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Dual interaction of agmatine with the rat α_{2D}-adrenoceptor: competitive antagonism and allosteric activation

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1 In segments of rat vena cava preincubated with [^{3}H]-noradrenaline and superfused with physiological salt solution, the influence of agmatine on the electrically evoked [^{3}H]-noradrenaline release, the EP_3 prostaglandin receptor-mediated and the α_{2D}-adrenoceptor-mediated inhibition of evoked [^{3}H]-noradrenaline release was investigated.

2 Agmatine (0.1–10 μM) by itself was without effect on evoked [^{3}H]-noradrenaline release. In the presence of 10 μM agmatine, the prostaglandin E_2(PGE_2)-induced EP_3-receptor-mediated inhibition of [^{3}H]-noradrenaline release was not modified, whereas the α_{2D}-adrenoceptor-mediated inhibition of [^{3}H]-noradrenaline release induced by noradrenaline, moxonidine or clonidine was more pronounced than in the absence of agmatine. However, 1 mM agmatine antagonized the moxonidine-induced inhibition of [^{3}H]-noradrenaline release.

3 Agmatine concentration-dependently inhibited the binding of [^{3}H]-clonidine and [^{3}H]-rauwolscine to rat brain cortex membranes (K_i values 6 μM and 12 μM, respectively). In addition, 30 and 100 μM agmatine increased the rate of association and decreased the rate of dissociation of [^{3}H]-clonidine resulting in an increased affinity of the radioligand for the α_{2D}-adrenoceptors.

4 [^{14}C]-agmatine labelled specific binding sites on rat brain cortex membranes. In competition experiments. [^{14}C]-agmatine was inhibited from binding to its specific recognition sites by unlabelled agmatine, but not by rauwolscine and moxonidine.

5 In conclusion, the present data indicate that agmatine both acts as an antagonist at the ligand recognition site of the α_{2D}-adrenoceptor and enhances the effects of α_2-adrenoceptor agonists probably by binding to an allosteric binding site of the α_{2D}-adrenoceptor which seems to be labelled by [^{14}C]-agmatine.

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Abbreviations: PGE_2, prostaglandin E_2; S_1–S_5, stimulation periods 1–5; t_2–t_5, collection period immediately before S1–S5, respectively

Introduction

Agmatine is a polycationic guanidine derivative synthesized from L-arginine by the enzyme arginine decarboxylase. In radioligand binding studies, agmatine exhibited affinity for all four subtypes (A–D) of the α_2-adrenoceptor (K_i 0.8–164 μM; Li *et al*., 1994; Piletz *et al*., 1995; Pinthong *et al*., 1995a,b; Regunathan & Reis, 1996) as well as for the non-adrenoceptor I_1 (0.03–0.7 μM) and I_2 (1–74 μM) imidazoline binding sites (Li *et al*., 1994; Piletz *et al*., 1995). It had no affinity for the α_1- and β-adrenoceptors, 5-HT_2 serotonin and D_2 dopamine binding sites (Li *et al*., 1994), κ opiod and adenosine A_1 receptors (Szabo *et al*., 1995). Agmatine is present in most mammalian tissues including brain and blood vessels (Raasch *et al*., 1995; Regunathan *et al*., 1996) and its distribution in the central nervous system suggests a role as a neurotransmitter (Otake *et al*., 1998). However, in most tissues in which functional tests have been carried out, agmatine failed to act as an agonist or antagonist at pre- and postsynaptic α_2-adrenoceptors (Pinthong *et al*., 1995b; Gonzalez *et al*., 1996; Jurkiewicz *et al*., 1996; Pineda *et al*., 1996). In contrast, agmatine is able to block nicotinic and 5-HT_3 receptor channels *via* an allosteric modulatory site (Loring 1990; Molderings *et al*., 1996).

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In the present study, the first aim was to investigate the influence of agmatine on the α_{2D}-autoreceptor-mediated (in comparison to the EP_3-receptor-mediated) modulation of noradrenaline release from perivascular sympathetic nerves in rat vena cava. The second aim was to examine whether agmatine interacts with an allosteric site associated with α_2-adrenoceptors. Since α_{2D}-adrenoceptors on cardiovascular sympathetic nerve terminals (inhibitory presynaptic autoreceptors) and in the central nervous system are identical, the latter can be used as a model for the former. Therefore, radioligand binding experiments were performed with [^{3}H]-rauwolscine, [^{3}H]-clonidine and [^{14}C]-agmatine in rat cerbral cortex membranes in which α_2-adrenoceptors mainly of the α_{2D}-subtype are known to be abundant (Greenberg *et al*., 1976, Bricca *et al*., 1989, Hussain *et al*., 1993).

