocked ICas at the level of the pore-forming protein. This effect of tBHQ on ICa should be considered when interpreting results obtained with tBHQ used on neuronal preparations. It also may be useful for developing new strategies for the generation of more potent intracellular calcium transient inhibitors.

Plasmalemmal voltage-gated calcium channels (VGCCs) initiate intracellular calcium transients and control many aspects of neuronal processes, including the generation of calcium-dependent action potentials, neurotransmitter release, regulation of neuronal death, synapse formation and elimination, phenotypic differentiation, and gene expression (Ghosh and Greenberg, 1995; Gu and Spitzer, 1997). These channels are activated by membrane depolarization, leading to a transmembrane calcium influx and a transient increase in cytoplasmic free calcium concentration. It has become clear, however, that these voltage-dependent calcium transients also are generated by mechanisms involving the release of calcium from intracellular stores in the sarco-endoplasmic reticulum. In neurons, at least two types of calcium stores have been identified in the sarco-endoplasmic reticulum, based on calcium-release channels: caffeine-ryanodine-sensitive and inositol-1,3,4-triphosphate-sensitive calcium channels. The relationships among voltage-activated calcium influx, release of calcium from cytoplasmic stores, and intracellular calcium transients are complex and are only just beginning to be understood.

Five VGCC subtypes (T, L, N, P/Q, and R) were initially defined according to electrophysiological and pharmacological characteristics (Birnbaumer et al., 1994) and, more recently, these definitions have been extended to take into account amino acid sequences (Perez-Reyes and Schneider, 1994; Perez-Reyes et al., 1998). Similarly, at least three intracellular calcium channel subtypes have been characterized at the molecular level for both the ryanodine and inositol-1,3,4-triphosphate receptors. Such diversity raises the possibility that calcium channels may combine in different ways in a single neuron, thereby controlling specific pathways for calcium signaling, which may in turn have different functions. Therefore, the pharmacological isolation of channels with specific agents is essential if we are to elucidate the

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ABBREVIATIONS: VGCC, voltage-gated calcium channel; tBHQ, 2,5-di(*tert*-butyl)-1,4-benzohydroquinone; SERCA, sarco-endoplasmic reticulum calcium ATPase; E15, embryonic day 15; DRG, dorsal root ganglion; NG108-15, mouse neuroblastoma and rat glioma hybrid cell line; ICa, Ca^{2+} current; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; DMSO, dimethyl sulfoxide; GVIA, ω-conotoxin GVIA; AgaIVA, ω-agatoxin IVA; CPA, cyclopiazonic acid; HP, holding potential.

roles of the various channel families in the regulation of neuronal calcium signaling.

The chemical 2,5-di(*tert*-butyl)-1,4-benzohydroquinone (tBHQ) belongs to a large family of synthetic phenolic antioxidants commonly used as food preservatives. In addition to this beneficial effect, these compounds are potentially carcinogenic and, paradoxically, play a significant role in oxidative stress (van Esch, 1986; Yu et al., 1997). Independent of its ability to modify the thiol groups of proteins (Moore et al., 1987), tBHQ also has been shown to inhibit the calcium ATPase of the sarco-endoplasmic reticulum (SERCA), which is involved in the refilling of $[Ca^{2+}]_i$ stores (Kostyuk and Verkhratsky, 1994). This inhibition of SERCA activity has been used in experiments designed to detect $[Ca^{2+}]_i$ stores in neuronal preparations under various experimental conditions and to evaluate their relative contributions. One study reported that tBHQ inhibited L-type calcium currents in neuroendocrine cells (Nelson et al., 1994), but no data are available concerning the effects of this compound on neuronal VGCCs. We show herein that, at concentrations used to block SERCA, tBHQ also inhibited N- and P/Q-type VGCCs in peripheral and central neurons without affecting neuronal T-, L-, and R-type VGCCs. This effect seemed to involve a direct action on VGCCs, and may generate new strategies for the development of new classes of "calcium antagonists."

Materials and Methods

Cell Cultures. Spinal motoneurons from embryonic day 15 (E15) Sprague-Dawley rat embryos were purified by a two-step metrizamide-panning method, as previously described (Camu et al., 1993). Briefly, ventral spinal cords were treated with trypsin, dissociated, and centrifuged on 6.5% metrizamide cushions. The yield of large cells, which were not dense enough to pass through the metrizamide, was increased by immunopanning on Petri dishes coated with an antibody directed against the p75 neurotrophin receptor (low-affinity nerve growth factor receptor), specifically expressed by motoneurons at this stage (Yan and Johnson, 1988).

Hippocampi from E17 Sprague-Dawley rat embryos were quickly removed under a binocular microscope and treated for 10 to 20 min at 37°C with trypsin. Hippocampal neurons were then dissociated with glass pipettes (Brewer et al., 1993). At this stage, cells were widely dispersed. Dorsal root ganglia (DGRs) were dissociated from E13 Swiss mice as previously described (Diochot et al., 1995; Hilaire et al., 1996). Cells were cultured in a defined medium (Neurobasal; Life Technologies, Cergy-Pointoise, France) supplemented with B27 (Life Technologies), 0.5 mM glutamine, and 25 μM glutamate) at 37°C in a 95% O_2, 5% CO_2 atmosphere before the experiments.

Mouse neuroblastoma × rat glioma hybrid cells (NG108-15) were obtained from Dr. P. Lory, Centre National de la Recherche Scientifique, Montpellier, France, and maintained in culture in Dulbecco's modified Eagle's medium (Eurobio, Paris, France) supplemented with 10% fetal bovine serum (Eurobio), 2% hypoxanthine-aminopterine-thymidine (Life Technologies), 2 mM glutamine, and 1% penicillin/streptomycin. Neuronal differentiation was induced by lowering the fetal bovine serum complement to 1% and adding dibutyril cAMP (1 mM final concentration). Differentiation occurred within 4 to 6 days as judged by the change in cell morphology as shown in Leuranguer et al. (1998).

Xenopus oocyte preparation and injection (5–10 nl of α_{1A}, α_{1B}, α_{1C}, or α_{1E} + $\alpha 2$-δ + β_1, β_2, β_3, or β_4 cDNAs at ~0.3 ng/nl) were performed as described in Cens et al. (1999). Oocytes were then incubated for 2 to 7 days at 19°C with gentle shaking before recording.

