α_1 -Adrenoceptors and calcium sources in adrenergic neurogenic contractions of rat vas deferens

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1 The involvement of α_1 -adrenoceptor subtypes in adrenergic neurogenic contractions of different type was studied in epididymal and prostatic portions of the rat vas deferens.

2 The adrenergic component of neurogenic contractions was isolated by suramin (300 μ M). Twitch-like and tonic contractions were elicited by appropriate pulse patterns of electrical field stimulation, and contractions relying on intracellular calcium mobilization and calcium entry were isolated by means of nifedipine (10 μ M) and ryanodine (20 μ M), respectively. Increasing concentrations of 2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane (WB 4101), α -ethyl-3,4,5-trimethoxy- α -(3-((2-(2-methoxyphenoxy)ethyl)-amino)-propyl)benzeneacetonitrile (HV 723), prazosin and 5-methylurapidil progressively, monophasically and with potency decreasing in that order reduced and finally abolished all types of contraction, with one exception: concentration-effect curves of 5-methylurapidil in epididymal segments in the presence of ryanodine levelled off at about 75% inhibition. In the presence of both nifedipine (10 μ M) and ryanodine (20 μ M), contractions were abolished.

3 Contractions elicited by exogenous noradrenaline were also studied in the presence of either nifedipine 10 μ M (prostatic segments) or ryanodine 20 μ M (epididymal segments). Increasing concentrations of tamsulosin, WB 4101, benoxathian, HV 723, prazosin, 5-methylurapidil and urapidil progressively, monophasically and with potency decreasing in that order reduced and eventually abolished both kinds of contraction, with two exceptions: in epididymal segments in the presence of ryanodine, the concentration-effect curve of 5-methylurapidil was biphasic and the curve of urapidil levelled off at only partial inhibition.

4 In slices prepared from the prostatic end and preincubated with [³H]-noradrenaline, WB 4101, HV 723, prazosin and 5-methylurapidil, at the highest concentrations tested against neurogenic contractions, increased only slightly the overflow of tritium elicited by trains of 50 pulses at 5 Hz.

5 It is concluded that two α_1 -adrenoceptor subtypes mediate adrenergic neurogenic contractions of rat vas deferens. The main one, pharmacologically α_{1A} , activates both calcium mobilization and entry. In addition there is a second receptor, not previously detected in the vas deferens and not corresponding to any named α_1 subtype, characterized by high and similar affinity for tamsulosin, WB 4101, benoxathian, HV 723 and prazosin and very low affinity for 5-methylurapidil and urapidil, and linked exclusively to calcium entry. Both subtypes and their respective transduction pathways also contribute to contractions elicited by exogenous noradrenaline. An α_{1B} -adrenoceptor-mediated contraction was not found under any experimental conditions.

Keywords: Rat vas deferens; α_1 -adrenoceptor subtypes; α_{1A} -adrenoceptor; α_{1B} -adrenoceptor; Ca^{2+} entry; intracellular Ca^{2+} mobilization; postganglionic sympathetic neurotransmission

Introduction

Two sources of calcium contribute to adrenergic, α_1 -adrenoceptor-mediated, neurogenic contractions of the rat vas deferens: mobilization inside the smooth muscle cells and entry into these cells from the extracellular space. Calcium mobilization predominates in the twitch contractions elicited by single electric pulses as well as in the initial twitch phase of contractions elicited by pulse trains; this component is resistant to the dihydropyridine derivative, nifedipine, which blocks L-type calcium channels (reviewed by Porzig, 1990), but suppressed by ryanodine, which depletes the intracellular calcium stores mobilized upon α_1 -adrenoceptor activation (Iino *et al.*, 1988; Itoh *et al.*, 1992). Calcium entry predominates in the late tonic contractions elicited by pulse trains; this component is blocked by nifedipine but is resistant to ryanodine (Blakeley *et al.*, 1981; Brown *et al.*, 1983; Amobi & Smith, 1987; Bourreau *et al.*, 1991; Bültmann *et al.*, 1993b).

The discovery of α_1 -adrenoceptor subtypes (reviewed by Minneman, 1988; Ruffolo *et al.*, 1991) opened up the possibility that different calcium sources might be linked to different subtypes. Experiments with exogenous nor-

adrenaline in rat vas deferens led to the view that the α_{1A} subtype activates calcium influx through dihydropyridine-sensitive channels whereas the α_{1B} subtype activates phosphatidylinositol breakdown followed by calcium mobilization from intracellular stores (Han *et al.*, 1987). The same relationship has recently been postulated for the effect of released endogenous noradrenaline in rat vas deferens (Mallard *et al.*, 1992a). The conclusion was based on experiments with chloroethylclonidine, a compound that blocks (irreversibly) α_{1B} but not α_{1A} -adrenoceptors (Minneman *et al.*, 1988). Chloroethylclonidine did not change the tonic phase of adrenergic neurogenic contractions but abolished single pulse-evoked adrenergic twitches as well as the twitch phase of contractions elicited by pulse trains, in accordance with the view that released noradrenaline triggers calcium entry (tonic contraction) via the α_{1A} and calcium mobilization (twitches) via the α_{1B} subtype (Mallard *et al.*, 1992a).

