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Blockade by agmatine of catecholamine release from chromaffin cells is unrelated to imidazoline receptors

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Abstract

The blockade of exocytosis induced by the putative endogenous ligand for imidazoline receptors, agmatine, was studied by using on-line measurement of catecholamine release in bovine adrenal medullary chromaffin cells. Agmatine inhibited the acetylcholine-evoked release of catecholamines in a concentration-dependent manner ($IC_{50} = 366$ μM); the K^+-evoked release of catecholamines was unaffected. Clonidine (100 μM) and moxonidine (100 μM) also inhibited by 75% and 50%, respectively, the acetylcholine-evoked response. In cells voltage-clamped at −80 mV, the intermittent application of acetylcholine pulses elicited whole-cell inward currents (I_{ACh}) that were blocked 63% by 1 mM agmatine. The onset of blockade was very fast ($\tau_{on} = 31$ ms); the recovery of the current after washout of agmatine also occurred very rapidly ($\tau_{off} = 39$ ms). Efaroxan (10 μM) did not affect the inhibition of I_{ACh} elicited by 1 mM agmatine. I_{ACh} was blocked 90% by 100 μM clonidine and 50% by 100 μM moxonidine. The concentration–response curve for acetylcholine to elicit inward currents was shifted to the right in a non-parallel manner by 300 μM agmatine. The blockade of I_{ACh} caused by agmatine (100 μM) was similar at various holding potentials, around 50%. When intracellularly applied, agmatine did not block I_{ACh}. At 1 mM, agmatine blocked I_{Na} by 23%, I_{Ba} by 14%, $I_{K(Ca)}$ by 16%, and $I_{K(VD)}$ by 18%. In conclusion, agmatine blocks exocytosis in chromaffin cells by blocking nicotinic acetylcholine receptor currents. In contrast to previous views, these effects seem to be unrelated to imidazoline receptors.

Keywords: Agmatine; Nicotinic receptor; Ca^{2+} channel; Chromaffin cell

1. Introduction

The idea of the existence of imidazoline receptors emerged from the observation that clonidine, an α_2-adrenoceptor agonist (Pineda et al., 1993), also binds to an additional site in brain membranes, termed the imidazoline receptor (Bousquet et al., 1984; Ernsberger et al., 1988). It is curious that bovine adrenal medulla chromaffin cells lack α_2-adrenoceptors, but express imidazoline receptors (Ernsberger et al., 1989; Regunathan et al., 1991; Wang et al., 1992). Clonidine potently blocks the release of catecholamines from isolated bovine chromaffin cells (Powis and Baker, 1986) and the perfused cat adrenal gland (Orts et al., 1987) by mechanisms unrelated to α_2-adrenoceptors. Hence, this blockade has been associated with an action of clonidine on imidazoline receptors of chromaffin cells (Ohara-Imaizumi and Kumakura, 1992).

Agmatine, a cationic amine, has been proposed to be the endogenous ligand for imidazoline receptors (Li et al., 1994). Agmatine has been identified in the brain (Feng et al., 1997) and is synthesised by arginine decarboxylase (Li et al., 1994), is stored in neurons, is released in a Ca^{2+}-dependent manner by depolarising stimuli, is metabolised and degraded to putrescine by agmatine ureohydrolase, and is taken up by synaptosomes (for a recent review, see Reis and Regunathan, 2000). Hence, agmatine seems to meet all the criteria of Henry Dale to be considered a brain neurotransmitter, although with dubious physiological functions

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as yet (Regunathan et al., 1991, 1996; Jurkiewicz et al., 1996; González et al., 1996).

Our interest in studying the mechanism of modulation by imidazoline receptors of exocytotic catecholamine release arose from the discovery that agmatine was endogenously synthesised and stored in bovine chromaffin cells (Wang et al., 1995; Raash et al., 1995), and that an imidazoline receptor protein was isolated from these cells (Wang et al., 1992). Hence, we reasoned that endogenously released agmatine could serve as a regulator of catecholamine release. So, we decided to study the effects of agmatine and related compounds that act at imidazoline receptors on catecholamine release from bovine chromaffin cells stimulated by neuronal nicotinic receptor agonists, or through cell depolarisation with high K^+. Additionally, we investigated the effects of agmatine on neuronal nicotinic receptor currents, Ca^{2+} channel currents, Na^+ channel currents, and K^+ channel currents in voltage-clamped bovine chromaffin cells. Contrary to previous conclusions that associated the functional effects of agmatine with imidazoline receptors (Regunathan et al., 1991; Pineda et al., 1993; Reis and Regunathan, 2000), our results suggest that agmatine interferes with catecholamine secretion by blocking directly and selectively the nicotinic acetylcholine receptor currents in chromaffin cells. The experiments presented here also help to understand the mechanism of blockade by agmatine of neuronal nicotinic acetylcholine receptors. Less well documented effects of agmatine on neuronal nicotinic acetylcholine receptors have previously been reported, showing a decrease in dimethylphenyl-piperazinium (DMPP)-evoked depolarisation of the optic nerve and the rat superior cervical ganglion elicited by the compound (Loring, 1990).

2. Materials and methods

2.1. Isolation and culture of adrenal medulla chromaffin cells

Bovine adrenal medulla chromaffin cells were isolated from adrenal glands of adult cows, following standard methods (Livett, 1984) with some modifications (Moro et al., 1990). Cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal calf serum, 10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, 50 IU ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Cells were plated on 10-cm diameter Petri dishes (5×10^6 cells in 10 ml of DMEM) for secretion experiments, and on 1-cm diameter glass coverslips at a density of 5×10^4 cells per coverslip for patch-clamp experiments.

2.2. On-line measurement of catecholamine release from chromaffin cells

Bovine chromaffin cells (5×10^6) were introduced in a microchamber for cell superfusion at room temperature (22–24°C) with Krebs–HEPES solution containing (in mM): NaCl 144, KCl 5.9, $MgCl_2$ 1.2, glucose 11, $CaCl_2$ 2, and HEPES 10 at pH 7.4. The perfusion rate was 2 ml min^{-1}. The liquid flowing from the perfusion chamber reached an electrochemical detector (model Metrohm AG CH-9100, Hersau) that monitors "on-line", under the amperometric mode, the amount of catecholamines secreted (Borges et al., 1986). The cells were stimulated with a Krebs–HEPES solution containing 100 μM acetylcholine or a depolarising solution (Krebs–HEPES containing 70 mM K^+, with isoosmotic reduction of NaCl), for 2 s at 5-min intervals by means of electrovalves.

