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Crosstalk between presynaptic angiotensin receptors, bradykinin receptors and α_2-autoreceptors in sympathetic neurons: a study in α_2-adrenoceptor-deficient mice

*,[1]Anne-Ulrike Trendelenburg, [1]Angelika Meyer, [1]Werner Klebroff, [2]Serafim Guimarães & [1]Klaus Starke

[1]Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Albertstrasse 25, D-79104 Freiburg i.Br., Germany and [2]Instituto de Farmacologia e Terapêutica, Faculdade de Medicina, Alameda Hemâni Monteiro, 4200-319 Porto, Portugal

1 In mouse atria, angiotensin II and bradykinin lose much or all of their noradrenaline release-enhancing effect when presynaptic α_2-autoinhibition does not operate either because of stimulation with very brief pulse trains or because of treatment with α_2 antagonists. We now studied this operational condition in α_2-adrenoceptor-deficient mice. Release of ^{3}H-noradrenaline was elicited by electrical stimulation.

2 In tissues from wild-type (WT) mice, angiotensin II and bradykinin increased the overflow of tritium evoked by 120 pulses at 3 Hz. This enhancement did not occur or was much reduced when tissues were stimulated by 120 pulses at 3 Hz in the presence of rauwolscine and phentolamine, or when they were stimulated by 20 pulses at 50 Hz.

3 In tissues from mice lacking the α_{2A}-adrenoceptor (α_{2A}KO) or the α_{2B}-adrenoceptor (α_{2B}KO), the concentration – response curves of angiotensin II and bradykinin (120 pulses at 3 Hz) were unchanged. In tissues from mice lacking the α_{2C}-adrenoceptor (α_{2C}KO) or both the α_{2A}- and the α_{2C}-adrenoceptor (α_{2AC}KO), the concentration – response curves were shifted to the same extent downwards.

4 As in WT tissues, angiotensin II and bradykinin lost most or all of their effect in α_{2A}KO and α_{2AC}KO tissues when rauwolscine and phentolamine were present or trains consisted of 20 pulses at 50 Hz.

5 Rauwolscine and phentolamine increased tritium overflow evoked by 120 pulses at 3 Hz up to seven-fold in WT and α_{2B}KO tissues, three-fold in α_{2A}KO and α_{2C}KO tissues, and two-fold in α_{2AC}KO tissues.

6 Results confirm that angiotensin II and bradykinin require ongoing α_2-autoinhibition for the full extent of their release-enhancing effect. Specifically, they require ongoing α_{2C}-autoinhibition. The peptide effects that remain in α_{2C}-autoreceptor-deficient mice seem to be because of α_{2B}-autoinhibition. The results hence also suggest that in addition to α_{2A}- and α_{2C}- mouse postganglionic sympathetic neurons possess α_{2B}-autoreceptors.

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Abbreviations: Dslet, [D-Ser^2]-leucine enkephalin-Thr^6; KO, knockout; WIN, 55,212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxan-6-yl]-1-naphthalenylmethanone mesylate; WT, wild type

Introduction

Angiotensin II and bradykinin increase exocytotic release of noradrenaline in many sympathetically innervated tissues (for a review, see Langer, 1977; Starke, 1977; Westfall, 1977; Fuder & Muscholl, 1995; Boehm & Kubista, 2002). Angiotensin II acts on presynaptic AT_1-receptors (Boehm & Kubista, 2002), which in some tissues differ from smooth muscle AT_1-receptors in their pharmacological properties (Guimarães *et al*., 2001; Pinheiro *et al*., 2002). Bradykinin acts on presynaptic B_2-receptors (Fuder & Muscholl, 1995; Boehm & Kubista, 2002). The ensuing presynaptic signal transduction probably involves G_q and activation of protein kinase C (Boehm & Kubista, 2002).

It was early observed that angiotensin II lost its release-enhancing effect in rabbit isolated hearts when the hearts had been pretreated with phenoxybenzamine, which by itself increased the release of noradrenaline because of irreversible α_2-autoreceptor blockade (Starke & Schümann, 1972). The observation lay dormant for many years. It was then extended in several ways. First, blockade of α_2-adrenoceptors also prevented or greatly attenuated the presynaptic effect of angiotensin II in guinea-pig atria (Brasch *et al*., 1995), mouse atria (Cox *et al*., 2000) and rat hearts (Kurz *et al*., 1997; Mota & Guimarães, 2002), and when the competitive α-adrenoceptor antagonists phentolamine, idazoxan or yohimbine were used instead of phenoxybenzamine (Brasch *et al*., 1995; Kurz *et al*.,

*Author for correspondence: Novartis Institutes for BioMedical Research, Opthalmology, WSJ-386.7.46, PO Box, CH-4002 Basel, Switzerland;
E-mail: anne-ulrike.trendelenburg@pharma.novartis.com

1997; Cox *et al*., 2000; Mota & Guimarães, 2002). Second, an analogous interaction was observed between α-adrenoceptor antagonists and bradykinin: like angiotensin II, bradykinin lost or almost lost its release-enhancing effect in mouse atria treated with phentolamine or the selective α_2-adrenoceptor antagonist rauwolscine (Cox *et al*., 2000). Third, the two peptides also failed to enhance noradrenaline release when, in the absence of any α_2 antagonist, the sympathetic axons were stimulated under α_2-autoinhibition-free or -poor conditions such as by brief high-frequency pulse trains (Brasch *et al*., 1995; Cox *et al*., 2000). Finally, when the effect of angiotensin II and bradykinin in mouse atria had been minimized by phentolamine, it could be restored by administration of neuropeptide Y or [D-Ser^2]-leucine enkephalin-Thr^6 (DSLET), which by themselves reduced noradrenaline release through presynaptic neuropeptide Y Y_2- and opioid OP_1-receptors, respectively (Cox *et al*., 2000).

From these results, it has been concluded that (1) both angiotensin II and bradykinin require ongoing α_2-autoinhibition for their full presynaptic release-facilitating effect; (2) in the absence of α_2-autoinhibition the effect of the peptides can be restored by activation of another $G_{i/o}$-coupled presynaptic receptor; and (3) protein kinase C, the likely effector of the AT_1- and B_2-receptors, increases noradrenaline release to a large extent by inactivating a component of the $G_{i/o}$ presynaptic inhibitory pathway (Brasch *et al*., 1995; Cox *et al*., 2000; see also Boehm & Kubista, 2002, Figure 5c).

There is a third approach to avoid α_2-autoinhibition, in addition to autoreceptor blockade and autoinhibition-free stimulation conditions, namely disruption of the α_2-autoreceptor genes. It is now established that the main α_2-autoreceptor is the α_{2A}-adrenoceptor (see Hein, 2001; Starke, 2001). However, especially in sympathetically innervated tissues the α_{2C}-adrenoceptor also functions as an autoreceptor; there is no firm evidence for α_{2B}-autoreceptors (Hein *et al*., 1999; see Hein, 2001; Starke, 2001).

The present experiments were devised to study the crosstalk between AT_1- and B_2-receptors on the one hand, and α_2-autoreceptors on the other by means of genetically manipulated mice: mice lacking the α_{2A}-adrenoceptor (α_{2A}KO, knockout), mice lacking the α_{2B}-adrenoceptor (α_{2B}KO), mice lacking the α_{2C}-adrenoceptor (α_{2C}KO), mice lacking both the α_{2A}- and the α_{2C}-adrenoceptor (α_{2AC}KO), and wild-type (WT) mice sharing the genetic background of the KO strains.

Since the crosstalk had been demonstrated previously only in the heart, we used in addition another tissue, the mouse vas deferens. Finally, we studied the interaction of bradykinin with α-adrenoceptor antagonists, previously tested in mouse atria only, in rat and rabbit atria. A mixture of phentolamine and rauwolscine was used to block α_2-autoreceptors when desired, because phentolamine is especially potent at rodent α_{2A}-adrenoceptors whereas rauwolscine is especially potent at α_{2C}-adrenoceptors, the two adrenoceptors known to operate physiologically as autoreceptors in mouse atria and vasa deferentia (Hein *et al*., 1999; Trendelenburg *et al*., 2001).

Methods

The α_2-adrenoceptor-deficient mice have been described previously (Altman *et al*., 1999; Hein *et al*., 1999). Male C57BL/6 × 129Sv (WT), α_{2A}KO, α_{2B}KO, α_{2C}KO and α_{2AC}KO mice aged 2 – 15 months, male Wistar rats (220 – 300 g) and male or female rabbits (1.8 – 2.7 kg) were killed by cervical dislocation. The wall of each pair of mouse, rat or rabbit atria was cut into pieces (mouse 6 – 8; rat 12 – 15; rabbit 20 – 30), and each mouse vas deferens was cut into six pieces. The tissue segments were preincubated in 2 ml medium containing 0.2 μM ^{3}H-noradrenaline for 30 min at 37°C. Segments were then placed in superfusion chambers between platinum electrodes, one segment per chamber, where they were superfused with ^{3}H-noradrenaline-free medium at a rate of 1.2 ml min^{-1}, also at 37°C. Successive 2-min samples of the superfusate were collected from $t = 50$ min onwards ($t = 0$ min being the start of superfusion). At the end of experiments, tissues were dissolved and tritium was determined in superfusate chambers and tissues.