However, this conclusion seems questionable. It has been shown that 2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane (WB 4101), a selective antagonist at α_{1A} -adrenoceptors, blocks adrenergic twitches and tonic contractions with the same potency (Mallard *et al.*, 1992b; Ohmura *et al.*, 1992). Moreover, WB 4101 blocks adrenergic twitches with higher potency than prazosin (Figure 1 of McGrath,

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1984; Ohmura *et al.*, 1992; Aboud *et al.*, 1993), which is the opposite order of potency to that at α_{1B} -adrenoceptors (e.g. Hanft & Gross, 1989). Chloroethylclonidine, the tool used by Mallard *et al.* (1992a), irreversibly activates prejunctional α_2 -autoreceptors in rat vas deferens, and evidence has been presented that prejunctional inhibition of noradrenaline release is responsible for the suppression of neurogenic twitches (Bültmann & Starke, 1993).

We re-investigated the coupling of calcium sources to specific α_1 -adrenoceptor subtypes in neurogenic contractions of rat vas deferens by means of four competitive antagonists, WB 4101, α -ethyl-3,4,5-trimethoxy- α -(3-((2-(2-methoxyphenoxy)ethyl)-amino)-propyl)benzeneacetonitrile (HV 723), prazosin and 5-methylurapidil. They distinguish between α_{1A} and α_{1B} subtypes (Morrow & Creese, 1986; Gross *et al.*, 1988) as well as between the α_{1H} , α_{1L} and α_{1N} subtypes of another recent subclassification system (Muramatsu *et al.*, 1990). Both epididymal and prostatic segments were studied because they differ in their α_1 -adrenoceptor mechanisms (Vardolov & Pennefather, 1976; Hay & Wadsworth, 1983). Adrenergic contractions relying on intracellular calcium mobilization were isolated by nifedipine and contractions relying on calcium entry were isolated by ryanodine (see Bourreau *et al.*, 1991; Bültmann *et al.*, 1993b). The interpretation of the results will be based on the accepted specificity of action of these two compounds. Some of these results have been communicated to the German Society for Pharmacology and Toxicology (Kurz *et al.*, 1993).

Methods

Male Wistar rats (240–360 g) were decapitated and the vasa deferentia removed, cleaned of adherent tissue and cut into three pieces equal in length of which the two outer ones were used as the epididymal and prostatic portion, respectively. The medium used for incubation and superfusion contained (mM): NaCl 118, KCl 4.8, CaCl₂ 2.5, KH₂PO₄ 0.9, NaHCO₃ 25, glucose 11, ascorbic acid 0.3 and disodium EDTA 0.03. It was saturated with 95% O₂/5% CO₂ and kept at 37°C.

Contraction

In neural stimulation experiments, the medium contained suramin (300 μ M) in order to suppress the purinergic contraction component (Mallard *et al.*, 1992b; Bültmann *et al.*, 1993a,b) and either nifedipine (10 μ M) or ryanodine (20 μ M) in order to isolate adrenergic contractions relying on calcium mobilization and calcium influx, respectively (Bourreau *et al.*, 1991; Bültmann *et al.*, 1993b). Prostatic or epididymal segments were suspended vertically in an organ bath, equilibrated and electrically stimulated as described (Bültmann *et al.*, 1993a,b; 0.3 ms pulse width, current strength 100 mA). There were up to 8 periods of electrical stimulation at 60 min intervals. Each stimulation period consisted of a single pulse, followed 10 s later by 3 pulses/100 Hz (3 p/100 Hz) and 5 min later by 50 p/5 Hz (Figure 1). Cumulative concentration-effect curves for α_1 -adrenoceptor antagonists were determined by stepwise concentration increases immediately after the first and each of the following stimulation periods.

When contractions induced by exogenous noradrenaline were examined, the medium contained nifedipine (10 μ M) or ryanodine (20 μ M) but no suramin. A fixed concentration of noradrenaline was added 8 to 11 times at 60 min intervals and left in the bath until the contraction had reached its maximum. Cumulative concentration-effect curves for α_1 -adrenoceptor antagonists were determined by concentration increases immediately after the first and each of the following responses to noradrenaline.

Contractions were measured at their maxima. They were expressed as a percentage of the first corresponding contraction in the same tissue and corrected for the mean corresponding percentage obtained in solvent-treated control

Figure 1 Adrenergic neurogenic contractions of epididymal (a,c) and prostatic (b) segments. The medium contained suramin 300 μ M and either nifedipine 10 μ M (a,b) or ryanodine 20 μ M (c) from the beginning. Shown are responses to the first stimulation period which consisted of a single pulse followed after 10 s by 3 p/100 Hz and after 5 min (break in tracings) by 50 p/5 Hz. Tissue tension was digitized at 100 Hz. In each experiment, the initial baseline tension was subtracted from all subsequent values. The tension values of the individual tissues thus obtained were then averaged at each time point sampled. Curves are the mean with the s.e.mean envelope from all control experiments ($n = 11$, 7 and 11 for a, b and c, respectively). In the presence of ryanodine, spontaneous contractions followed the neurogenic contractions proper.