Electrophysiological Recordings. Ca^{2+} current (ICa) from dorsal root ganglions (DRGs) was recorded 2 h after dissociation. For motoneurons, ICa was recorded after 1 or 2 days in culture. ICa in hippocampal pyramidal cell-like neurons was recorded after 3 days in culture. ICa in NG108-15 cells was recorded at 4 or 5 days of differentiation. Whole-cell recordings were made at 20–22°C under conditions optimized so as to ensure the isolation of ICa from other voltage-activated currents. The bathing solution contained 115 mM tetraethylammonium chloride, 5 mM $BaCl_2$ (10 mM for NG108-15 cells), 10 mM HEPES, 10 mM glucose, and 1 μM tetrodotoxin with the pH adjusted to 7.35 with CsOH. Recording pipettes were filled with the following solution: 135 mM CsCl, 20 mM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), 10 mM HEPES, 3 mM Mg-ATP, 1 mM Mg-GTP, and 10 mM glucose, pH 7.35 (adjusted with CsOH). The pipette resistance was ≤4 MΩ. Whole-cell currents were recorded with a Bio-Logic RK300 patch-clamp amplifier (Claix, France). After seal formation and membrane disruption, series resistance was estimated from the capacitative transient evoked by a +10-mV test pulse. Membrane capacity was calculated as Cm = τ/Rs, with τ being the time constant of capacitative transient and Rs the series resistance. Because current amplitudes were always ≤1 nA, voltage errors due to uncompensated series resistance were negligible (<4 mV). All experimental parameters were controlled with a computer equipped with a DigiData 1200 analog interface (Axon Instruments, Foster City, CA). Data acquisition and analysis were performed with the pClamp software (version 6.03; Axon Instruments). Current signals were sampled at 5 kHz and filtered at 3 kHz. They were then digitized and stored.

For oocytes, whole-cell Ba^{2+} currents were recorded under two-electrode voltage-clamp with the GeneClamp 500 amplifier (Axon Instruments). Electrodes (<1 MΩ) were filled with 2.8 M CsCl and 10 mM BAPTA, pH = 7.2 (adjusted with CsOH). Ba^{2+} and ICa were recorded after injection of BAPTA (one or two 40- to 70-ms injections at 1 bar of 100 mM BAPTA free acid, 10 mM CsOH, 10 mM HEPES, pH 7.2). The recording solution had the following composition: 10 mM BaOH, 20 mM tetraethylammonium hydroxide, 50 mM *N*-methyl-D-glucamine, 2 mM CsOH, and 10 mM HEPES, pH 7.2 (adjusted with methane-sulfonic acid). Currents were filtered and digitized with a DMA-Tecmar Labmaster, and were subsequently stored on an IPC 486 personal computer with version 6.02 of pClamp (Axon Instruments). Currents were recorded during a typical test pulse from −80 to +10 mV of 0.4-s duration. tBHQ (30 μM) was prepared from a stock solution [0.1 M in dimethyl sulfoxide (DMSO)] by appropriate dilution with the BaOH recording solution. DMSO had no effect at this concentration (0.03%; data not shown). All currents analyzed in this study had an amplitude of 0.5 to 4 μA, when recorded 1 to 4 days after injections. Currents were repeatedly recorded, with all combinations of the subunits tested ($\alpha 2$-δ and β_1, β_2, β_3, or β_4).

Dye Loading and Measurements of $[Ca^{2+}]_i$. Dye loading and measurement of $[Ca^{2+}]_i$ was performed as previously described (Dayanithi et al., 1996). Briefly, the culture dishes were washed and loaded by incubation with 2.5 μM Fura-2-AM and 0.05% w/v Pluronic F-127 (Molecular Probes, Eugene, OR) in Locke buffer (140 mM NaCl, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 10 mM glucose, 5 mM KH_2PO_4, and 10 mM HEPES-NaOH, pH 7.25) at 34°C for 40 min. Loaded cells were washed with Locke buffer and fluorescence measurements were performed at room temperature. $[Ca^{2+}]_i$ in single cells was measured with the digital imaging microfluorimetry system (Axon Instruments) based on an inverted microscope equipped with epifluorescence optics (Nikon, Champigny-sur-Marne, France). Interference filters of 340/10 nm and 380/10 nm were alternately mounted on the filter wheel and the excitation light beam was deflected through an oil-immersion objective (40× 0.75 numerical aperture; Nikon). Fluorescence measurements were converted to $[Ca^{2+}]_i$ with a standard equation (Grynkiewicz et al., 1985) for ratiometric dyes $[Ca^{2+}]_i = K_d \times (S_{f2}/S_{b2}) \times (R - R_{min})/(R_{max} - R)$ where K_d is the dissociation constant (0.145 μM for Fura-2; Molecular Probes); S_{f2}/S_{b2} is the ratio of the signals obtained at 380 nm in the nominal absence and presence of saturating concentrations of Ca^{2+}, respectively; R_{min} and R_{max} are the fluorescence ratios deter-

mined in the nominal absence and presence of saturating concentrations of Ca^{2+}; and R is the fluorescence ratio measured. For 11 motoneurons, S_{f2}/S_{b2} was 2.7 ± 0.1, R_{min} was 0.45± 0.01, and R_{max} was 2.6 ± 0.1.

Drugs. Nitrendipine (Bayer AG, Wuppertal, Germany), tBHQ, cyclopiazonic acid (CPA), and thapsigargin (Sigma, Saint Quentin Fallavier, France) were dissolved in DMSO to make concentrated stock solutions (10, 100, and 1 mM, respectively) and stored at −20°C. Controls showed that the solvent had no effect on ICa at the maximal final dilutions used herein (<0.05%). ω-Conotoxin GVIA (GVIA; Sigma) and ω-agatoxin IVA (AgaIVA; Peptide International, Louisville, KY, and Pfizer, New York, NY) were dissolved in double distilled water at 0.1 and 1 mg/ml, respectively, to give stock solutions. Stock solutions of $NiCl_2$ and $CdCl_2$ were prepared in double distilled water at 10 mM. Test solutions were prepared daily with aliquots from frozen stocks to obtain the working concentrations. Results are expressed as mean ± S.D. Means were compared with Student's t test.

Results

tBHQ and Thapsigargin Do Not Reduce $[Ca^{2+}]_i$ to the Same Extent in Cultured Motoneurons. The basal $[Ca^{2+}]_i$ of rat embryonic motoneurons was 36 ± 2 nM and application of 100 mM K^+ induced an increase in $[Ca^{2+}]_i$ due to the opening of calcium channels, with the concentration reaching 475 ± 63 nM (n = 43; Fig. 1). To evaluate the spontaneous run-down of the response, a second depolarization with K^+ was applied 10 min after the first. The run-down after the second application of K^+ amounted to a 20 ± 2% decrease in the peak $[Ca^{2+}]_i$ (n = 30; Fig. 1A). In another series of experiments, cells were incubated for 10 min with 250 nM thapsigargin or 10 μM CPA, the maximal inhibitory doses known to completely inhibit SERCA (Thastrup et al., 1990). Thapsigargin decreased peak $[Ca^{2+}]_i$ by 51 ± 2% (n = 25) and CPA by 50 ± 2% (n = 17). These values were significantly different from those obtained for run-down ($P < .001$). After correction for run-down, thapsigargin was found to have caused a 31 ± 2% (n = 25) decrease in maximal amplitude of the intracellular calcium transient and CPA at 30 ± 3% (n = 17; Fig. 1B). This suggests that intracellular stores and calcium influx contributed 30 and 70%, respectively, of the intracellular calcium transient induced by depolarizing stimuli. Application of 10 μM tBHQ, which gives maximal inhibition of SERCA (Moore et al., 1987), resulted in a 60 ± 3% decrease in the intracellular calcium transient (n = 15; $P < .001$ relative to run-down). Corrected for run-down, tBHQ gave 40 ± 3% inhibition (n = 15; Fig. 1C), and therefore had a significantly stronger effect than thapsigargin ($P < .05$).