The superfusion medium contained (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5 unless stated otherwise, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, ascorbic acid 0.57, disodium EDTA 0.03 and desipramine 0.001. The medium for preincubation with ^{3}H-noradrenaline contained no desipramine and only 0.2 mM $CaCl_2$ (Limberger *et al*., 1992).

There were seven periods of electrical stimulation. Stimulation consisted of rectangular pulses of 1 ms width and 47 V cm^{-1} voltage drop between the electrodes of each chamber, yielding a current strength of 80 mA. The first stimulation period (180 pulses at 3 Hz) was delivered at $t = 30$ min and was not used for the determination of tritium overflow. The subsequent stimulation periods ($S_1 - S_6$) were applied at $t = 54$, 72, 90, 108, 126 and 144 min. They consisted of either 120 pulses at 3 Hz, 36 pulses at 3 Hz or 20 pulses at 50 Hz. Concentration – response curves for the release-enhancing effect of angiotensin II and bradykinin were determined by the addition of the peptides at increasing concentrations 12 min before $S_2 - S_6$.

The outflow of tritium was calculated as a fraction of the tritium content of the tissue at the onset of the respective collection period (fractional rate; min^{-1}). The overflow elicited by electrical stimulation was calculated as the difference 'total tritium outflow during and after stimulation' minus 'basal outflow', and was then expressed as a percentage of the tritium content of the tissue at the onset of stimulation (see Trendelenburg *et al*., 1997). For further evaluation, overflow ratios S_n/S_1 were calculated. Overflow ratios obtained after addition of a drug were also calculated as a percentage of the corresponding ratio in controls in which no drug was added. Effects of drugs on basal tritium efflux were evaluated similarly.

Results are expressed as arithmetic means ± s.e.m. Groups were tested for significant differences with the Mann – Whitney test and Bonferroni correction. An error probability $P < 0.05$ was taken to be a significant difference, n is the number of tissue pieces.

Drugs

(−)-[Ring-2,5,6-^{3}H]-noradrenaline, specific activity 51.8 – 56.0 Ci mmol^{-1}, was from DuPont, Dreieich, Germany; angiotensin II (human), bradykinin, desipramine hydrochloride, rauwolscine hydrochloride and (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxan-6-yl]-1-naphthalenylmethanone mesylate (WIN 55,212-2) were from Sigma, Deisenhofen, Germany; phentolamine hydro-

Table 1 Electrically evoked overflow of tritium from mouse, rat and rabbit tissues

Species	*Mouse strain*	*Tissue*	*Overflow (% of tissue tritium) evoked by*		
			120 pulses/3 Hz	*20 pulses/50 Hz*	*120 pulses/3 Hz (+phentolamine 1 μM+rauwolscine 1 μM)*
Mouse	WT	Atria	0.984±0.042 ($n=77$)	0.367±0.013 ($n=29$)	4.314±0.134** ($n=24$)
	α_{2A}KO		1.428±0.086* ($n=63$)	0.494±0.032* ($n=12$)	4.451±0.181** ($n=18$)
	α_{2B}KO		0.965±0.071 ($n=40$)		
	α_{2C}KO		1.284±0.067* ($n=32$)		
	α_{2AC}KO		2.154±0.068* ($n=30$)	0.323±0.023 ($n=15$)	2.121±0.124 ($n=24$)
Mouse	WT	Vas deferens	0.313±0.008 ($n=90$)	0.388±0.018 ($n=27$)	2.314±0.174**[a] ($n=23$)
	α_{2A}KO		0.522±0.024* ($n=61$)	0.438±0.020 ($n=15$)	2.045±0.239** ($n=25$)
	α_{2B}KO		0.275±0.011* ($n=51$)		
	α_{2C}KO		0.450±0.027* ($n=37$)		
	α_{2AC}KO		0.651±0.029* ($n=48$)	0.439±0.018 ($n=35$)	1.543±0.143** ($n=19$)
Rat		Atria	1.302±0.069 ($n=34$)	0.214±0.009 ($n=22$)	4.055±0.132** ($n=38$)
Rabbit		Atria	0.630±0.045 ($n=39$)	0.287±0.025 ($n=24$)	2.266±0.165** ($n=25$)

Preparations were incubated with ^{3}H-noradrenaline and then superfused with a medium containing desipramine 1 μM and in some cases also other drugs as indicated. Values represent the overflow of tritium evoked by the first stimulation period, S_1. Means±s.e.m. from n superfusion chambers. [a]In the presence of WIN 55,212-2 0.1 μM: 0.486±0.036 ($n=23$); at a lower Ca^{2+}-concentration (0.65 instead of 2.5 mM): 0.286±0.024 ($n=24$); when S_1 consisted of 36 pulses/3 Hz instead of 120 pulses/3 Hz: 0.589±0.036 ($n=24$). *Signficant differences ($P<0.05$) from WT. **Significant differences ($P<0.05$) from absence of phentolamine 1 μM and rauwolscine 1 μM.

chloride was a gift from Ciba-Geigy, Basel, Switzerland. Drugs were dissolved in water except WIN 55,212-2 (dimethylsulfoxide).

Results

The primary results from experiments of this kind are tritium efflux-*versus*-time curves with the characteristic peaks caused by electrical stimulation. Such curves have been published from our laboratory for all tissues examined (mouse atria Wahl *et al*. (1996), mouse vas deferens Altman *et al*. (1999), rat atria Limberger *et al*. (1992), rabbit atria Limberger *et al*. (1995)). The fractional rates of basal tritium efflux and the size of the stimulation peaks were similar to the previous work. Values for the overflow of tritium elicited by S_1 are summarized in Table 1. In control experiments without drug administration after S_1, the peaks remained similar from S_1 to S_6 ($n=8-27$). None of the drugs changed the basal efflux of tritium.

Peptide effects in WT mouse tissues

Initial experiments were carried out with tissues from WT mice. When these tissues were stimulated by trains of 120 pulses at 3 Hz, marked autoinhibition developed as shown by the large increases of S_1 overflow values caused by the combined administration of phentolamine 1 μM and rauwolscine 1 μM throughout superfusion: a more than four-fold increase in WT atria and a more than seven-fold increase in WT vas deferens (Table 1).

Under these conditions (and in the absence of α-adrenoceptor antagonists), angiotensin II and bradykinin greatly increased the evoked overflow of tritium (filled circles in Figure 1). The maximum increase caused by angiotensin II in atria was by about 80%, and the EC_{50}, that is the concentration causing a 40% increase, was 0.21 nM (calculated by interpolation from the nearest points of the concentration – response curve; Figure 1a). The maximum increase caused by bradykinin in atria also was by about 80%, and the EC_{50} 0.16 nM (Figure 1b). The maximum increase caused by angiotensin II in the vas deferens was by about 60%, and the EC_{50} 0.08 nM (Figure 1c). The maximum increase caused by bradykinin in the vas deferens was by about 80%, also with an EC_{50} of 0.08 nM (Figure 1d). The values are similar to previously determined values in atria and vasa deferentia of NMRI mice (Cox *et al*. (2000) and Trendelenburg *et al*. (2000); no previous values available for angiotensin II in the vas deferens).

As in our previous work on atria of NMRI mice (Cox *et al*., 2000), the release-enhancing effects of angiotensin II and bradykinin in atria as well as the vas deferens of WT mice were significantly and greatly reduced or even abolished when either, in experiments with 120 pulses at 3 Hz, α_2-autoreceptors were blocked by addition of phentolamine 1 μM and rauwolscine 1 μM throughout superfusion (empty diamonds in Figure 1), or when the tissues were stimulated by trains of 20 pulses at 50 Hz (empty squares in Figure 1). Only minor α_2-autoinhibition develops during the latter brief high-frequency pulse trains (Trendelenburg *et al*., 1999; 2000; 2001; Cox *et al*., 2000).

In the atria of NMRI mice stimulated by trains of 120 pulses at 3 Hz and treated with phentolamine, the effects of angiotensin II and bradykinin fully reappeared when the atria were additionally treated with neuropeptide Y or DSLET (Cox *et al*., 2000). We carried out an analogous experiment in the WT vas deferens, activating another $G_{i/o}$-coupled receptor, the cannabinoid CB_1-receptor, by the selective agonist WIN 55,212-2 (see Trendelenburg *et al*., 2000). WIN 55,212-2 indeed completely re-established the facilitation by angiotensin II and bradykinin (Figure 2).