tissues. For evaluation of concentration-effect relationships, logistic curves were fitted to the weighted mean percentage contractions using equation No. 25 of Waud (1976) and non-linear regression analysis, with the minimal drug effect fixed to zero (contraction 100%). The calculation yielded the maximal inhibition, the IC₅₀, i.e. the concentration of antagonist producing 50% of the maximal inhibition, and a slope value. In the case of 5-methylurapidil, the sum of two logistic functions was also fitted to the data, and the two fits (one logistic function *versus* the sum of two) were compared (p. 371 of Motulsky & Ransnas, 1987). Curves in Figures 2 to 5 are the fitted curves.

Tritium overflow

Experiments with [³H]-noradrenaline were carried out as described by Bültmann *et al.* (1993b). Briefly, slices of the prostatic portion were preincubated with [³H]-noradrenaline and then superfused. The medium did not contain suramin. The stimulation periods (S₁ to S₆) consisted of either 6 p/100 Hz or 50 p/5 Hz. Drugs or solvent were added after S₃. For evaluation of their effects on basal tritium efflux, ratios were calculated as efflux in the 2 min before S₄ over efflux in the 2 min before S₁. For evaluation of effects on

stimulation-evoked tritium overflow, the sum of the overflow elicited by S_1 to S_3 (S_{1-3}) and the sum of the overflow elicited by S_4 to S_6 (S_{4-6}) were obtained and the ratio S_{4-6}/S_{1-3} was then calculated.

Materials

Suramin hexasodium salt was a gift from Bayer (Wuppertal, Germany); (–)-tamsulosin HCl ((–)-YM-12617) from Dr C. Korstanje (Brocades, Delft, The Netherlands); α -ethyl-3,4,5-trimethoxy- α -(3-((2-(2-methoxyphenoxy)ethyl)-amino)-propyl)benzeneacetonitrile fumarate (HV 723) from Dr I. Muramatsu (Fukui, Japan); prazosin HCl from Pfizer (Karlsruhe, Germany); 5-methylurapidil from Dr K.H. Sanders (Byk Gulden, Konstanz, Germany). The following compounds were purchased: (±)-benoxathian HCl, ryanodine, (±)-2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane HCl (WB 4101), urapidil HCl (Biotrend, Köln, Germany); rauwolscine HCl (Roth, Karlsruhe, Germany); nifedipine, (–)-noradrenaline di-(+)-tartrate, tetrodotoxin (Sigma, Deisenhofen, Germany); (–)-[ring-2,5,6- 3 H]-noradrenaline, specific activity 56.9 Ci mmol $^{-1}$ (Du Pont, Dreieich, Germany). Nifedipine and ryanodine were dissolved in absolute ethanol (maximal final bath concentration below 0.1%), WB 4101 and 5-methylurapidil in 1 mM HCl, tetrodotoxin in 0.1 M sodium acetate buffer pH 4.85 and other drugs in distilled water. Experiments with nifedipine were carried out in near darkness or under sodium light.

Statistics

The arithmetic mean and s.e.mean are given throughout. Differences between means were tested for significance by the Mann-Whitney test. Differences with error probabilities <0.05 were taken to be statistically significant.

Results

Adrenergic neurogenic contractions

Neurogenic contractions were elicited in medium containing suramin 300 μ M in order to isolate the adrenergic component.

In the presence of nifedipine (10 μ M) to block calcium entry, all responses of the epididymal segment were twitch-like, even in the case of 50 p/5 Hz (i.e., the tonic contraction normally following the twitch during the 50 p/5 Hz train was suppressed by nifedipine; averaged responses to first stimula-

tion period in Figure 1a). The contractions remained approximately constant or declined slightly when stimulation periods were repeated at 60 min intervals in controls: in the last (7th) stimulation period they were $85.6 \pm 2.6\%$, $98.4 \pm 1.6\%$ and $103.2 \pm 1.7\%$ of those in the first period for single pulses, 3 p/100 Hz and 50 p/5 Hz, respectively ($n = 11$). Cumulative addition of increasing concentrations of WB 4101, HV 723, prazosin and 5-methylurapidil progressively and monophasically reduced and finally abolished contractions elicited by all three modes of stimulation (Figure 2; IC $_{50}$ values in Table 1). Rauwolscine 100 nM, when added after the first stimulation period for one further period, did not alter the twitch elicited by a single pulse but slightly increased the twitch elicited by the 3 p/100 Hz train 10 s later and also that elicited by 50 p/5 Hz (by 8 ± 1 and $17 \pm 1\%$, respectively; $n = 6$).