tBHQ Reduced ICa in Cultured Motoneurons. It has been reported that tBHQ inhibits L-type ICa in non-neuronal preparations. We therefore used the patch-clamp technique to evaluate and to compare the effects of thapsigargin and tBHQ on the voltage-activated ICa of embryonic motoneurons. ICa were recorded with a 300-ms depolarization to 0 or +10 mV from a −100-mV holding potential (HP) to ensure maximal amplitude. At this voltage, ICa was composed of a sustained and a transient component. The sustained component was quantified by measuring the absolute amplitude of ICa at 250 ms, whereas the transient component was calculated as the difference between peak inward current and sustained current. After breaking the membrane patch, repetitive cell stimulation every 10 s initially induced an increase in ICa amplitude, ICa run-up, as is usually reported in cardiac as well other neuronal cells (Tiaho et al., 1993; Diochot et al., 1995). Once ICa had reached a steady amplitude, on four cells tested, the application of thapsigargin (250 nM) had no effect (Fig. 2A). In seven other cells, the application of 10 μM tBHQ induced a decrease in ICa amplitude corresponding to 24 ± 8% inhibition for the transient component and 13 ± 4% inhibition for the sustained component (n = 7; Fig. 2B). The onset of ICa inhibition was fast and complete within 10 s. Dose-response curves for tBHQ were constructed from the transient and sustained components of macroscopic ICa. tBHQ inhibited both the transient and sustained components of ICa with similar half-maximal inhibitory concentrations, IC_{50} = 35 μM, and the maximum inhibition was 75 to 80% of macroscopic ICa (Fig. 2C).

tBHQ Selectively Inhibited N- and P-Types of ICa But Did Not Affect L- and R-Types of ICa in Motoneurons. In a previous study, we showed that the macroscopic ICa of E15 rat motoneurons consists of four high voltage-activated ICa, the N, P, L, and R-type ICa**,** and we characterized these currents pharmacologically, kinetically, and functionally (Scamps et al., 1998; Table 1). A low voltage-

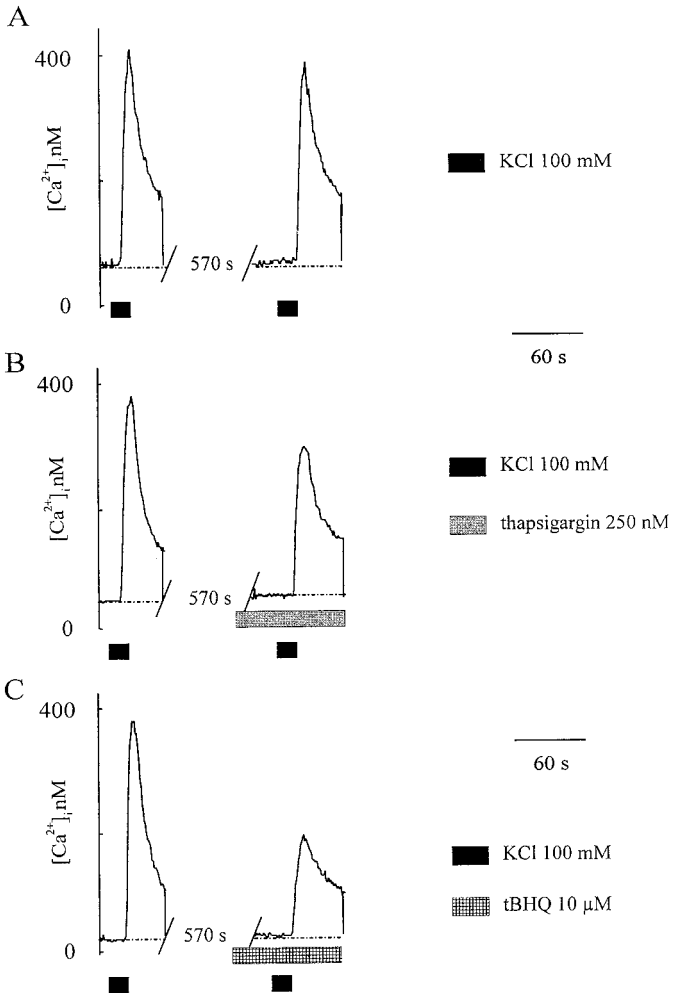

Fig. 1. Effects of SERCA pump inhibitors on K^+-induced $[Ca^{2+}]_i$ responses in embryonic rat motoneurons. A, effects of two successive applications of 100 mM K^+ on the $[Ca^{2+}]_i$ responses are illustrated. The cell was allowed to recover for 10 min between each depolarization. B and C, effects of 10-min incubation of SERCA pumps inhibitors thapsigargin and tBHQ, respectively, on K^+-induced $[Ca^{2+}]_i$ responses.

activated, T-type current was observed in 10% of the cells studied with a negligible amplitude as previously reported (Magnelli et al., 1998) for a similar preparation after 7 days in culture. Because 75% of macroscopic ICa were sensitive to tBHQ, we investigated whether tBHQ had some specificity with respect to calcium channel types. After application of a supramaximal concentration of tBHQ (300 μM), we evaluated the effects of 3 μM GVIA, 1 μM nitrendipine, and 50 nM AgaIVA as specific antagonists of the N-, L-, and P type channels, respectively (Fig. 3A). In the presence of 300 μM tBHQ, GVIA and AgaIVA had no effect (n = 4), but nitrendipine induced a 27 ± 3% decrease relative to the sustained ICa amplitude, an effect similar to that obtained under control conditions (n = 7; Fig. 3B). These results suggest that tBHQ totally inhibits the N- and P-type channels without affecting L- and R-type channels.

N-, P-, and Q-Type ICa But Not T-, L-, and R-Type ICa Is Inhibited by tBHQ in Hippocampal and DRG Neurons. We checked whether the observed effects were tissue-specific with two other preparations, originating from the central nervous system (hippocampus) and from the peripheral nervous system (DRGs). After 3 days in culture, the macroscopic ICa of the pyramidal cells from E17 hippocampus consisted of Q-, L-, and R-type channels because these neurons did not respond to the application of 3 μM GVIA (n = 6) and were sensitive only to a high concentration (250–500 nM) of AgaIVA (45 ± 6% inhibition; n = 5) and to 1 μM nitrendipine (35 ± 3% inhibition; n = 6; Fig. $4A_1$). The R-type current accounted for ~10 to 20% of the sustained macroscopic current. A T-type current was observed in 40% of the cells studied and its amplitude was no >50 pA. This preparation was therefore of value for studying specifically the effects of tBHQ on the Q-type ICa. Superfusion with 300 μM tBHQ decreases sustained ICa amplitude by 61 ± 10% (n = 5). Under these conditions, AgaIVA had no effect, nitrendipine induced a decrease in current amplitude, and an R-type current was detected in the presence of these agents (Fig. $4A_2$).