Methods

Superfusion studies

The experiments were carried out on segments of vena cava from adult Wistar rats. The segments were incubated for 30 min in 1.5 ml physiological salt solution (37°C, composition see below) containing (−)-[2,5,6-^{3}H]-noradrenaline 0.1 μM). Subsequently, they were mounted vertically in an

organ bath (tension adjusted to 2 g) between two parallel platinum electrodes (1.5 cm long) and superfused with [^{3}H]-noradrenaline-free physiological salt solution of 37°C at a rate of 2 ml min^{-1}. The composition of the solution was (mM): NaCl 118, Na_2HPO_4 1.2, $NaHCO_3$ 25, KCl 4.7, $CaCl_2$ 1.6, $MgSO_4$ 1.2, glucose 11, ascorbic acid 0.3, Na_2EDTA 0.038, aerated with 95% O_2 and 5% CO_2. Throughout superfusion of the blood vessels this solution contained desipramine (0.6 μM) and corticosterone (40 μM) to block neuronal and extraneuronal noradrenaline uptake.

For transmural electrical stimulation, rectangular pulses of 0.3 ms duration and 150 mA were delivered to the segments at frequencies of 2 Hz during up to five 3-min periods after 93 (S_1), 117 (S_2), 141 (S_3), 165 (S_4) and 189 (S_5) min of superfusion. The superfusate was continuously collected in 3- or 6-min fractions. At the end of superfusion the blood vessels were solubilized with Soluene®. The radioactivity in the superfusate samples and blood vessels was determined by liquid scintillation counting.

Unless stated otherwise, the receptor agonists were applied at concentrations increasing by a factor of 10 from 9 min before until 15 min after the onset of S_3, S_4 and S_5. Separate control experiments (no agonists applied) were carried out for each series of experiments. In interaction experiments, the interacting drug (agmatine, clonidine or rauwolscine) was applied from 13 min before S_1 until the end of superfusion.

Tritium efflux was calculated as the fraction of tritium present in the strip at the onset of the respective collection period. Basal tritium efflux was expressed as the ratio of the fractional efflux during the collection period immediately before S_3, S_4 or S_5 (t_3, t_4, t_5) over that immediately before S_2 (t_2); S_1 (and t_1 correspondingly) was not included in the evaluation, since it was applied as a conditioning stimulation period. Stimulation-evoked tritium overflow was calculated by subtraction of the basal efflux from the total efflux during the 12 min subsequent to the onset of stimulation; basal efflux was assumed to decrease linearly from the collection period before to that 12–15 min after onset of stimulation. Evoked tritium overflow was calculated as a percentage of tissue tritium at the onset of stimulation, and the ratios of the overflow evoked by S_3, S_4 or S_5 over that evoked by S_2 were determined.

Results are given as means ± s.e.mean. Students' *t*-test for unpaired data was used for comparison of mean values. Apparent pA_2 values were determined according to formula (4) of Furchgott (1972). As an estimate of potency the concentration that reduced evoked tritium overflow by 50% ($IC_{50\%}$) was determined by interpolation between the two nearest points of the concentration-response curve for mean values. $pIC_{50\%}$ values are the negative logarithms of these concentrations.

Binding studies

Binding experiments were carried out in principle as described by Bylund *et al.* (1992) with slight modifications. Cerebral cortices obtained from male Wistar rats were homogenized (Potter-Elvehjem; 10 up and down strokes during 1 min) in 25 volumes of ice-cold Tris-HCl buffer (Tris 50 mM, pH 8.0; EDTA 5 mM; sucrose 10.27%) and centrifuged at $1000 \times g$ for 10 min (4°C). The supernatant was centrifuged at $35{,}000 \times g$ for 10 min and the pellet was resuspended in 10 volumes of Tris-HCl buffer and frozen at −80°C.

In the competition experiments, a 400-μl aliquot of the membranes (containing 0.2–0.3 mg protein) was incubated for 30 min with 1 nM [^{3}H]-rauwolscine or 364 nM [^{14}C]-agmatine and for 45 min with 5 nM [^{3}H]-clonidine (25 μl each), respectively, at room temperature in a final volume of 0.5 ml. In the experiments with [^{3}H]-clonidine, 1 μM cimetidine which at that concentration binds to I_1 imidazoline binding sites (Ernsberger *et al.*, 1990) was added to the assay to prevent the radioligand from binding to I_1 imidazoline binding sites. The reaction was stopped by rapid vacuum filtration with a Brandel cell harvester through Whatman GF/C glass-fibre filters presoaked with polyethylenimine 0.5 M followed by rapid washing of the incubation tubes and filters with 10 ml ice-cold buffer. Filters were placed in 6 ml of scintillation fluid and shaken overnight, and the radioactivity was determined by liquid scintillation counting at 44% ([^{3}H]) and 100% ([^{14}C] efficiency. Non-specific binding was defined as [^{3}H]-rauwolscine and [^{3}H]-clonidine binding in the presence of 10 μM noradrenaline (40% and 36%, respectively). [^{14}C]-Agmatine binding in the presence of 1 mM unlabelled agmatine revealed nonsaturable [^{14}C]-agmatine binding sites (17%, filter binding amounted to 56% of total binding).

