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ω-Agatoxin-IVA-sensitive calcium channels in bovine chromaffin cells

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A large component of the whole-cell currents through Ca^{2+} channels in bovine adrenomedullary chromaffin cells has been shown to be insensitive to both L-type and N-type Ca^{2+} channel blockers, suggesting the existence of a third type of Ca^{2+} channel. In the present paper, ω-agatoxin-IVA (AgTx), a selective blocker of P-type Ca^{2+} channels in mammalian neurons, has been used to investigate the presence of this subtype of Ca^{2+} channel in bovine chromaffin cells. Barium currents (I_{Ba}) through Ca^{2+} channels were recorded in whole-cell patch-clamped bovine chromaffin cells. I_{Ba} was blocked by AgTx in a dose-dependent and irreversible manner. At the maximal concentration used (1 μM), AgTx inhibited I_{Ba} by 49.5 ± 3%. Such a blockade was also present when bovine chromaffin cells were pretreated with 10 μM furnidipine, a novel 1,4-dihydropyridine L-type channel blocker, and after treatment with 1 μM of the N-type channel blocker, ω-conotoxin GVIA (CgTx). A combination of these three types of Ca^{2+} channel blockers suppressed the macroscopic Ba^{2+} currents by 88%. We conclude that bovine chromaffin cells, in addition to N- and L-type Ca^{2+} channels, possess a P-like component in their whole-cell currents through the Ca^{2+} channels.

P-type Ca^{2+} channel; Chromaffin cell; ω-Agatoxin-IVA

1. INTRODUCTION

Among the different subtypes of voltage-dependent Ca^{2+} channels that have been described in neurons [1,2], studies in bovine chromaffin cells seem to indicate that only high voltage-activated Ca^{2+} channels (HVA; L-and N-type) are present, while low voltage-activated (LVA; T-type) Ca^{2+} channels do not appear to be present in these cells [3–6]. Recent studies on whole-cell Ca^{2+} currents in bovine chromaffin cells have shown that these cells contain dihydropyridine (DHP)-sensitive [3,7] as well as ω-conotoxin GVIA (CgTx)-sensitive components [8–10], but a large third component, DHP- and CgTx-resistant is always present. The pharmacology of this channel remains unknown.

On the other hand, a new class of voltage-sensitive Ca^{2+} channel, P-type, has been described in neurons and characterized by its sensitivity to the toxin fraction (FTX) of the poison from the funnel web spider *Agelenopsis aperta* [11] and to the purified peptide ω-agatoxin-IVA (AgTx) [12,13]. By using FTX or its synthetic analogue (sFTX 3:3), on catecholamine secretion experiments [14] and on whole-cell recordings of Ba^{2+} currents [10], the existence of this type of Ca^{2+} channels in bovine chromaffin cells has recently been suggested. Because the specificity of sFTX 3:3 to block only P-type Ca^{2+} channels has recently been challenged [15], we have attempted to characterize the presence of P-type Ca^{2+} channels in bovine chromaffin cells by using the peptide fraction AgTx from the funnel web spider venom, which specifically blocks this type of voltage-dependent Ca^{2+} channels in neurons [12,13].

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2. MATERIALS AND METHODS

Chromaffin cells were enzymatically isolated from adult bovine adrenal medullae following methods previously described [16]. Cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 50 IU·ml^{-1} penicillin and 50 μg·ml^{-1} streptomycin, at a density of 50×10^3 cells·ml^{-1}. Cells were plated on glass coverslips and maintained in an incubator under an atmosphere of 95% air/5% CO_2 at 37°C. Experiments were performed in cells from 2 to 8 days in culture. All experiments were done at room temperature (22–25°C).

Ba^{2+} currents in chromaffin cells were recorded using the whole-cell configuration of the patch-clamp technique [17]. Coverslips containing the cells were placed on an experimental chamber mounted on the stage of a Nikon Diaphot inverted microscope. The chamber was continuously perfused with a control Krebs solution containing (in mM): 145 NaCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES/NaOH, 0.002 tetrodotoxin (TTX), pH 7.4. For current recordings, 10 mM Ba^{2+} (instead of 2 mM Ca^{2+}) was used as the charge carrier. Cells were dialyzed with an intracellular solution containing (in mM): 10 NaCl, 100 CsCl, 20 TEA.Cl, 5 Mg.ATP, 14 EGTA, 20 HEPES/CsOH, pH 7.2.

Whole-cell recordings were made with fire-polished electrodes (resistance 2–5 MΩ) 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, Inc.) were used to acquire and analyze the data. Solutions were exchanged using a multibarreled concentration-clamp device, the common outlet of which was placed within 100 μm of the cell to be patched.