DRG neurons isolated from E13 embryonic mice have been reported to possess well developed P/Q- and N-type ICa and almost no L-type current (Diochot et al., 1995; Table 1). This preparation was therefore suitable for evaluating the sensitivity of each type of calcium channel to tBHQ. These neurons also have a well resolved T-type current, unlike motoneurons and hippocampal pyramidal neurons (Table 1). A representative distribution of ICa obtained by depolarization to 0 mV from a −100-mV HP in E13 DRG neurons is shown Fig. $4B_1$. For simplicity, the P and Q types were studied together by a single superfusion of 500 nM AgaIVA. Figure $4B_2$ illustrates the effects of tBHQ in DRG neurons. Application of 300 μM tBHQ induced a 75 ± 3% decrease in sustained ICa (n = 5). Application of 3 μM GVIA induced an 11 ± 2% decrease in ICa amplitude (n = 3), whereas 500 nM AgaIVA had no effect (n = 3). For motoneurons and pyramidal neurons, L-type current was not inhibited by tBHQ.

We evaluated the sensitivity of N-, P/Q-, and T-type ICa to

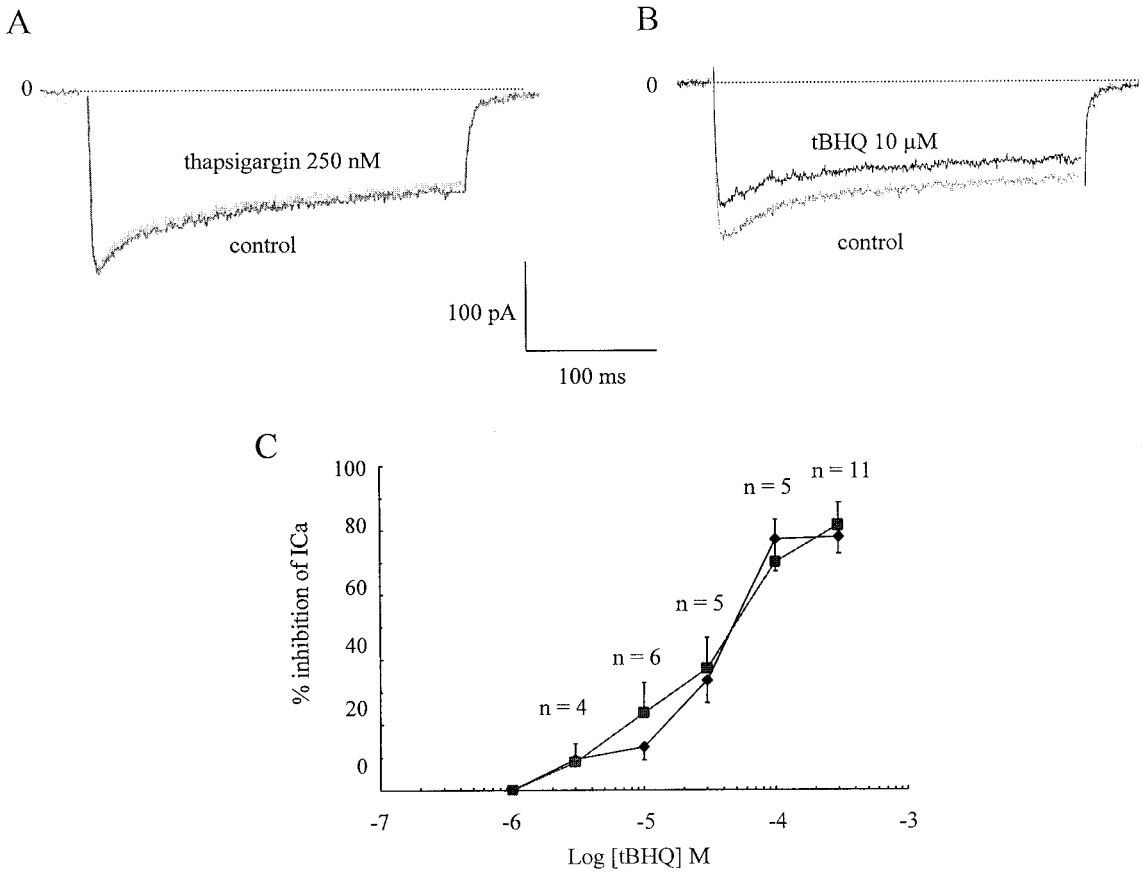

Fig. 2. Effects of SERCA pump inhibitors on the ICa amplitude of embryonic rat motoneurons. A, superfusion of 250 nM thapsigargin had no effects on ICa amplitude. B, at 10 μM, tBHQ induced a substantial decrease in ICa amplitude. C, dose-response curves for tBHQ inhibition of transient (■) and sustained (♦) components of macroscopic ICa. Current recorded from a HP of −100 mV to a depolarization to +10 mV, every 10 s.

tBHQ by applying a two-pulse protocol to a DRG neuron. The cell was first depolarized for 150 ms to −40 mV from a −100-mV HP to elicit mainly the T-type ICa. It was then followed 1 s later by a 300-ms to 0-mV depolarization. The effects of 30 and 100 μM tBHQ are illustrated in Fig. 5A$_1$ on P/Q-type ICa (in the presence of 3 μM GVIA and 250 nM nitrendipine) and in Fig. 5A$_2$ on N-type ICa (in the presence of 250 nM AgaIVA and 250 nM nitrendipine). The percentage inhibition of 30 μM tBHQ was calculated relative to the current remaining in the presence of 100 μM tBHQ (100%). At 30 μM tBHQ induced a 39 ± 3 (n = 6) and a 35 ± 3% (n = 7) decrease in P/Q-type and N-type ICa amplitude, respectively. T-type ICa was not inhibited by tBHQ even at 300 μM (Fig. 5A). The inhibitory effects of tBHQ were rapid and reversible.

TABLE 1

Contribution of each subtype of calcium channel to the macroscopic ICa in central and peripheral neurons and sensitivity to tBHQ

Subtype	Motoneuron[a]	Hippocampal Neuron	DRG Neuron[b]	% Inhibition at 30 μM tBHQ (DRG Neuron)
	%	%	%	
L	28 ± 17 (n = 8)	35 ± 3 (n = 6)	<5 (n = 5)	no
N	48 ± 12 (n = 10	no	47 ± 10 (n = 9)	35 ± 3 (n = 7)
P	24 ± 9 (n =8)	no	27 ± 5 (n = 9)	39 ± 3 n = 6)
Q	no	45 ± 6 (n = 5)	21 ± 10 (n = 5)	39 ± 3 (n = 6)
R	~10 (n = 10)	~20 (n = 5)	~15 (n = 5)	no
T	small (10% of cells)	small (40% of cells)	yes (all cells)	no

[a] From Scamps et al. (1998).
[b] From Diochot et al. (1995).

