Calcium-Dependent Inhibition of L, N, and P/Q Ca^{2+} Channels in Chromaffin Cells: Role of Mitochondria

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The hypothesis that the buffering of Ca^{2+} by mitochondria could affect the Ca^{2+}-dependent inhibition of voltage-activated Ca^{2+} channels, (I_{Ca}), was tested in voltage-clamped bovine adrenal chromaffin cells. The protonophore carbonyl cyanide m-chlorophenyl-hydrazone (CCCP), the blocker of the Ca^{2+} uniporter ruthenium red (RR), and a combination of oligomycin plus rotenone were used to interfere with mitochondrial Ca^{2+} buffering. In cells dialyzed with an EGTA-free solution, peak I_{Ca} generated by 20 msec pulses to 0 or +10 mV, applied at 15 sec intervals, from a holding potential of −80 mV, decayed rapidly after superfusion of cells with 2 μM CCCP (τ = 16.7 ± 3 sec; n = 8). In cells dialyzed with 14 mM EGTA, CCCP did not provoke I_{Ca} loss. Cell dialysis with 4 μM ruthenium red or cell superfusion with oligomycin (3 μM) plus rotenone (4 μM) also accelerated the decay of I_{Ca}. After treatment with CCCP, decay of N- and P/Q-type Ca^{2+} channel currents occurred faster than that of L-type Ca^{2+} channel currents. These data are compatible with the idea that the elevation of the bulk cytosolic Ca^{2+} concentration, $[Ca^{2+}]_c$, causes the inhibition of L- and N- as well as P/Q-type Ca^{2+} channels expressed by bovine chromaffin cells. This $[Ca^{2+}]_c$ signal appears to be tightly regulated by rapid Ca^{2+} uptake into mitochondria. Thus, it is plausible that mitochondria might efficiently regulate the activity of L, N, and P/Q Ca^{2+} channels under physiological stimulation conditions of the cell.

Key words: mitochondrial Ca^{2+}; Ca^{2+} channels; Ca^{2+}-dependent inhibition of Ca^{2+} channels; chromaffin cells; L-type Ca^{2+} channels; N-type Ca^{2+} channels; P/Q-type Ca^{2+} channels

The Ca^{2+}-dependent inactivation of high-threshold voltage-dependent Ca^{2+} channels was first suggested in the pioneering work of Hagiwara and Nakajima (1966), subsequently proven by Brehm and Eckert (1978) and Tillotson (1979), and later on extended to various excitable cells (Hagiwara and Byerly, 1981). Since then, various Ca^{2+} channel subtypes (L, N, P/Q, R) have been identified (García et al., 2000), but it is unclear whether all of them are equally prone to inactivation by Ca^{2+} entry or the elevation of the cytosolic concentration of Ca^{2+}, $[Ca^{2+}]_c$. For instance, the cardiac and smooth muscle L-type Ca^{2+} channel is quickly inactivated after depolarization, with a time constant in the range of 50–100 msec (Yue et al., 1990; Giannattasio et al., 1991; Neely et al., 1994). However, the neuronal L-type Ca^{2+} channel is inactivated more slowly by the increase of $[Ca^{2+}]_c$ (von Gersdorff and Matthews, 1996). The bovine adrenal chromaffin cell is a suitable model to reexamine this problem for two reasons: (1) this cell expresses L-, N- and P/Q-types of Ca^{2+} channels (Albillos et al., 1996), and (2) its mitochondria undergo large Ca^{2+} transients and hence its manipulation might produce drastic local changes of $[Ca^{2+}]_c$ to cause the inhibition of Ca^{2+} channels.

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On the other hand, the concept that mitochondria can act as rapid and reversible Ca^{2+} buffers during depolarization of neurons (Werth and Thayer, 1994; White and Reynolds, 1997; Pivovarova et al., 1999; Colegrove et al., 2000) and chromaffin cells (Herrington et al., 1996; Park et al., 1996; Babcock et al., 1997) is a recent one. Mitochondria can certainly sense the $[Ca^{2+}]_c$ transients generated at subplasmalemmal domains (Rizzuto et al., 1994; Budd and Nicholls, 1996; Lawrie et al., 1996; Pivovarova et al., 1999), and reciprocally, Ca^{2+} uptake or release from mitochondria generate changes in local $[Ca^{2+}]_c$ that modulate Ca^{2+} entry (Budd and Nicholls, 1996). However, the measured elevations of mitochondrial Ca^{2+} concentrations, $[Ca^{2+}]_m$, were only in the submicromolar or the low micromolar range (Rizzuto et al., 1994; Babcock et al., 1997; Brini et al., 1997). These small $[Ca^{2+}]_m$ transients could not account for the efficacy of mitochondria to sense the local $[Ca^{2+}]_c$ of 20–50 μM likely occurring in the vicinity of Ca^{2+} channels in bovine chromaffin cells (Neher, 1998). This issue has been clarified recently by using mitochondrially targeted aequorins of low Ca^{2+} affinity; we observed that mitochondria undergo surprising, rapid near-millimolar $[Ca^{2+}]_m$ transients on activation of Ca^{2+} entry through Ca^{2+} channels into bovine chromaffin cells stimulated with short pulses of acetylcholine (ACh) or high K^+ (Montero et al., 2000). These large $[Ca^{2+}]_m$ can already account for the large and rapid changes of $[Ca^{2+}]_c$ taking place at subplasmalemmal sites during cell depolarization.

In this context, we thought that if we impaired the rapid sequestration of Ca^{2+} by mitochondria of the Ca^{2+} entering the cell during its depolarization, we could provoke larger and long-lasting $[Ca^{2+}]_c$ at subplasmalemmal sites; in this manner, we

should be able to study how these local $[Ca^{2+}]_c$ elevations would affect the Ca^{2+} current through each Ca^{2+} channel subtype (L, N, or P/Q) generated by test depolarizing pulses applied to voltage-clamped bovine chromaffin cells. By following this strategy, we have found that the three Ca^{2+} channel subtypes can undergo full inhibition by local $[Ca^{2+}]_c$ elevations, although at different rates. We report here the experiments leading to these conclusions.

MATERIALS AND METHODS

Isolation and culture of adrenal medulla chromaffin cells. Bovine adrenal medulla chromaffin cells were isolated following standard methods (Livett, 1984) with some modifications (Moro et al., 1990). Cells were suspended in DMEM supplemented with 5% fetal calf serum, 10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. They were plated on 1-cm-diameter glass coverslips at low density (5×10^4 cells per coverslip).