Contractions also of the prostatic end in the presence of nifedipine (10 μ M) were all twitch-like (Figure 1b). In control experiments amplitudes in the last (8th) stimulation period were $75.4 \pm 6.6\%$, $88.7 \pm 3.4\%$ and $84.1 \pm 4.4\%$ of those in

Figure 2 Inhibition by WB 4101 (■), HV 723 (○), prazosin (□) or 5-methylurapidil (●) of adrenergic neurogenic contractions in epididymal segments in the presence of suramin (300 μ M) and nifedipine (10 μ M). Shown is the inhibition of the twitch-like contraction elicited by single pulses (a), 3 p/100 Hz (b) and 50 p/5 Hz (c). Abscissae, α -adrenoceptor antagonist concentration. Ordinates show contraction as a percentage of pre-antagonist contraction, corrected for any change observed in controls. Means from 5 or 6 experiments. Standard errors of means did not exceed 6% of pre-antagonist contractions.

Table 1 IC $_{50}$ values (nM) for inhibition by α -adrenoceptor antagonists of adrenergic neurogenic contractions

Antagonist	Contractions elicited by		
	1 pulse	3 p/100 Hz	50 p/5 Hz
<i>Epididymal segments, nifedipine (10 μM) present</i>			
WB 4101	1.4 $\pm$ 0.1	6.0 $\pm$ 0.5	2.9 $\pm$ 0.1 (5)
HV 723	4.1 $\pm$ 0.3	16.8 $\pm$ 1.4	8.0 $\pm$ 0.2 (5)
Prazosin	9.0 $\pm$ 0.7	68.2 $\pm$ 4.3	18.1 $\pm$ 0.8 (6)
5-Methylurapidil	14.6 $\pm$ 0.1	44.2 $\pm$ 1.8	26.9 $\pm$ 1.4 (6)
<i>Prostatic segments, nifedipine (10 μM) present</i>			
WB 4101	0.5 $\pm$ 0.1	1.7 $\pm$ 0.3	0.8 $\pm$ 0.1 (4)
HV 723	2.6 $\pm$ 0.5	20.9 $\pm$ 3.5	4.2 $\pm$ 0.9 (4)
Prazosin	2.2 $\pm$ 0.1	22.0 $\pm$ 1.2	3.2 $\pm$ 0.4 (4)
5-Methylurapidil	16.0 $\pm$ 0.1	39.3 $\pm$ 4.1	25.9 $\pm$ 2.0 (3)
<i>Epididymal segments, ryanodine (20 μM) present</i>			
WB 4101		0.7 $\pm$ 0.1	0.8 $\pm$ 0.3 (5)
HV 723		2.7 $\pm$ 0.7	5.5 $\pm$ 0.3 (6)
Prazosin		2.7 $\pm$ 0.5	4.6 $\pm$ 0.3 (6)
5-Methylurapidil		20.4 $\pm$ 3.3	26.1 $\pm$ 4.4 (6)

IC $_{50}$ values were estimated from experiments shown in Figures 2, 3 and 4. Means $\pm$ s.e.mean from (n) experiments.

the first period for single pulses, 3 p/100 Hz and 50 p/5 Hz, respectively ($n = 7$). Responses were progressively and monophasically reduced and eventually abolished by increasing concentrations of WB 4101, HV 723, prazosin and 5-methylurapidil, for all three modes of stimulation (Figure 3; IC_{50} values in Table 1). Rauwolsine 100 nM, when added as mentioned above, did not alter the twitch elicited by a single pulse or 3 p/100 Hz but slightly increased the twitch elicited by 50 p/5 Hz (by $25 \pm 1\%$; $n = 5$).

In the presence of ryanodine ($20 \mu M$) to deplete intracellular calcium stores in epididymal portions, the only large responses were the twitch elicited by 3 p/100 Hz 10 s after the single pulse and a tonic response to 50 p/5 Hz (i.e., the twitches normally produced by the single pulse and initially during the 50 p/5 Hz train were suppressed by ryanodine; Figure 1c). In control experiments, amplitudes in the last (7th) stimulation period in the presence of solvent were $105.3 \pm 9.9\%$ and $80.7 \pm 13.6\%$ of those in the first for 3 p/100 Hz and 50 p/5 Hz, respectively ($n = 11$). Increasing concentrations of WB 4101, HV 723, prazosin and 5-methyl-

urapidil again caused progressive inhibition. However, whereas WB 4101, HV 723 and prazosin at sufficient concentrations produced complete blockade, only about 84% of the twitches elicited by 3 p/100 Hz and 69% of the tonic contractions elicited by 50 p/5 Hz were sensitive to 5-methylurapidil (Figure 4; percentages from curve fitting; IC_{50} values in Table 1). Rauwolsine (100 nM), when added as mentioned, did not significantly alter the twitch elicited by 3 p/100 Hz but slightly increased the tonic contractions at 50 p/5 Hz (by $26 \pm 7\%$; $n = 6$).

The only contraction in prostatic segments in the presence of ryanodine ($20 \mu M$) was a twitch at 3 p/100 Hz (not shown). Its inhibition by α -adrenoceptor antagonists was not investigated.

When ryanodine ($20 \mu M$) was added to tissue pre-exposed to nifedipine ($10 \mu M$) or *vice versa*, contractions were abolished ($n = 2$ for either sequence and for both epididymal and prostatic portions). Tetrodotoxin ($0.5 \mu M$) abolished all contractions ($n = 2$).