WIN 55,212-2 had a second effect: it greatly reduced the evoked overflow of tritium from the high level attained in the presence of phentolamine and rauwolscine (S_1; footnote 'a' in Table 1). We wished to make sure that it was the presence of

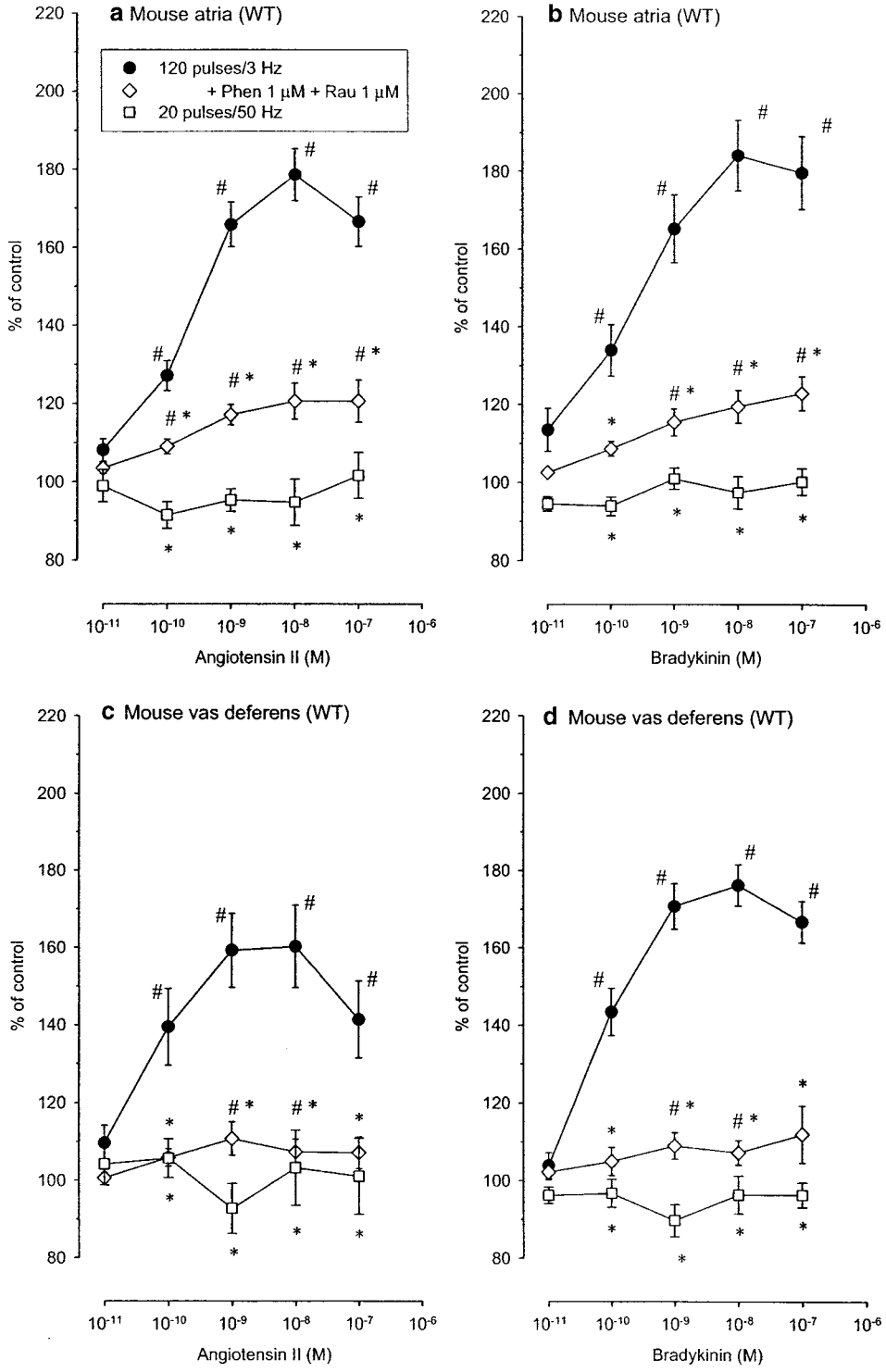

Figure 1 Effect of angiotensin II and bradykinin on the evoked overflow of tritium from WT mouse atria (a, b) and vasa deferentia (c, d) stimulated under different degrees of α_2-autoinhibition. The preparations were stimulated for six periods ($S_1 - S_6$) by either 120 pulses at 3 Hz or 20 pulses at 50 Hz. Angiotensin II and bradykinin were added at increasing concentrations 12 min before $S_2 - S_6$. In some experiments, phentolamine 1 μM and rauwolscine 1 μM were present throughout superfusion. Symbols represent the evoked overflow of tritium, expressed as a percentage of control (no peptide added). Values are means $\pm$ s.e.m. from 9 to 29 tissue pieces. Significant differences from control, no peptide: $\#P<0.05$. Significant differences from 120 pulses/3 Hz without α-adrenoceptor antagonists: $*P<0.05$.

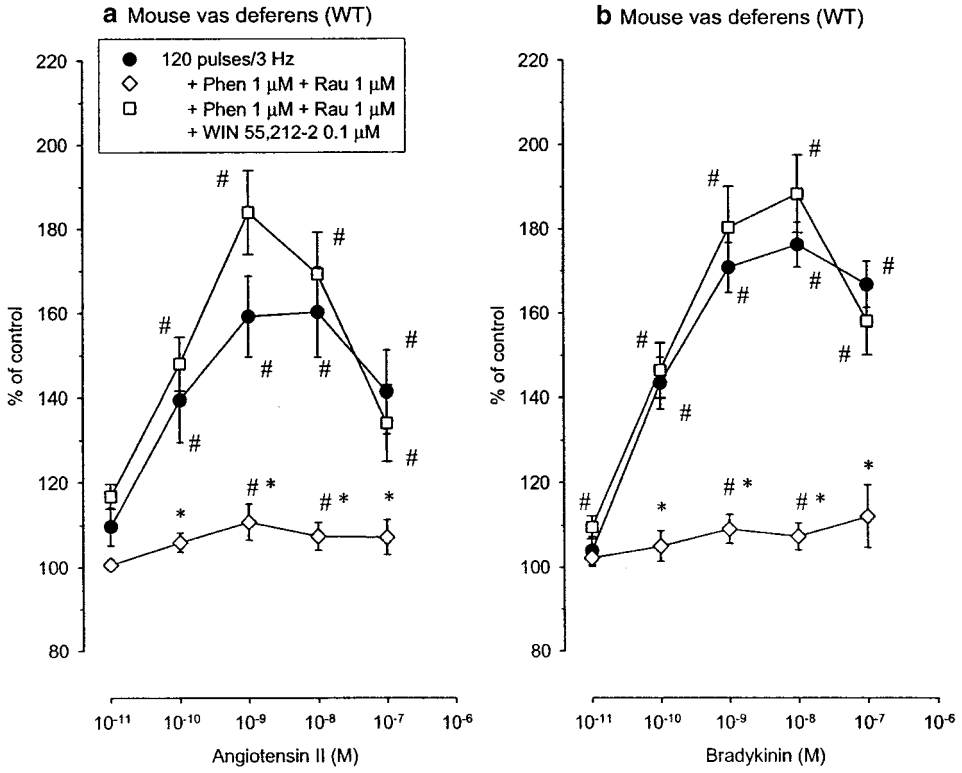

Figure 2 Effect of angiotensin II and bradykinin on the evoked overflow of tritium from WT mouse vasa deferentia in the presence of α-adrenoceptor antagonists: influence of WIN 55,212-2. The preparations were stimulated for six periods ($S_1 - S_6$) by 120 pulses at 3 Hz. Angiotensin II and bradykinin were added at increasing concentrations 12 min before $S_2 - S_6$. In some experiments, phentolamine 1 μM and rauwolscine 1 μM, in other experiments these two drugs and in addition WIN 55,212-2 0.1 μM were present throughout superfusion. Symbols represent the evoked overflow of tritium, expressed as a percentage of control (no peptide added). Values are means ± s.e.m. from 8 to 29 tissue pieces. Significant differences from control, no peptide: $\#P<0.05$. Significant differences from 120 pulses/3 Hz without α-adrenoceptor antagonists and WIN 55,212-2: $*P<0.05$.

WIN 55,212-2 and not just this reduction in overflow that re-established the effects of the peptides. To this end, we lowered the evoked overflow in two other ways: by a decrease in the concentration of Ca^{2+} in the medium to 0.65 mM, and by a decrease in the number of pulses per train to 36. In both cases the overflow at S_1, in the presence of phentolamine and rauwolscine, was close to the overflow evoked by 120 pulses at 3 Hz in normal Ca^{2+} in the presence of phentolamine, rauwolscine and WIN 55,212-2 (S_1; footnote 'a' in Table 1). In contrast to WIN 55,2122 (Figure 2), neither low Ca^{2+} (Figure 3) nor stimulation with 36 pulses per train (not shown; $n=8$) re-established the effects of angiotensin II and bradykinin: the interaction with WIN 55,212-2 was a specific one.