Kinetic experiments were carried out with 5 nM [^{3}H]-clonidine. Association was initiated by addition of membranes and dissociation by addition of 10 μM noradrenaline after 45 min of incubation of membranes with the radioligand. Reactions were stopped at points of time between 30 s and 60 min by rapid filtration and washing with ice-cold buffer.

All experiments were carried out in triplicate. Data from the kinetic and competition experiments were analysed using the least-squares fitting program PRISM (GraphPad Software Inc., San Diego, U.S.A.). The affinity (K_d value) of agmatine in the homologous competitive binding experiments was calculated by the formulas (3) and (5) of DeBlasi *et al.* (1989). Results are expressed as mean values ± s.e.mean. The statistical significance of differences was analysed by Dunnett's test and Bonferroni's test.

Drugs used

(−)-[2,5,6-^{3}H]-noradrenaline, specific activity 43.7–55 Ci mmol^{-1}; [benzene ring ^{3}H]-clonidine, specific activity 60 Ci mmol^{-1} (New England Nuclear, Dreieich, Germany); [O-methyl-^{3}H]-rauwolscine, specific activity 76 Ci mmol^{-1} (Amersham, Braunschweig, Germany); [^{14}C]-agmatine, specific activity 55 mCi mmol^{-1} (American Radiolabeled Chem. Inc, St. Louis, U.S.A.); moxonidine (Beiersdorf, Hamburg, Germany); corticosterone, noradrenaline base, agmatine sulphate, rauwolscine hydrochloride, prostaglandin E_2 (Sigma, München, Germany); clonidine hydrochloride (Boehringer, Ingelheim, Germany); desipramine hydrochloride (Ciba-Geigy, Wehr, Germany); cirazoline hydrochloride (Synthélabo, Paris, France); propranolol hydrochloride (ICI, Planckstadt, Germany). Drugs were dissolved in saline with the following exceptions: noradrenaline base was dissolved in HCl (0.01 M) and corticosterone in propandiol-1,2. The stock solutions were further diluted in saline.

Results

Superfusion experiments in rat vena cava

Basal tritium efflux In control experiments in the absence of test drugs, basal tritium efflux during t_2 from segments of the rat vena cava preincubated with [^{3}H]-noradrenaline was 0.22 ± 0.03 nCi min^{-1} ($n = 7$; in a representative control series), corresponding to a fractional rate of efflux of 0.00059 ± 0.00002 min^{-1}. It did not significantly differ from that in the presence of 10–1000 μM agmatine and/or 0.3 μM

rauwolscine from 13 min before S_1 until the end of the experiments. Basal efflux decreased with time, as reflected by t_n/t_2 ratios which, in the control experiments, declined from t_3/t_2 (0.86 ± 0.04) to t_5/t_2 (0.77 ± 0.02). The t_n/t_2 values were not altered by 10 to 1000 μM agmatine, 10 μM clonidine and/or by 0.3 μM rauwolscine present from 13 min before S_1 until the end of the experiments (controls for the interaction experiments of these drugs with other compounds) nor by noradrenaline, moxonidine, clonidine and PGE_2 (see Figure 1 for concentrations and time schedule).

Evoked tritium overflow under control conditions In a representative series of control experiments in the absence of test drugs from 13 min before S_1 until the end of the experiments, the tritium overflow evoked at 2 Hz by S_2 amounted to 7.77 ± 0.38 nCi (corresponding to $1.45 \pm 0.30\%$ of tissue tritium; $n = 7$); evoked tritium overflow remained constant (S_3/S_2: 0.90 ± 0.04; S_4/S_2: 0.90 ± 0.05; S_5/S_2: 0.86 ± 0.05). These findings were not significantly different from the values determined in control experiments in which 10 – 1000 μM agmatine, 10 μM clonidine and/or 0.3 μM rauwolscine was/were present from 13 min before S_1 until the end of the experiments.