Current measurements and analysis. Ca^{2+} (I_{Ca}), Ba^{2+} (I_{Ba}), Na^+ (I_{Na}), and acetylcholine (I_{ACh}) currents were recorded using the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Coverslips containing the cells were placed on an experimental chamber mounted on the stage of a Nikon Diaphot inverted microscope. During the preparation of the seal with the patch pipette, the chamber was continuously perfused with a control Tyrode solution containing (in mM): 137 NaCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES/NaOH, 0.005 tetrodotoxin (TTX), pH 7.4 (no TTX was added when measuring I_{Na}). Once the patch membrane was ruptured and the whole-cell configuration of the patch-clamp technique established, the cell being recorded was locally, rapidly, and continuously superfused with an extracellular solution of a composition similar to the chamber solution, but containing nominally 0 mM Ca^{2+} (no EGTA added; I_{Na}), 10 mM Ca^{2+} (I_{Ca}), or 10 mM Ba^{2+} (I_{Ba}) as charge carriers (see Results for specific experimental protocols). External solutions were rapidly exchanged using electronically driven miniature solenoid valves coupled to a multi-barrel concentration-clamp device, the common outlet of which was placed within 100 μm of the cell to be patched. The flow rate was ~1 ml/min and regulated by gravity. Experiments were performed at room temperature (22–24°C). Cells were dialyzed with an intracellular solution containing (in mM): 10 NaCl, 100 CsCl, 20 TEA.Cl, 5 Mg.ATP, 0.3 Na.GTP, 20 HEPES/CsOH, pH 7.2; in some experiments (see Results) 14 mM EGTA was also included in the pipette solution.

Whole-cell recordings were made with fire-polished electrodes (resistance 2–5 MΩ when filled with the standard Cs^+/TEA intracellular solution) mounted on the headstage of a DAGAN 8900 patch-clamp amplifier, allowing cancellation of capacitative transient and compensation of series resistance. A Labmaster data acquisition and analysis board and a 386-based microcomputer with pCLAMP software (Axon Instruments, Foster City, CA) were used to acquire and analyze the data.

Cells were clamped at −80 mV holding potential (HP). Step depolarization to 0, +10, or +20 mV from this HP of different duration, were applied at various time intervals (see Results). Cells with pronounced rundown of I_{Ca} or I_{Ba} were discarded (Fenwick et al., 1982). Leak and capacitative currents were subtracted by using currents elicited by small hyperpolarizing pulses.

Measurements of changes of $[Ca^{2+}]_c$ in fura-2-loaded chromaffin cells. 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): 145 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 10 Na-HEPES, 10 glucose. The loading incubation was terminated by washing several times the coverslip containing the attached cells, using Krebs-HEPES. The cells were then kept at 37°C in the incubator for 15–30 min. The fluorescence of fura-2 in single cells was measured with the 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). Experiments were performed at room temperature (22–24°C).

Chemicals. Collagenase type A was purchased from Boehringer Mannheim (Madrid, Spain). DMEM, fetal calf serum, penicillin, and streptomycin were purchased from Life Technologies (Madrid, Spain). BSA, TTX, carbonyl cyanide m-chlorophenyl-hydrazone (CCCP), ruthenium red, oligomycin, and rotenone were purchased from Sigma (Madrid, Spain). Other chemicals were obtained from either Sigma or Merck (Madrid, Spain).

Statistical analysis. Results are expressed as means ± SEM. The statistical differences between means of two experimental results were assessed by Student's *t* test or one-factor ANOVA by Scheffe *F* test. A value of $p \leq 0.05$ was taken as the limit of significance.

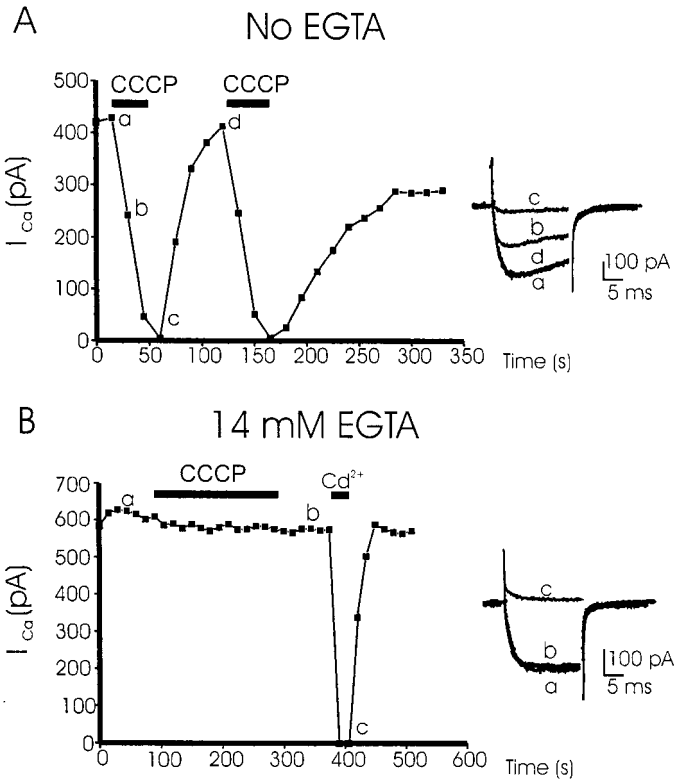

Figure 1. CCCP provokes the inhibition of I_{Ca} in cells dialyzed with an EGTA-free intracellular solution, but not in the presence of EGTA. The two cells of *A* and *B* were voltage clamped at −80 mV and dialyzed with 14 mM EGTA (*B*) or with an intracellular solution without EGTA (*A*). To generate inward whole-cell Ca^{2+} channel currents (10 mM Ca^{2+} in the extracellular solution), cells were stimulated with 20 msec test depolarizing pulses to +10 mV at 15 sec intervals. Each *black square* shows the amplitude of peak I_{Ca} (ordinates) as a function of the time course shown in the abscissa. CCCP (2 μM) and Cd^{2+} (100 μM) were added with the extracellular solution (that continuously superfused the cells) during the time periods indicated by the *horizontal black bars. Insets* at the *right* in *A* and *B* show original current traces taken at the times shown by *small letters*. These are original records of two typical experiments, of eight for each protocol (see Results for averaged data).

RESULTS

Effects of CCCP on I_{Ca} in cells dialyzed with intracellular solutions with or without EGTA

Experiments were designed to test how interference with the ability of mitochondria to sequester Ca^{2+} during cell activation modified the amplitude of peak inward Ca^{2+} channel currents. I_{Ca} was measured as indicated in Materials and Methods. In cells dialyzed with an EGTA-free intracellular solution, the current declined slowly and stabilized after 3–4 min. In eight cells, the current loss amounted to 35 ± 3% and had a time constant (τ) of 78 ± 11 sec. Cells with initial current decline that was not stabilized after 3 min were discarded. The cell of Figure 1*A* shows an already stabilized initial I_{Ca} of 420 pA (peak current).