2.3. Electrophysiological recordings

Inward currents through ion channels 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. The chamber was continuously perfused with a control Tyrode solution containing (in mM): 137 NaCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES/NaOH, pH 7.4. Soft glass patch-clamp electrodes were filled with a standard intracellular solution containing (in mM): 10 NaCl, 100 CsCl, 20 tetraethylammonium · Cl, 5 Mg · ATP, 14 EGTA, 0.3 Na · GTP and 20 HEPES–KOH, pH 7.2. Experiments were performed at room temperature (22–24°C).

External solutions were 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 low (0.5–1 ml min^{-1}) and regulated by gravity. Nicotinic receptor currents (I_{ACh}) were usually elicited by fast application of 100 μM acetylcholine dissolved in the Tyrode solution described above, through this superfusion pipette.

For recording of voltage-gated Ca^{2+} channel currents, cells were internally dialysed with the same intracellular solution as above; the extracellular solution contained 10 mM Ba^{2+} (instead of Ca^{2+}) as charge carrier, and 5 μM tetrodotoxin. The cells were clamped at −80 mV holding potential; step depolarisations to different test potentials from this holding potential lasted 50 ms and were applied at 0.1 Hz. For recording of currents through voltage-dependent Na^+ channels the extracellular solution was Tyrode without $CaCl_2$. Cells were voltage-clamped at −80 mV and depolarised to −10 mV during 14 ms at 10-s intervals. For recording of K^+ currents, cells were internally dialysed with a solution containing (in mM): NaCl 10, KCl 130, Mg · ATP 5, EGTA 14, and 20 HEPES–KOH, pH 7.2. The cells were clamped at −80 mV holding potential; step depolarisations to different test potentials from this resting potential lasted 200 ms and were applied at 0.1 Hz.

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 transients 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, USA) were used to acquire and analyse the data. Leak and capacitative currents were subtracted by using currents elicited by small hyperpolarising pulses.

2.4. Materials and solutions

The following materials were used: collagenase from *Clostridium histolyticum* (Boehringer-Manheim); bovine serum albumin fraction V, cytosine arabinoside, fluorodeoxyuridine, acetylcholine, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), clonidine and ethylene glycol-bis(β-aminoethyl-ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (Sigma); foetal calf serum, penicillin and streptomycin (GIBCO); moxonidine (kindly provided by Ely Lilly, Brazil and Spain); and efaroxan (kindly provided by Dr. J.A. García-Sevilla, Universidad de Baleares, Spain). All other chemicals were reagent grade from Sigma, Merck or Bio-Rad.

Agmatine and efaroxan were dissolved in distilled water at 10^{-1} M and diluted in saline solution to the desired concentrations. Moxonidine was dissolved in 10% DMSO at 10^{-2} M.

2.5. Statistical analysis

Results are expressed as means ± S.E.M. The statistical differences between means of two experimental results were assessed by Student's *t*-test. A value of p equal to or smaller than 0.05 was taken as the limit of significance. The IC_{50} for blockade of secretion was estimated through non-linear regression analysis by ISI software for a PC computer. To calculate the time constant (τ) for blockade and recovery of acetylcholine currents, records were fitted to a single exponential curve.

3. Results

3.1. Agmatine blocks the nicotinic receptor-mediated catecholamine release response, but not the K^+-evoked response

The study of catecholamine release responses can satisfactorily be performed through repeated challenge with secretagogues of fast-superfused chromaffin cell populations. In the experiment shown in Fig. 1A, cells superfused with Krebs–HEPES solution had a basal steady state spontaneous catecholamine release of about 20 nA. Upon challenge with 5-s pulses of a 70 mM K^+ solution, secretion peaks of around 500–700 nA were obtained; these peaks were very reproducible when the K^+ pulses were repeated at 5-min intervals. In this prototype experiment, the superfusion of the cells with agmatine (1 mM), from 5 min before and during the K^+ pulses (P4 and P5), did not appreciably affect the size of the secretory peaks. In five experiments, the release obtained in the presence of 1 mM agmatine (P5) amounted to 76.7 ± 5.7% of that obtained with the pulse (P3) immediately preceding the introduction of the compound, a value similar to that obtained in control paired experiments in the absence of agmatine (83.5 ± 8.5%).

With this protocol, but challenging the cells with the nicotinic agonist dimethylphenylpiperazinium (DMPP), secretory peaks of similar magnitude to those of K^+ were obtained (Fig. 1B). Sequential 5-s pulses of 100 μM DMPP, applied at 5-min intervals, produced catecholamine release responses that tended to gradually decline, likely due to desensitisation of nicotinic receptors (Schiavone and Kirpekar, 1982). Thus, at P7 the size of the secretion peak was 72.5 ± 2.4% of that at P1 ($n = 4$ experiments). In the prototype experiment shown in Fig. 1B, the introduction of 1 mM agmatine from 5 min before and during the P4 and P5 pulses drastically reduced the secretory response. In four experiments, the response in P5 was only 18.2% of the response obtained in P3 immediately preceding the introduction of agmatine. Note also in Fig. 1B that, upon wash out of agmatine, the secretory response recovered quickly but partially, likely due to desensitisation of neuronal nicotinic acetylcholine receptors, as pointed out above.

In view of the powerful blockade of the DMPP response, and considering that agmatine is an endogenous compound with unknown physiological functions, it was desirable to test its effects on responses evoked by acetylcholine, the physiological neurotransmitter at cholinergic synapses. All subsequent experiments involving neuronal nicotinic acetylcholine receptor activation were, therefore, performed with acetylcholine (5-s pulses of 100 μM acetylcholine). The effects of increasing concentrations of agmatine on acetylcholine-evoked secretion were tested (Fig. 1C). The threshold agmatine concentration for blockade of catecholamine release was 30 μM; 1 mM agmatine caused maximum inhibition of acetylcholine responses (about 70%). Note also in Fig. 1C the quick recovery of the response upon agmatine washout. An IC_{50} of 366 μM was calculated for the agmatine-induced blockade of acetylcholine-induced secretion (Fig. 1D).

