Br. J. Pharmacol. (1988), **94**, 890–900

Muscarinic inhibition of [^{3}H]-noradrenaline release on rabbit iris *in vitro*: effects of stimulation conditions on intrinsic activity of methacholine and pilocarpine

[1]Irene T. Bognar, Sylvia Pallas, Hermann Fuder & Erich Muscholl

Pharmakologisches Institut der Universität, Obere Zahlbacher Strasse 67, D-6500 Mainz, Federal Republic of Germany

1 Rabbit isolated irides were loaded with [^{3}H]-noradrenaline and superfused with Tyrode solution. The inhibition by the muscarinic agonists (±)-methacholine and pilocarpine of the [^{3}H]-noradrenaline overflow into the superfusate evoked by field stimulation (pulses of 1 ms duration, 75 mA) was measured as an index of activation of presynaptic muscarinic receptors.

2 The fractional rate of release per pulse during the first stimulation period (S1) was low with 360 pulses at 3 Hz, intermediate with 360 pulses at 10 Hz and high with 1200 pulses at 10 Hz. Upon repetitive stimulation (7 periods at 20 min intervals), the fractional rates of release per pulse during S7 no longer differed, suggesting a 'long-term' regulation of [^{3}H]-noradrenaline release depending on the stimulation conditions.

3 The evoked [^{3}H]-noradrenaline overflow was depressed by (±)-methacholine in a concentration-dependent manner. The EC_{50} ranged from 0.29 to 0.42 μM. Methacholine nearly abolished the transmitter release evoked at 3 Hz but reduced that induced at 10 Hz by only 50%. Under the latter condition the methacholine concentration-inhibition curve was bell-shaped and no muscarinic inhibition was observed in the presence of methacholine 30 μM. After washout of methacholine the evoked [^{3}H]-noradrenaline release was temporarily enhanced.

4 Atropine 0.1 μM enhanced the [^{3}H]-noradrenaline overflow (evoked by stimulation with 360 or 1200 pulses at 10 Hz), probably antagonizing a presynaptic inhibition by endogenous acetylcholine. The inhibition by methacholine was competitively antagonized by atropine 0.1 μM (apparent $-\log K_B = 8.5$–9.0).

5 Depending on the concentration, pilocarpine reduced the [^{3}H]-noradrenaline overflow evoked by 360 pulses at 3 Hz up to 63%. However, at 10 Hz stimulation frequency the compound was inactive as an agonist but competitively antagonized the presynaptic inhibition induced by methacholine. The K_B under the latter condition (0.95 μM) was very close to the EC_{50} value determined at 3 Hz (0.85 μM).

6 The results demonstrate a muscarinic inhibition of noradrenaline release from the rabbit isolated iris. The activation by pilocarpine of the presynaptic receptors provides an alternative explanation for the miosis induced in the rabbit *in vivo*, which might be the result of a decreased sympathetic tone in the iris dilator muscle.

Introduction

It is generally accepted that the miosis induced by topical application of parasympathomimetic agents is due to stimulation of postsynaptic muscarinic receptors on the iris sphincter muscle. This view is supported by the finding that muscarinic agonists contract the isolated iris sphincter muscle *in vitro* in an atropine-sensitive, concentration-dependent manner (Boros & Takáts, 1961; Yamauchi *et al.*, 1973; Salazar *et al.*, 1976). Pilocarpine, however, showed a very low intrinsic activity in contracting rabbit iris sphincter strips and competitively antagonized the contractions elicited by the full agonist carbachol (Akesson *et al.*, 1983). This observation markedly contrasts with the effectiveness of pilocar-

[1]Author for correspondence.

pine in reducing the pupillary size of the rabbit eye *in vivo* (Bynke *et al.*, 1985) and raises the question about the mechanism underlying the miotic effect of this compound. It was suggested that pilocarpine may act indirectly by reducing the sympathetic tone of the iris dilator (Akesson *et al.*, 1983). This suggestion was supported by unpublished results obtained by the same group: in rabbits pretreated with reserpine pilocarpine not only failed to produce a miosis but even caused a slight mydriasis.

The occurrence of presynaptic muscarinic receptors inhibiting noradrenaline release has been established in virtually all peripheral tissues tested so far (Starke, 1977; Muscholl, 1980). A physiological role of the muscarinic inhibition is likely, at least in some organs in which the stimulation of parasympathetic nerves reduces the release of noradrenaline elicited by simultaneously applied sympathetic nerve stimulation (for review see Muscholl, 1980).

