ω-Agatoxin IVA blocks nicotinic receptor channels in bovine chromaffin cells

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Abstract We have studied the contribution of P-type voltage-dependent Ca^{2+} channels to both catacholamine (CA) and ATP secretion from bovine chromaffin cells induced by high K^+ or nicotine using ω-agatoxin IVA, a selective blocker of P-type voltage-dependent Ca^{2+} channels. We found that high K^+ (75 mM) induced the release of about 13% of norepinephrine, 5% epinephrine and 11% ATP, and that ω-agatoxin (100 nM) did not affect this secretion. However, both nicotine-induced CA and ATP secretion were significantly blocked (about 50%) by ω-agatoxin IVA (100 nM). In addition, this toxin also reversibly blocked (about 70%) the inward current induced by nicotine in bovine chromaffin cells. The results suggest that, besides its known action of blocking P-type voltage-dependent channels, ω-agatoxin is a potent and reversible blocker of the nicotinic receptor channel in chromaffin cells, and that this action would explain the blockade of nicotine-induced secretion.

Key words: Calcium channel; P-type; Nicotinic receptor; ω-Agatoxin IVA; Secretion; Chromaffin cell

1. Introduction

Adrenomedullary chromaffin cells secrete catecholamines in response to nicotinic cholinergic agonists [1–3]. Following activation of the nicotinic receptor, a non-selective cation channel, associated to the receptor, opens [4,5], and the membrane of chromaffin cells is depolarized [6–8]. This depolarization leads to the opening of voltage-dependent Na^+ and Ca^{2+} channels located on the plasma membrane that elevate $[Ca^{2+}]_i$, which induces catecholamine (CA) secretion. Using electrophysiological techniques, the presence of L- [9,10], N- [11] and P- [12] type voltage-dependent Ca^{2+} channels located on the plasma membrane of chromaffin cells has been demonstrated.

P-Type voltage-dependent Ca^{2+} channels have been described to carry a significant amount of voltage-activated Ca^{2+} current in different neural cells [13–15], and have also been implicated in neurotransmitter secretion in different systems [16,17]. In adrenergic tissues, P-type voltage-dependent Ca^{2+} channels seem to contribute significantly to Ca^{2+} currents in response to depolarization [12]. However, its participation in the secretory process is less clear, although a fraction of the funnel-web spider poison (FTX) can partially block both the increase in intracellular Ca^{2+} levels and CA secretion in response to K^+-induced depolarization [18].

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In the present work, we have attempted to characterize the contribution of P-type voltage-dependent Ca^{2+} channels to CA and ATP secretion from bovine chromaffin cells induced by either depolarization with high extracellular K^+ or by nicotinic receptor activation, by using the toxin ω-agatoxin IVA (ω-AgaTx), obtained from a peptide fraction of the funnel-web spider, *Agenelopsis aperta*, that is considered to be a specific blocker of P-type voltage-dependent Ca^{2+} channels [13].

2. Materials and methods

2.1. Cell culture

Chromaffin cells were isolated as previously described [19] with minor modifications. Briefly, glands were perfused through the adrenolumbar vein with Ca^{2+}-free Locke's solution of the following composition (in mmol/l): NaCl, 154; KCl, 5.6; $MgCl_2$, 1; NaH_2PO_4, 3; HEPES, 10; and glucose, 10. The pH of the solution was adjusted to 7.4 with NaOH. The glands were then digested with Locke's solution containing 0.2% collagenase (Boehringer-Mannheim, Indianapolis, IN) and 0.5% bovine serum albumin (Calbiochem, La Jolla, CA) and incubated for 45 min at 37°C. Next, the medulla was separated from the cortex, minced with scissors and further digested in the presence of collagenase at 37°C for an additional 30 min. After filtering through a nylon mesh, chromaffin cells were separated from erythrocytes using a Percoll gradient. The yield varied between 20 and 30×10^6 cells per gland and the viability was greater than 95% as defined by exclusion of the biological dye Trypan blue. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) under an atmosphere of 5% CO_2.