3.2. Effects of clonidine and moxonidine on acetylcholine-evoked secretion

Since agmatine, the putative endogenous ligand for imidazoline receptors, blocked the acetylcholine secretory responses, it was reasonable to test whether other exogenous imidazoline receptor ligands, i.e. clonidine (Bousquet et al., 1984; Ernsberger et al., 1988) and moxonidine (Ernsberger et al., 1993), were able to mimic the effects of

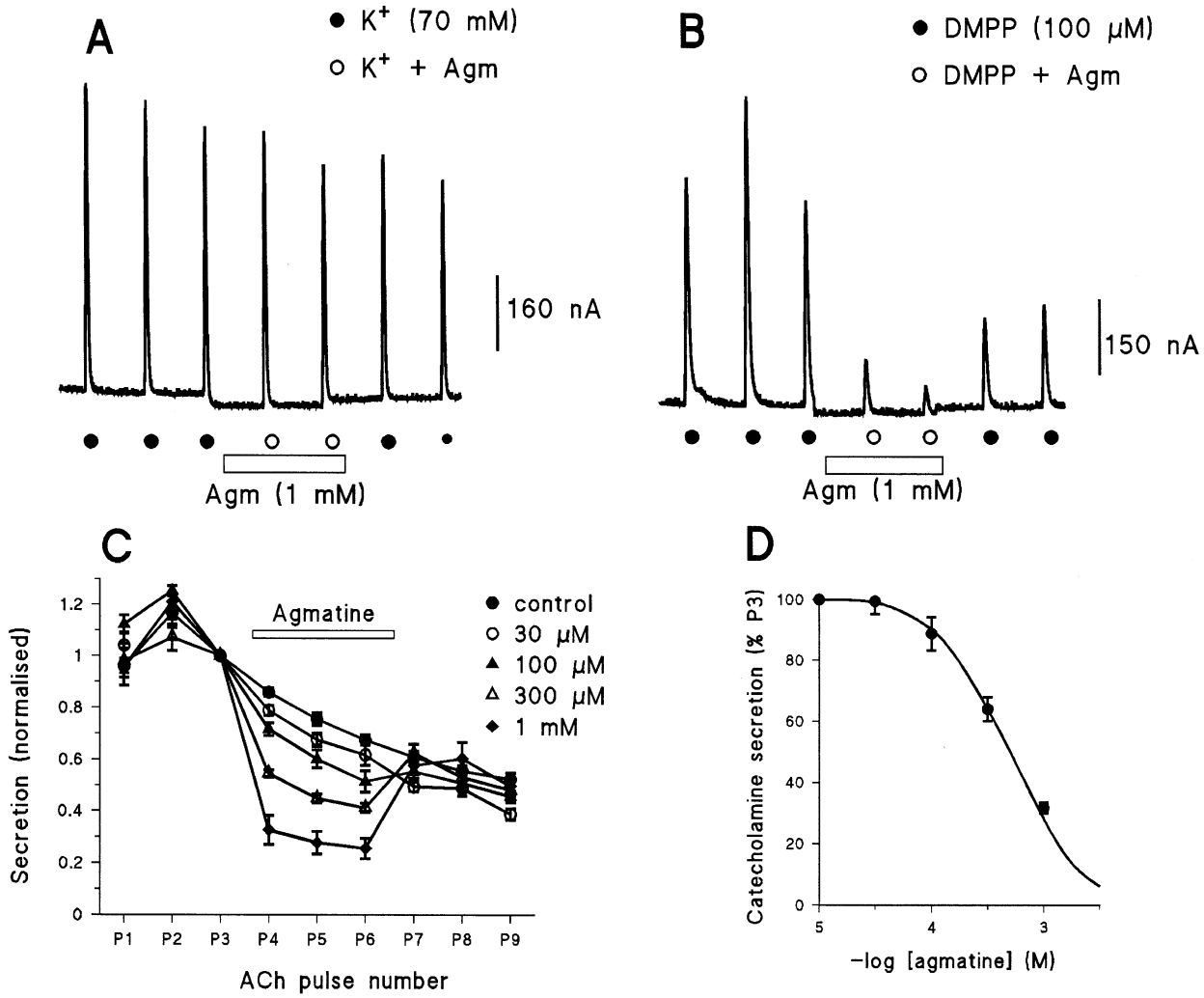

Fig. 1. Agmatine blocks the catecholamine release responses obtained in fast-superfused cells challenged with pulses of nicotinic receptor agonists, but not those triggered by K^+ stimulation. Cells were superfused with Krebs–HEPES solution containing 2 mM Ca^{2+} and stimulated at 5-min intervals with 5-s pulses of 70 mM K^+ (panel A) or with 100 μM dimethylphenylpiperazinium (DMPP, panel B). Agmatine was present as indicated by the horizontal bars. In panel C, averaged results on the inhibitory effects of increasing concentrations of agmatine on acetylcholine-evoked secretion are shown. Data are means ± S.E.M. of 6–8 experiments for each agmatine concentration.

agmatine. Hence, the effects of clonidine and moxonidine on catecholamine secretion were investigated in experiments with protocols similar to those described in Fig. 1, that is, with the application of eight sequential pulses of acetylcholine (100 μM, 5-s, 5-min intervals) and assessment of the blocking effects of clonidine (100 μM). Clonidine was applied from 5 min before and during the acetylcholine pulses, in P4 and P5. In these experiments, clonidine blocked the acetylcholine response by 79.1 ± 5.2% at P4 and by 81.8 ± 5% at P5 ($n = 4$). Washout of clonidine led to a prompt recovery of the response to nearby its control value. Moxonidine also blocked acetylcholine-evoked secretion. At 100 μM, moxonidine caused a blockade of 42.5 ± 7.4% at P4 and of 63.5 ± 5.3% at P5 ($n = 4$ experiments), substantially smaller than that induced by 100 μM clonidine ($p < 0.05$, comparing blockade at P4 caused by clonidine and moxonidine). At 1 mM, moxonidine did not induce a further blockade of the acetylcholine response (not shown). As for clonidine, the acetylcholine response rapidly recovered to control levels upon moxonidine washout.

3.3. Blockade by agmatine, clonidine and moxonidine of nicotinic receptor currents

The blockade by agmatine of acetylcholine-evoked secretion, and its lack of action on K^+-evoked secretion, suggested a direct action on neuronal nicotinic acetylcholine receptors rather than on voltage-dependent Ca^{2+} channels. To clarify this dilemma with more direct approaches, we measured acetylcholine-evoked inward currents under conditions in which voltage-dependent ion currents were excluded, using the whole-cell configuration of the patch-clamp technique.