Superfusion of the cell with CCCP caused almost full loss of I_{Ca} in 30 sec. The current quickly recovered during CCCP washout (in ~30 sec). A second addition of CCCP again caused a loss of I_{Ca} in a reversible manner, but the recovery was partial, likely because of the absence of EGTA in the pipette that favors the rundown of Ca^{2+} channels (Fenwick et al., 1982). Original traces in the *inset* show that contrary to the cell dialyzed with 14 mM EGTA (*B, trace a, inset*), this cell exhibited clear current inactivation during the depolarizing pulse, before (*A, trace a, inset*) and during CCCP treatment (*A, trace b, inset*). The averaged initial I_{Ca} amplitude was 415 ± 68 pA (n = 8 cells). In the presence of CCCP, I_{Ca} was fully blocked with the much faster τ of 16.7 ± 3.4 sec. From now on we will call this I_{Ca} loss inhibition, to distinguish this effect from the classic Ca^{2+}-dependent current inactivation occurring during cell depolarization (Hagiwara and Byerly, 1981).

The second cell (Fig. 1*B*) was dialyzed with an intracellular solution containing 14 mM EGTA to prevent surges of $[Ca^{2+}]_c$ during cell activation. The initial peak I_{Ca} was >600 pA, and the current in this cell was fairly stable, both before and during the application for 3 min of the mitochondrial protonophore CCCP (2 μM). Applied at the end of the recording period, Cd^{2+} (100 μM) fully and reversibly inhibited I_{Ca}. Original I_{Ca} traces showed no obvious inactivation (Fig. 1B, *inset*). Averaged results obtained in cells dialyzed with EGTA using this protocol showed an initial I_{Ca} amplitude of 608.5 ± 4 pA (n = 8 cells); in the presence of CCCP, peak I_{Ca} decreased by only 5.32 ± 2% (n = 8).

Similar experiments were performed using 10 mM Ba^{2+} as charge carrier. As shown previously, Ba^{2+} generated greater peak currents than Ca^{2+} (Albillos et al., 1994): although the initial I_{Ca} in 20 cells averaged 493 ± 48 pA, the initial I_{Ba} of another 17 cells amounted to 854 ± 94 pA. The cell of Figure 2*A* was dialyzed with an EGTA-free intracellular solution. The initial I_{Ba} of 1300 pA was reduced in CCCP to 900 pA in 1 min. Washout of CCCP allowed a partial gradual recovery of the current, which was fully blocked by Cd^{2+}. Averaged results from nine cells gave an initial I_{Ba} amplitude of 783 ± 140 pA, which was reduced by 39 ± 4.9% in CCCP (τ of 76.9 ± 13 sec). In the cell of Figure 2*B* that was dialyzed with 14 mM EGTA, the initial I_{Ba} amounted to 1200 pA. With 2 μM CCCP, the current measured along a 5 min period of cell superfusion remained unchanged. Cd^{2+} (100 μM) fully and reversibly blocked such current. In eight cells the initial I_{Ba} in the absence of CCCP was 933 ± 136 pA, and in its presence I_{Ba} decreased by only 5.3 ± 4%. Original current traces (*insets* at the *right* in *A* and *B*) show that I_{Ba} did not suffer inactivation during the depolarizing pulse, neither in the cell dialyzed with EGTA (*B*) nor in the cell dialyzed with an EGTA-free solution (*A*).

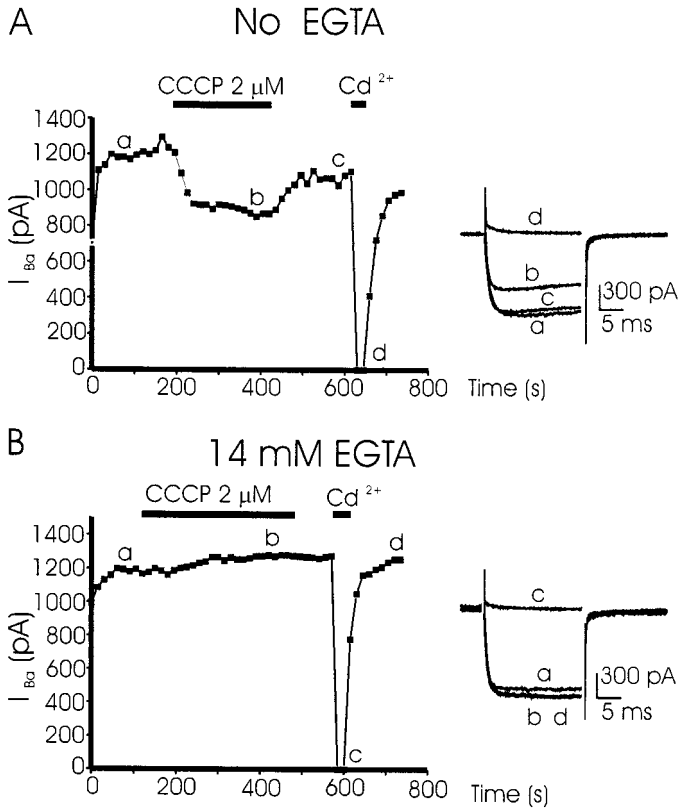

Figure 2. CCCP affects little the amplitudes of whole-cell Ba^{2+} currents (I_{Ba}), independently of cell dialysis with EGTA or with an EGTA-free intracellular solution. The cell of *B* was dialyzed with 14 mM EGTA and that of *A* with a solution deprived of EGTA. Cells were superfused with 10 mM Ba^{2+} as charge carrier and stimulated with 20 msec test depolarizing pulses to +10 mV at 15 sec intervals to study the time course of their peak I_{Ba}. CCCP (2 μM) or Cd^{2+} (100 μM) was applied during the time periods shown by the *horizontal black bars*. Original traces at the *right* in *A* and *B* were taken at the times shown by *small letters*. Nine cells were tested for each protocol. Here we present the results obtained in one prototypical cell with each protocol. See Results for averaged results.