tBHQ Inhibits Currents Generated by Injection of cDNAs Encoding α1A, α1B, α1C, and α1E into Oocytes. To determine more accurately the site of action of tBHQ, we injected the α-subunits encoded by the A, B, C, and E genes into oocytes. tBHQ at 30 μM inhibited the α1A, α1B, α1C, and α1E subunit currents, suggesting that this compound acts specifically on the pore-forming subunit of the calcium channel (Fig. 6A). At 30 μM, tBHQ inhibits barium currents recorded from oocytes expressing the α1A, α1B, α1C, or α1E calcium channel subunits by 58 ± 14 (n = 8), 64 ± 10 (n = 4),

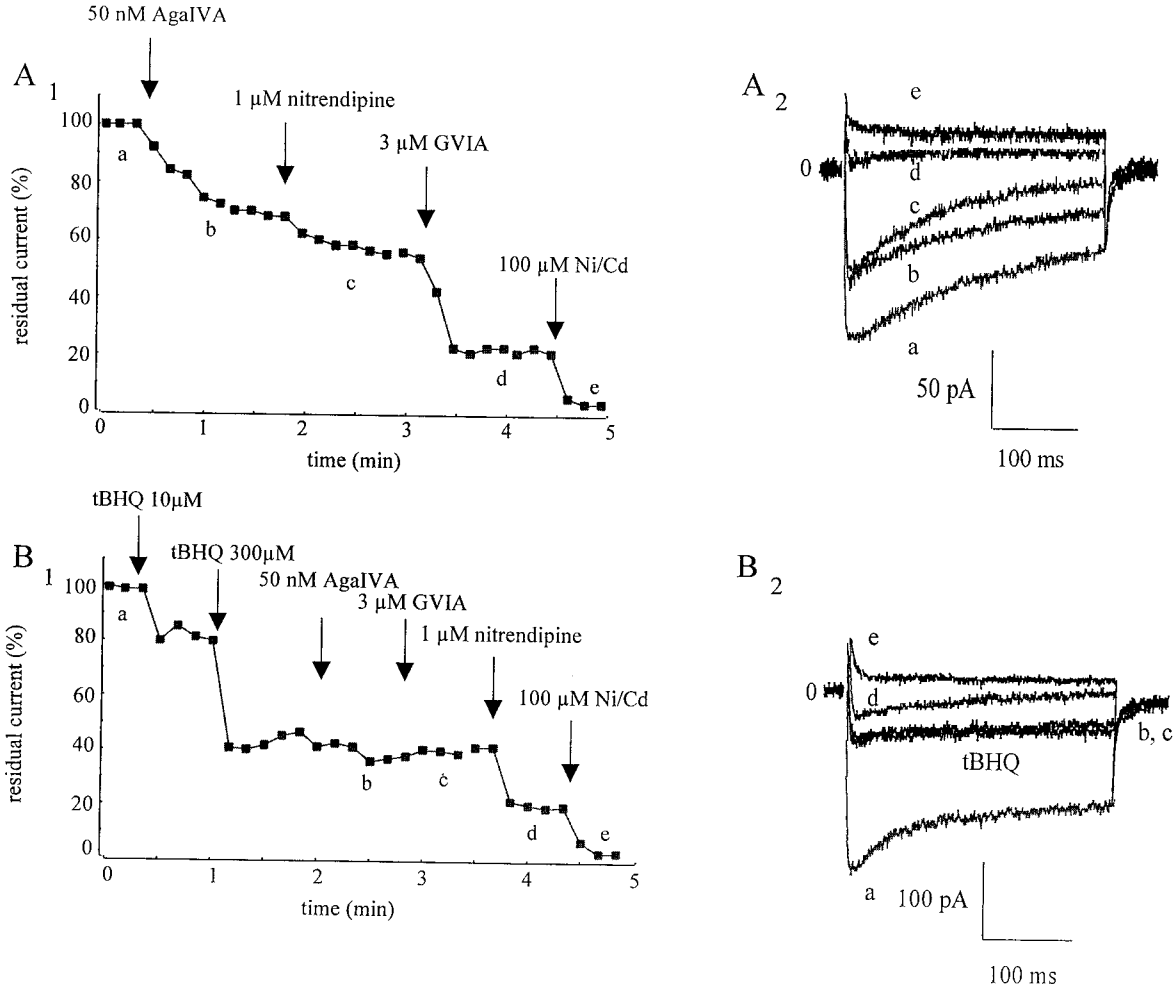

Fig. 3. Specificity of tBHQ inhibition of Ca^{2+} channel type in embryonic rat motoneurons. A, macroscopic high voltage-activated ICa of E15 rat embryonic motoneuron is composed of the P-, N-, L-, and R-type Ca^{2+} channels as shown by the use of 50 nM AgaIVA, 3 μM GVIA, and 1 μM nitrendipine, respectively. Nickel/cadmium (Ni/Cd; 100 μM) was added to evaluate the true zero current (A$_1$, time course of ICa inhibition; A$_2$, corresponding current traces). B, application of a supramaximal dose of tBHQ (300 μM) decreased ICa amplitude and prevented the effects of the specific inhibitors of the P- and N-type channels (B$_1$, time course effect of tBHQ and subsequent application of the specific Ca^{2+} channels inhibitors; B$_2$, corresponding current traces). Same protocol as in Fig. 2.

30 ± 10 (n = 6), and 67 ± 13% (n = 6), respectively, when coexpressed with the α2-δ and β1 auxiliary subunits. Therefore the order of potency of tBHQ was α1E > α1B > α1A > α1C. As with the native proteins, the kinetics of tBHQ inhibition was rapid (Fig. 6B) and reversible. The insensitivity of the L- and R-type channels in neurons is in contradiction with the results obtained after expression of the α1C and specifically of the α1E subunits. One possible explanation of this discrepancy is the expression of specific combinations of auxiliary subunits with the α1E subunits in DRG, hippocampal neurons, and motoneurons. We have therefore tested the effects of coexpression of the β2, β3, and β4 subunits with the α1E and α1C subunits on the inhibition by tBHQ. As seen in Table 2, block of the α1E or α1C subunits by tBHQ was statistically (P > .05) identical for all subunit combinations tested.

tBHQ Inhibits L-Type ICa Recorded in Differentiated Mouse Neuroblastoma and Rat Glioma Hybrid Cell Line. In an attempt to elucidate whether L-type ICa generated by the α1D subunit gene was sensitive to tBHQ, as suggested by Nelson et al. (1994) in neuroendocrine cells, we used a cell line that expresses the α1D subunit gene on neuronal differentiation, the mouse neuroblastoma and rat glioma hybrid cell line NG108-15 (Kamp et al., 1995). Cell depolarization from a −40-mV HP to 0 mV elicited a sustained inward current that was sensitive to 1 μM nitrendipine in 50% of the cell population (four of eight cells). The nitrendipine-sensitive current amounted to 29 ± 10% of the macroscopic ICa (n = 4; Fig. 7A). In another series of experiments, application of 300 μM tBHQ induced an 84 ± 5% decrease in macroscopic ICa, and addition of 1 μM nitrendipine to tBHQ did not induce a further decrease (n = 10; Fig. 7B).