The experiment shown in Fig. 2A was done with a chromaffin cell whose membrane potential was held at −80 mV; the cell was fast-superfused with an extracellular Tyrode solution similar to that used for the secretion experiments described above (see Section 2). Acetylcholine pulses (100 μM, 1 s) were applied at 30-s intervals. This generated an inward current (I_{ACh}) that peaked at around 1500 pA (1532 ± 139 pA in 21 cells) and quickly desensitised during the acetylcholine pulse. The amplitude of this current, however, was fairly constant upon sequential application of 15–20 acetylcholine pulses at 30-s intervals (not shown). When the acetylcholine pulses were co-applied with 1 mM agmatine, the size of the current was reduced in a single-step to 36% of the initial current (pulses P3, P4 and P5 of Fig. 2A). The subsequent application of acetylcholine alone produced again a current similar to the initial I_{ACh}. This behaviour was reproduced once more in the same cell (pulses P11, P12 and P13 of Fig. 2A).

Using this type of experiment, we decided to study whether different modes of application of agmatine would affect I_{ACh}. For instance, when 1 mM agmatine was given only during the 30-s period preceding the acetylcholine pulse, but was absent during the pulse, only 5.8 ± 2.6% blockade of the current was produced ($n = 7$; Fig. 2A, first top horizontal bar, and Fig. 2B, column labelled "pre"). In contrast, when agmatine was absent during the 30-s period preceding the acetylcholine pulse, but was present only during the 1-s acetylcholine pulse, then 63.4 ± 3.7% blockade of I_{ACh} was induced ($n = 20$; Fig. 2B, column labelled "co"). Finally, with pre- plus co-application of agmatine, 66.9 ± 9.1% ($n = 5$) blockade of I_{ACh} was elicited ($n = 5$; Fig. 2A, second horizontal black bar, and Fig. 2B, column labelled "pre/co").

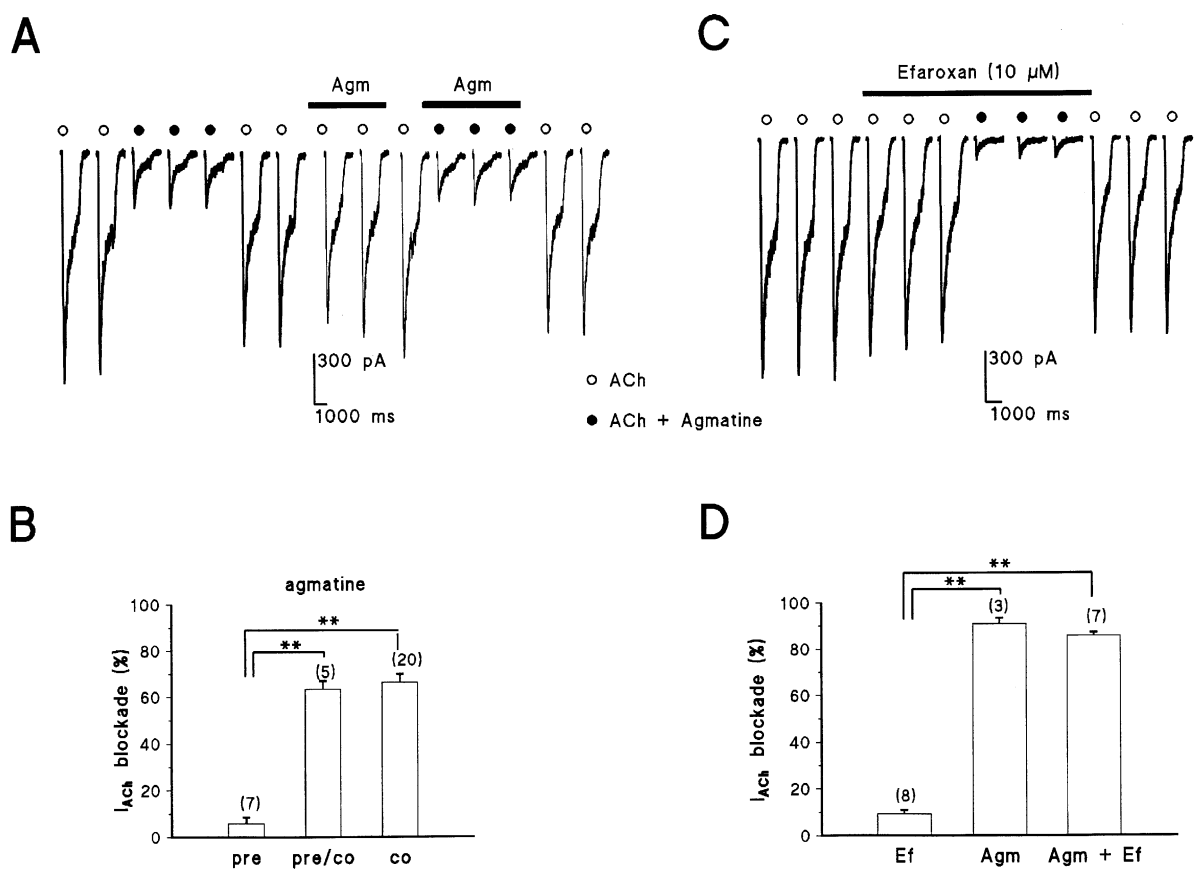

Fig. 2. Agmatine blocks the inward current through nicotinic acetylcholine receptors (I_{ACh}). The original I_{ACh} traces of panel A were obtained in a cell voltage-clamped at −80 mV and continuously superfused with a local superfusion pipette. Acetylcholine pulses (100 μM, 1-s duration) were regularly applied at 30-s intervals, either in the absence (white dots) or in the presence of 1 mM agmatine (black dots). When indicated by the top horizontal bars, agmatine (1 mM) was superfused in the intervals between the acetylcholine pulses. Panel B shows quantitative averaged data for the % blockade of I_{ACh} elicited by different modes of agmatine (1 mM) application, i.e. only during the 30 s preceding the acetylcholine pulse ("pre", pre-application mode), only during the application of the acetylcholine pulse ("co", co-application mode), or before and during the acetylcholine pulse ("pre/co"). Panels C and D show that efaroxan does not prevent the blockade of I_{ACh} elicited by agmatine in a chromaffin cell voltage-clamped at −80 mV and challenged with 1-s pulses of 100 μM acetylcholine, given at 30-s intervals. Acetylcholine pulses were applied in the absence (white circles) or the presence of 1 mM agmatine (black circles). Efaroxan was superfused as shown by the top horizontal bar. Panel D shows averaged results of the number of cells shown in parentheses on top of each column. Blockade of I_{ACh} in each situation (efaroxan alone, EF; agmatine alone, Agm; or efaroxan + agmatine given during the acetylcholine pulse) is normalized as % of blockade of the initial I_{ACh} current (ordinate). $^{**}p < 0.01$ with respect to efaroxan alone.