Time course of CCCP-induced inhibition of I_{Ca} and of its recovery, studied at high-frequency stimulation

Under physiological conditions in the intact adrenal gland, chromaffin cell Ca^{2+} channels are likely recruited at intervals of 1 sec, because splanchnic nerves fire action potentials at ~1 Hz. Hence, experiments were performed to study I_{Ca} decline during repetitive pulsing. Cells were dialyzed with an EGTA-free intracellular solution. In these experiments, control currents showed an initial decay of their amplitude during repetitive pulsing at 1 Hz; the peak I_{Ca} decayed slightly to reach a steady state with a τ of 4.3 sec (n = 50 cells). Once I_{Ca} stabilized (in ~15 sec), CCCP (0.02–20 μM) was given for 45 sec (Fig. 3*A*). As shown in the Figure, the rate of I_{Ca} inhibition was dependent on the concentration of CCCP applied. Thus, at 0.02 μM, I_{Ca} peak was inhibited by only 6.3% at the end of the application period (45 sec). At 0.2 μM CCCP, I_{Ca} declined slowly (τ of 21.1 sec). At 2 and 20 μM CCCP, I_{Ca} declined much faster (τ of 6.5 sec at 2 μM and 3.6 sec at 20 μM CCCP). The highest concentrations used, 2 and 20 μM, inhibited I_{Ca} by 100%, with an average τ of 8.6 ± 1.8 sec (n = 15 cells) and 6 ± 0.7 sec (n = 18 cells), respectively. Blockade induced by 0.2 μM CCCP amounted to 52.1 ± 8.9%, with a τ of 25 ± 8.1 sec (n = 11 cells). No significant blockade of I_{Ca} was seen with 0.02 μM CCCP (6.3 ± 4.1% blockade; n = 6 cells). The one-factor ANOVA by Scheffe F test showed statistically significant differences between the low and high CCCP concentrations.

To study I_{Ca} recovery after blockade by CCCP, cells were superfused with 2 μM CCCP, which suppressed fully the current after 29 sec of pulsing. Then, CCCP was removed form the superfusion, and I_{Ca} was tested at different time intervals (5–240 sec) (Fig. 3*B*). At 5 sec there was no current recovery, but at 30 sec a sizable I_{Ca} was measured; after 2 min, most of the current was recovered. The current is expressed as a fraction of the initial I_{Ca}; the recovery curve could be well fitted to a single exponential, showing a τ of 73.6 sec (n = 18 cells) (Fig. 3*B*).

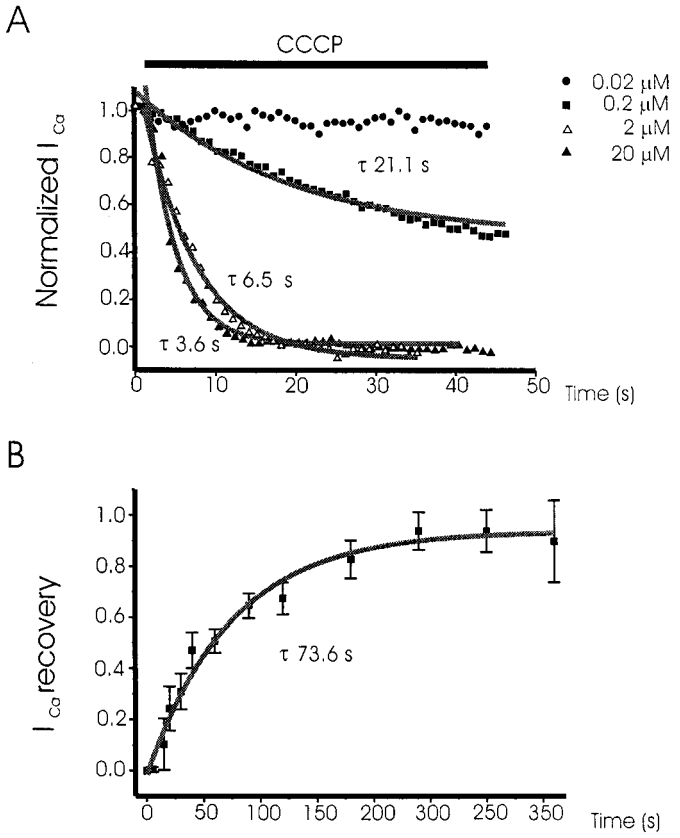

Figure 3. I_{Ca} generated by depolarizing test pulses applied at high frequency: inhibition by CCCP and recovery after its washout. Cells were dialyzed with an EGTA-free intracellular solution and superfused with a 10 mM Ca^{2+}-based extracellular solution. They were voltage clamped at −80 mV and 10 msec pulses to +10 mV applied at 1 sec intervals. *A* shows experiments performed with the concentrations of CCCP shown at the *right*. I_{Ca} was normalized as a fraction of the initial current peak. In *B*, I_{Ca} was elicited at 1 sec intervals by applying 20 msec depolarizing test pulses to +20 mV. Superfusion of CCCP (2 μM) provoked a progressive decline of peak I_{Ca} amplitude (data not shown). Currents were measured at the times shown in the abscissa after washout of CCCP. I_{Ca} that recovered after each washout time period was normalized as a fraction of the initial I_{Ca} before CCCP (ordinate). Data are means ± SEM of 18 cells; τ for current recovery was 73.6 sec (n = 18 cells).

Effects on I_{Ca} of the intracellular application of ruthenium red

Another means of blocking Ca^{2+} sequestration by mitochondria was the direct inhibition of the Ca^{2+} uniporter by RR (Montero et al., 2000). Because it is cell impermeant, this compound was given intracellularly by dialysis with the patch pipette. Figure 4*A* shows averaged results obtained in chromaffin cells dialyzed with an EGTA-free solution in the absence (control) or the presence of RR (4 μM). The τ for the decay of I_{Ca} in control cells was 1110 sec (n = 10 cells), whereas that of cells dialyzed with ruthenium red was 228 sec (n = 11 cells). The inhibition of peak current after 5 min recording was 24.5 ± 4.5% in control cells and 55.5 ± 6.5% in cells dialyzed with ruthenium red ($p < 0.01$). When EGTA (14 mM) was present in the intracellular solution, either with or without ruthenium red, no current inhibition was observed after 5 min stimulation (data not shown).

Effects of oligomycin plus rotenone on I_{Ca} amplitude in cells dialyzed with or without EGTA

Ca^{2+} uptake into mitochondria can be also blocked by using a combination of oligomycin plus rotenone. Oligomycin blocks mitochondrial ATP production by direct inhibition of the ATP synthase, whereas rotenone is a specific inhibitor of the electron transport chain that blocks electron transfer and proton extrusion mechanisms, thereby decreasing mitochondrial membrane poten-