Contractions elicited by exogenous noradrenaline

Experiments using exogenous noradrenaline were conducted in suramin-free medium. Three additional α -adrenoceptor antagonists were tested, tamsulosin, benoxathian and urapidil. The former two were chosen for their ability to distinguish between α_{1A} and α_{1B} subtypes (Han *et al.*, 1987; Michel *et al.*, 1989; Büscher *et al.*, 1992), the latter for its structural similarity to 5-methylurapidil.

Noradrenaline failed to contract the epididymal portion in the presence of nifedipine $10 \mu M$ (cf. Hay & Wadsworth, 1983). In the prostatic portion, noradrenaline $30 \mu M$ elicited rapid and transient contractions of 3.8 ± 0.2 mN in the

Figure 3 Inhibition of WB 4101 (■), HV 723 (○), prazosin (□) or 5-methylurapidil (●) of adrenergic neurogenic contractions in prostatic segments in the presence suramin ($300 \mu M$) and nifedipine ($10 \mu M$). Shown is the inhibition of the twitch-like contraction elicited by single pulses (a), 3 p/100 Hz (b) and 50 p/5 Hz (c). Abscissae, α -adrenoceptor antagonist concentration. Ordinates show contraction as a percentage of pre-antagonist contraction, corrected for any change observed in controls. Means from 3 or 4 experiments. Standard errors of means did not exceed 12% of pre-antagonist contractions.

Figure 4 Inhibition by WB 4101 (■), HV 723 (○), prazosin (□) or 5-methylurapidil (●) of adrenergic neurogenic contractions in epididymal segments in the presence of suramin ($300 \mu M$) and ryanodine ($20 \mu M$). Shown is the inhibition of the twitch-like contraction elicited by 3 p/100 Hz (a) and the tonic contraction elicited by 50 p/5 Hz (b). Abscissae, α -adrenoceptor antagonist concentration. Ordinates show contraction as a percentage of pre-antagonist contraction, corrected for any change observed in controls. Means from 5 or 6 experiments. Standard errors of means did not exceed 12% of pre-antagonist contractions.

Table 2 IC_{50} values (nM) for inhibition by α -adrenoceptor antagonists of contractions elicited by exogenous noradrenaline

Antagonist	Prostatic segments, nifedipine (10 μ M) present	Epididymal segments, ryanodine (20 μ M) present
Tamsulosin	0.7 $\pm$ 0.1 (5)	1.0 $\pm$ 0.1 (5)
WB 4101	1.6 $\pm$ 0.2 (6)	1.3 $\pm$ 0.4 (5)
Benoxathian	6.6 $\pm$ 1.3 (5)	2.4 $\pm$ 0.4 (5)
HV 723	7.4 $\pm$ 0.9 (6)	5.6 $\pm$ 0.5 (5)
Prazosin	7.4 $\pm$ 2.1 (6)	7.0 $\pm$ 1.9 (5)
5-Methylurapidil	20.9 $\pm$ 3.2 (5)	26.9 $\pm$ 8.6* (7)
Urapidil	1583 $\pm$ 283 (4)	602 $\pm$ 144 (4)

IC_{50} values for WB 4101, HV 723, prazosin and 5-methylurapidil were estimated from experiments shown in Figure 5. Means $\pm$ s.e.mean from (n) experiments.

* IC_{50} for high affinity site; IC_{50} for low affinity site was $9.1 \pm 1.4 \mu$ M.

Figure 5 Inhibition by WB 4101 (■), HV 723 (○), prazosin (□) or 5-methylurapidil (●) of contractions elicited by exogenous noradrenaline. (a) Contractions of prostatic segments in the presence of nifedipine (10 μ M); noradrenaline concentration 30 μ M. (b) Contractions of epididymal segments in the presence of ryanodine (20 μ M); noradrenaline concentration 3 μ M. Abscissae, α -adrenoceptor antagonist concentration. Ordinates show contraction as a percentage of pre-antagonist contraction, corrected for any change observed in controls. Means from 5 to 7 experiments. Standard errors of means did not exceed 12% of pre-antagonist contractions.

presence of nifedipine 10 μ M ($n = 46$); noradrenaline 30 μ M was approximately an EC_{50} under these conditions (Bültmann, unpublished data). The contractions remained approximately constant when noradrenaline was added every 60 min in controls: upon the last (8th) addition they were $96.0 \pm 4.9\%$ of those obtained upon the first addition ($n = 9$). WB 4101, HV 723, prazosin and 5-methylurapidil progressively and monophasically reduced and finally abolished the contractions (Figure 5a). The same was true for tamsulosin, benoxathian and urapidil. IC_{50} values are in Table 2.