DMEM, fetal calf serum, penicillin and streptomycin were purchased from Gibco, Madrid, Spain. TTX and CgTx were purchased from Sigma, Madrid, Spain. AgTx was purchased from SMA (Scientific Marketing Associates, Barnet, Herts, EN5 5SU, UK). Furni-

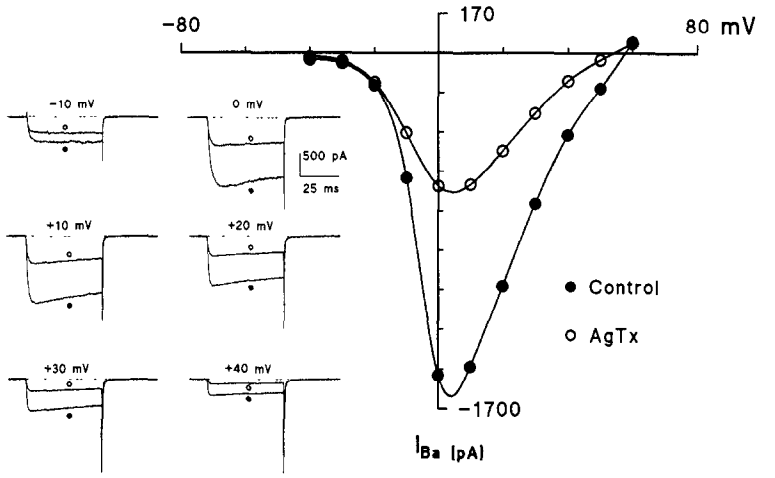

Fig. 1. Whole-cell peak current–voltage relationship for I_{Ba} recorded in a patch-clamped bovine chromaffin cell before and after the superfusion with an extracellular solution containing AgTx (1 μM). Currents were elicited by 50 ms depolarizing pulses from a holding potential of −70 mV applied at 0.2 Hz in 10 mV steps. Current traces were obtained before (control) and after superfusion of the cell with AgTx (1 μM). Each concentration of toxin was present at least 2 min before the application of the depolarizing pulse. Insets show original current traces recorded at the indicated test potentials.

dipine was supplied by Alter S.A., Madrid, Spain. sFTX was supplied by Eli-Lilly, UK. Other chemicals were obtained either from Sigma or Merck, Madrid, Spain.

3. RESULTS

3.1. *AgTx blocks a component of the whole-cell Ba^{2+} current*

Ba^{2+} currents were elicited by 50 ms depolarizing pulses applied from a holding potential of −70 or −80 mV in 10 mV steps. Other ionic currents were suppressed by dialyzing the chromaffin cells with the Cs^{+}-based intracellular solution and by superfusing them with an extracellular solution containing TTX (2 μM).

Superfusion of the cells with an extracellular solution containing increasing concentrations of AgTx led to a dose-dependent decrease of the barium current. At the concentration of 0.3 μM, I_{Ba} was inhibited 25 ± 4% (n = 7), from an average control value of 619 ± 85 pA, to 463 ± 67 pA. At 1 μM, the current was further decreased to 49.5 ± 3% (n = 17; Fig. 1). Not all cells responded to AgTx with a similar degree of blockade, suggesting that different cells contain different proportions of the various subpopulations of Ca^{2+} channels.

The effects of AgTx were irreversible upon washing out (5–10 min) the toxin from the extracellular medium, and were not accompanied by significant alterations of the voltage-dependence of I_{Ba} (Fig. 1). AgTx suppressed I_{Ba} at all potentials tested without significantly shifting the I–V curve and without appreciable alterations in the activation and inactivation kinetics of the current (Fig. 1, insets).

3.2. *Effects of AgTx in cells pretreated with other Ca^{2+} channel blockers*

In order to test the specificity of AgTx, experiments were performed in chromaffin cells pretreated with furnidipine, a specific DHP L-type Ca^{2+} channel blocker [18] or CgTx, a N-type Ca^{2+} channel blocker. In these experiments, AgTx was applied either before or after the other blockers. Fig. 2 shows the time course for the blocking effects observed in a cell upon superfusion with an extracellular solution containing CgTx (1 μM). Under these conditions I_{Ba} was blocked 43 ± 3% (n = 31). Upon washing out CgTx, blockade did not reverse. Once the maximum blockade of CgTx was reached and reversibility of this blockade was not observed, AgTx (1 μM) was added to the perfusion solution; an additional blockade of I_{Ba} could then be appreciated (Fig. 2). As seen with CgTx, washout of AgTx did not result in recovery of the current. Further addition of furnidipine (10 μM) accounted for an additional