Actions of α_2-adrenoceptor agonists, agmatine and PGE_2 Agmatine in concentrations up to 1 mM tended to increase the electrically-evoked tritium overflow (0.1 μM: $93.5 \pm 7.2\%$; 1 μM: $88.6 \pm 5.8\%$; 10 μM: $84.6 \pm 9.3\%$; 100 μM: $134.9 \pm$

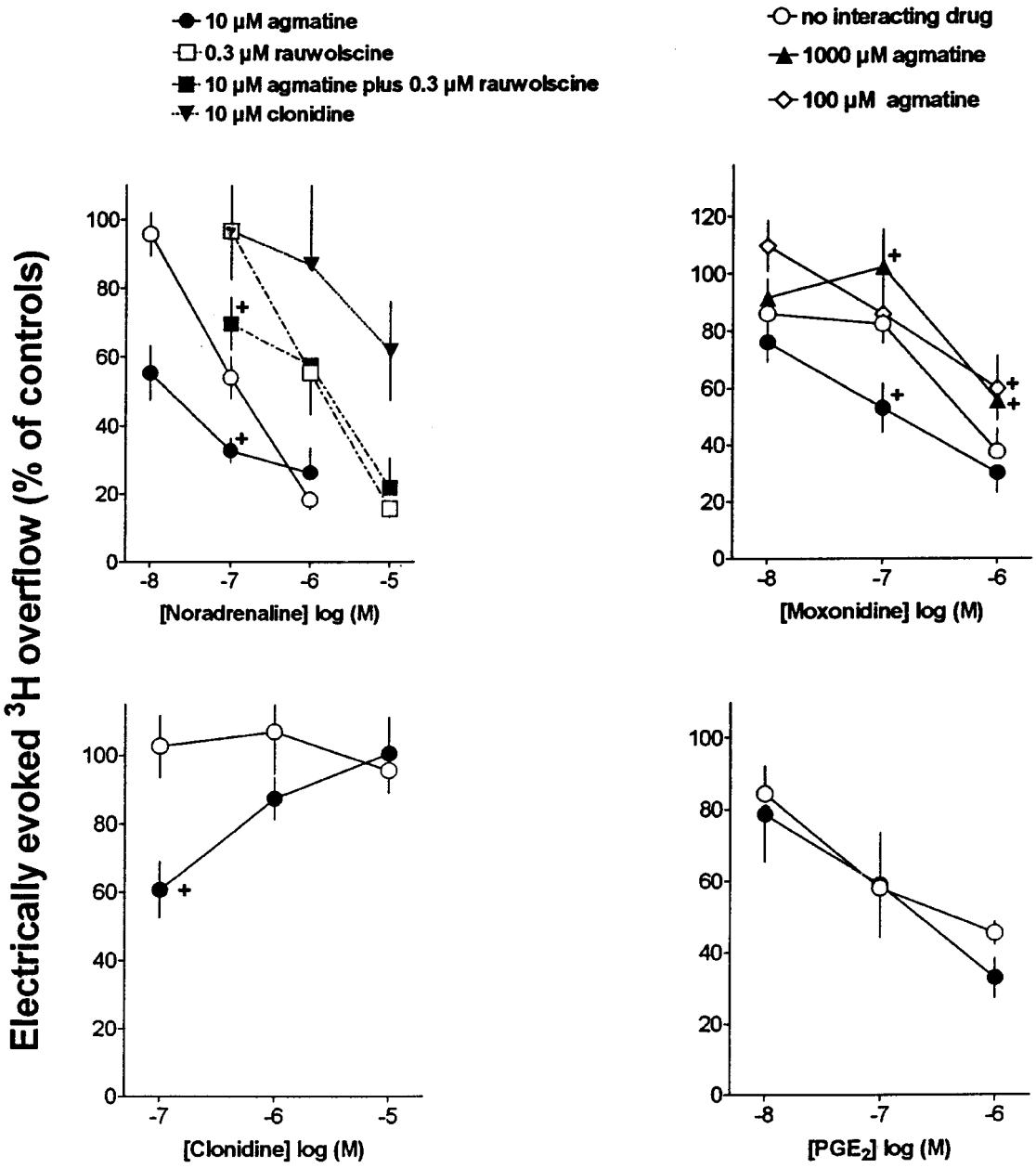

Figure 1 Effects of noradrenaline, moxonidine, clonidine and PGE_2 on electrically evoked tritium overflow from segments of the rat vena cava preincubated with [^{3}H]-noradrenaline and interaction with agmatine, clonidine and/or rauwolscine. Interacting drugs were present from 13 min before S_1 until the end of the experiments. The experiments with noradrenaline and the corresponding controls were carried out in the presence of 1 μM propranolol throughout superfusion. Ordinate, S_3/S_2, S_4/S_2 and S_5/S_2 overflow ratios, expressed as percentage of ratios in corresponding control experiments without noradrenaline, moxonidine, clonidine or PGE_2. Means $\pm$ s.e.mean from 5 – 12 tissue segments. All S_n/S_2 ratios below 75% of the corresponding S_n/S_2 ratios in the respective controls were significantly different from these control ratios (i.e. in the absence of the respective agonist). + $P < 0.05$, compared with the effect of the corresponding agonist concentration in the absence of agmatine.