Histochemical studies on irides from several species have shown that both dilator and sphincter muscles receive a dual autonomic innervation (Laties & Jacobowitz, 1964; Nilsson, 1964; Richardson, 1964; Hökfelt & Nilsson, 1965; Ehinger, 1966). The sympathetic terminals in the iris sphincter of the guinea-pig, for instance, represent about 15% of the axons present in the tissue (Nishida & Sears, 1969a), whereas the non-sympathetic nerve fibres in the dilator muscle represent about 33% of the total number of nerves (Nishida & Sears, 1969b). Moreover, Ehinger *et al.* (1970) observed membrane to membrane contact sites between adjacent adrenergic and cholinergic varicosities in the rat iris.

A detailed pharmacological analysis of muscarinic modulation of the noradrenergic transmission in the iris, however, is lacking. To our knowledge, only one brief study provided evidence that sympathetic nerve endings in the rabbit iris-ciliary body are endowed with presynaptic muscarinic receptors, which undergo considerable tonic activation by the endogenous acetylcholine released during field stimulation (Jumblatt & North, 1986).

In the present study we investigated the effects of the muscarinic agonists methacholine and pilocarpine and those of atropine on the [^{3}H]-noradrenaline overflow evoked by field stimulation in rabbit isolated irides. The tissue used included the dilator as well as the sphincter muscles but was free from the surrounding ciliary body.

Preliminary accounts of some of this work have been given (Fuder *et al.*, 1986; Bognar *et al.*, 1987).

Methods

Preparation of rabbit isolated irides

Rabbits of mixed strains (albino and non-albino) and either sex (1.6–3.1 kg weight) were killed by a sharp blow to the back of the neck and exsanguinated via the left carotid artery. The eyes were quickly removed and the irides, excluding the ciliary bodies, dissected free as a ring containing sphincter and dilator. For labelling of the noradrenaline stores, irides were suspended for 30 min in a glass tube filled with 5 ml of Tyrode solution (composition in mM: NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHCO_3$ 11.9, NaH_2PO_4 0.42, D-glucose 5.6, (+)-ascorbic acid 0.057) containing 1 μCi ml^{-1} of (−)-[7,8^{3}H]-noradrenaline (specific activity: 11–15 Ci mmol^{-1}). The tissues were then rinsed and placed horizontally between two parallel platinum wire electrodes (2 mm apart, 10 mm length) in a 2 ml jacketed organ bath. The irides were superfused with Tyrode solution at a constant rate of 2 ml min^{-1}. The tubes, solution reservoirs and the bath were gassed continuously with 95% O_2 plus 5% CO_2 and kept at 37°C.

Design of experiments and determination of stimulation-evoked [^{3}H]-noradrenaline overflow

In order to wash out radioactivity not bound to synaptic vesicles, the tissue was superfused for 65 min before the first field stimulation period (S1) was applied. A 'conditioning stimulation' (S_0; same stimulation parameters as in the rest of the experiment) was performed 45 min after the beginning of superfusion. The release evoked by S_0 was not determined. Generally, 7 field stimulation periods were carried out at intervals of 20 min (S1–S7), each consisting of 360 or 1200 square-wave pulses (1 ms duration, 75 mA current strength) delivered by a Grass S6 stimulator at a frequency of 3 or 10 Hz. The impulse flow was monitored on an oscilloscope.

For the measurement of tritium overflow, the superfusate was collected continuously in 5 min samples commencing 5 min before S1. The tubes contained 1 M HCl (to adjust the pH to 2–3) and 1 mg ascorbic acid. One ml of the fluid was used to determine the total tritium content and the remainder was subjected to column chromatography according to Graefe *et al.* (1973) to separate [^{3}H]-noradrenaline from its tritiated metabolites. Occasionally, the quantities of tritiated metabolites were determined. The tritium activity was measured by liquid scintillation spectrometry and corrected for the counting efficiency by an external standardization method (scintillation cocktail: Instagel, Packard Instruments). The values for [^{3}H]-noradrenaline and metabolite contents were corrected for minor cross contaminations (Fuder *et al.*, 1982) and expressed as fmol referring to the specific activity of the [^{3}H]-noradrenaline used for loading. The stimulation-evoked efflux of tritium and [^{3}H]-noradrenaline (in fmol per stimulation period)

was calculated by subtracting the mean basal overflow (calculated from the 5 min samples immediately preceding and 15 min after the onset of stimulation) from the overflow contained in the two samples (10 min) collected during and after the stimulation period. The overflow of radioactivity evoked by S2–S7 was expressed as a percentage of that evoked by S1.