Similar experiments were done to study the blockade by clonidine and moxonidine of I_{ACh}. In these experiments, pre-application of 100 μM clonidine caused only 11.9 ± 5.7% ($n = 3$) blockade of I_{ACh}. However, its co-application with the acetylcholine pulse produced 88.1 ± 1.3% blockade ($n = 3$); the combined pre- plus co-application provoked 88.6 ± 1.9% inhibition of I_{ACh} ($n = 4$). Comparable results were obtained with 100 μM moxonidine: no blockade with pre-application (4.3 ± 6.1%; $n = 4$), 57.1 ± 7.7% blockade with co-application ($n = 4$) and 50.7 ± 6.4% blockade with combined pre- plus co-application ($n = 4$).

3.4. Efaroxan does not modify the blocking effects of agmatine on I_{ACh}

Efaroxan, at a concentration (10 μM) that blocks imidazoline receptors (Piletz et al., 1996), did not affect I_{ACh} (Fig. 2C). I_{ACh} amplitude amounted to 1179 ± 119 pA ($n = 8$ cells) in the absence of efaroxan and to 1052 ± 89 pA in its presence ($n = 8$ cells); thus, blockade of I_{ACh} by efaroxan amounted to only 9.7 ± 1.7% ($n = 8$ cells; Fig. 2D). Blockade of I_{ACh} elicited by agmatine amounted, however, to 90.8 ± 2.5% ($n = 3$ cells); in the presence of efaroxan, such blockade reached 85.8 ± 1.3% ($n = 7$ cells; Fig. 2D).

3.5. An extracellular binding site for agmatine on neuronal nicotinic acetylcholine receptors

In this series of experiments agmatine (1 mM) was sequentially given, first intracellularly and subsequently extracellularly. After the cell was pierced with a patch pipette filled with 1 mM agmatine, I_{ACh} peaked at 1500 pA (Fig. 3A). This value was similar to that obtained in cells dialysed with an intracellular solution free of agmatine (Fig. 2A). Thus, it seemed that after 5 min dialysis with 1 mM agmatine, the current elicited by 1-s acetylcholine pulses (100 μM acetylcholine) remained fully unblocked. This finding was corroborated by the results obtained with the subsequent application of 1 mM agmatine to the same cells that were previously dialysed with the compound. The external co-application of agmatine led to a pronounced fast blockade of I_{ACh} (Fig. 3A); the blockade amounted to 65.2 ± 4.6% ($n = 9$ cells; Fig. 3B), a figure very similar to that obtained in cells not dialysed with agmatine (Fig. 2A). These experiments strongly support the idea that agmatine blocks neuronal nicotinic acetylcholine receptors through its binding to an extracellular site.

3.6. Onset and offset of I_{ACh} blockade elicited by agmatine, clonidine and moxonidine

The co-application experiments described above suggested that the blockade by agmatine of I_{ACh} was very rapid, and was established in less than a second. To study the rate of blockade and its reversal in more detail, we used a highly efficient device for rapid local superfusion of the chromaffin cells. With this device, complete replacement of one solution by another is achieved in few milliseconds (Gandía et al., 1993). Hence, the introduction and removal of agmatine in the middle of a short pulse of acetylcholine was possible. In this manner, the rate of onset and offset of I_{ACh} blockade by agmatine could be studied in the millisecond time range.

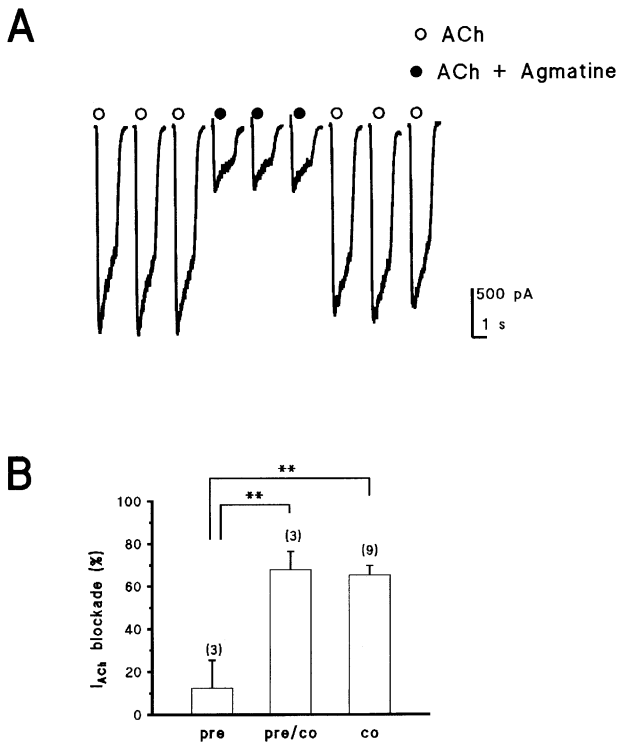

Fig. 3. Dialysis of the cells with agmatine (1 mM) did not block I_{ACh} or prevent its blockade by extracellular application of agmatine. After 5 min of dialysis with the standard intracellular solution containing 1 mM agmatine, a chromaffin cell voltage-clamped at −80 mV was stimulated with 1-s pulses of 100 μM acetylcholine, at 30-s intervals. Acetylcholine pulses were applied in the absence (white circles) or the presence of 1 mM agmatine (black circles). Panel B shows quantitative averaged data on the % blockade of I_{ACh} elicited by different modes of extracellular agmatine (1 mM) application as in Fig. 2. Data are means ± S.E.M. of the number of cells shown on top of each column. $^{**}p < 0.01$ compared with the pre-application mode.