14.9%; 300 μM: 121.1±15.5%; 1000 μM: 112.3±9.9%; $n=6-7$; values represent percentages of the overflow at S_3, S_4 and S_5, respectively, in control experiments in the absence of agmatine); however, due to a high variance of the data the facilitatory effect did not reach the level of significance.

In the absence of agmatine, the full α_2-adrenoceptor agonists noradrenaline and moxonidine and the EP_3 receptor agonist PGE_2 inhibited the electrically evoked tritium overflow (Figure 1, open circles; $pIC_{50\%}$ values of noradrenaline, moxonidine and PGE_2: 6.89, 6.44, and 6.37, respectively). In contrast, clonidine which is a partial agonist with low intrinsic activity at α_2-adrenoceptors (Jasper *et al*., 1998) failed to inhibit evoked tritium overflow (Figure 1, open circles).

In the presence of 10 μM agmatine from 13 min before S_1 until the end of the experiments, the concentration-response curves of noradrenaline and moxonidine for their inhibitory effect on evoked tritium overflow were shifted to the left (Figure 1, closed circles; $pIC_{50\%}$ values of noradrenaline and moxonidine: 7.76 and 6.86, respectively). However, in the presence of 100 μM agmatine, the concentration response-curve of moxonidine tended to be shifted to the right (Figure 1). A more clear-cut rightward shift of the concentration-response curve of moxonidine was determined in the presence of 1000 μM agmatine (Figure 1, closed triangles up).

In the presence of 10 μM agmatine, clonidine 0.1 μM significantly inhibited evoked tritium overflow, whereas higher concentrations did not (Figure 1, closed circles). The concentration-response curve of PGE_2 was not altered in the presence of 10 μM agmatine (Figure 1, closed circles).

Rauwolscine 0.3 μM present in the superfusion fluid from 13 min before S_1 until the end of the experiments, shifted the concentration-response curve of noradrenaline to the right yielding an apparent pA_2 value of 7.31 (Figure 1, open squares). In the presence of both 0.3 μM rauwolscine and 10 μM agmatine, 0.1 μM noradrenaline inhibited the evoked tritium overflow (Figure 1, closed squares), whereas in the presence of rauwolscine alone (i.e. in the absence of agmatine) it was without effect (Figure 1, open squares). The apparent pA_2 value for rauwolscine determined in the presence of 10 μM agmatine amounted to 8.49. Clonidine 10 μM present in the superfusion fluid from 13 min before S_1 until the end of the experiments, also shifted the concentration-response curve of noradrenaline to the right (Figure 1, closed triangles) yielding an apparent pA_2 value of 7.05 (determined at the level of $IC_{30\%}$).

Radioligand binding experiments

The specific binding of [^{3}H]-clonidine (5 nM) increased with time and reached equilibrium already after about 20 min (not shown). The time $t_{1/2}$ (0.693/observed rate of association) at which half of the equilibrium specific binding of [^{3}H]-clonidine was reached amounted to 2.15 min (Table 1). Agmatine 30 μM did not significantly change the association of the radioligand, whereas association was significantly enhanced in the presence of 100 μM agmatine ($t_{1/2}=0.85$ min; Table 1).

The dissociation of [^{3}H]-clonidine from its binding sites (induced by 10 μM noradrenaline) was monoexponential with a dissociation rate constant of 0.084 ± 0.011 min^{-1} ($t_{1/2}=7.24$ min; see Table 1). In the presence of 30 and 100 μM agmatine the dissociation of the radioligand from its specific binding sites was significantly delayed (Table 1). The K_d values calculated from the kinetic experiments decreased with increasing agmatine concentration in the incubation buffer (Table 1). Higher agmatine concentrations could not be investigated, since at those concentrations agmatine inhibited [^{3}H]-clonidine binding nearly complete (see Figure 2); hence, no specific binding could be reliably detected in respective kinetic experiments.

In competition experiments of agmatine with [^{3}H]-clonidine at 5 nM, agmatine induced a monophasic inhibition ($n_{Hill}=-0.97$, not significantly different from unity; Figure 2). The K_i-value of agmatine amounted to 5.8 ± 1.7 μM.

In competition experiments of agmatine with 1 nM [^{3}H]-rauwolscine, agmatine also produced a monophasic inhibition ($n_{Hill}=-0.95$, not significantly different from unity; Figure 2). The K_i-value of agmatine amounted to 12.1 ± 2.5 μM.

To rule out that [^{14}C]-agmatine retained in the brain membranes after the incubation period was due to an incorporation into vesicles by an agmatine transporter (recently described by Sastre *et al*., 1997), the time course of dissociation of [^{14}C]-agmatine was studied. The dissociation of [^{14}C]-agmatine from its binding sites (induced by 1 mM agmatine) was monoexponential with a dissociation rate constant $k_{-1}=0.107\pm0.101$ min^{-1} ($n=4$). Hence, it appears justified to assume that [^{14}C]-agmatine labelled specific binding sites in the membranes rather than being transported into the vesicles. Homologous competition experiments of [^{14}C]-agmatine with unlabelled agmatine revealed a reaction with one binding site with a K_d value of 158.7 ± 100.5 μM (Figure 3). Cirazoline also weakly inhibited specific binding of [^{14}C]-agmatine in a concentration-dependent manner. Cirazoline at 100 μM (i.e. the highest concentration tested) produced an inhibition by 33% (extrapolated K_i value 199 μM; Figure 3).