The testing of drugs and analysis of data

To test the effects of the muscarinic agonists methacholine and pilocarpine on the evoked overflow, the drugs were introduced (2–3 concentrations increasing by a factor of 3 or 10) into the Tyrode solution 10 min before the respective stimulation period (S3–S4 or S3–S5) and remained present for 20 min. Thereafter, the irides were again superfused with drug-free solution until the end of the experiment. The evoked overflows at S1, S2 and S7 were displayed in a co-ordinate system and fitted to a straight line by regression analysis. Predicted overflows at S3 to S6 in the absence of agonist (predicted individual control values) were estimated from the regression lines. Concentration-response curves for the inhibition of evoked overflow by muscarinic agonists were constructed by expressing the evoked overflow values at a given stimulation period in the presence of agonist as a percentage of the predicted individual control values. Total suppression of evoked overflow corresponded to 100% inhibition. The concentration of agonist at half-maximal inhibition (EC_{50} values of the agonist) was estimated graphically from the mean curves.

In an additional series of experiments, methacholine concentration-inhibition curves were obtained in the presence of atropine (0.1 μM) or pilocarpine (50 μM). The antagonists were added to the superfusion solution 10 min before S3 and remained throughout the experiment. Two or three concentrations of agonist were tested at S4–S5 or S4–S6, respectively, in one preparation. Predicted individual control values in the presence of antagonist were derived from the regression lines between S3 and S7. The EC_{50} values for methacholine in the presence and absence of antagonist were estimated as described above, and from the dose-ratio an 'apparent dissociation constant' (K_B) for each antagonist was calculated according to the following equation: K_B = antagonist concentration/(dose-ratio − 1) (Furchgott, 1972).

Drug effects on the spontaneous overflow of [^{3}H]-noradrenaline were investigated by comparing the relative changes in basal overflow (with respect to the first sample of spontaneous overflow) occurring in the presence of drugs with those observed under control conditions (drugs not added).

Extraction of tritium and [^{3}H]-noradrenaline from the irides

In order to calculate the fractional rate of ^{3}H-transmitter release per pulse, the [^{3}H]-noradrenaline content of the tissues was determined after the end of experiments. In the first series of experiments, the irides were homogenized in 3 ml $HClO_4$ 0.4 M (3 × 1 ml) for 1 min with an Ultraturrax at maximal setting. After centrifugation (6,000 r.p.m. for 10 min), the total tritium and [^{3}H]-noradrenaline content of the supernatant was determined. In agreement with previous observations on the rat iris (Jonsson *et al.*, 1969) the [^{3}H]-noradrenaline in the rabbit irides represented 95.2 ± 1.4% (n = 7) of the total tritium recovered by this procedure. This percentage was used to calculate the [^{3}H]-noradrenaline content in further experiments in which the irides were immersed overnight in 5 ml $HClO_4$ (0.4 M) and only the total tritium content of the extract was measured. The amount of tritium recovered after one overnight extraction represented 99.8 ± 0.02% (n = 5) of the total tissue tritium i.e. the yield was increased only negligibly by repeating the extraction and measurement twice, followed by homogenization of the extracted iris and determination of the remaining tritium as described above.

The mean [^{3}H]-noradrenaline content of the irides was 15.9 ± 0.2 pmol per iris (n = 14) under various control conditions and did not differ significantly between groups of different treatment. The wet weight of an iris determined at the end of the experiments ranged from 10 to 22 mg.

Statistical analysis

Results are given as the mean ± s.e. mean. Statistical differences between means were determined by use of Student's t test for unpaired observations and, if more than one group of treatments was to be compared, by analysis of variance followed by Newman-Keuls test (for simultaneous comparisons) or Dunnett's test (comparison with one control group). Statistical differences between series of points in mean concentration-inhibition curves were assessed by modified t test according to Bonferroni (Wallenstein *et al.*, 1980).