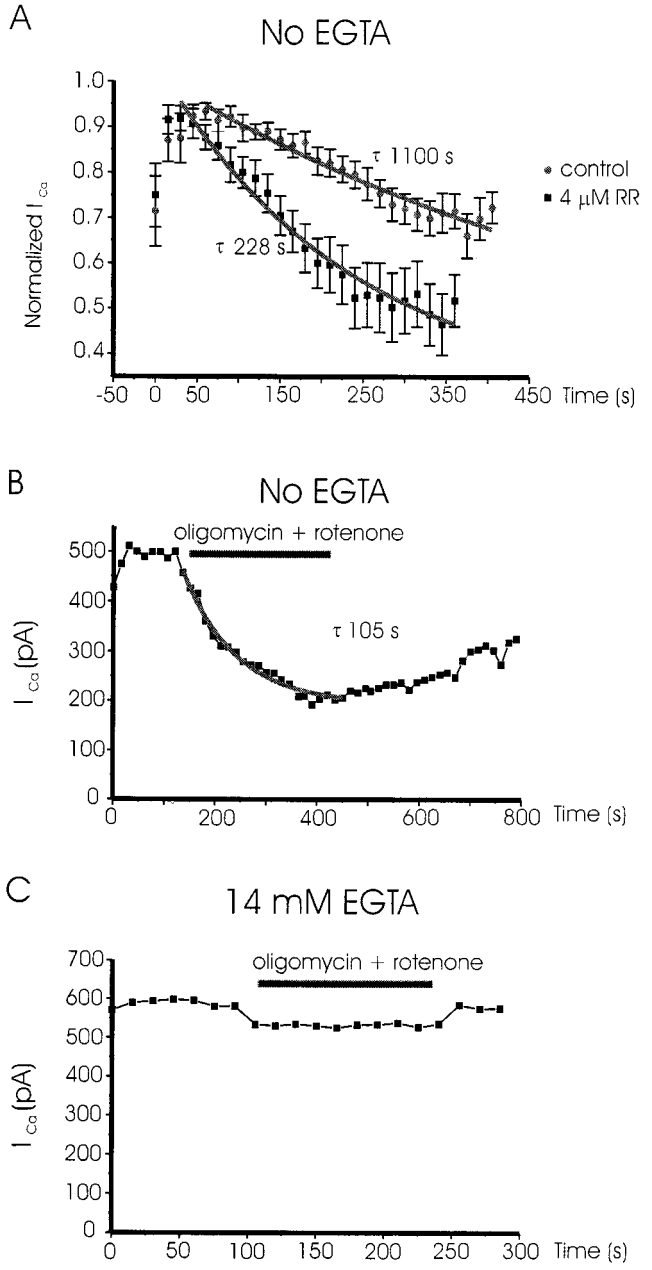

Figure 4. RR and oligomycin plus rotenone accelerated the inhibition of I_{Ca} with repetitive depolarizing pulsing. *A* shows averaged results (± SEM) obtained in 10 control cells (no RR) and in 11 cells dialyzed with RR. Cells were voltage clamped at −80 mV, dialyzed with an EGTA-free intracellular solution, and superfused with 10 mM Ca^{2+} as charge carrier. I_{Ca} was elicited by 20 msec depolarizing test pulses to +10 mV, applied at 15 sec intervals. *B* and *C* show the effects of the superfusion with a solution containing oligomycin (3 μM) plus rotenone (4 μM) on two different cells dialyzed with 14 mM EGTA (*C*) or with an intracellular solution without EGTA (*B*). The cells were stimulated with 20 msec test depolarizing pulses to +10 mV at 15 sec intervals using 10 mM Ca^{2+} as charge carrier. Each *black square* shows the amplitude of peak I_{Ca} as a function of the time course shown in the abscissa. Oligomycin plus rotenone was applied with the continued extracellular perfusion during the time periods indicated by the *horizontal black bars*.

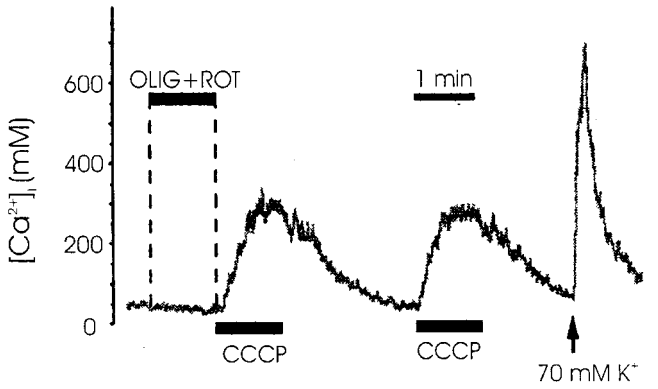

Figure 5. Changes in the $[Ca^{2+}]_c$ in fura-2-loaded cells induced by CCCP, oligomycin, and rotenone. Typical records of changes in the $[Ca^{2+}]_c$ in one fura-2-loaded chromaffin cell are shown. The cell was superfused for 90 sec with CCCP (2 μM) or a mixture of oligomycin (1 μM) plus rotenone (4 μM), as shown by the *horizontal bars*. At the end of the experiment, after the washout of CCCP, a K^+ pulse (70 mM isotonic K^+, 2.5 mM Ca^{2+}, 5 sec) was applied as shown by the *arrow* to assess the viability of the cell. The $[Ca^{2+}]_c$ is expressed in nanomolar (ordinate). Similar results were obtained in the other eight cells (see Results for averaged results).

tial and ATP synthesis. Thus, the dissipation of the negative membrane potential of the mitochondria will prevent the Ca^{2+} uptake by the uniporter, leading Ca^{2+} to accumulate near the Ca^{2+} channels.

The cell shown in Figure 4*B* was dialyzed with an intracellular EGTA-free solution. Once I_{Ca} reached a stable value (~500 pA), superfusion of the cell with a mixture of oligomycin (3 μM) plus rotenone (4 μM) caused the loss of ~60% of the current in 3 min. After washout of the drugs, I_{Ca} recovered slowly and partially. In 11 cells, the superfusion of the cell with oligomycin plus rotenone induced a 67 ± 10% inhibition of I_{Ca}, with an averaged τ of 102 ± 12 sec.

Figure 4*C* shows a similar experiment in one cell dialyzed with an intracellular solution containing 14 mM EGTA. In this cell, superfusion with the mixture of oligomycin plus rotenone caused a small inhibition of I_{Ca}, which recovered fully after washout of the drugs. Averaged results obtained in EGTA-dialyzed cells shows an inhibition of 17 ± 1% of I_{Ca} (n = 3 cells). This inhibition of I_{Ca} in cells dialyzed with EGTA-containing solutions seems to be related to oligomycin, as proven by the fact that superfusion of cells with only oligomycin induced a similar inhibition (19 ± 4%; n = 4 cells), whereas superfusion with rotenone alone had no effect on I_{Ca} in cells dialyzed with EGTA (data not shown).