Epididymal segments were chosen for experiments with ryanodine, since it was in this portion that the 5-methylurapidil resistance of neurogenic contractions had been observed (Figure 4). In the presence of ryanodine (20 μ M), exogenous noradrenaline 3 μ M caused tonic contractions similar in height (9.2 ± 0.5 mN; $n = 45$) and shape to

Table 3 Effect of α -adrenoceptor antagonists on evoked tritium overflow from vas deferens slices preincubated with [3 H]-noradrenaline

Drug present from S_4 to S_6 (μ M)	S_4 - S_6 / S_1 - S_3 ratio	
	6 p/100 Hz	50 p/5 Hz
Solvent	0.99 $\pm$ 0.04 (12)	1.08 $\pm$ 0.04 (14)
Tetrodotoxin 0.5	0.86 $\pm$ 0.04 (5)*	
WB 4101 0.032	0.10 $\pm$ 0.02* (4)*	0.06 $\pm$ 0.02* (3)
HV 723 0.32	0.94 $\pm$ 0.08 (4)	1.52 $\pm$ 0.13* (4)
Prazosin 0.32	0.90 $\pm$ 0.09 (4)	1.74 $\pm$ 0.06* (4)
Prazosin 0.32	0.99 $\pm$ 0.09 (4)	1.66 $\pm$ 0.06* (4)
5-Methylurapidil 1	1.07 $\pm$ 0.08 (4)	1.67 $\pm$ 0.07* (4)
Rauwolscine 0.1	0.94 $\pm$ 0.09 (4)*	2.60 $\pm$ 0.19* (4)

Stimulation periods (S_1 - S_6) consisted either of 6 p/100 Hz or of 50 p/5 Hz. Solvent or drug was added after S_3 , 64 min before S_4 , and kept for the remainder of the experiment. The fractional rate of tritium efflux immediately before S_1 was $0.00192 \pm 0.00004 \text{ min}^{-1}$ ($n = 65$). The overflow evoked by S_1 to S_3 (sum of S_1 , S_2 and S_3) was $0.129 \pm 0.005\%$ of tissue tritium for 6 p/100 Hz ($n = 28$) and $0.245 \pm 0.087\%$ for 50 p/5 Hz ($n = 37$). S_4 - S_6 / S_1 - S_3 values are ratios of overflow elicited by S_4 to S_6 over overflow elicited by S_1 to S_3 . Means and s.e.mean from (n) slices.

*Significantly different from solvent controls ($P < 0.05$).

*From Bültmann *et al.* (1993b).

contractions elicited by 50 p/5 Hz in the presence of ryanodine (Figure 1c). Noradrenaline is a more potent agonist in epididymal than prostatic portions (Vardolov & Pennefather, 1976; Sallés & Badia, 1991), and 3 μ M was approximately an EC_{50} (Bültmann, unpublished data). Contractions produced by the last (11th) exposure to noradrenaline were $105.3 \pm 7.6\%$ of first contractions in controls ($n = 8$). WB 4101, HV 723 and prazosin progressively and monophasically reduced and finally abolished noradrenaline-induced contractions (Figure 5b), as did tamsulosin and benoxathian. The concentration-effect relationship of 5-methylurapidil, in contrast, was significantly ($P < 0.05$) better fit by a biphasic curve. Only about 60% of noradrenaline-induced contractions were sensitive to low concentrations of 5-methylurapidil (percentage from curve fitting), and the two IC_{50} values differed by a factor of about 340 (Figure 5b; IC_{50} values in Table 2). The concentration-effect curve of urapidil resembled the left-hand part of the curve of 5-methylurapidil: it levelled off at about 78% inhibition (percentage from curve fitting; Table 2).

Tritium overflow

Experiments on vas deferens slices preincubated with [3 H]-noradrenaline were carried out in order to detect possible presynaptic effects of the α -adrenoceptor antagonists. Release of [3 H]-noradrenaline was measured as overflow of total

tritiated compounds. In solvent controls, the evoked overflow of tritium remained approximately constant (Table 3). Tetrodotoxin practically abolished the evoked overflow. WB 4101, HV 723, prazosin and 5-methylurapidil, when given at the highest concentrations used in neurogenic contraction experiments, did not change the overflow of tritium elicited by 6 p/100 Hz, and the same was true for rauwolsine (100 nM). All antagonists, however, increased the overflow elicited by 50 p/5 Hz. The effect was marked for rauwolsine and smaller for the other antagonists. HV 723, prazosin and 5-methylurapidil accelerated the basal outflow of tritium.

Discussion

Nature of contractions

The neurogenic contractions elicited in this study were adrenergic since they were abolished by α -adrenoceptor antagonists. The postjunctional receptors involved were only α_1 since the α_2 -adrenoceptor antagonist, rauwolsine, did not cause any inhibition of the responses (in contrast to mouse *vas deferens*: Bültmann *et al.*, 1991). Contractions remaining in the presence of nifedipine were abolished by ryanodine, and contractions remaining in the presence of ryanodine were abolished by nifedipine. Under the premise stated in the Introduction, concerning the specificity of action of nifedipine and ryanodine, this observation shows that contractions remaining in the presence of nifedipine were due to intracellular calcium mobilization, whereas contractions remaining in the presence of ryanodine were due to calcium entry.