2.2. Catecholamine secretion

After 3–7 days of culture, chromaffin cells were washed for 60 min (12×5 min) with 800 μl Krebs-HEPES (K-H) of the following composition (in mmol/l): NaCl, 140; KCl, 5.9; $MgSO_4$, 1.2; $CaCl_2$, 2.5; HEPES, 15; and glucose, 11; pH was adjusted to a value between 7.3 and 7.4. After washing, a 5 min sample was taken to determine basal CA release. The solution in the well was changed, for 5 min, to one containing the desired secretagogue, either high K^+ or nicotine. A final 5 min sample was taken following the 5 min period of stimulation. Samples were kept on ice-cold water and quickly acidified with perchloric acid (PCA) to a final concentration of 0.05 N. To determine total CA cell content at the end of the experiment, 800 μl of PCA (0.05 N) was added to each well. High K^+ solution was prepared by isosmotically substituting NaCl by KCl. ω-Agatoxin IVA, when present, was added 5 min before, and maintained through, the stimulus. Fractional release was calculated by dividing the total amount of either norepinephrine (NE) or epinephrine (E) released during a 5 min period by the total amount of that amine present at the beginning of that particular period of time. Individual net fractional release was calculated for each period either during the secretory stimulus or 5 min after the stimulus, by subtracting the release during the basal 5 min period. Total net fractional release was calculated by adding individual net fractional releases during and 5 min after K^+ stimulation.

CA release was determined using a HPLC system with electrochemical detection, provided with two Gilson pumps (model 303), two Gilson automatic injectors, two amperometric detectors (Model LC4B, Bioanalytical Systems, West Lafayette, IN and model 656, Metrohm,

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Switzerland with voltages set at 0.65 V and 0.9 V, respectively). Signals recorded from the detectors were integrated, the area under the peaks measured and the CA concentration calculated from internal standards using a Gilson software (model 714). The precolumn and the analytical columns were identical in both systems and consisted of a replaceable cartridge reverse-phase C_{18} precolumn (5 μm particle size, 30 × 4 mm i.d.; Macherey-Nagel, Duren, Germany) and an analytical reverse-phase C_{18} replaceable cartridge column (5 μm particle size, 100 × 4 mm i.d.; Machinery-Nagel, Duren, Germany). Flow was set at 1 ml/min and 100 μl samples were injected. The mobile phase was similar to the one previously described [20] and had the following composition (in mmol/l): NaH_2PO_4, 80; EDTA, 0.13; heptane sulfonic acid, 5; methanol 10%; the pH was adjusted to 3 with orthophosphoric acid. Retention times were 5.1 min for NE and 7.9 min for E.

2.3. Electrophysiological recording

Recording of chromaffin cell nicotinic receptor currents under voltage-clamp was done as previously described [4] with some modifications. Chromaffin cells were bathed in a solution with the following ionic composition (in mmol/l): NaCl, 140; KCl, 5.9; $CaCl_2$, 2.5; $MgCl_2$, 1; HEPES, 10; glucose, 11 (pH 7.4). The ionic composition of the pipette solution was the following (in mmol/l): KCl, 40; K_2SO_4, 50; $MgSO_4$, 7; HEPES, 10 and nystatin, 250 μg/ml (pH 7.2). A high resistance seal was established in the cell-attached configuration (open tip resistance of the pipette ranged between 3 and 5 MΩ). Capacity transients were canceled using the built-in circuitry of the patch-clamp amplifier (EPC-7; List, Darmstadt, Germany) and the pipette potential set at −55 mV. After 2 or 3 min a small capacity transient indicating electrical continuity between the cell interior and pipette solution was observed. The size of the transient stabilized about 5 min after formation of the gigaseal. Nicotine (10 μM) was applied for 3.5 s using a fast perfusion system (DAD-12; Adams & List, NY) 10 min after gigaseal formation. Nicotine (10 μM) application was repeated 3 times (S_1, S_2, S_3) with an interval of 15 min. The average capacitance of the cells was 11.1 ± 0.99 pF and the series resistance (R_s) 9.8 ± 0.9 MΩ (n = 12). No compensation for R_s was used. When used, ω-AgaTx or D-tubocurarine were perfused to the cell 5 s before and during S_2.