Peptide effects in KO mouse tissues

We then proceeded to the receptor-deficient mice. Tissues were initially stimulated by trains of 120 pulses at 3 Hz in the absence of α-adrenoceptor antagonists. The S_1 overflow responses under these conditions were greater in atria and vasa deferentia from α_{2A}KO, α_{2C}KO and α_{AC}KO mice than in WT tissues (Table 1), in accord with the physiological function of α_{2A}- and α_{2C}-adrenoceptors as autoreceptors. Neither deletion of the α_{2A}-adrenoceptor nor deletion of the α_{2B}-adrenoceptor changed the effects of angiotensin II and bradykinin: the peptide concentration – response curves in the α_{2A}KO and α_{2B}KO tissues were practically identical with the curves in the WT tissues, with no significant difference from the latter (Figure 4). Only one of the single-gene disruptions, that of the α_{2C} gene, caused a change: the concentration – response curves of angiotensin II and bradykinin in α_{2C}KO atria and vasa deferentia were consistently shifted downwards to reach lower maxima. Elimination of the α_{2A}-adrenoceptor in addition to the α_{2C}-receptor produced no further change: the peptide concentration – response curves in α_{2AC}KO tissues were superimposed on the curves in the α_{2C}KO tissues (Figure 4).

The question arose whether the facilitatory effects of the peptides that remained when one or both of the autoreceptors were deleted were independent of α_2-autoinhibition. We therefore stimulated the α_{2A}KO and α_{2AC}KO tissues also by trains of 120 pulses at 3 Hz in the presence of phentolamine and rauwolscine, or by trains of 20 pulses at 50 Hz. As shown in Table 1, the antagonist mixture increased S_1 overflow values in α_{2A}KO atria, α_{2A}KO vasa deferentia and α_{2AC}KO vasa deferentia although not in α_{2AC}KO atria, indicating the operation of autoinhibition in the former three tissues. The effects of angiotensin II and bradykinin behaved like in WT tissues: in α_{2A}KO atria and vasa deferentia (Figure 5) as well as in α_{2AC}KO atria and vasa deferentia (Figure 6) the peptide effects were attenuated or abolished when either, in experiments with 120 pulses at 3 Hz, α_2-adrenoceptors were blocked (empty diamonds in Figures 5 and 6) or when, in the absence of blockers, stimulation was changed to trains of 20 pulses at

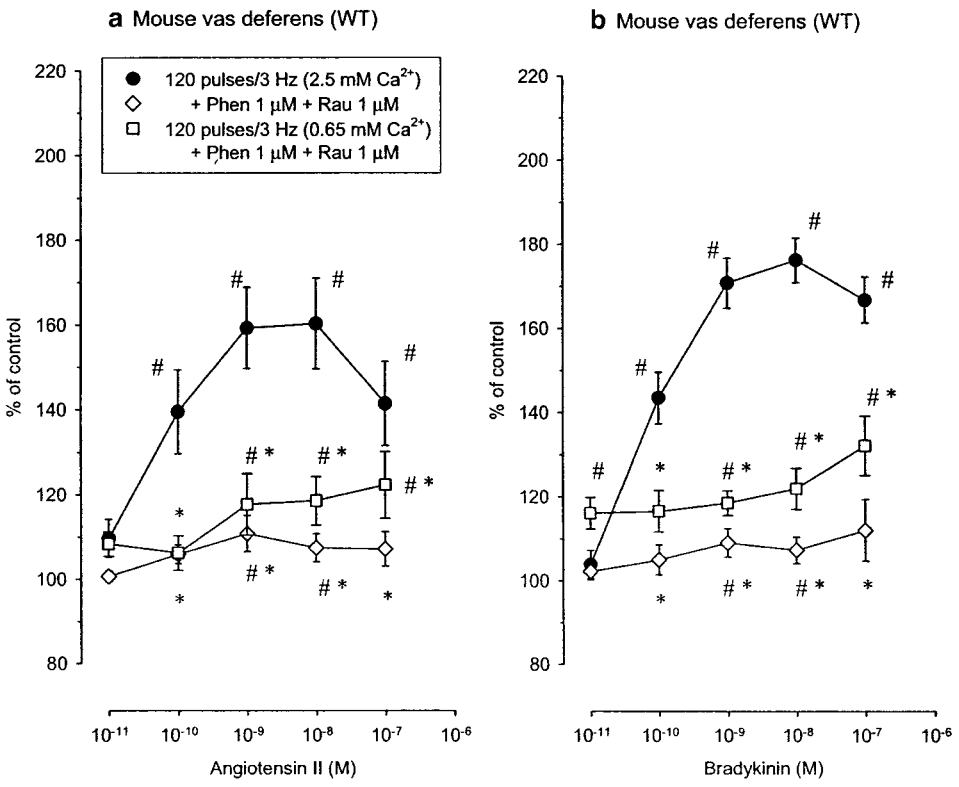

Figure 3 Effect of angiotensin II and bradykinin on the evoked overflow of tritium from WT mouse vasa deferentia in the presence of α-adrenoceptor antagonists: influence of the concentration of Ca^{2+}. The preparations were stimulated for six periods ($S_1 - S_6$) by 120 pulses at 3 Hz. Angiotensin II and bradykinin were added at increasing concentrations 12 min before $S_2 - S_6$. In some experiments, phentolamine 1 μM and rauwolscine 1 μM were present throughout superfusion. The superfusion medium contained either the normal (2.5 mM) or a lower concentration of Ca^{2+} (0.65 mM). Symbols represent the evoked overflow of tritium, expressed as a percentage of control (no peptide added). Values are means ± s.e.m. from 8 to 29 tissue pieces. Significant differences from control, no peptide: $\#P<0.05$. Significant differences from 120 pulses/3 Hz, 2.5 mM Ca^{2+}, without α-adrenoceptor antagonists: $*P<0.05$.

50 Hz (empty squares in Figures 5 and 6). There was one exception: the α antagonists did not reduce the effect of angiotensin II in the α_{2AC}KO vas deferens, possibly because the effect was small in that tissue even in the absence of the blockers (Figure 6c; but angiotensin II also did not cause significant facilitation in the α-adrenoceptor-blocked α_{2AC}KO vas deferens as compared to the control without peptide).

Assessment of α_2-autoinhibition in mouse tissues

The degree of α_2-autoinhibition can be estimated from the increase of S_1 overflow values caused by α_2 antagonists that are present throughout superfusion (Table 1). However, this procedure is not very reliable (see Limberger *et al.*, 1995, p. 32). A more accurate measure of autoinhibition is the increase in overflow caused by α_2 antagonists when they are added after S_2, that is their effect on $S_3 - S_6$. With this protocol, each tissue segment serves as its own control. When added together after S_2, phentolamine 1 μM and rauwolscine 1 μM increased the overflow of tritium evoked by 120 pulses at 3 Hz in all five mouse strains (Figure 7). The increase was greatest in WT tissues: three- to four-fold in atria and more than seven-fold in the vas deferens (Figure 7a). Deletion of the α_{2B}-adrenoceptor caused no significant change (Figure 7c). Deletion of both the α_{2A}- and the α_{2C}-adrenoceptor alone significantly reduced or tended to reduce the effect of the antagonists, but in either case an approximately three-fold increase remained (Figure 7b and d). While these results had been expected (see Trendelenburg *et al.*, 2001), it was surprising to see that the antagonists continued to cause some increase, about two-fold, even in the α_{2AC}KO tissues, that is when both known α_2-autoreceptors had been deleted (Figure 7e). Antagonist addition after S_2 thus revealed α_2-autoinhibition also in α_{2AC}KO atria (filled diamonds in Figure 7e) in which the S_1 comparison had not detected it (Table 1; see above).

Rat and rabbit atria

Marked autoinhibition developed in rat and rabbit atria when they were stimulated by trains of 120 pulses at 3 Hz: combined administration of phentolamine 1 μM and rauwolscine 1 μM throughout superfusion increased the overflow at S_1 three-fold in the rat and three- to four-fold in the rabbit (Table 1).