Drugs

The following drugs were used: atropine sulphate (Boehringer, Ingelheim); (−)-[7,8-^{3}H]-noradrenaline

(Amersham-Buchler, Braunschweig); (±)-methacholine chloride (Sigma, St. Louis); pilocarpine hydrochloride (Boehringer, Ingelheim) and tetrodotoxin (Sigma, St. Louis).

Results

Isolated irides preloaded with [^{3}H]-noradrenaline were subjected to 7 stimulation periods which consisted of 1200 pulses at a frequency of 10 Hz or of 360 pulses at either 3 or 10 Hz. Albino as well as pigmented irides were used in all experimental groups. Neither the basal nor the evoked overflow of tritium compounds under control conditions, nor the effects of the various muscarinic drugs differed between the two types of irides. Hence, the results obtained with albino and pigmented irides were pooled.

Overflow of ^{3}H-compounds from the resting and stimulated irides

In the present study, the [^{3}H]-noradrenaline overflow was chosen as an index of neurotransmitter release from rabbit isolated irides. The total tritium content of the samples, however, was always determined in order to detect any changes in the overflow of the metabolites caused by the various drugs under study. Additionally, the contribution to the total radioactivity of individual tritiated compounds was determined for the first three stimulation periods in some of the control series of experiments. As an example, the pattern of metabolites in the overflow from irides stimulated with 1200 pulses at 10 Hz is shown in Figure 1.

The spontaneous overflow of [^{3}H]-noradrenaline in the collection period immediately before S1 accounted for 15.9 ± 2.6% ($n = 14$, mean value pooled from control groups) of the total tritium overflow, although for an unknown reason the variation between different experimental groups was rather large (Figures 1 and 2). ^{3}H-O-methylated deaminated metabolites (^{3}H-OMDA) and [^{3}H]-3,4-dihydroxyphenylglycol ([^{3}H]-DOPEG) comprised 40 and 30%, respectively, of the sum of all the ^{3}H-compounds under resting conditions . [^{3}H]-3,4-dihydroxymandelic acid ([^{3}H]-DOMA) and [^{3}H]-normetanephrine ([^{3}H]-NMN) were only minor metabolites, each contributing to the total spontaneous tritium overflow by at the most 5%.

Field stimulation of the irides caused a marked increase in tritium overflow which consisted mainly of unmetabolized [^{3}H]-noradrenaline. Upon stimulation at 10 Hz, [^{3}H]-noradrenaline contributed about 80% to the evoked increase in tritium overflow. The efflux of the main tritiated metabolites