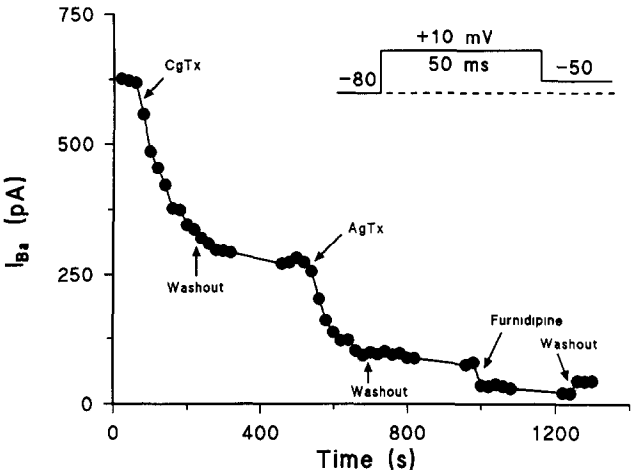

Fig. 2. Time course for the blocking effects of CgTx (1 μM), AgTx (1 μM) and furnidipine (10 μM) on I_{Ba}. Currents were elicited by 50 ms depolarizing pulses to 0 mV from a holding potential of −80 mV, applied at 20 s intervals (see protocol at inset).

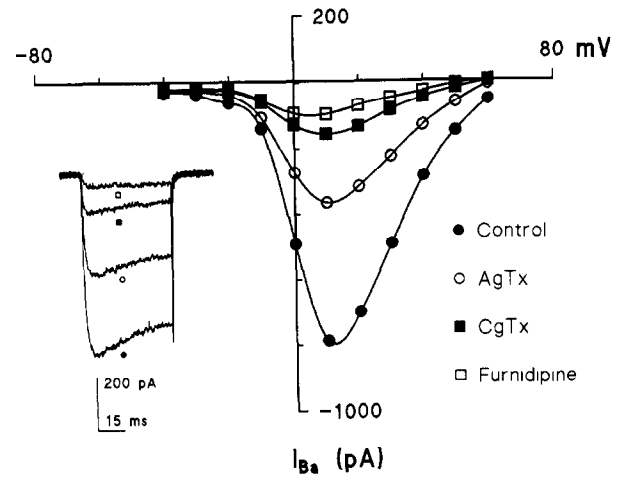

Fig. 3. Effects of different types of Ca^{2+} channel blockers on chromaffin cell macroscopic Ba^{2+} currents. Depolarizing pulses were applied in the absence (control) and the presence of AgTx (1 μM), CgTx (1 μM) and furnidipine (10 μM). I_{Ba} was elicited as in Fig. 1. The inset shows capacitance and linear leak subtracted current traces elicited by a 50 ms test pulse to 0 mV from a holding potential of −80 mV, under the different perfusion conditions indicated in the figure.

blockade of the small current left. The effects of furnidipine were reversible upon removing the DHP from the perfusion solution. In some cells, a small current remained with all three blockers present. Averaged data from experiments in which combination of these three types of Ca^{2+} channel blockers were used show 88 ± 2% blockade (n = 14).

The effects of the combination of these Ca^{2+} channel blockers were also studied on the availability of the channels. Fig. 3 shows the effects of AgTx, CgTx and furnidipine on the peak current-voltage relationships obtained both in the absence or upon application of the blockers. No significant shift of the curves were observed with any of the blockers used. In this cell, AgTx was applied before CgTx, and a similar degree of blockade was observed. Maximal block by AgTx did not prevent further inhibition by additional application of CgTx and furnidipine, showing that N- and L-type currents remained intact after AgTx treatment.

3.3. *Specificity of AgTx vs sFTX*

Experiments were performed to test whether AgTx and the polyamine sFTX were both acting on the same type of Ca^{2+} channels. For these experiments we took advantage of the reversibility of the sFTX blocking effects of whole cell currents previously described [10] versus the irreversibility of the blocking effects of AgTx described above. We were expecting that if both toxins were acting on the same target, binding of sFTX could prevent the binding of AgTx, protecting the AgTx 'receptor' against irreversible blockade.

Fig. 4 shows a representative experiment where superfusion of a chromaffin cell with a solution containing both sFTX (1 mM) and AgTx (1 μM) led to the expected 50% decrease of I_{Ba}. These blocking effects had a fast onset, in accordance with the fast blocking effects of sFTX [10] and in opposition to the slow effect observed when only AgTx was perfused (Fig. 2). When toxins were removed from the extracellular solution, blockade was reversed. Further addition of AgTx led to the slow and irreversible effect of this toxin. These results suggest that addition of sFTX prevents the binding of AgTx to its 'receptor'.