Under these conditions (and in the absence of α-adrenoceptor antagonists), angiotensin II and bradykinin greatly increased the evoked overflow of tritium from rat atria (filled circles in Figure 8). The maximum increase caused by angiotensin II was by about 60%, and the EC_{50}, that is the concentration causing a 30% increase, was 0.30 nM (calculated by interpolation from the nearest points of the concentration – response curve; Figure 8a). The maximum increase caused by bradykinin in rat atria also was by about 60%, and the EC_{50} was 0.05 nM (Figure 8b). Still under these conditions, angiotensin II also greatly increased the evoked overflow of

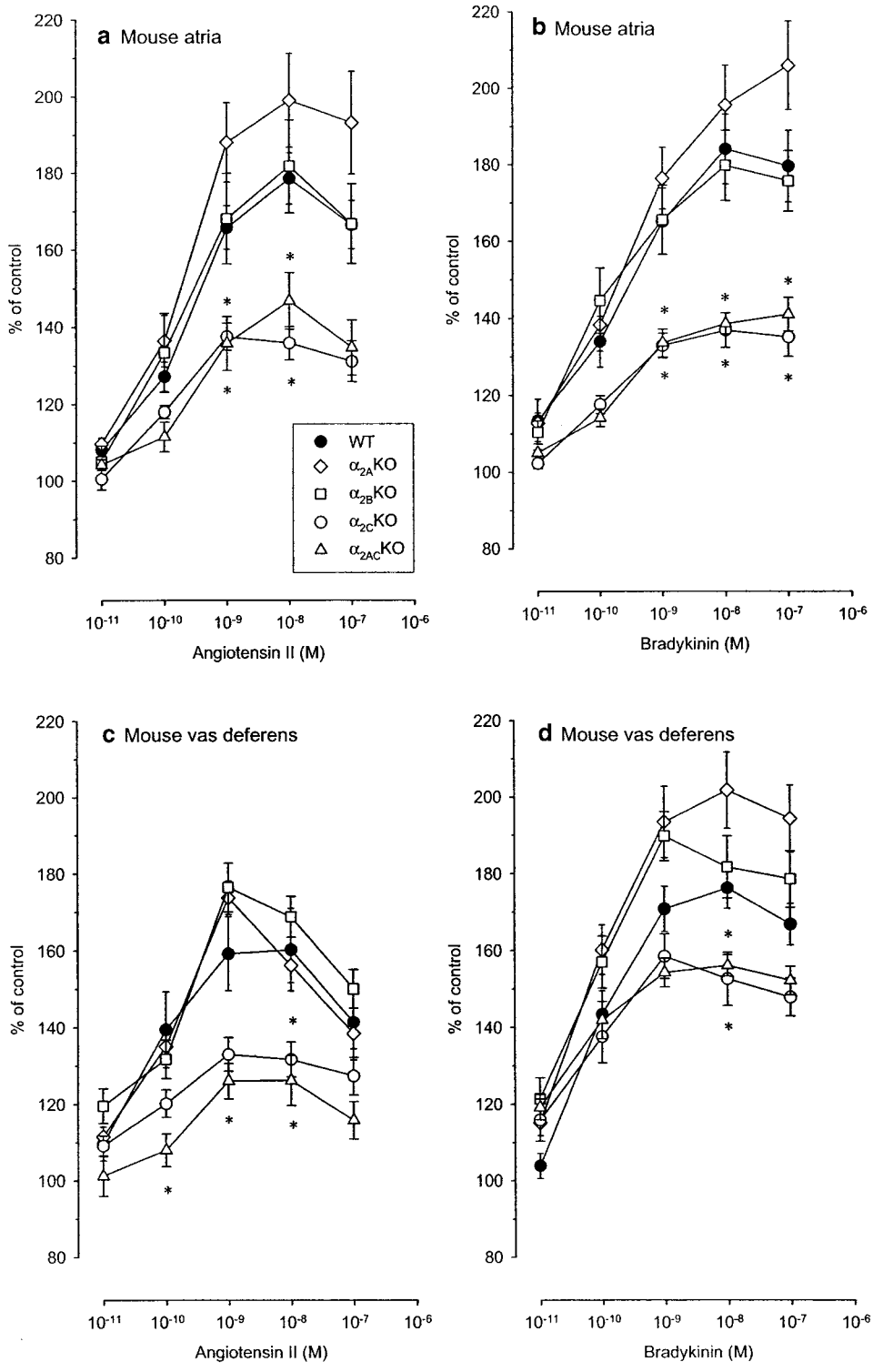

Figure 4 Effect of angiotensin II and bradykinin on the evoked overflow of tritium from atria (a, b) and vasa deferentia (c, d) taken from WT and various α_2-adrenoceptor-deficient mice. The preparations were stimulated for six periods ($S_1 - S_6$) by 120 pulses at 3 Hz. Angiotensin II and bradykinin were added at increasing concentrations 12 min before $S_2 - S_6$. Symbols represent the evoked overflow of tritium, expressed as a percentage of control (no peptide added). Values are means ± s.e.m. from 5 to 29 tissue pieces. All peptide effects were significant ($P<0.05$) *versus* control, no peptide, except in some cases at 0.01 and 0.1 nM. Significant differences from WT: $^*P<0.05$.

tritium from rabbit atria, with maximal enhancement by 130% and an EC_{50} of 0.27 nM (filled circles in Figure 9a). Bradykinin, in contrast, failed to cause any change in rabbit atria (filled circles in Figure 9b).

As in the WT mouse tissues, the release-enhancing effects of angiotensin II and bradykinin in rat atria were greatly reduced or even abolished when either, in experiments with 120 pulses at 3 Hz, α_2-autoreceptors were blocked by addition of a

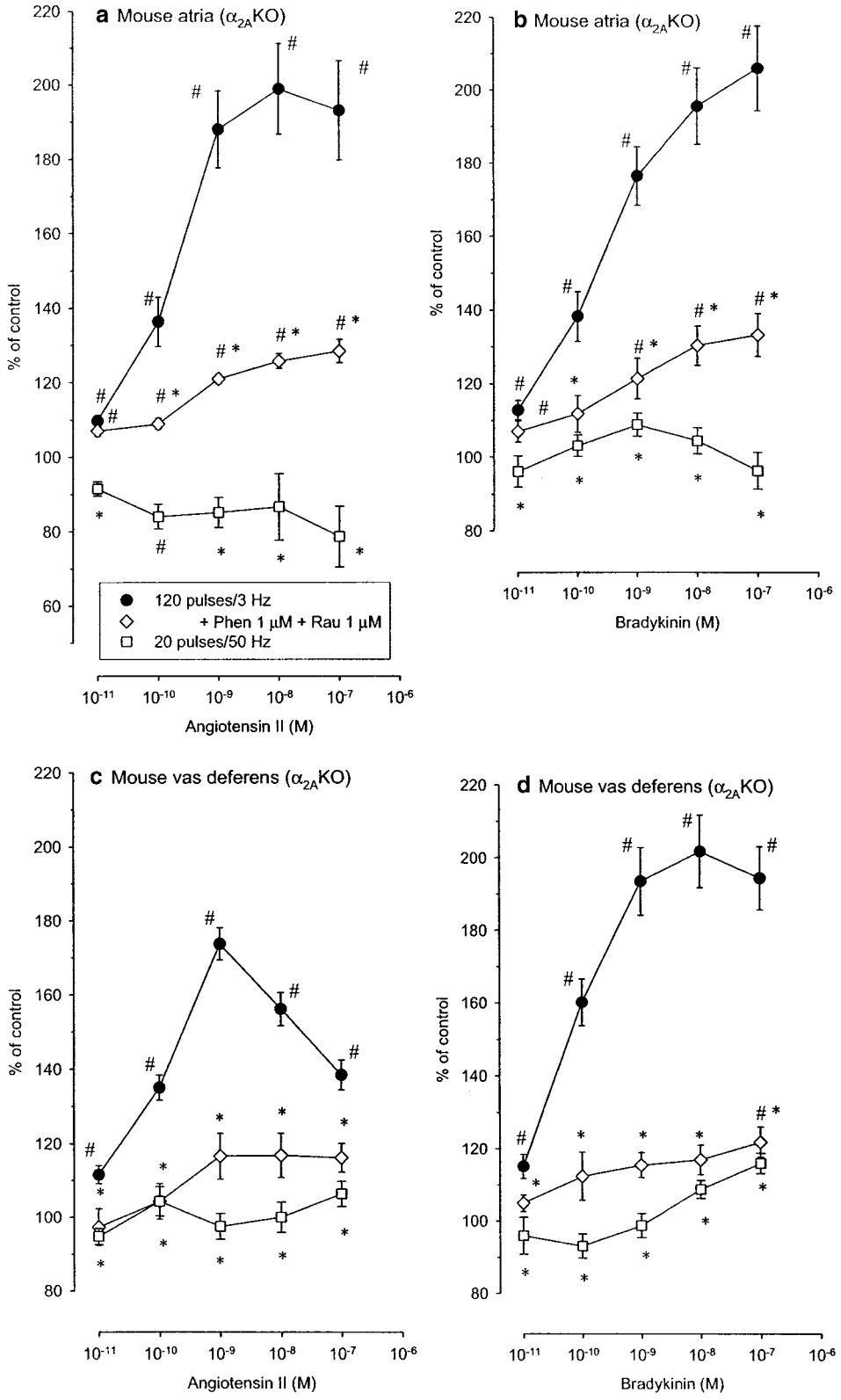

Figure 5 Effect of angiotensin II and bradykinin on the evoked overflow of tritium from α_{2A}KO mouse atria (a, b) and vasa deferentia (c, d) stimulated under different degrees of α_2-autoinhibition. The preparations were stimulated for six periods ($S_1 - S_6$) by either 120 pulses at 3 Hz or 20 pulses at 50 Hz. Angiotensin II and bradykinin were added at increasing concentrations 12 min before $S_2 - S_6$. In some experiments, phentolamine 1 μM and rauwolscine 1 μM were present throughout superfusion. Symbols represent the evoked overflow of tritium, expressed as a percentage of control (no peptide added). Values are means $\pm$ s.e.m. from 5 to 19 tissue pieces. Significant differences from control, no peptide: $\#P<0.05$. Significant differences from 120 pulses/3 Hz without α-adrenoceptor antagonists: $*P<0.05$.