Table 1 Half life ($t_{1/2}$) of dissociation and association and K_d of [^{3}H]-clonidine derived from the respective kinetic data in the absence and presence of agmatine in the incubation buffer. Number of experiments in parentheses

Agmatine (μM)	*$t_{1/2}$ dissociation* (min)	*$t_{1/2}$ association* (min)	K_d *value* (nM)
0	7.24±0.56 (4)	2.15±0.19 (4)	2.1
30	9.28±0.56 (3)*	2.95±0.48 (4)	1.4
100	10.79±1.01 (4)**	0.85±0.13 (8)**	0.3

*$P<0.05$, **$P<0.01$ (compared with the values determined in the absence of agmatine).

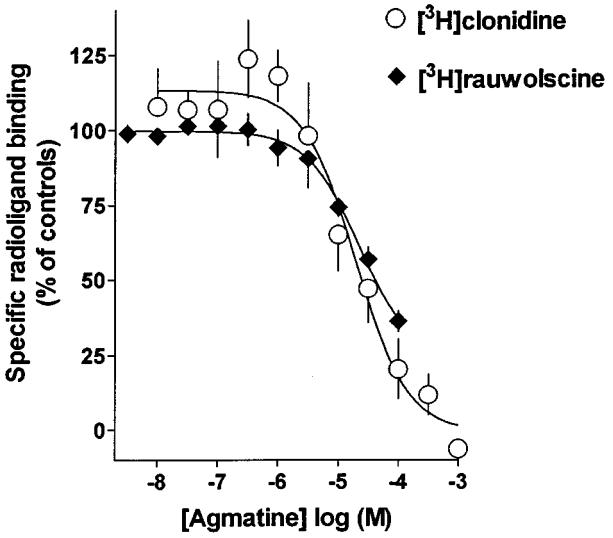

Figure 2 Competition of agmatine with 5 nM [^{3}H]-clonidine or 1 nM [^{3}H]-rauwolscine for their specific binding sites in rat cerebral cortex membranes. Each point is the mean of 3–4 experiments performed in triplicate.

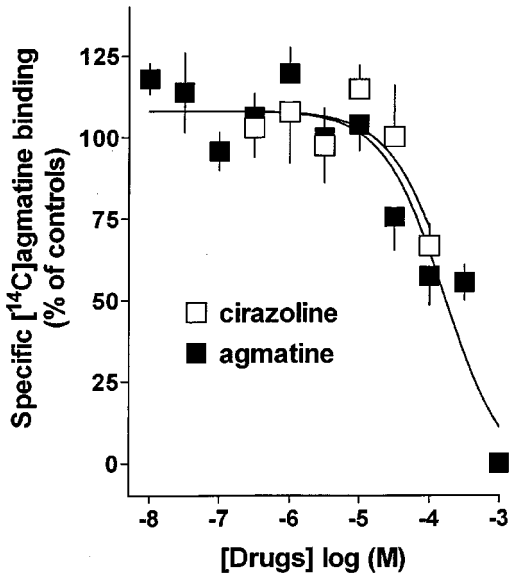

Figure 3 Competition of unlabelled agmatine and cirazoline with [^{14}C]-agmatine (364 nM) for its specific binding sites in rat cerebral cortex membranes. Each point is the mean of 5–7 experiments performed in triplicate.

Rauwolscine and moxonidine failed to inhibit specific [^{14}C]-agmatine binding at concentrations up to 100 μM (not shown).

Discussion

One of the aims of the present study was to investigate the influence of agmatine on presynaptic α_{2D}-autoreceptors of the sympathetic nerves in the rat vena cava (Göthert *et al*., 1986; subclassification by Trendelenburg *et al*., 1997). For this purpose, the effects of the full α_2-adrenoceptor agonists noradrenaline and moxonidine and of the partial α_2-adrenoceptor agonist clonidine on the electrically evoked tritium overflow from the rat vena cava preincubated with [^{3}H]-noradenaline was investigated in the absence and presence of agmatine. Under the conditions applied (i.e. presence of neuronal and extraneuronal uptake inhibitors), evoked tritium overflow reflects action-potential-induced release of tritiated and unlabelled noradrenaline from the sympathetic axon terminals contained in the blood vessel wall (for further details, see Göthert *et al*., 1986).