The complex stimulation protocol needs a brief comment. One aim was to study both twitch-like and tonic contractions, another to obtain calcium mobilization-mediated and calcium entry-mediated contractions of similar size and shape. Calcium mobilization and entry usually lead to responses of different shape, twitch-like and tonic, respectively (see Introduction). However, application of a 3 p/100 Hz train 10 s after a single pulse elicited a sizeable twitch in the presence of ryanodine (i.e. calcium entry-mediated), possibly due to the increase in calcium entry that has been suggested to occur when a second neurogenic contraction follows a priming contraction within a few seconds (Bültmann *et al.*, 1993b).

Rauwolsine did not change contractions elicited by single pulses but occasionally increased contractions elicited 10 s later by 3 p/100 Hz and always increased the response to 50 p/5 Hz. Moreover, rauwolsine did not change the release of [3 H]-noradrenaline elicited by 6 p/100 Hz but greatly increased the release elicited by 50 p/5 Hz. This is the pattern of effect expected from the operation of prejunctional α_2 -autoreceptors (see Starke *et al.*, 1989) and indicates, moreover, that postjunctional consequences of prejunctional α_2 -autoinhibition were small under the conditions used. The increase in [3 H]-noradrenaline release (50 p/5 Hz) produced by WB 4101, HV 723, prazosin and 5-methylurapidil was similar and less than in the case of rauwolsine. Prejunctional α_2 -autoreceptor blockade by the four drugs, therefore, did not interfere with the comparison of their effects at postjunctional α_1 -adrenoceptors.

α_1 -Adrenoceptors and calcium source-specific contractions

Our results indicate that two types of α_1 -adrenoceptor are involved in adrenergic neurogenic contractions of rat *vas deferens*. The first, main one mediates all calcium mobilization-dependent contractions (isolated by nifedipine) and the major part of calcium entry-dependent contractions (isolated by ryanodine); it corresponds to the α_{1A} subtype. The second receptor mediates a minor part of calcium entry-

dependent contractions; it does not correspond to any named α_1 subtype.

The common receptor for calcium mobilization and entry

In support of one common receptor for calcium mobilization and entry, four antagonists displayed a similar rank order of potency against twitches elicited by single pulses in the presence of nifedipine (Figure 2a, 3a); twitches elicited by 3 p/100 Hz in the presence of nifedipine (Figure 2b, 3b); twitches that developed initially in response to 50 p/5 Hz in the presence of nifedipine (Figure 2c, 3c); twitches elicited by 3 p/100 Hz in the presence of ryanodine (Figure 4a); and tonic contractions in response to 50 p/5 Hz in the presence of ryanodine (Figure 4b). The pulse patterns applied created different extracellular concentrations of noradrenaline, and this probably accounts for the fact that the antagonists usually were very effective against single pulse responses, less effective against responses to 50 p/5 Hz and least effective against 3 p/100 Hz (Table 1). The rank order of potency, however, was common to all pulse patterns. It has been suggested that twitches are mediated by junctional receptors and tonic contractions by extrajunctional receptors (Brown *et al.*, 1983), and these have been equated with α_{1B} and α_{1A} , respectively (Mallard *et al.*, 1992a). However, we infer from our experiments that junctional and extrajunctional receptors are pharmacologically alike.

In radioligand binding studies in rat tissues, α_{1A} binding sites are characterized by affinity ratios prazosin/WB 4101 = 0.4–2.5 and prazosin/5-methylurapidil = 2.5–16, whereas at α_{1B} sites these affinity ratios are prazosin/WB 4101 = 10–50 and prazosin/5-methylurapidil = 100–1200 (Morrow & Creese, 1986; p. 110 of Minneman, 1988; Gross *et al.*, 1988; Hanft & Gross, 1989; Michel *et al.*, 1989; Michel *et al.*, 1990). The corresponding ratios for the main α_1 -adrenoceptor mediating neurogenic contractions in rat *vas deferens* were prazosin/WB 4101 = 0.1–0.25 and prazosin/5-methylurapidil = 0.6–8.1 (calculated from the IC_{50} values of Table 1). Both ratios are close to, or overlapping with, the corresponding α_{1A} but far from the corresponding α_{1B} ratios. The relatively low prazosin/5-methylurapidil affinity ratio of 0.6–8.1 also distinguishes the *vas deferens* receptor from cloned α_1 sites (Perez *et al.*, 1991; Schwinn & Lomasney, 1992). In terms of the $\alpha_{1A/B}$ classification system, the common receptor involved in all neurogenic contraction types, calcium mobilization- as well as calcium entry-mediated, corresponds best to α_{1A} . α_{1A} -Adrenoceptors have previously been suggested to mediate the tonic phase of adrenergic neurogenic contractions (Mallard *et al.*, 1992a), in accord with the present results. The twitch-like phase was suggested to be α_{1B} -mediated (Mallard *et al.*, 1992a). Here we disagree: the rank order of antagonist potency, in accordance with previous observations (see Introduction), indicates an α_{1A} character; the inhibition of the twitches by chloroethylclonidine (Mallard *et al.*, 1992a) presumably was due to activation of prejunctional α_2 - rather than blockade of postjunctional α_{1B} -adrenoceptors (Bültmann & Starke, 1993). Since this paper was submitted, Aboud *et al.* (1993) suggested that adrenergic twitches of rat *vas deferens* were mediated by a non- α_{1A} , non- α_{1B} -adrenoceptors because they were (i) reduced by chloroethylclonidine (arguing against α_{1A}) and (ii) blocked by competitive antagonists such as WB 4101 with high potency (arguing against α_{1B}); if one assumes a prejunctional explanation for the effect of chloroethylclonidine, the results of these authors are compatible with our α_{1A} postulate.