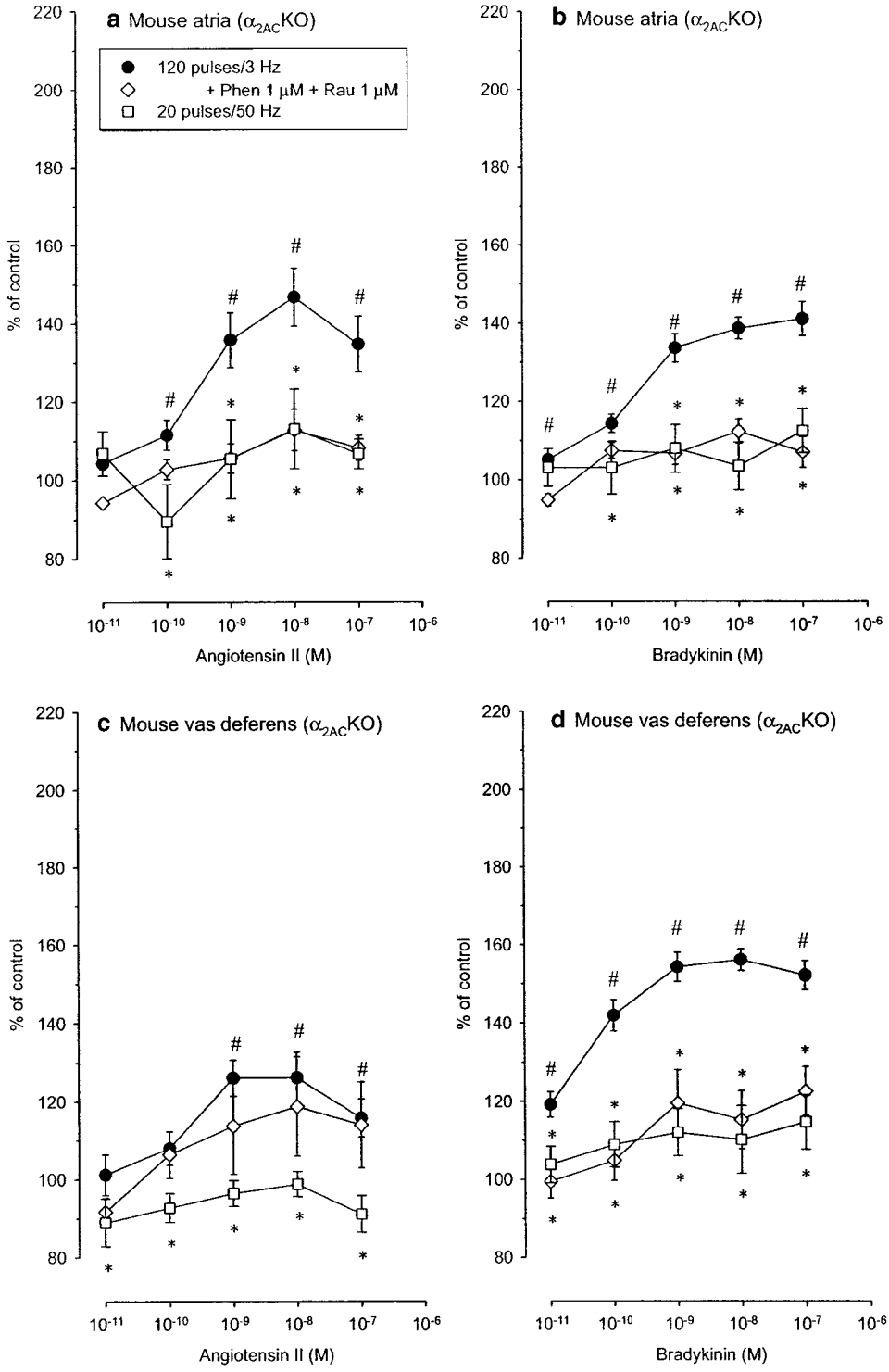

Figure 6 Effect of angiotensin II and bradykinin on the evoked overflow of tritium from α_{2AC}KO mouse atria (a, b) and vasa deferentia (c, d) stimulated under different degrees of α_2-autoinhibition. The preparations were stimulated for six periods ($S_1 - S_6$) by either 120 pulses at 3 Hz or 20 pulses at 50 Hz. Angiotensin II and bradykinin were added at increasing concentrations 12 min before $S_2 - S_6$. In some experiments, phentolamine 1 μM and rauwolscine 1 μM were present throughout superfusion. Symbols represent the evoked overflow of tritium, expressed as a percentage of control (no peptide added). Values are means $\pm$ s.e.m. from 5 to 10 tissue pieces. Significant differences from control, no peptide: $\#P<0.05$. Significant differences from 120 pulses/3 Hz without α-adrenoceptor antagonists: $*P<0.05$.

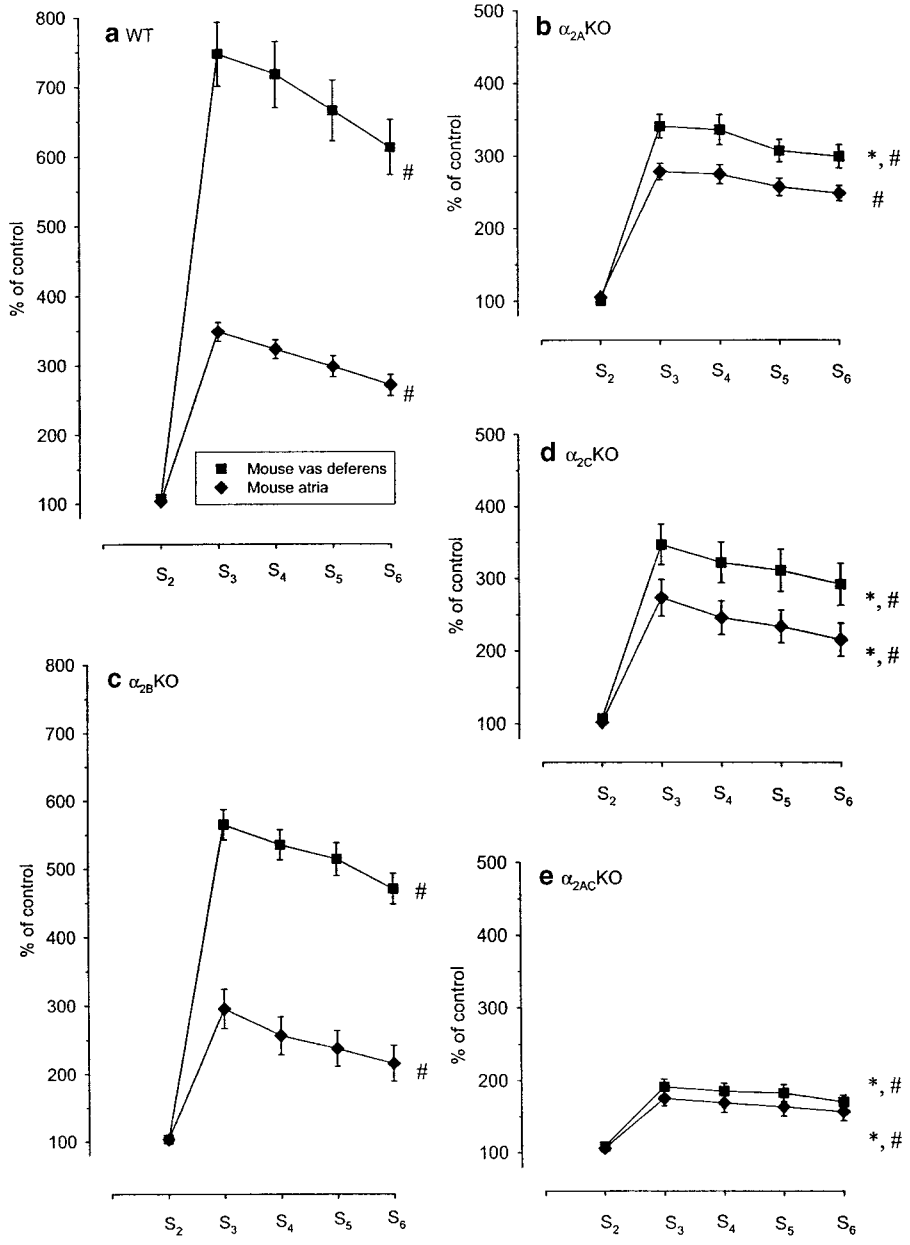

Figure 7 Effect of a mixture of phentolamine 1 μM and rauwolscine 1 μM on the evoked overflow of tritium from atria and vasa deferentia taken from WT and various α_2-adrenoceptor-deficient mice. The preparations were stimulated for six periods ($S_1 - S_6$) by 120 pulses at 3 Hz. Phentolamine 1 μM and rauwolscine 1 μM were added simultaneously 12 min before S_3. Symbols represent the evoked overflow of tritium, expressed as a percentage of control (no α antagonist added). Values are means $\pm$ s.e.m. from 5 to 12 tissue pieces. Significant differences from control, no α antagonist: $\#P<0.05$. Significant differences from WT: $*P<0.05$. The significance symbols refer to $S_3 - S_6$.

mixture of phentolamine 1 μM and rauwolscine 1 μM throughout superfusion (empty diamonds in Figure 8), or when the tissues were stimulated by trains of 20 pulses at 50 Hz (empty squares in Figure 8). The effect of angiotensin II in rabbit atria equally was much depressed under these conditions (empty symbols in Figure 9a). Bradykinin remained without effect in rabbit atria (empty symbols in Figure 9b).