The main aim of this investigation is based on the suggestion that agmatine is a novel neurotransmitter capable of recognizing both α_2-adrenoceptors and non-adrenoceptor imidazoline binding sites (Reis & Regunathan, 1999). Agmatine binds to imidazoline sites with high to moderate affinity in radioligand binding studies (Li *et al*., 1994; Piletz *et al*., 1995) and exhibits intrinsic activity in functional assays for imidazoline recognition sites in the brain and periphery (for review, see Regunathan & Reis, 1996; Molderings 1997). However, no α_2-adrenoceptor-mediated function could be reliably ascribed to agmatine in several α_2-adrenoceptor containing preparations of various species (for references, see Introduction). In accordance with those findings, agmatine failed to significantly modulate the electrically evoked noradrenaline release in the present experiments on the rat vena cava, only 100 μM agmatine tended to facilitate evoked noradrenaline release, whereas at higher concentrations no enhancement was observed. The same result has been obtained in the rabbit pulmonary artery and aorta (unpublished observations). Obviously, agmatine influences sympathetic neurotransmission in the rat vena cava in a more complex manner. The modification of the concentration-response curves for the full α_2-adrenoceptor agonists noradrenaline and moxonidine by the relatively low agmatine concentration of 10 μM basically differs from the change of the moxonidine concentration-response curve produced by 1 mM agmatine. Whereas the latter concentration produced a rightward shift of the concentration-response curve, which can be most plausibly explained by a weak competitive antagonism, application of the lower agmatine concentration resulted in a higher potency of the α_2-adrenoceptor agonists (leftward shift by 0.87 log unit). In the presence of 100 μM agmatine the facilitatory and inhibitory effects of agmatine seemed to be balanced, since the rightward shift of concentration-response curve of moxonidine was not as clear-cut as that in the presence of 1 mM agmatine. The potentiating effect of agmatine appears to be due to an influence on α_2-adrenoceptors because the concentration-response curve of PGE_2 which inhibited evoked noradrenaline release from the sympathetic nerves *via* presynaptic EP_3 receptors (Molderings *et al*., 1992) to the same extent as noradrenaline and moxonidine, was not altered by agmatine (Figure 1). The results of the interaction experiments of 10 μM agmatine with noradrenaline and moxonidine suggest that agmatine may be a positive allosteric modulator at the presynaptic α_{2D}-adrenoceptor in rat vena cava leading to an increased affinity of α_2-adrenoceptor agonists for the α_2-adrenoceptor. It is compatible with the assumption of an allosteric modulation that also the potency of rauwolscine to antagonize the noradrenaline-induced inhibition of noradrenaline release was higher in the presence than in the absence of agmatine (difference in apparent pA_2 value: 1.18 log unit).

The agmatine-induced modification of the effect of clonidine can also be explained on the basis of a positive allosteric modulation by the polyamine. Clonidine has been reported to be a partial agonist with low intrinsic activity at α_{2D}-adrenoceptors (Jasper *et al*., 1998). In accordance with this finding, clonidine markedly shifted the concentration-response curve of noradrenaline to the right suggesting an estimate of affinity for the presynaptic α_{2D}-autoreceptors (apparent pA_2 value) of about 7.00. Since the intrinsic activity of the partial agonist clonidine is relatively small, the error factor introduced into the estimate because of its agonistic effect is also small. In the absence of agmatine, clonidine at all concentrations investigated did not modify noradrenaline release; this can be explained by the suggestion that an inhibitory effect of clonidine due to its agonistic property is compensated by its antagonism against endogenous noradrenaline released by electrical stimulation. However, when 100 nM clonidine was applied in the presence of 10 μM agmatine, the positive allosteric effect of agmatine appears to result in a shift in the equilibrium between agonistic and antagonistic properties of clonidine towards agonism, leading to receptor activation. At the higher clonidine concentrations, the agonism unmasked by the positive allosteric effect of agmatine is re-balanced by the increased ability of clonidine to antagonize the effect of endogenous noradrenaline. Finally, a simultaneous action of clonidine at presynaptic imidazoline recognition sites present in the rat vena cava (Molderings & Göthert 1998) at which the drug is an agonist, might also influence the effect of clonidine.

Taken together, the data from our superfusion experiments suggest that agmatine enhances α_2-adrenoceptor-mediated inhibition of noradrenaline release from presynaptic nerve terminals *via* an allosteric binding site and that at higher concentration the compound acts as an antagonist at the ligand recognition site of the α_2-adrenoceptor. Since the

presynaptic α_{2D}-autoreceptors in the cardiovascular system are identical with the postsynaptic α_{2D}-adrenoceptors in the brain cortex, the latter can be used as a model for the former. Therefore, radioligand binding experiments were carried out with the radiolabelled partial α_{2D}-adrenoceptor agonist [^{3}H]-clonidine (in the presence of 1 μM cimetidine to mask I_1 imidazoline binding sites; Ernsberger *et al*., 1990), the non-selective α_2-adrenoceptor antagonist [^{3}H]-rauwolscine and with [^{14}C]-agmatine on rat cerebral cortex membranes. An interference of [^{3}H]-clonidine with presynaptic imidazoline recognition sites is not to be expected because we previously failed to demonstrate the presence of presynaptic imidazoline recognition sites in the rat brain cortex (Schlicker *et al*., 1997).