CCCP increased the $[Ca^{2+}]_c$ in resting cells, whereas oligomycin plus rotenone did not

The differences observed between CCCP effects and those of oligomycin plus rotenone could be attributed to the ability of the former compound to release Ca^{2+} from mitochondria and other intracellular stores, in addition to its effect of preventing Ca^{2+} uptake into mitochondria. To test this possibility, we studied the effects of these compounds on the $[Ca^{2+}]_c$ levels in single fura-2-loaded bovine chromaffin cells. In the cell shown in Figure 5, superfusion of the cell with a solution containing oligomycin (1 μM) plus rotenone (4 μM) did not induce any significant change on $[Ca^{2+}]_c$. However, superfusion of the cells with 2 μM CCCP during 90 sec induced a fast increase of $[Ca^{2+}]_c$ to approximately three to four times the basal levels (basal level amounted to 55 ± 1 nM; n = 10 cells). As shown in the Figure, effects of CCCP were reversible and reproducible after a second application of the compound. Similar results were obtained in the other eight cells, in which application of CCCP induced an increase of $[Ca^{2+}]_c$ levels to a peak of 187 ± 19 nM.

Different effects of CCCP on the various subtypes of Ca^{2+} channels in chromaffin cells

We have studied the effects of the superfusion with CCCP (2 μM) on L-, N-, and P/Q-type Ca^{2+} channels in bovine chromaffin cells. In these experiments, cells were dialyzed with an intracellular EGTA-free solution. To isolate P/Q-type Ca^{2+} channels, cells were superfused with a combination of nisoldipine (an L-type Ca^{2+} channel blocker; 3 μM) plus ω-agatoxin GVIA (an N-type Ca^{2+} channel blocker; 1 μM). To isolate N-type Ca^{2+} channels, cells were superfused with a combination of nisoldipine plus ω-agatoxin IVA (a P/Q-type Ca^{2+} channel blocker; 2 μM). To isolate L-type Ca^{2+} channels, cells were superfused with ω-conotoxin GVIA (1 μM) plus ω-agatoxin IVA (2 μM).

Figure 6*A* shows a typical experiment in a cell treated with nisoldipine plus ω-conotoxin GVIA. Once the current stabilized, we studied the effects of CCCP on the remaining current, mostly P/Q Ca^{2+} channel current, by superfusing the cell with 2 μM CCCP. This treatment led to a prompt decrease of I_{Ca}, which slowly and partially recovered after washout of CCCP. Averaged results showed that under these conditions, I_{Ca} was inhibited by 86.4 ± 3% (n = 10) in the first 10 sec of superfusion with CCCP (Fig. 6*E*). Inhibition of I_{Ca} was almost complete and developed with a τ of 7.2 ± 1 sec (n = 10 cells) (Fig. 6*D,F*). When cells were treated with a combination of ω-conotoxin GVIA plus ω-agatoxin IVA to isolate the L-type Ca^{2+} channel current (Fig. 6*B*), superfusion of the cells with 2 μM CCCP induced a slower blockade of the remaining L-type Ca^{2+} channel current, with a τ of 19.1 ± 2 sec (n = 10 cells) (Fig. 6*D,F*). Only 54 ± 8% (n = 10 cells) of the I_{Ca} was blocked during the first depolarizing pulse in the presence of CCCP (Fig. 6*E*). Finally, in cells treated with ω-agatoxin IVA plus nisoldipine to isolate N-type channel currents, superfusion of the cells with 2 μM CCCP induced a fast (τ of 11.4 ± 1 sec; n = 14 cells) (Fig. 6*C,F*) and almost complete blockade of I_{Ca} of 80.5 ± 4% in the first depolarizing pulse (n = 14 cells) (Fig. 6*E*).

Effects of CCCP on Na^+ channel currents and nicotinic receptor currents

It was of interest to test the specificity of the effects of CCCP on Ca^{2+} channel currents; thus, its effects on Na^+ channel currents and nicotinic receptor currents were also studied. Figure 7*A* shows the time course of peak I_{Na} in a chromaffin cell dialyzed with an EGTA-free solution. Test pulses to −10 mV produced initial I_{Na} of near 1200 pA amplitude. Addition of 2 μM CCCP for 2 min did not affect significantly the amplitude of the current; neither the kinetics of activation nor the kinetics of inactivation of the current was affected, as seen in the original I_{Na} traces shown in the *inset*. The τ for inactivation of I_{Na} before CCCP was 1 msec, and after 90 sec superfusion with CCCP the τ amounted to 0.998 msec (*inset*). Averaged results obtained with this protocol show a peak I_{Na} amplitude of 851 ± 119 pA (10 cells) and 800 ± 113 pA (10 cells), respectively, before and during CCCP superfusion (6.1% of current inhibition); the τ values for I_{Na} inactivation were 0.997 ± 0.001 and 0.998 ± 0.001 msec, respectively.

The protocol used to explore sequentially the effects of CCCP on Ca^{2+} channels and nicotinic receptor channel currents is