Discussion

Our results confirm that angiotensin II and bradykinin can enhance exocytotic release of noradrenaline from the sympathetic neurons innervating the mouse heart, the rat heart and the mouse vas deferens, and that angiotensin II can enhance noradrenaline release in the rabbit heart (see Starke (1977), Fuder & Muscholl (1995) and Boehm & Kubista (2002); for mouse vas deferens: Trendelenburg *et al.*, (2000) and Schelb *et al.* (2001)). The lack of any effect of bradykinin in rabbit atria even under autoinhibition-rich conditions (Figure 9b) may be because of a dual action. Bradykinin reduces the release of noradrenaline in the rabbit perfused heart and in other rabbit tissues by promotion of the synthesis of prostaglandins, which then cause presynaptic inhibition (Starke *et al.*, 1977; Rónai, 1991). This component may have counteracted any possible direct facilitatory effect of

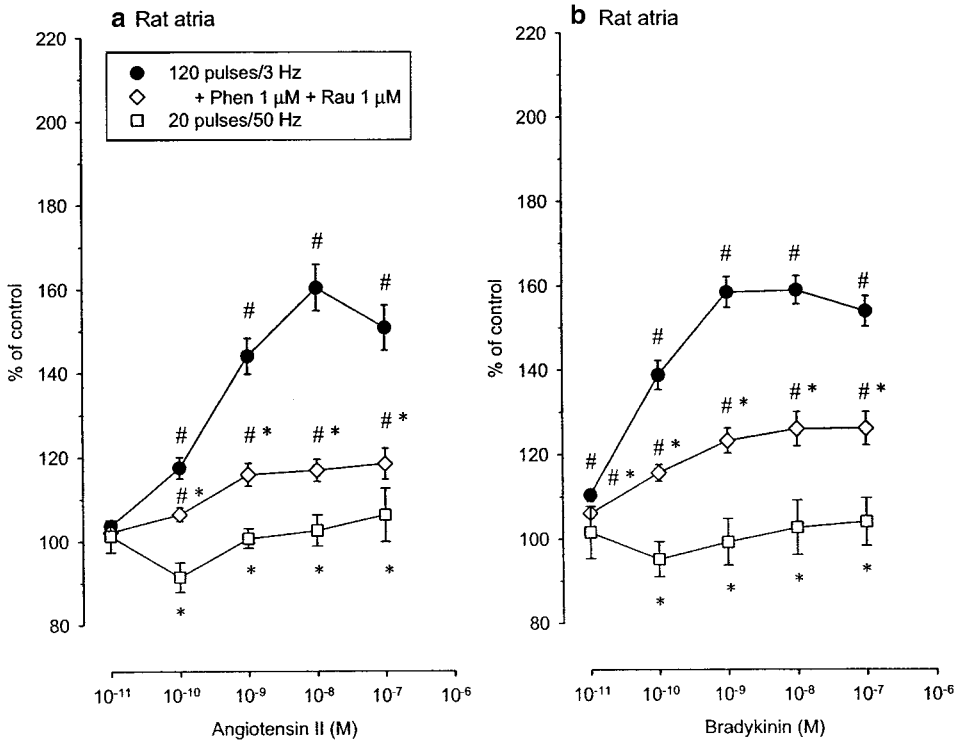

Figure 8 Effect of angiotensin II and bradykinin on the evoked overflow of tritium from rat atria stimulated under different degrees of α_2-autoinhibition. The preparations were stimulated for six periods ($S_1 - S_6$) by either 120 pulses at 3 Hz or 20 pulses at 50 Hz. Angiotensin II and bradykinin were added at increasing concentrations 12 min before $S_2 - S_6$. In some experiments, phentolamine 1 μM and rauwolscine 1 μM were present throughout superfusion. Symbols represent the evoked overflow of tritium, expressed as a percentage of control (no peptide added). Values are means ± s.e.m. from 8 to 12 tissue pieces. Significant differences from control, no peptide: #$P<0.05$. Significant differences from 120 pulses/3 Hz without α-adrenoceptor antagonists: *$P<0.05$.

bradykinin in the present experiments on superfused atrial segments, thus leading to a zero net effect. The effects of angiotensin II and bradykinin in mouse atria and vasa deferentia and rat atria are mediated by AT_1- and B_2-receptors, respectively (see references above). The type of presynaptic angiotensin receptor in the rabbit heart does not seem to have been identified.

Our results also confirm that angiotensin II loses much or all of its presynaptic facilitatory effect in mouse, rat and rabbit cardiac preparations when either α_2-autoreceptors are pharmacologically blocked or the sympathetic nerves stimulated in a manner leading to no or little autoinhibition, and that the same loss occurs with the facilitatory effect of bradykinin in mouse atria (Starke & Schümann, 1972; Kurz *et al*., 1997; Cox *et al*., 2000; Mota & Guimarães, 2002). To these instances, our experiments add the demonstration of analogous conditions for the effects of angiotensin II and bradykinin in the mouse vas deferens and for the effect of bradykinin in rat atria. It should be noted that we used NMRI mice in our previous study (Cox *et al*., 2000) but a different strain, the WT of the genetically engineered mice, in the present work: results were the same.

We have suggested that angiotensin II and bradykinin increase the release of noradrenaline at least to a large extent by interrupting an ongoing α_2-autoinhibition. More specifically, we have suggested that one effector of AT_1- and B_2-receptors, namely protein kinase C, inactivates a protein involved in the presynaptic α_2-autoreceptor $\rightarrow G_{i/o} \rightarrow$ presynaptic calcium channel inhibitory pathway (Cox *et al*., 2000). In support of this view, when α_2-autoinhibition was absent, activation of other $G_{i/o}$-coupled presynaptic receptors, namely neuropeptide Y Y_2- and opioid OP_1-receptors, enabled angiotensin II and bradykinin to produce their full scope of facilitation (Cox *et al*., 2000). Again we now add a further example: in the mouse vas deferens, cannabinoids reduce the release of noradrenaline by the activation of $G_{i/o}$-coupled CB_1-receptors, and exposure to the CB_1 agonist WIN 55,212-2 restored the full extent of facilitation by angiotensin II and bradykinin (Figure 2). Presynaptic CB_1-receptor do not operate in mouse atria (Trendelenburg *et al*., 2000), so the interaction experiment had to be limited to the vas deferens. It has been shown a number of years ago that angiotensin II caused a greater increase in noradrenaline release in rabbit atria when presynaptic muscarinic receptors were activated simultaneously (Garcia-Sevilla *et al*., 1985). Perhaps, this was because of an analogous mechanism, that is in this case muscarinic stimulation of the presynaptic $G_{i/o}$ pathway, thus giving angiotensin II greater space for disinhibition.