In competition experiments agmatine inhibited [^{3}H]-clonidine and [^{3}H]-rauwolscine binding to sites, previously shown to be α_2-adrenoceptors (Greenberg *et al*., 1976; Bricca *et al*., 1989; Hussain *et al*., 1993), with a potency (K_i values 6 μM and 12 μM, respectively) comparable to that reported in the literature (4 μM, Li *et al*., 1994; 8–17 μM, Pinthong *et al*., 1995a,b, 26–164 μM, Piletz *et al*., 1995). It can be concluded from this finding that agmatine binds with μmolar affinity to the ligand recognition site of α_2-adrenoceptor. This inhibition of radioligand binding to the α_2-adrenoceptor may be assumed to reflect the antagonistic property of agmatine at these receptors in the superfusion experiments (see above).

To identify the postulated allosteric modulatory site for agmatine at α_2-adrenoceptors in radioligand binding experiments, association and dissociation kinetics of [^{3}H]-clonidine in the absence and presence of increasing concentrations of agmatine were determined. These experiments revealed that agmatine concentration-dependently accelerates association of the radioligand to and delayed dissociation from the specific binding sites (Table 1). Accordingly, the affinity of [^{3}H]-clonidine for the α_2-adrenoceptors was 7 fold increased by 100 μM agmatine. These findings strongly supported the suggestion that agmatine acts at an allosteric site of the α_{2D}-adrenoceptor. This allosteric site may be assumed to represent the allosteric site of action of agmatine in the superfusion experiments. Yet it should be noted that agmatine concentrations as high as 100 μM were necessary to markedly increase the affinity of clonidine for the α_2-adrenoceptor in the radioligand binding experiments, whereas the increase in potency of the ligands in the functional experiments was detected at 10 times lower agmatine concentration. However, this quantitative difference may be related to differences in the experimental procedures: a drop in potency is not unusual when results of experiments obtained in isolated organs are compared with those observed in more artificial systems such as radioligand binding experiments. In contrast to the latter experiments, the physiological readout in isolated veins involves a whole sequence of events from activation of the α_{2D}-adrenoceptors, Ca^{2+} influx and modulation of adenylate cyclase. Thus, the differences in potency between these bioassay systems probably reflect, among others, differences in signal amplification.

Finally an attempt was made to identify the allosteric modulatory site for agmatine in binding experiments with [^{14}C]-agmatine. Due to the expected low affinity of agmatine for this allosteric site, we used a relatively high concentration of the radioligand. Since specific binding in absolute terms was low, the data can be interpreted in qualitative but not in quantitative terms. It can be derived from the data shown in Figure 2 that at this radioligand concentration only 3–6% of the ligand recognition sites of the α_2-adrenoceptors are labelled by [^{14}C]-agmatine. Hence, specific binding of [^{14}C]-agmatine must represent another ligand binding site. Accordingly, neither rauwolscine (α_2-adrenoceptor antagonist) nor moxonidine (α_2-adrenoceptor/I_1-imidazoline recognition site agonist) inhibited [^{14}C]-agmatine binding, indicating that under the present conditions [^{14}C]-agmatine did indeed not label α_2-adrenoceptors or I_1-imidazoline binding sites. In addition, cirazoline which is a high affinity ligand at α_1-adrenoceptors and I_2-imidazoline binding sites, up to 100 μM only produced a very weak inhibition of [^{14}C]-agmatine binding, ruling out substantial binding to α_1-adrenoceptors and I_2-imidazoline binding sites. Hence, it seems justified to assume that [^{14}C]-agmatine bound to the allosteric modulatory site at α_2-adrenoceptors postulated on the basis of the superfusion experiments. Whether or not the clearly lower affinity of agmatine for the [^{14}C]-agmatine binding sites found in the homologous displacement experiments than its potency to enhance the inhibitory effect of the α_2-adrenoceptor agonists in the superfusion experiments reflects the true affinity of agmatine for the modulatory site remains to be established in future experiments.

In conclusion, our data suggest that agmatine is a positive modulator at an allosteric site of α_2-adrenoceptors and an antagonist at the ligand recognition site of the α_2-adrenoceptor. This pattern of effects may explain why agmatine either failed to act at α_2-adrenoceptors or yielded inconsistent results in the respective previous studies.

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