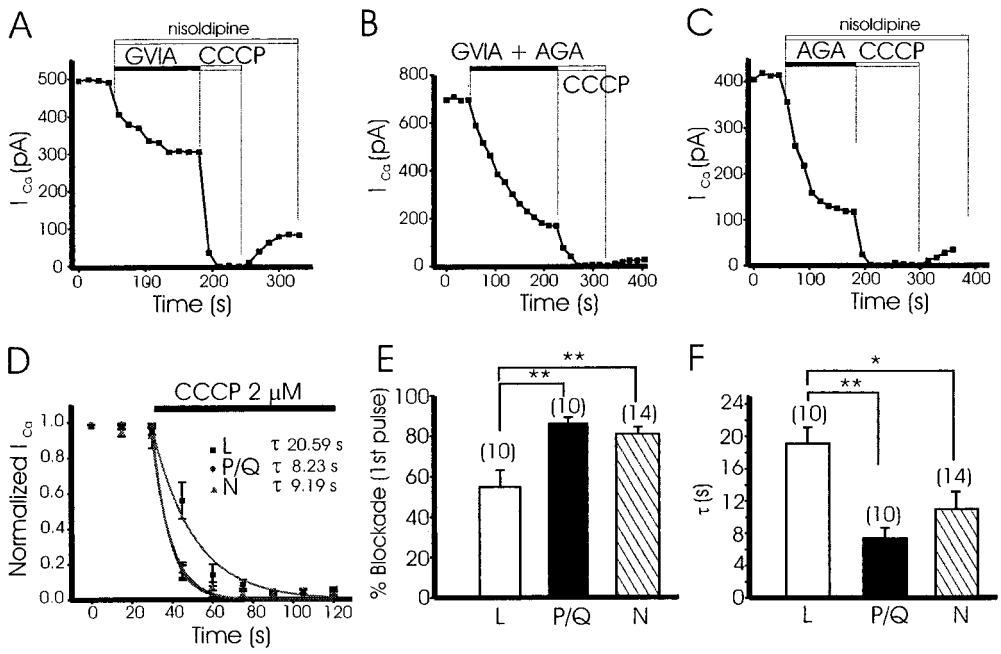

Figure 6. CCCP caused a faster inhibition of I_{Ca} through non-L, as compared with L-type Ca^{2+} channels. Cells were voltage clamped at −80 mV and stimulated with 20 msec test depolarizing pulses to +10 mV at 15 sec intervals using 10 mM Ca^{2+} as charge carrier and dialyzed without EGTA. *A* shows the time course of peak I_{Ca} recorded in one cell superfused with nisoldipine (3 μM) and ω-conotoxin GVIA (GVIA; 1 μM) to block L- and N-type Ca^{2+} channels, as well as the blocking effects of CCCP (2 μM) of the remaining current. *B* shows a similar experiment in which N- and P/Q-type Ca^{2+} channels were blocked by superfusing the cell with GVIA and ω-agatoxin IVA (AGA; 2 μM); after this, CCCP was applied at 2 μM. *C* shows an experiment in which L- and P/Q-type Ca^{2+} channels were blocked by superfusing the cell with nisoldipine and ω-agatoxin IVA; after this, CCCP was applied at 2 μM. *D* shows the normalized averaged time course of CCCP effects under these conditions. Blockade of N- and P/Q-type Ca^{2+} channels developed faster, with a τ of 9.2 sec ($n = 14$) and 8.2 sec ($n = 10$ cells), respectively, than that of L-type Ca^{2+} channels (τ = 20.6 sec; $n = 10$ cells) as shown in *F*. *E* shows averaged data of the blockade of the first depolarizing pulse induced by CCCP under each experimental condition. The data are means ± SEM of 10–14 cells. $^{*}p < 0.05$, $^{**}p < 0.001$.

shown at the top of Figure 7*B*. The control trace shows first the I_{Ca} generated by a long (1 sec) depolarizing pulse to +20 mV, followed by the current induced by 500 msec application of 100 μM ACh (I_{ACh}). I_{Ca} (372 pA) inactivated with a τ of 120.2 msec. I_{ACh} had a greater amplitude (1160 pA) and desensitized with a τ of 98.5 msec. In the presence of 2 μM CCCP (120 sec superfusion), peak I_{Ca} was greatly inhibited (20.5 pA) but I_{ACh} was unaffected (1049 pA), showing a τ for its desensitization of 92.3 msec. In 17 cells, I_{Ca} (in 10 mM Ca^{2+}) amounted to 284 ± 36 pA and was reduced to 63 ± 88 pA in the presence of CCCP (75% current inhibition). However, I_{ACh} was 1091 ± 98 and 972 ± 95 pA, before and after superfusion of the cells with 2 μM CCCP (~11% of current loss).

DISCUSSION

The central finding of this study was the inhibition by CCCP of the amplitude of peak I_{Ca} generated by repeated depolarizing pulses of voltage-clamped chromaffin cells. This effect seemed to be quite selective for Ca^{2+} channel currents. In fact, other inward currents through voltage-dependent Na^{+} channels or nicotinic receptor channels were scarcely affected by CCCP.

Because it is an uncoupler of oxidative phosphorylation, CCCP could cause ATP depletion and a deficit of energy supply to Ca^{2+}-ATPases and hence inhibition of I_{Ca}. However, the intracellular pipette solution contained 5 mM ATP that surely served to fuel intracellular as well as plasmalemmal Ca^{2+}-ATPases. Another possibility is that CCCP causes direct pharmacological inhibition of Ca^{2+} channels, as reported by Stapleton et al. (1994) and Park et al. (1996). This is also unlikely because a compound causing direct blockade of Ca^{2+} channels will do so regardless of the use of Ca^{2+} or Ba^{2+} as charge carrier, or regardless of the presence or absence of EGTA in the intracellular solution. This is the case, for instance, for the neuroprotectant lubeluzole, which blocks L- and N- as well as P/Q-type Ca^{2+} channel currents regardless of the use of Ca^{2+} or Ba^{2+} as charge carrier (Hernández-Guijo et al., 1997). This was not the case for CCCP, which caused the quick inhibition of I_{Ca} (Fig. 1*A*) but only a mild inhibition of I_{Ba} (Fig. 2*A*). In addition, the inhibition of I_{Ca} was fully prevented by intracellular EGTA.

Rather, we attribute the effects of CCCP on I_{Ca} to its well known effects on mitochondria. CCCP collapses the proton gradient and the electrical potential, causing mitochondrial depolarization. This has two immediate consequences: blockade of Ca^{2+} uptake by the mitochondrial uniporter and the release of any stored mitochondrial Ca^{2+}. This will cause an increase of the bulk $[Ca^{2+}]_c$ (Fig. 5) that will reach tenths of micromolar at subplasmalemmal sites, after cell depolarization, as we recently demonstrated using mitochondrially targeted aequorin and electroporated bovine chromaffin cells (Montero et al., 2000). We believe that this high local $[Ca^{2+}]_c$ is responsible for the inhibition of I_{Ca}. Such inhibition is gradual during repeated application of depolarizing pulses at 1 Hz (Fig. 3). This was likely caused by the progressive elevation of $[Ca^{2+}]_c$ near the Ca^{2+} channels, because Ca^{2+} enters the cell through those channels during each pulse, and Ca^{2+} cannot be buffered by CCCP-poisoned mitochondria. A subpopulation of mitochondria is capable of taking up vast amounts of Ca^{2+}, which reach mitochondrial $[Ca^{2+}]$ near the millimolar. These mitochondria can sense up to 50 μM $[Ca^{2+}]_c$, and hence they must be located close to the plasmalemma, where such large $[Ca^{2+}]_c$ gradients are possible (Montero et al., 2000).