The experiments discussed so far rule out a selective coupling of α_{1A} -adrenoceptors to calcium entry and α_{1B} -adrenoceptors to calcium mobilization in neurogenic contractions. Does the differential link hypothesis hold true for exogenous noradrenaline, as originally suggested (Han *et al.*, 1987)? This seems also unlikely. All seven antagonists attenuated the effect of exogenous noradrenaline with the same potency

rank order in the presence of nifedipine (Figure 5a) and in the presence of ryanodine (Figure 5b); even the two absolute IC_{50} values for any given antagonist agreed well (Table 2). For WB 4101, HV 723, prazosin and 5-methylurapidil the rank order was identical with that against neurogenic contractions, indicating an α_{1A} -adrenoceptor, as discussed, a classification confirmed by the high potency of tamsulosin and benoxathian (Han *et al.*, 1987; Michel *et al.*, 1989; Büscher *et al.*, 1992). Our results do not exclude the possibility that noradrenaline, acting at α_{1B} -adrenoceptors, can elicit calcium mobilization and ensuing contractions in rat vas deferens (Han *et al.*, 1987; Martinotti *et al.*, 1991; Aboud *et al.*, 1993). However, no indication for an α_{1B} component was found under the present conditions.

Effects of WB 4101, HV 723 and prazosin on adrenergic neurogenic contractions of rat vas deferens have also been reported by Ohmura *et al.* (1992). The authors used one pulse pattern only and did not isolate calcium entry-dependent responses. However, their antagonist affinity rank order and their conclusion – one main receptor for twitches and tonic contractions and for calcium mobilization and entry – are identical to ours. Ohmura *et al.* (1992) classified the receptor as α_{1L} in terms of the $\alpha_{1H/L/N}$ proposal (Muramatsu *et al.*, 1990). Given the excellent agreement with our values, the receptor characterized in the present study also fits the α_{1L} type. Decision between the α_{1A} and α_{1L} designations has to await clarification of the relationship between the $\alpha_{1A/B}$ and $\alpha_{1H/L/N}$ nomenclature (but see the criticism of the latter scheme by Esbenshade *et al.*, 1993).

The receptor selective for calcium entry

Unfortunately, the conclusion from the present study is not a 'one physiological receptor' concept. Concentration-effect curves in epididymal portions, in the presence of ryanodine to isolate calcium entry, showed that part of the effect of both endogenous (Figure 4) and exogenous noradrenaline (Figure 5b) was resistant to moderate concentrations of 5-methylurapidil. The same was true for urapidil (tested against exogenous noradrenaline only). The remaining contractions were α_1 -adrenoceptor-mediated since no resistant component was observed with any other antagonist. Hence, there is a second α_1 -adrenoceptor. It is characterized by similar and

high affinity for tamsulosin, WB 4101, benoxathian, HV 723 and prazosin but very low affinity for 5-methylurapidil and urapidil, with a prazosin/WB 4101 affinity ratio of 0.2 (identical to the α_{1A} receptor) but a prazosin/5-methylurapidil affinity ratio of 1,300 (from IC_{50} values in Table 2). Obviously, it is neither α_{1A} nor α_{1B} . It also differs from the cloned α_1 sites mentioned. It resembles, however, a [3H]-prazosin binding site in rat lung that resists inactivation by chloroethylclonidine and possesses a prazosin/WB 4101 affinity ratio of 0.8 and a prazosin/5-methylurapidil ratio of 140 (Hiramatsu *et al.*, 1992; see Han & Minneman, 1991, for possibly similar sites in rat heart and hippocampus). No 5-methylurapidil or urapidil resistance was observed in the presence of nifedipine, suggesting that the second α_1 -adrenoceptor in rat vas deferens operates exclusively through activation of calcium entry.

Conclusion

The study suggests some revision of the current view of α_1 -adrenoceptor subtypes and calcium sources in adrenergic neurogenic contractions of the rat vas deferens. Two α_1 -adrenoceptor subtypes mediate these contractions. The main one, pharmacologically α_{1A} (or α_{1L}), activates both calcium entry and mobilization. A second receptor, not previously detected in the vas deferens and not corresponding to any named α_1 subtype, is linked exclusively to calcium entry. Both subtypes and their respective transduction pathways similarly contribute to contractions elicited by exogenous noradrenaline. α_{1B} -Adrenoceptors did not mediate contraction under the present experimental conditions, irrespective of whether the contractions were twitch-like or tonic, neurogenic or caused by exogenous noradrenaline, due to calcium entry or due to intracellular calcium mobilization.

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