Fig. 4A shows original I_{ACh} traces sequentially obtained in the same voltage-clamped cell. Pulses of 1-s duration of a low concentration of acetylcholine (30 μM) were applied in order to prevent excessive current desensitisation. During trace 1, I_{ACh} peaked at 1495 pA and subsequently relaxed to 573 pA at the end of the pulse. Trace 2, obtained 2 min later with a second acetylcholine pulse, perfectly matched the peak and late currents of trace 1. Agmatine (1 mM), introduced during the period 250–750

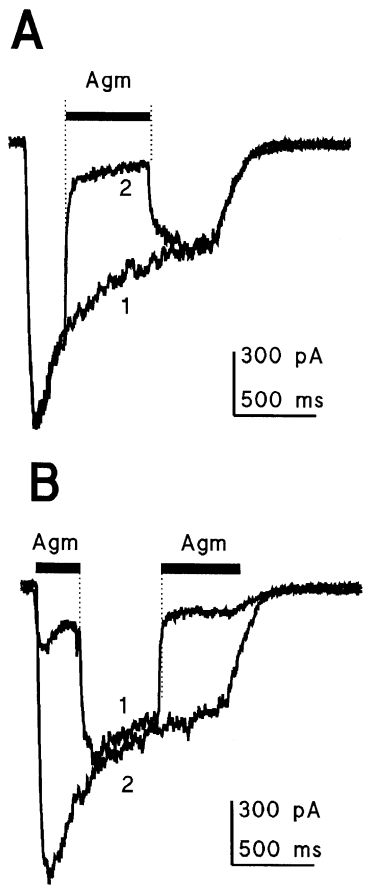

Fig. 4. Blockade of I_{ACh} by agmatine develops in the millisecond time range. Each pair of I_{ACh} traces shown in panels A and B were obtained in four separate cells voltage-clamped at −80 mV. In trace 1, acetylcholine was applied alone for 1 s; in trace 2, 1 mM agmatine was applied within the 1-s acetylcholine pulse, during the time period shown by the top horizontal bars. These are examples of typical experiments; values for τ_{on} and τ_{off} of I_{ACh} blockade elicited by agmatine and other compounds, obtained from pooled data of various cells, are given in the text.

ms of this second acetylcholine pulse, caused a fast blockade of I_{ACh}. The current recovered its control level quickly after removal of agmatine. In different cells, the τ_{on} for 1 mM agmatine blockade was 31.1 ± 3.8 ms, and the τ_{off} was 38.6 ± 6.3 ms ($n = 18$ cells). Blockade induced by 100 μM agmatine exhibited a τ_{on} of 51.5 ± 15.9 ms and a τ_{off} of 73.7 ± 13.5 ms ($n = 10$). In a second protocol (Fig. 4B), acetylcholine plus agmatine were initially given together, and during the period 250–750 ms of the 1-s acetylcholine pulse, agmatine was removed. The initial current, which was blocked by 80%, quickly recovered to control levels upon agmatine removal, with a τ of 34 ± 10.7 ms. The current was again quickly blocked upon agmatine reintroduction, with a τ of 48.8 ± 14.7 ms ($n = 5$ cells).

To define the rate of onset and offset of I_{ACh} blockade elicited by clonidine and moxonidine, experiments similar to those of Fig. 4A were performed. In these experiments, 100 μM clonidine also caused a fast 91% blockade of I_{ACh} that quickly but partially reversed upon removal of the drug. In six cells, I_{ACh} blockade by clonidine exhibited a τ_{on} of 13.3 ± 1.5 ms and a τ_{off} of 43.8 ± 1.4 ms (not shown). Blockade by moxonidine (100 μM) of I_{ACh} showed a τ_{on} of 64.6 ± 8.9 ms and a τ_{off} of 31.3 ± 4.4 ms ($n = 14$ cells).

3.7. Agmatine interacts with the nicotinic receptor in a non-competitive mode with acetylcholine

To gain insight into the mechanism of interaction of agmatine at the nicotinic receptor, the following experiment was performed. Voltage-clamped cells were stimulated with 1-s pulses of increasing concentrations of acetylcholine (0.03–3 mM). At 30 μM, acetylcholine elicited a peak current of 369 ± 14 pA ($n = 13$ cells); the maximum current was obtained with 1 mM acetylcholine. An EC_{50} of around 200 μM was obtained from the concentration–response curve shown in Fig. 5.

The concentration–response curve for acetylcholine was repeated in the presence of a concentration of agmatine, 300 μM, close to its IC_{50} to block catecholamine release (see Fig. 1D). Now, the curve was shifted slightly to the right but in a non-parallel manner. In the presence of agmatine, increasing concentrations of acetylcholine did not yield the maximal amplitude of I_{ACh}. This is better seen when the relative blockade achieved by agmatine at each acetylcholine concentration is expressed as % of the

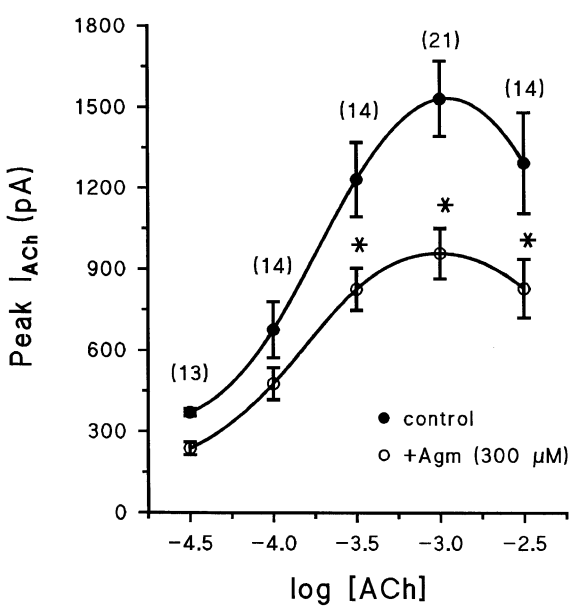

Fig. 5. Non-competitive blockade of I_{ACh} elicited by agmatine in chromaffin cells voltage-clamped at −80 mV. In each individual cell, three concentrations of acetylcholine were tested, first in the absence of agmatine (black circles) and subsequently in the presence of 300 μM of this compound (white circles). The concentration–response curves for I_{ACh} (ordinate, in pA) are elicited by increasing concentrations of acetylcholine (abscissa). Data are means ± S.E.M. of the number of cells shown in parentheses.

current obtained in the absence of agmatine. The current blockade exerted by agmatine amounted to about 40% at all acetylcholine concentrations tested. This was true with the co-application and with the combined pre- plus co-application of agmatine.

3.8. Voltage-independent blockade by agmatine of I_{ACh}

Experiments with protocols similar to those described in the legend to Fig. 4 were performed in cells voltage-clamped at various holding potentials. Acetylcholine pulses (100 μM each) were applied and agmatine (100 μM) was given for half a second in the middle of the pulse. In these experiments a rapid blockade and recovery of the current was observed upon agmatine application to and removal from cells voltage-clamped at −110, −80, −60 or −40 mV. Averaged results from seven cells showed that the blockade of I_{ACh} elicited by agmatine (around 50–60%) was similar at all voltages tested (not shown).