A second mechanism relates to the release of mitochondrial Ca^{2+} as a consequence of the dissipation of the proton gradient. If CCCP is applied when mitochondria is still full of Ca^{2+}, then CCCP will induce a prompt release of mitochondrial Ca^{2+} that generates the increase in local $[Ca^{2+}]_c$ near the internal mouth of the Ca^{2+} channel (Fig. 5). This increase will promote the Ca^{2+}-induced inhibition of Ca^{2+}. This second mechanism can also explain the differences observed between CCCP and the other

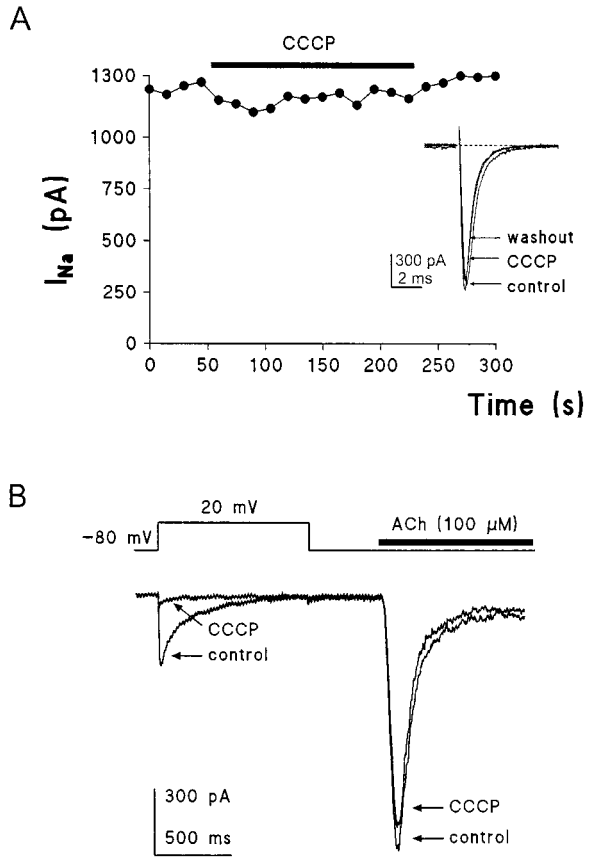

Figure 7. Inward currents through voltage-dependent Na^+ channels (I_{Na}) and through nicotinic receptor channels (I_{ACh}) were not affected by CCCP. *A* shows the time course of peak I_{Na} elicited by 16 msec depolarizing test pulses to −10 mV, applied at 15 sec intervals to a cell voltage clamped at −80 mV. The cell was superfused with an extracellular solution containing 137 mM Na^+ and 0 mM Ca^{2+} and dialyzed with an intracellular EGTA-free solution. CCCP was superfused as shown by the *black horizontal bar. Insets* show original traces taken immediately before (*control*), at 90 sec of superfusion with CCCP, and 2 min after washout of CCCP. Similar results were obtained in the other nine cells; averaged values of I_{Na} for all cells are given in Results. The stimulation protocol for recording of I_{ACh} is shown at the top of *B*. The cell was voltage clamped at −80 mV, and then stimulation was applied at 120 sec intervals as follows. First, a 1 sec depolarizing pulse to +20 mV was applied, and then, after 300 msec, an acetylcholine (*ACh*) pulse of 500 msec duration was given. Typical current traces obtained before (*control*) and at the 90 sec of superfusion with 2 μM CCCP are shown.

agents used to prevent Ca^{2+} uptake into mitochondria, i.e., slower and partial I_{Ca} decay after ruthenium red dialysis or extracellular superfusion with oligomycin plus rotenone. As shown in Figure 5, CCCP favors Ca^{2+} release from mitochondria and other intracellular store in fura-2-loaded chromaffin cells, whereas oligomycin plus rotenone did not.

That the dialysis of the cells with 14 mM EGTA completely prevented the current inhibition by CCCP (Fig. 1*B*) strongly supports the hypothesis that the sequestration of Ca^{2+} by mitochondria plays a major role in maintaining functional Ca^{2+} channels during repeated stimulation of chromaffin cells. This was corroborated also by three additional experimental findings. First, ruthenium red, which blocks the Ca^{2+} uniporter thus preventing mitochondrial Ca^{2+} uptake, also caused the gradual inhibition of I_{Ca} during repeated depolarization pulses (Fig. 4*A*). Second, combined oligomycin plus rotenone, which also collapses the mitochondrial potential, also caused gradual I_{Ca} inhibition (Fig. 4*B*), which was again prevented by 14 mM intracellular EGTA (Fig. 4*C*). Third, when Ba^{2+} was used as charge carrier instead of Ca^{2+}, CCCP caused little inhibition of I_{Ba}. This might be explained by the fact that Ba^{2+} is a poor substrate for the Ca^{2+} transport systems (Schilling et al., 1989; Wagner-Mann et al., 1992). In addition, Ba^{2+} has also been described as inducing the inactivation of Ca^{2+} channels, although with an affinity for the inactivation site on the Ca^{2+} channels 100 times slower than that of Ca^{2+} (Ferreira et al., 1997). The partial inhibition of I_{Ba} induced by CCCP could be also attributed to Ba^{2+}-induced release of Ca^{2+} from intracellular stores or binding sites (von Rüden et al., 1993). It is plausible that this Ca^{2+} may be taken up by mitochondria in control conditions (Montero et al., 2000). However, treatment of the cells with CCCP will preclude this mitochondrial Ca^{2+} removal, and this Ca^{2+} might thus contribute to the partial blockade of Ca^{2+} channel currents seen when Ba^{2+} is used as charge carrier.

We believe that the full inhibition of I_{Ca} by CCCP, indicating that L- and N- as well as P/Q-type Ca^{2+} channels were affected, is a most interesting finding (Fig. 6). Modulation by Ca^{2+} of L-type channels is well illustrated; however, little evidence is available for N and P/Q channels. The Ca^{2+}-dependent inactivation of Ca^{2+} channels seems to be more sensitive to Ca^{2+} in the case of L-type Ca^{2+} channels, as compared with non-L-type Ca^{2+} channels (Plant, 1988; Kasai and Neher, 1992). However, we find here that N and P/Q channels were inhibited faster than L channels after repeated application of depolarizing pulses to CCCP-treated cells (Fig. 6*D*). This raises the question of whether the classic Ca^{2+}-dependent inactivation of Ca^{2+} channel currents (Hagiwara and Byerly, 1981) and the Ca^{2+}-dependent inhibition of such currents (this work) are different manifestations of the same underlying mechanism of modulation of the channels by Ca^{2+} ions.

In any case, the idea that clearly emerges from this study is that as on other cell types (Bassani et al., 1992; Rizzuto et al., 1992; Friel and Tsien, 1994; Drummond and Fay, 1996; Park et al., 1996; Greenwood et al., 1997), mitochondria have an important role in shaping the $[Ca^{2+}]_c$ transients generated under our experimental conditions (i.e., brief depolarizing pulses applied at 0.1–1 Hz to dialyzed cells). It might be that under more physiological stimulation conditions of chromaffin cells (i.e., by short-cut repeated depolarizing pulses), mitochondria could sense the high $[Ca^{2+}]_c$ that can be reached only underneath the plasma membrane (Montero et al., 2000). Hence, we believe that by limiting the extent and duration of such large local $[Ca^{2+}]_c$ transients, mitochondria strategically located near the plasma membrane play the important function of maintaining the L, N, and P/Q Ca^{2+} channels ready to be recruited under stressful conditions that cause repetitive stimulation of chromaffin cells, by endogenously released acetylcholine in the intact adrenal gland.

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