Discussion

The main finding of this study is that tBHQ, a well characterized inhibitor of SERCA, reversibly blocks neuronal ICa at a range of concentrations overlapping that used to inhibit mobilization of $[Ca^{2+}]_i$. This VGCC block by tBHQ is not typical of other SERCA pump antagonists such as thapsigargin and CPA, and is specific with respect to ICa subtypes. tBHQ specifically inhibited N-, P-, and Q-type VGCCs without affecting the T, L, or R types. This effect appears to be a general feature of the action of tBHQ in the nervous system because tBHQ-induced VGCC inhibition was effective in neurons from both the peripheral (sensory neurons) and central (hippocampal and motor neurons) nervous systems. The effects of tBHQ may be due to direct, specific binding sites on VGCCs as suggested by VGCC α1-subunit expression studies. The specificity of this compound should be taken into account when assessing the physiological roles of endoplasmic reticulum calcium stores in neuronal preparations. It opens up the possibility of developing novel selective VGCC

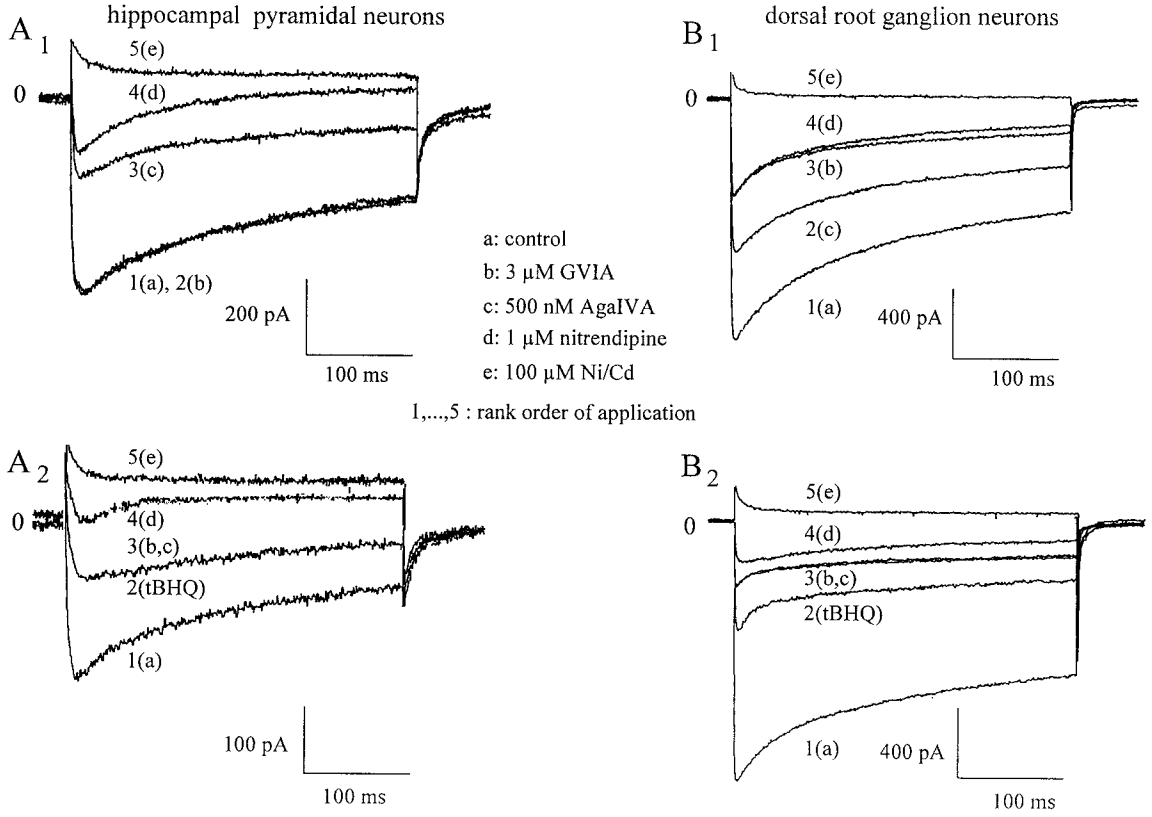

Fig. 4. Specificity of tBHQ inhibition of Ca^{2+} channel type in embryonic hippocampal pyramidal neurons and DRG neurons. High voltage-activated ICa of E17 embryonic pyramidal neurons is composed of the Q-, L-, and R-type Ca^{2+} channels (A_1), as determined by the use of AgaIVA and nitrendipine, respectively. Application of 300 μM tBHQ decreased ICa amplitude and prevented the effects of AgaIVA, but not of nitrendipine (A_2). High voltage-activated ICa of E13 embryonic DRG neurons is composed of the P-, Q-, N-, L-, and R-type Ca^{2+} channels (B_1), as determined by the use of AgaIVA [50 nM (P type) + 500 nM (Q type; data not shown)], GVIA, and nitrendipine, respectively. Application of 300 μM tBHQ decreased ICa amplitude and partially prevented the effects of GVIA while totally blocking the effects of AgaIVA (B_2). For other tissues, the effects of nitrendipine were not affected despite a low contribution of the L-type channel to the macroscopic current.

antagonists or broad-specificity intracellular calcium transient inhibitors.

For motoneurons, the IC_{50} for tBHQ effects was 35 μM and the saturating concentration was in the 100 μM range. The maximal inhibitory effect of tBHQ amounted to 75% of the macroscopic HVA ICa, which suggests some specificity with respect to ICa types. Testing the selective blocking by tBHQ of N-, P-, and Q-type channels and not of T-, L-, and R-type VGCCs requires that the different components of the ICa be clearly distinguished. As previously shown, N-type GVIA-sensitive and P/Q-type AgaIVA-sensitive VGCCs are distinct, nonoverlapping ICa components that differ from the dihydropyridine-sensitive L-type and GVIA-, AgaIVA-, dihydropyridine-insensitive R-type current components, both in the DRG and motoneurons (Diochot et al., 1995; Scamps et al., 1998). Based on this pharmacological dissection of the components of macroscopic ICa (GVIA, AgaIVA, and nitrendipine for the N, P/Q, and L types, respectively) and on the differential expression of channel types in the preparations (N, L, P, and R types in motoneurons; L, Q, and R types in hippocampal neurons; and T, N, L, P, Q, and R types in DRG neurons), we demonstrated that a supramaximal dose of tBHQ specifically inhibited N-, P-, and Q-type ICa without affecting other VGCC subtypes. In addition, the N- and P/Q-type ICa displayed similar sensitivity over the range of tBHQ inhibition. However, it should be noted that in DRG neurons, the GVIA induced a further 10% decrease in ICa amplitude in the presence of a supramaximal inhibitory dose of tBHQ, an effect not found in motoneurons (Figs. 3 and 4). Two hypotheses may be put forward to explain these pharmacological differences. First, this effect may be related to a nonspecific effect of GVIA (at this concentration) toward non-N-type ICa in DRG neurons (i.e., T-, L-, and R-type ICa). It has been consistently reported that the L- and T-type ICa were partially inhibited by GVIA in various cell types, including DRG neurons (Kasai et al., 1987; McCleskey et al., 1987). Second, pharmacological differences between N-type ICa recorded in mouse DRG and rat motoneurons also may exist as a result of either interspecies variations in the amino acid sequence of the pore-forming α1B subunit of the N-type channels or differential expression of specific splice variants of the α1B subunit in DRG and motoneurons (Lu and Dunlap, 1999). The low voltage-activated T-type ICa, which has been shown to be clearly different from the R-type current and to be more frequently found in DRG neurons than in motoneurons and hippocampal neurons (Hilaire et al., 1997), also was insensitive to tBHQ.