3.9. Effects of agmatine on Na^+ channel currents, Ca^{2+} channel currents and K^+ channel currents

Is agmatine a specific blocker of neuronal nicotinic acetylcholine receptors channels or does the compound also target other type of voltage-gated channels? To answer this question we tested its effects on voltage-dependent Na^+, Ca^{2+} and K^+ channel currents, as well as Ca^{2+}-dependent K^+ channels. Inward currents through voltage-dependent Na^+ channels (I_{Na}) peaked at test depolarising pulses to −10 mV, applied from a holding potential of −80 mV (*I–V* curves not shown). Fig. 6A shows the time course of peak I_{Na} in a chromaffin cell dialysed with the standard intracellular solution and superfused with

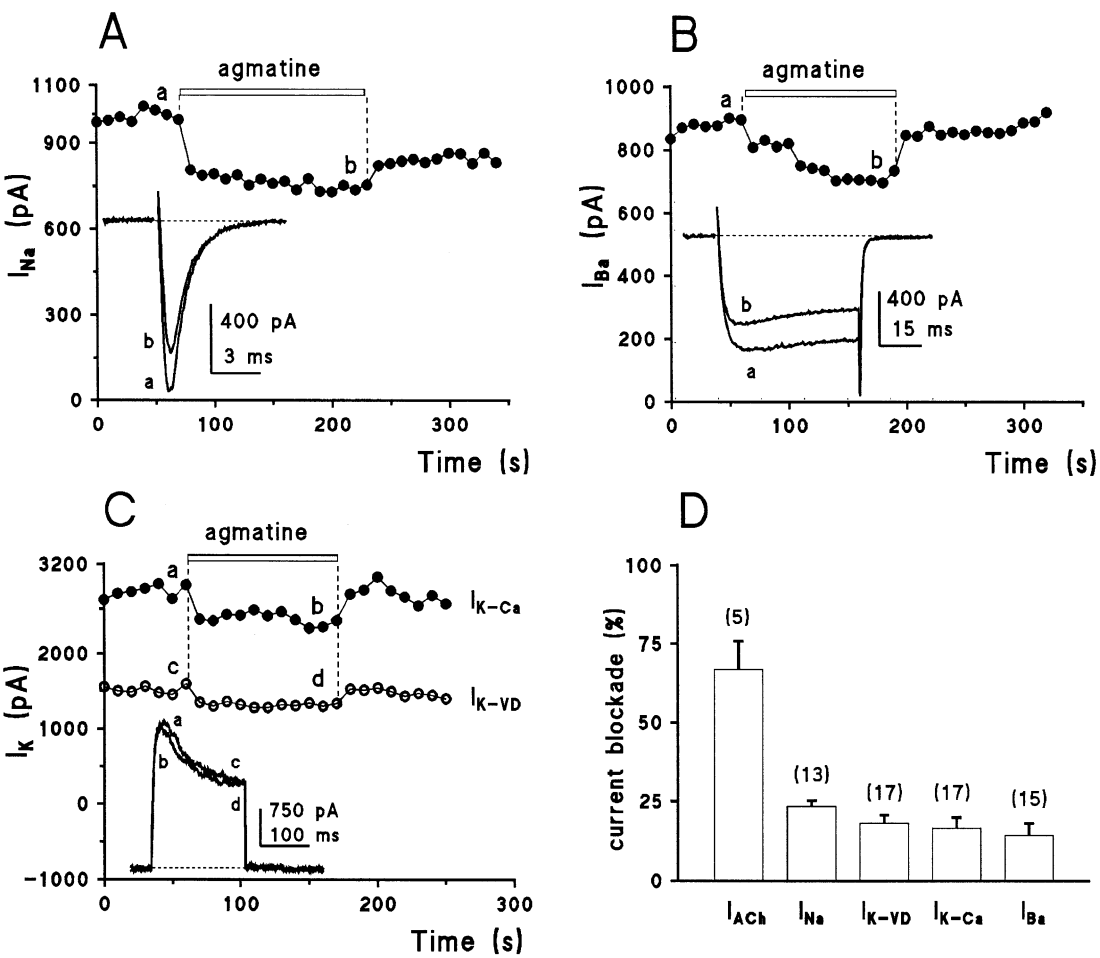

Fig. 6. Effects of agmatine on Na^+, Ca^{2+} and K^+ channel currents. Cells were voltage-clamped at −80 mV and dialysed and superfused with appropiate intracellular and extracellular solutions (see Section 2) in order to isolate each specific current. Panel A shows the time course of the peak inward Na^+ current (I_{Na}) obtained through the application of 14-ms test depolarising pulses to 0 mV, given at 10-s intervals. Panel B shows the time course of the peak inward Ca^{2+} channel current (10 mM Ba^{2+} was used as charge carrier; I_{Ba}) obtained through the application of 50-ms test depolarising pulses to 0 mV, given at 10-s intervals. Panel C shows the time course of the peak $I_{K(Ca)}$ and the late plateau outward K^+ current $I_{K(VD)}$ obtained through the application of 200-ms test depolarising pulses to 0 mV, given at 10-s intervals. Insets show typical current traces, taken as indicated by small letters in the time course curve. Agmatine was applied as shown by the top horizontal bar in each panel. Panel D shows the blockade by agmatine of each current. For comparative purposes, data of I_{ACh} blockade are also included. Data are means ± S.E.M. of the number of cells shown in parentheses.

a Tyrode solution without Ca^{2+} (see Section 2). In this cell stimulated with 14-ms test depolarising pulses to −10 mV applied at 10-s intervals, the initial peak I_{Na} reached about 1000 pA. Agmatine (1 mM) reversibly reduced I_{Na} to 700 pA. The average blockade of I_{Na} amounted to 23.3 ± 1.9% ($n = 13$ cells; Fig. 6D). The kinetics of activation or inactivation of the current were not affected by agmatine, as seen in the original current traces shown in the inset to Fig. 6A.

To test the effects of agmatine on the Ca^{2+} channel current (I_{Ba}), cells were dialysed with the standard intracellular solution and superfused extracellularly with a Tyrode solution containing 10 mM Ba^{2+} (instead of Ca^{2+}). I_{Ba} was elicited by applying 50-ms test depolarising pulses to 0 mV, from a holding potential of −80 mV, applied at 10-s intervals. In the cell shown in Fig. 6B, the initial I_{Ba} had an amplitude of near 800 pA. Superfusion of the cell with agmatine (1 mM) reversibly reduced I_{Ba} to about 700 pA; in 17 cells, the blockade amounted to 14.2 ± 3.9% (Fig. 6D). I_{Ba} exhibited little inactivation (see inset to Fig. 6B), both in the absence and in the presence of agmatine.