In these experiments, another interesting feature of sFTX was observed. Once the maximum effects of a supramaximal concentration of AgTx were reached, and hence AgTx-sensitive Ca^{2+} channels were fully blocked, application of sFTX, either alone or in combination with AgTx, led to a further decrease of I_{Ba} (Fig. 4), suggesting that sFTX, in addition to the blockade of P-Type Ca^{2+} channels, could block another component of the whole-cell currents through Ca^{2+} channels. This add evidence supporting the suspected unspecificity of sFTX to only block the P-type Ca^{2+} channels [15].

4. DISCUSSION

In contrast to cat chromaffin cells where an L-type Ca^{2+} channel seems to dominate the control of catecholamine release, the bovine chromaffin cell offers a more complex picture. Early experiments on Ca^{2+} uptake into K^+-depolarized bovine chromaffin cells already suggested that extracellular Ca^{2+} could enter the cells through both DHP- as well as CgTx-sensitive pathways [19]. In addition, a DHP- and CgTx-resistant component of Ca^{2+} uptake was also described [20]. The existence of different subtypes of Ca^{2+} channels was corroborated through the recording of whole-cell and single

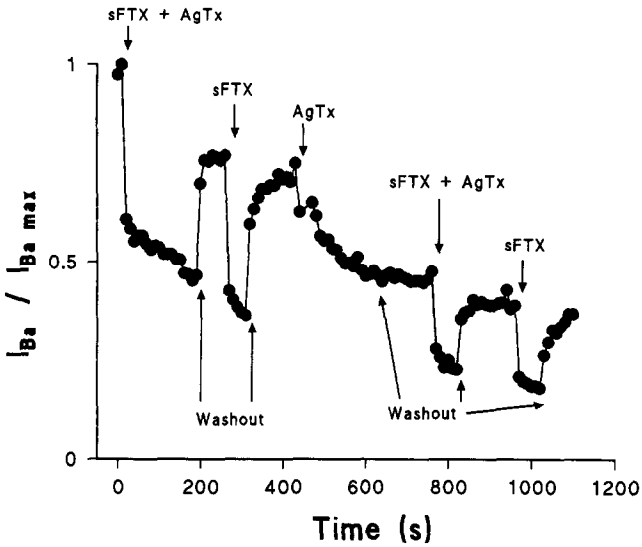

Fig. 4. Time course for the blocking effects of sFTX (1 mM) and AgTx (1 μM) on I_{Ba}. When indicated (arrows) toxins were applied (either alone or in combination) or removed from the extracellular solution (washout). Cell was held at −70 mV and 50 ms pulses to 0 mV were applied at 10 s intervals.

Ca^{2+} channel activity [3–6]. However, a third type of HVA Ca^{2+} channel, insensitive to both DHPs and CgTx seemed to be present in bovine cells; its identity remained unknown. The results of this study strongly suggest that the third component of the whole-cell I_{Ba} is certainly associated to a P-type-like Ca^{2+} channel. The following arguments support this contention. (i) AgTx halves I_{Ba} in an irreversible manner. This behaviour reminds the effects of the toxin on neurones endowed with P-type Ca^{2+} channels [12,13]. (ii) A common site for sFTX and AgTx exists in bovine chromaffin cells, as proven by the fact that sFTX prevents the irreversible blockade of I_{Ba} by AgTx; though apparently less specific, sFTX is however being considered as a P-type channel blocker [11]. (iii) In Purkinje neurons, which only contain P-type Ca^{2+} channels, AgTx fully block them at concentrations below 300 nM [12,13]. It should be noted that in contrast with Purkinje cells, higher concentrations of AgTx were required in order to block the DHP- and CgTx-resistant component of the whole cell Ba^{2+} current in bovine chromaffin cells. Two reasons could be argued to explain this difference: first, we are using a synthetic batch of the toxin instead of the natural toxin used in Purkinje experiments [12,13] and its affinity for the receptor could be different. Second, a non P-type Ca^{2+} channel that binds AgTx with lower affinity than the Purkinje cell might exist in bovine chromaffin cells. Therefore, we will refer to this component as a P-type-like Ca^{2+} channel. (iv) After treating bovine chromaffin cells with supramaximal AgTx concentrations (1 μM), CgTx- and DHP-sensitive components of I_{Ba} still remained. And vice versa, after using supramaximal concentrations of CgTx to block N channels, or furnidipine to block L channels, a large component of I_{Ba} sensitive to AgTx still remained.

We conclude that P-type-like Ca^{2+} channels, similar to those described in neurons are present in cultured bovine chromaffin cells. CgTx does not block catecholamine release in response to chromaffin cell stimulation with acetylcholine or high K^+ concentrations, and DHPs antagonists block only partially such response [21]. It is plausible that P-like Ca^{2+} channels strategically located near the plasmalemmal exocytotic sites might play an important role in regulating the access of extracellular Ca^{2+} to the intracellular secretory machinery.

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