The center of the present work, the experiments on α_2-adrenoceptor-deficient tissues, gave both expected and unexpected results. Since there is no firm evidence for α_{2B}-autoreceptors, we did not expect changes in the effects of angiotensin II and bradykinin in the α_{2B}KO tissues: and in fact, there was no change (Figure 4). Since the α_{2A}-adrenoceptor is generally considered to be the major α_2-autoreceptor, we had expected a clear attenuation of the peptide effects in the α_{2A}KO tissues; however, here also no change occurred (Figure 4). Of the single deletions, only deletion of the α_{2C}-adrenoceptor

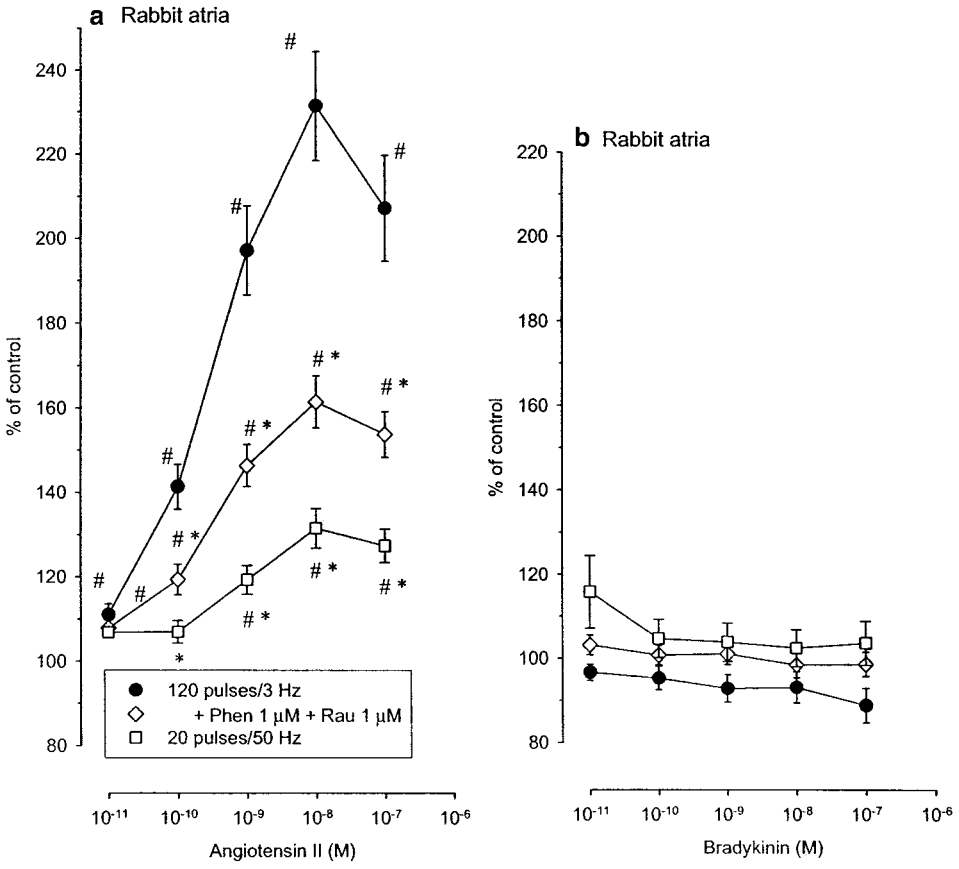

Figure 9 Effect of angiotensin II and bradykinin on the evoked overflow of tritium from rabbit atria stimulated under different degrees of α_2-autoinhibition. The preparations were stimulated for six periods ($S_1 - S_6$) by either 120 pulses at 3 Hz or 20 pulses at 50 Hz. Angiotensin II and bradykinin were added at increasing concentrations 12 min before $S_2 - S_6$. In some experiments, phentolamine 1 μM and rauwolscine 1 μM were present throughout superfusion. Symbols represent the evoked overflow of tritium, expressed as a percentage of control (no peptide added). Values are means ± s.e.m. from 8 to 11 tissue pieces. Significant differences from control, no peptide: $\#P<0.05$. Significant differences from 120 pulses/3 Hz without α-adrenoceptor antagonists: $*P<0.05$.

blunted the effects of the peptides (Figure 4). The most unexpected findings were those in the α_{2AC}KO tissues. Even when both known autoreceptors had been deleted, angiotensin II and bradykinin still facilitated the release of noradrenaline, and did so to no smaller extent than after deletion of the α_{2C}-adrenoceptor alone (Figure 4). All effects of the peptides remaining after gene deletion, in the α_{2A}KO as well as in the α_{2AC}KO tissues, continued to depend on an ongoing α_2-autoinhibition: when there was no autoinhibition, the effects shrank or disappeared (Figures 5 and 6). Finally, this remaining autoinhibition was demonstrated directly: although reduced, it clearly operated in α_{2A}KO, α_{2C}KO and even α_{2AC}KO tissues (Figure 7). It should also be noted that the degree of autoinhibition in α_{2A}KO and α_{2C}KO tissues was very similar (Figure 7b and d), indicating that the selective attenuation of the peptide effects in α_{2C}KO but not the α_{2A}KO tissues was not because of a lower overall autoinhibitory tone in the former.

These results tell us something on the prerequisites for AT_1- and B_2-receptor-mediated presynaptic facilitation. They simultaneously change our concept of α_2-autoinhibition.

The first conclusion is that although the main autoreceptor is α_{2A} (see Hein, 2001; Starke, 2001), it is the α_{2C}- rather than α_{2A}-autoinhibition that permits or promotes the presynaptic effects of angiotensin II and bradykinin: deletion of the α_{2A}-adrenoceptor caused no change in the peptide effects, neither when it was the only deletion (compare α_{2A}KO to WT in Figure 4) nor when the α_{2C}-adrenoceptor was also deleted (compared α_{2AC}KO to α_{2C}KO in Figure 4). It cannot be excluded that α_{2A}- and α_{2C}-autoreceptors couple to different transduction pathways, above all to different $G_{i/o}$ heterotrimers (see Hein, 2001; Starke, 2001). The present results may be an incitement to search for the difference that makes α_{2C} signal transduction selectively sensitive to angiotensin II and bradykinin.

The second conclusion, rather provocative for autoreceptor theory, is that there must be another autoreceptor that permits facilitation by angiotensin II and bradykinin: the autoreceptor responsible for the remaining facilitation in the α_{2AC}KO tissues (Figure 4). It should be remembered that this remaining facilitation was α_2-autoinhibition dependent (Figure 6) and that α_2-autoinhibition was indeed present (Figure 7e). Given the fact that the receptor cannot be α_{2A} or α_{2C}, and given the belief that only three α_2-adrenoceptor types exist (see Hein, 2001): are there α_{2B}-autoreceptors, and is the transduction pathway behind these, like the α_{2C} transduction pathway, sensitive to angiotensin II and bradykinin?

Two contra arguments immediately come to mind: deletion of the α_{2B} gene alone changed neither the degree of autoinhibition (Figure 7c) nor the concentration – response

curves of angiotensin II and bradykinin (Figure 4). However, these arguments do not exclude the existence of α_{2B}-autoreceptors. There was a similar series of findings when the α_{2C}-autoreceptors were discovered. Initial experiments had shown that some noradrenaline release-inhibiting effect of α_2-adrenoceptor agonists persisted after α_{2A}-adrenoceptors had been genetically deleted (Altman *et al*., 1999). Deletion of α_{2C}-adrenoceptors alone did not change the effect of the agonists, and this seemed to argue against α_{2C}-autoreceptors (Hein *et al*., 1999; Trendelenburg *et al*., 1999). It was only when Hein *et al*. (1999) generated the double KO, α_{2AC}KO, that the non-α_{2A}-autoreceptors were identified as α_{2C}: deletion of the α_{2C}- in addition ot the α_{2A}-adrenoceptor abolished the remaining inhibition by α_2 agonists (Hein *et al*., 1999). Perhaps, certain functions that depend on two α_2-autoreceptor types remain unchanged when only one contributing type is deleted (the α_{2C}-adrenoceptor in the previous work on α_2 agonists, the α_{2B}-adrenoceptor in the present work): the remaining contributing type compensates for the loss. The α_{2B}-adrenoceptor gene is expressed in the rat superior cervical ganglion (Vidovic & Hill, 1997). This gives some support to the idea of α_{2B}-autoreceptors. In fact, in a study of the influence of α_2-autoinhibition on the stimulation frequency – noradrenaline release relation, the possibility of α_{2B}-autoreceptors has already cautiously been raised (Scheibner *et al*., 2001).

In conclusion, we haved extended the evidence for a crosstalk between presynaptic α_2-autoreceptors, angiotensin AT_1- and bradykinin B_2-receptors to further tissues. The crosstalk is a frequent phenomenon. We have also extended the evidence that stimulation of the presynaptic $G_{i/o}$ inhibitory pathway through other receptors, namely neuropeptide Y Y_2-, opioid OP_1- and – this is the new finding – cannabinoid CB_1-receptors can replace $G_{i/o}$ stimulation through α_2-autoreceptors to permit full presynaptic facilitation by angiotensin II and bradykinin. The results should not be interpreted as indicating that interruption of α_2-autoinhibition is the sole presynaptic mode of action of angiotensin II and bradykinin; there may be α_2-autoinhibition-independent mechanisms (see Costa & Majewki, 1988; Mota *et al*., 2000); indeed, small facilitatory effects of the two peptides in the presence of phentolamine plus rauwolscine or during brief high-frequency stimulation were occasionally seen throughout the present work (e.g. Figures 1, 8 and 9); however, in the preparations examined the interruption of autoinhibition prevailed. Experiments on α_2-adrenoceptor subtype-deficient mice indicate that it is the α_{2C}-autoreceptor and not the α_{2A}-autoreceptor that interacts with the AT_1- and B_2-receptors. However, α_{2B}-autoreceptors may also contribute to the crosstalk. The existence of α_{2B}- in addition to the established α_{2A}- and α_{2C}-autoreceptors is a somewhat provocative perspective opened by our study.

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