Finally, the K^+ channel currents were studied in cells dialysed with an intracellular solution containing KCl (see Section 2) and superfused with Tyrode containing 2 mM Ca^{2+}. Cells were stimulated with 200-ms depolarising pulses to +20 mV. The outward K^+ current was separated into its Ca^{2+}-dependent component ($I_{K(Ca)}$; Marty, 1981) and its voltage-dependent component ($I_{K(VD)}$); Sala and Soria, 1991). $I_{K(Ca)}$ was estimated by measuring the peak outward current at the beginning of the depolarising pulse and $I_{K(VD)}$ was estimated by measuring the outward current at the end of the 200-ms depolarising pulse, as indicated in Fig. 6C. The initial peak $I_{K(Ca)}$ was 1850 ± 264 pA and the blockade induced by 1 mM agmatine amounted to 16.5 ± 3.4% ($n = 17$ cells; Fig. 6D). The control $I_{K(VD)}$ was 1136 ± 136 pA and blockade by agmatine (1 mM) amounted to 18.2 ± 2.5% ($n = 17$ cells; Fig. 6D).

4. Discussion

The pivotal finding in this study is that the endogenous neurotransmitter candidate agmatine blocks the Ca^{2+}-dependent exocytotic release of catecholamines from fast-superfused bovine chromaffin cells. This finding is not only of academic interest—it is of potential physiological importance, inasmuch as agmatine selectively blocked the secretory response to acetylcholine but not that evoked by depolarisation of chromaffin cells with high K^+ concentrations. This selectivity of action on the secretory response is consistent with the findings on current measurements, which indicated the clear blocking effects of agmatine on I_{ACh} and the poor blockade of voltage-gated Na^+, K^+ and Ca^{2+}-channel currents. These findings prompted the hypothesis that, by acting on neuronal nicotinic acetylcholine receptors, endogenously released agmatine could modulate the release of catecholamines in chromaffin cells. Attempts were made to clarify the nature of the interaction of agmatine with the neuronal nicotinic acetylcholine receptors, and the results were interpreted as follows.

The non-competitive nature of I_{ACh} blockade induced by agmatine (Fig. 5) suggests a binding site for agmatine other than the acetylcholine binding site on the α subunit of the receptor (Changeux et al., 1992). This site must be extracellularly located, since the intracellular application of agmatine with patch pipettes did not cause I_{ACh} blockade (Fig. 3). As the blockade was non-competitive, the possibility exists that the agmatine binding site might be located deep within the pore itself, as proposed for other non-competitive blockers such as hexamethonium, amantadine, nemantadine and TMB8 (Buisson and Bertrand, 1998). However, the lack of voltage dependence of the agmatine-induced I_{ACh} blockade does not support this hypothesis. Rather, it seems that agmatine binds to a more superficially located, readily accessible site. The fast blockade of I_{ACh} (τ_{on} 30 ms, Fig. 4) exerted by agmatine supports this contention.

It is most unfortunate that our findings do not fit with the attractive hypothesis of putative imidazoline receptors in chromaffin cells (Wang et al., 1992) or in brain synapses (Feng et al., 1997). We could not find any effect of efaroxan, an imidazoline receptor blocker (Piletz et al., 1996), on the I_{ACh} inhibition induced by agmatine. Hence, all our findings on the effects of agmatine on exocytosis can be best explained through its actions on neuronal nicotinic acetylcholine receptors. In chromaffin cells, $\alpha_3\beta_4$ (Criado et al., 1992), α_5 (Campos-Caro et al., 1997) and α_7 (García-Guzmán et al., 1995) neuronal nicotinic acetylcholine receptors are likely expressed, and thus agmatine could act on some or all of these receptors. The exocytotic response is mediated at least by $\alpha_3\beta_4$ and α_7 receptors (López et al., 1998), and thus at least these receptor subtypes must recognise agmatine.

A final question arises on whether agmatine could act as a non-selective blocker of all types of ligand-gated or voltage-gated ion channels. For instance, agmatine also blocks the NMDA subtype of glutamate receptor channel of rat hippocampal neurons (Yang and Reis, 1998); however, Loring (1990) did not see significant effects of agmatine on responses induced by glutamate analogues. It seems that agmatine can also cause some blockade of voltage-gated Na^+, Ca^{2+} and K^+ channels; however, this blockade was only seen at a high concentration of agmatine (1 mM) and accounted for only 10–20% (Fig. 6).

The physiological significance of these findings can be discussed in the following context. In the adrenal medulla of mammals, chromaffin cells adopt a columnar disposition around a small capillary vessel; the cells in the intact gland are believed to be polarised (Carmichael, 1986; Cuchillo-Ibáñez et al., 1999). Recent experiments with adrenal medulla slices show that chromaffin cells in situ are grouped in clusters with diameters of 80 μm; cells in a

cluster are excited simultaneously, suggesting that the adrenal medulla is organised into discrete cell complexes with a common innervation (Kajiwara et al., 1997). Chromaffin cell surfaces are exposed to the secretory materials released from the cells (i.e. ATP, opiates, chromogranin, and likely agmatine). Under resting conditions, the splanchnic nerves fire action potentials at a low frequency (less than 1 Hz). As a result, acetylcholine is released into the cholinergic-chromaffin cell synapse and activates neuronal nicotinic acetylcholine receptors. This causes cell depolarisation, Ca^{2+} channel opening, Ca^{2+} entry, catecholamine release and a concomitant elevation of agmatine in the immediate vicinity of the cell secretory surface, leading to partial blockade of neuronal nicotinic acetylcholine receptors. Thus, fine-tuning of the rate of catecholamine release at each moment will be achieved through the interplay between the degree of activation of neuronal nicotinic acetylcholine receptors by acetylcholine and the blockade exerted by the endogenously released agmatine. Since agmatine has also been found in the brain of mammals (Feng et al., 1997), this modulatory mechanism could also be exerted at central neuronal nicotinic acetylcholine receptors. Such modulation seems to be physiologically relevant even for a rapid cholinergic synapse since, as we demonstrate here, it is exerted in the millisecond time range, and it reverses also in an ultrafast mode.

In conclusion, we present here the first study showing that agmatine, the putative endogenous ligand for imidazoline receptors, blocks exocytosis by selectively modulating the activity of neuronal nicotinic acetylcholine receptors. This action could have physiological, physiopathological and pharmaco-therapeutic relevance, for instance in neurodegenerative diseases (i.e. Parkinson, Alzheimer) where cholinergic neurotransmission is altered (Schneider, 1996).

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