2.4. ATP determination

ATP release in response to high K^+ or nicotine was determined using the luciferin luciferase method as previously described [3]. Total ATP content in the cell was determined by solubilizing the cells using Extralight (Analytical Luminescence, San Diego, CA).

2.5. Chemicals

Culture reagents were obtained from Gibco. Nicotine was purchased from Sigma and ω-Agatoxin IVA was obtained from RBI (Natick, MA). All other reagents were obtained from commercial sources and were of the highest quality available.

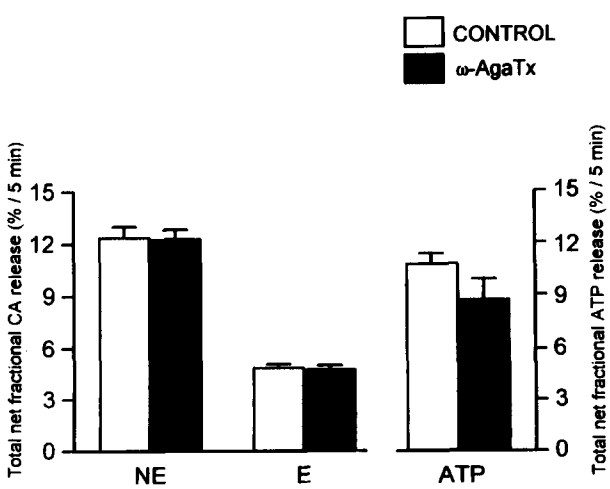

Fig. 1. Effect of ω-agatoxin IVA on high K^+-induced NE, E and ATP secretion. Secretion of norepinephrine (NE), epinephrine (E) and ATP after exposure of bovine chromaffin cells to high extracellular K^+ (75 mM) for 5 min in the absence (open bars) and in the presence (filled bars) of ω-agatoxin (100 nM). Data represent mean ± S.E.M. of 7 experiments for NE and E secretion and 28 for ATP.

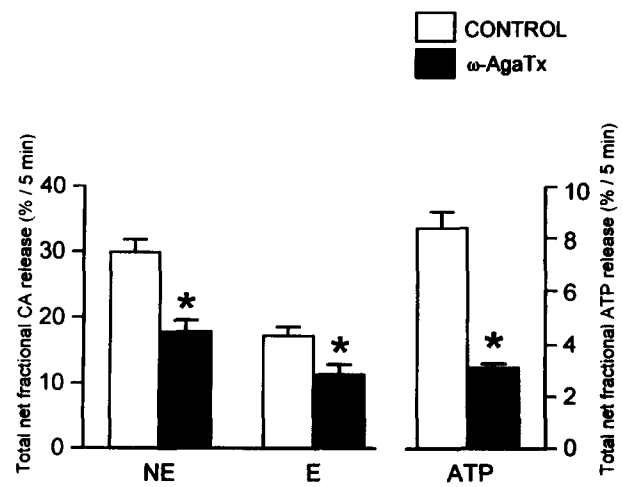

Fig. 2. Effect of ω-agatoxin IVA on nicotine-induced NE, E and ATP secretion. Secretion of norepinephrine (NE), epinephrine (E) and ATP after exposure of bovine chromaffin cells to nicotine (10 μM) for 5 min in the absence (open bars) and in the presence (filled bars) of ω-agatoxin IVA (100 nM). Data represent mean ± S.E.M. of 8 experiments. *$P < 0.01$ as compared to control.

3. Results and discussion

Two different populations of chromaffin cells preferentially secreting NE and E have been described in bovine adrenal medulla [21,22]. Exposure of bovine chromaffin cells in culture to high extracellular K^+ (75 mM) induced the release of about 13% of the total NE and 5% of total E content in the cells (n = 7) (Fig. 1). High K^+ (75 mM) induced the release of 12% of total ATP present in chromaffin cells (n = 28). There is a good correlation between CA and ATP secretion, as would be expected since the more likely source of released ATP in response to depolarization is the chromaffin granule where it is co-stored with CA [23,24]. In the presence of 100 nM of ω-AgaTx, a dose high enough to completely block P-type voltage-dependent Ca^{2+} channels [25], CA or ATP secretion induced by high K^+ (75 mM) were not affected (Fig. 1). This suggests that although about 50% of the Ca^{2+} entering bovine chromaffin cells following a depolarizing pulse is sensitive to ω-AgaTx [12], P-type Ca^{2+} channels are not involved in either CA or ATP secretion in response to depolarization.