Interestingly, we observed that tBHQ did not inhibit the neuronal L-type ICa, whereas it inhibited non-neuronal L-type ICa (i.e., in neuroendocrine cells; Nelson et al., 1994), probably in smooth muscle cells (Philippe et al., 1995) and L-type ICa in differentiated NG108-15 cells (present study). Moreover, the poor sensitivity of the neuronal α1C subunit to tBHQ in the oocyte expression system appears to be consistent with the inefficacy of this drug in the various neuronal preparations tested. The difference between neuronal and non-neuronal cells may be due to L-type ICa diversity. It has been reported that at least four different genes encode the

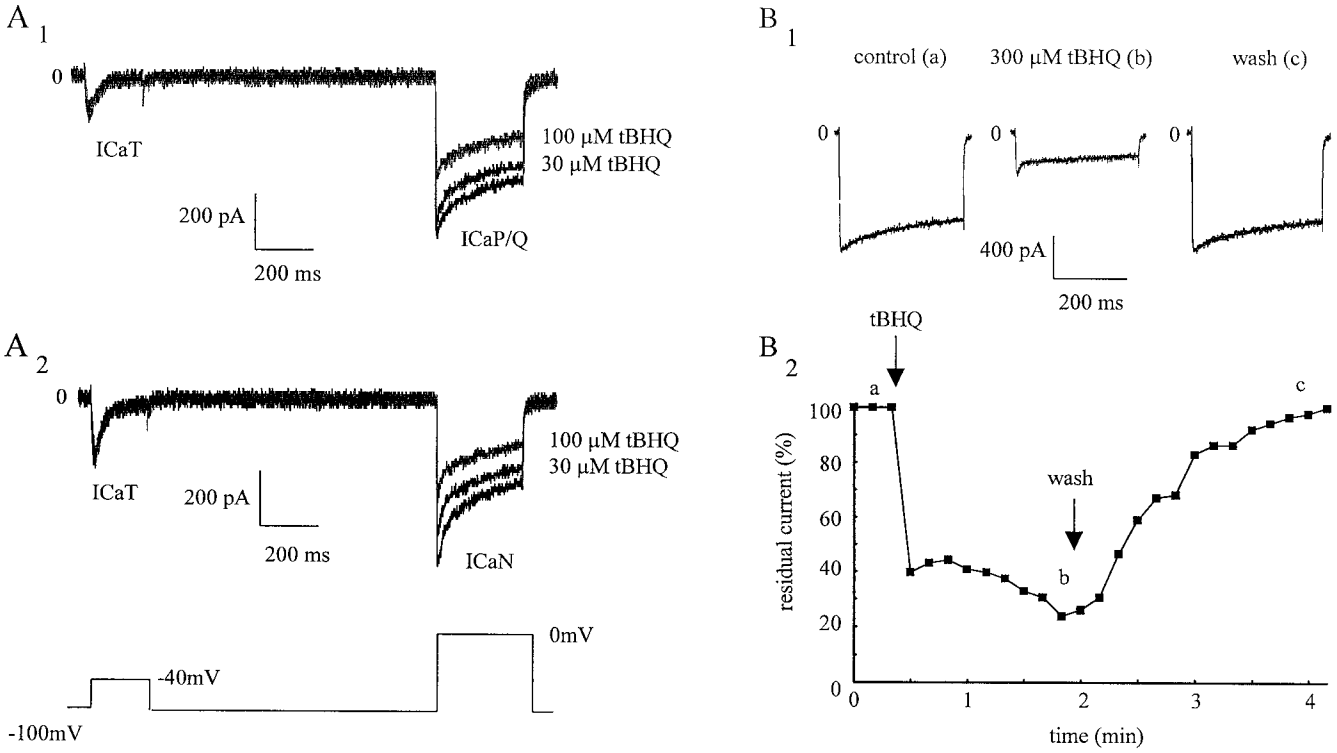

Fig. 5. Sensitivity of T-type ICa and of P/Q- and N-type ICa to tBHQ in a DRG neuron. Kinetics of tBHQ effects. A, effects of tBHQ on the T-type ICa and on the P/Q- and N-type ICa. A_1, to record the P/Q-type ICa, the cell was incubated in the presence of 250 nM nitrendipine and 3 μM GVIA. A_2, to record the N-type ICa, the cell was incubated in the presence of 250 nM nitrendipine and 250 nM AgaIVA. As shown below current traces, a two-pulse protocol was applied to record T-type ICa at a −40-mV depolarization and the drug-selected, high voltage-activated ICa at a 0-mV depolarization, 1-s interpulse every 10 s, −100-mV HP. Concentrations of 30 and 100 μM tBHQ were cumulatively applied to the cell. B, kinetics of tBHQ inhibition and reversibility. Application of supramaximal dose of tBHQ induced a rapid inhibition of macroscopic ICa recorded from a DRG neuron (no inhibitors). Washout of the drug allows for complete recovery of inhibition.

pore-forming α1-subunits responsible for the L-type current: α1S mainly present in skeletal muscle (Tanabe et al., 1987); α1C found in cardiac muscle, smooth muscle (Lory et al., 1991), and neurons (Hell et al., 1993); α1D predominantly found in neurosecretory cells (Chin et al., 1992) but also in neurons, cardiac myocytes, and differentiated NG108-15 cells (Hell et al., 1993; Kamp et al., 1995; Wyatt et al., 1997); and a recently described retina-specific α1F (Strom et al., 1998). Selectivity with respect to the L-type ICa was reported for the calcium channel antagonists (Hockerman et al., 1997; Striessnig et al., 1998). It also has been reported that different splice variants of the α1C gene confer tissue-specific dihydropyridine sensitivity on the channel (Welling et al., 1997) and that alternative splicing of the α1A gene results in channels with different pharmacological properties (Bourinet et al., 1999). Therefore, the differential expression of α1 isoforms (we show that the auxiliary β1, β2, β3, and β4 do not account for the weak α1C inhibition by tBHQ) may confer a specific pharmacological profile, accounting for differences in the effects of tBHQ on different L types of ICa. Overall, these data support the notion that in situ neuronal and non-neuronal L-type VGCCs differ in their interactions with tBHQ.

The α1E subunit appeared clearly to be more sensitive to tBHQ inhibition than the native R-type ICa. We have no experimental data to explain this difference in sensitivity, which is not due to the composition of the auxiliary β-subunits. Splice variants could presumably modulate the effects of tBHQ on the α1E subunit, but we cannot rule out the possibility that the R-type ICa that we record in our preparations does not belong to the gene family coding for the α1E subunit.