The physiological stimulus for CA and ATP secretion in the adrenal medulla is acetylcholine that activates cholinergic nicotinic receptors [1]. Exposure of bovine chromaffin cells to the nicotinic cholinergic agonist nicotine (10 μM) induced the secretion of 30 ± 1.8% of total NE and 17.9 ± 1.6% of total E present in chromaffin cells at the beginning of the stimulation (n = 8) (Fig. 2). Nicotine treatment also induced the release of 8.4 ± 0.6% of total ATP present in the cells (n = 8) (Fig. 2). ω-AgaTx (100 nM) decreased by about 40% nicotine-induced NE and E release (Fig. 2). In addition, nicotine-induced ATP secretion was significantly blocked (about 60%) by ω-AgaTx (100 nM) (Fig. 2). These results suggest that voltage-dependent P-type Ca^{2+} channels are selectively coupled, in bovine chro-

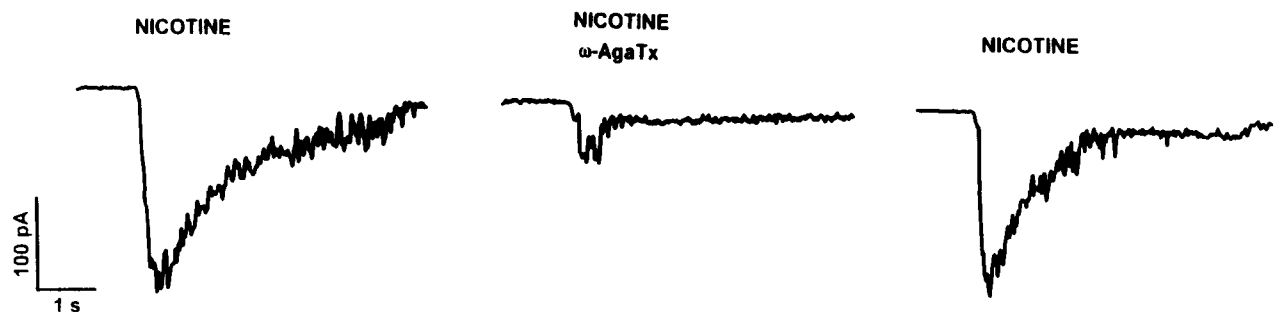

Fig. 3. Effect of ω-agatoxin IVA on nicotine-induced currents. Inward current induced by 3 consecutive applications of nicotine (10 μM) with an interval of 15 min as described in section 2. The second application of nicotine was done in the presence of ω-agatoxin IVA (100 nM). The figure represents a typical experiment that was repeated 4 times with similar results.

maffin cells, to nicotine-induced secretion. This indicates that P channels are located in the vicinity of the nicotinic receptor and so would be preferentially activated by nicotinic stimulation. This suggests that P-type voltage-dependent Ca^{2+} channels may play a role in secretion in chromaffin cells similar to other systems [26,27]. However, since ω-AgaTx did not affect high K^+-induced secretion, an effect of the toxin on a target different from voltage-dependent P-type Ca^{2+} channels should be excluded.

Nicotine-induced secretion from chromaffin cells begins by activation of nicotinic receptors that increase the permeability to Na^+ and K^+ ions and very little, under physiological concentrations of extracellular Na^+, to Ca^{2+} [4]. This change in permeability depolarizes chromaffin cells and produces opening of voltage-dependent Ca^{2+} channels. Although ω-AgaTx is considered a highly selective blocker of P-type voltage-dependent Ca^{2+} channels [13], we decided to investigate the effect of this toxin on the current induced by activation of nicotinic receptors to exclude the possibility that the nicotinic receptor could be a primary target for ω-AgaTx. If this is the case it could explain the selective inhibitory action of the toxin on nicotine-induced secretion without affecting high K^+-induced secretion.