These data suggest that tBHQ acts directly on the channel. The effect of tBHQ on endogenous N-, P-, and Q-type currents was not an indirect result of depleting intracellular pools because this effect was not shared with thapsigargin and our whole-cell recording conditions do not permit changes of $[Ca^{2+}]_i$. The onset of ICa inhibition by tBHQ was rapid. Its effects were reproduced at the level of the expressed proteins, as seen by injecting the α1A and α1B genes into oocytes. So the site of action of tBHQ on the N-, P-, and Q-type currents was probably the pore-forming protein. However we did not

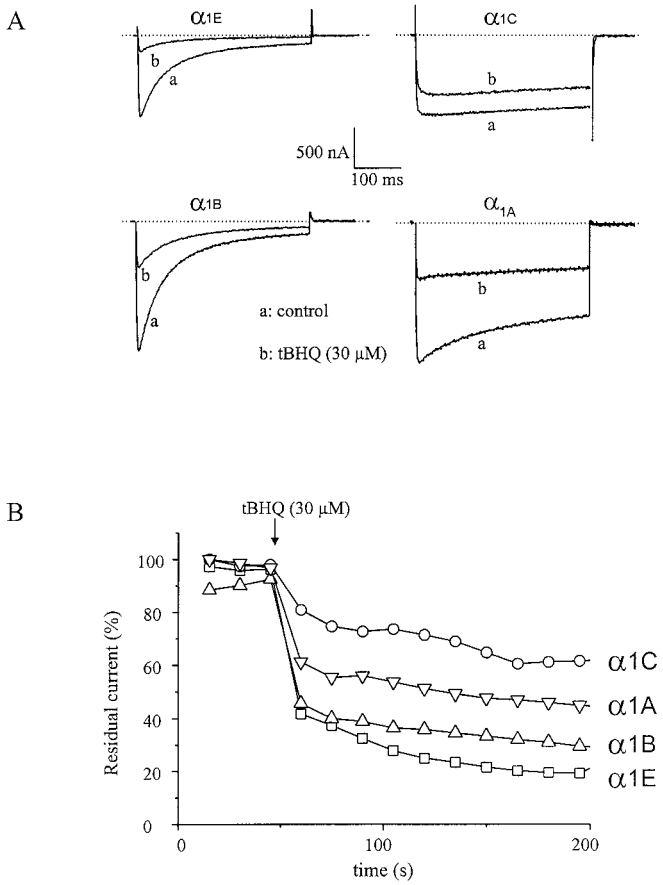

Fig. 6. tBHQ blocks neuronal VGCCs expressed in *Xenopus* oocytes. A, typical current traces showing steady-state inhibition of α1E, α1C, α1B, and α1A calcium channels by 30 μM tBHQ. Each α1 Ca^{2+} channel subunit was expressed with the subunits cDNA α2-δ and β1 (α1E, α1B, and α1C) or β2 (α1A). Currents were recorded during a typical depolarization at +10 mV from an HP of −80 mV in a Cl-free, 10 mM Ba^{2+}-containing solution. B, time course of the Ba^{2+} current inhibition induced by perfusion of tBHQ. tBHQ (30 μM) was applied by superfusion to oocytes injected with α1E, α1C, α1B, or α1A. Peak currents were recorded during a typical depolarization from −80 to +10 mV applied every 15 s, and subsequently normalized according to the current recorded just before tBHQ application. Note the lower sensitivity of the α1C combination.

TABLE 2

Percentage of inhibition of tBHQ on Ba^{2+} currents recorded from oocytes expressing α1C or α1E calcium channel subunit with the α2-δ and β1, β2, β3, or β4 auxiliary subunits

[tBHQ] = 30 μM	α1C	α1E
	%	%
β1	30 ± 10 (n = 6)	67 ± 13 (n = 6)
β2	17 ± 16 (n = 8)	60 ± 19 (n = 8)
β3	25 ± 11 (n = 3)	76 ± 12 (n = 3)
β4	25 ± 11 (n = 5)	72 ± 16 (n = 6)

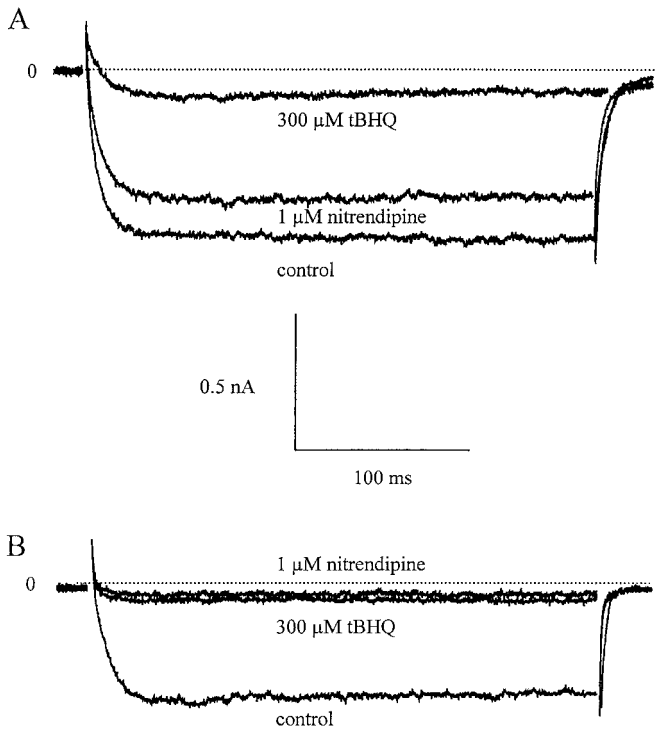

Fig. 7. tBHQ inhibits the L-type ICa in NG108-15 cells. A, NG108-15 cells possess a nitrendipine-sensitive ICa. Currents were recorded during a depolarization from a −40-mV HP to 0 mV, every 10 s. B, at 300 μM, tBHQ induced a decrease in macroscopic ICa. Application of nitrendipine did not induce a further decrease.

determine whether the inhibitory effects of tBHQ were related to its ability to modify the thiol groups of the pore-forming proteins. This is relevant given that this is the first report showing that tBHQ blocks neuronal ICa at concentrations in the range at which it is active both against the SERCA and in the control of cell redox state.

These data demonstrate for the first time that micromolar concentrations of tBHQ inhibit voltage-gated ICa in neurons, in addition to SERCA. This newly identified site of action could lead to misinterpretation or overestimation of data relating to variation in $[Ca^{2+}]_i$ movement and may therefore explain some of the conflicting results in the literature (for review, see Taylor and Broad, 1998). It also opens up the possibility of developing new calcium channel antagonists with more potent and selective activity against either VGCCs or SERCA. Finally, calcium overload is deleterious to cells in pathological conditions such as epilepsy and ischemia. So, the design of mixed calcium channel antagonists that inhibit both calcium influx and $[Ca^{2+}]_i$ release may be an important strategy in the treatment of damage to the central nervous system.

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