To explore this possibility we decide to use nystatin-perforated patch-clamp recordings to preserve physiological regulation of the cell, and use a holding potential of −55 mV that is the resting membrane potential measured under current-clamp conditions (data not shown). Perfusion of bovine chromaffin cells under voltage-clamp with nicotine (10 μM) for 3.5 s induced an inward current that amounted to −204 ± 25 pA (n = 23) (Fig. 3). That current inactivated quickly with a time constant of about 1 s. Peak current was very little affected by a second exposure of the cell to nicotine (10 μM), with a S_2/S_1 ratio of 0.81 ± 0.12 (Table 1). However, in the presence of ω-AgaTx, peak current was markedly decreased in a dose-dependent manner (S_2/S_1 ratio of 0.34 ± 0.04 in the presence of ω-AgaTx 100 nM; Fig. 3). In addition, nicotine-evoked inward current was almost completely blocked by the nicotinic receptor antagonist D-tubocurarine (Table 1). The time integral on the current gives the total amount of charge entering the cell during the first nicotine stimulation (S_1) as 256.5 ± 19.2 pC (n = 23). When nicotine perfusion was repeated 15 min later, about 35% of inactivation was observed (S_2/S_1 ratio for the total charge of 0.55 ± 0.05). However, when, after an initial exposure to nicotine (10 μM) for 3.5 s, a second perfusion with nicotine was repeated 15 min later in the presence of ω-AgaTx, a dose-dependent blocking action of ω-AgaTx could be observed (Table 1). Consistent with its effect on peak current, the nicotinic receptor blocker D-tubocurarine blocked net charge influx in response to nicotine. The blocking effect of both ω-AgaTx (Fig. 3) and D-tubocurarine (data not shown) could be partially reverted by washing out the toxin or the drug.

The data indicate that besides its known action as a blocker of P-type voltage-dependent Ca^{2+} channels [14,25], ω-AgaTx is a very potent blocker of the neuronal cholinergic nicotinic receptor present in bovine chromaffin cells and that its blocking actions on nicotine-induced secretion might be explained, not by a specific location of P-type voltage-dependent Ca^{2+} channels close to nicotinic receptors, but rather by a specific reversible blockade of agonist-induced current through the nicotinic receptor channels. The blocking effect of ω-AgaTx on nicotinic receptor channels is shared by different compounds, including somatostatin [28], methoxyverapamil [29] and atropine [30], suggesting that nicotinic receptor function might be regulated by multiple pathways.

Table 1
Effect of agatoxin IVA and D-tubocurarine on nicotine-induced inward currents and net charge influx in bovine chromaffin cells

Drug added together with nicotine during S_2	n	Peak current (S_2/S_1 ratio)	Total net charge influx (S_2/S_1 ratio)
None	10	0.81 ± 0.12	0.55 ± 0.05
ω-AgaTx 1 nM	3	0.48 ± 0.06*	0.39 ± 0.05*
ω-AgaTx 10 nM	4	0.26 ± 0.07**	0.15 ± 0.06**
ω-AgaTx 100 nM	4	0.34 ± 0.04**	0.18 ± 0.04**
D-Tubocurarine 10 μM	3	0.10 ± 0.02***	0.09 ± 0.04***

Bovine chromaffin cells were exposed for 3.5 s to nicotine (10 μM) as stated in section 2. Nicotine stimulation was repeated 3 times (S_1, S_2, S_3) at 15 min interval. D-Tubocurarine or ω-AgaTx IVA were perfused to the cell 5 s before and during the second (S_2) nicotine stimulation. The average value for inward peak current and total net charge influx during S_1 were −204 ± 25 pA (n = 23) and 256 ± 19 pC (n = 23). Data represent mean ± S.E.M. *P < 0.05; **P < 0.005; ***P < 0.001 as compared to control.

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