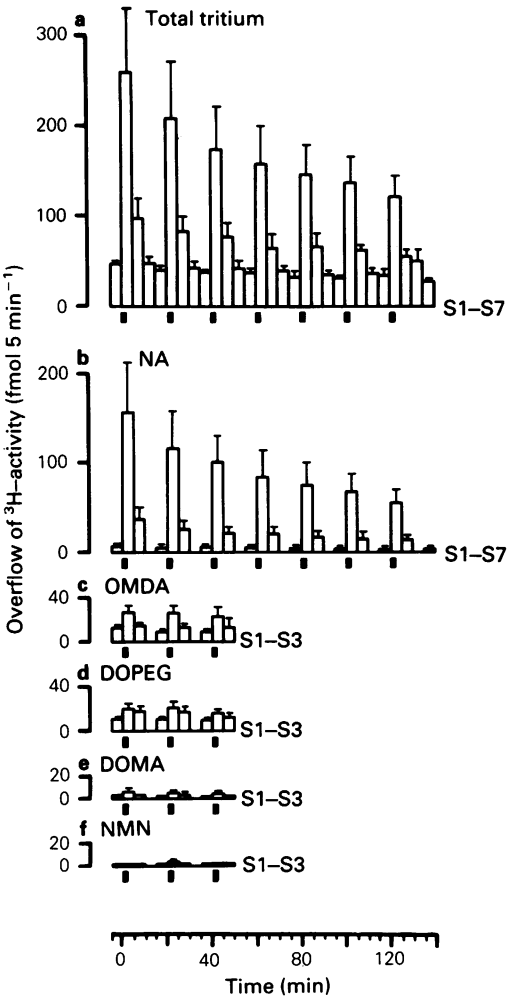

Figure 1 The effects of 7 successive field stimulation periods (S1–S7) on the overflow of ^{3}H-activity from 4 rabbit isolated irides. Time (min, abscissa scale) '0' corresponds to 70 min after the end of the loading procedure (1 μCi ml^{-1} [^{3}H]-noradrenaline for 30 min). The irides were then superfused with Tyrode solution at a rate of 2 ml min^{-1}. When indicated by filled squares 1200 pulses (1 ms duration, 75 mA current strength) were delivered at a frequency of 10 Hz. Values are means (vertical lines indicate s.e. mean) of the amounts of (a) total tritium, (b) noradrenaline and (c–f) ^{3}H-compounds in the superfusate (5 min collection periods) expressed as fmol per 5 min referring to the specific activity of the [^{3}H]-noradrenaline used for labelling of the noradrenaline stores. Abbreviations: NA = noradrenaline; OMDA = O-methylated deaminated metabolites (sum of 3-methoxy-4-hydroxyphenylglycol and 3-methoxy-4-hydroxymandelic acid); DOPEG = 3,4-dihydroxyphenylglycol; DOMA = 3,4-dihydroxymandelic acid; NMN = normetanephrine.

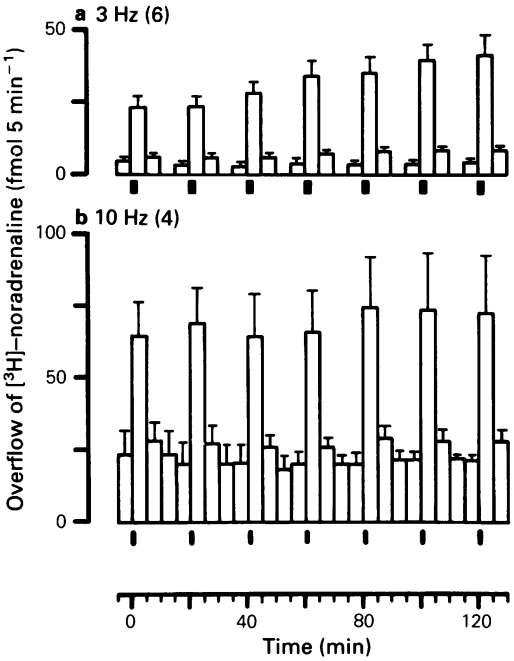

Figure 2 The effects of 7 successive field stimulation periods (S1–S7) on the [^{3}H]-noradrenaline overflow from superfused rabbit isolated irides. When indicated by filled squares (■, ▮) 360 pulses (1 ms duration, 75 mA current strength) were delivered at a frequency of (a) 3 Hz or (b) 10 Hz. Values are means (vertical lines indicate s.e. mean) of the number of experiments in parentheses. For further explanations see legend to Figure 1.

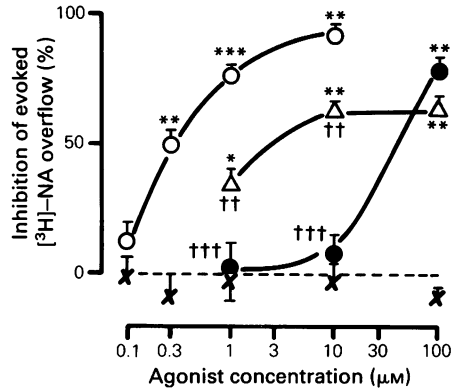

Figure 3 The inhibition of the stimulation-evoked (360 pulses, 3 Hz) [^{3}H]-noradrenaline ([^{3}H]-NA) overflow from the superfused rabbit isolated irides by (±)-methacholine in the absence (○) and presence (●) of atropine 0.1 μM, and by pilocarpine (△). The inhibition is expressed as a percentage of total suppression of the evoked [^{3}H]-NA release. The predicted individual control values (X) in experiments with stimulation in the absence of agonist (controls) did not differ from 0 (broken line). Each value is the mean of 3–7 individual observations; vertical lines indicate s.e. mean. Statistical comparison between curves, (Bonferroni test) *, † $P < 0.05$, **, †† $P < 0.01$, ***, ††† $P < 0.001$: *compared to controls, †compared to methacholine alone.

OMDA and DOPEG was also increased (Figure 1). Stimulation at 3 Hz, in contrast, failed to affect the overflow of the various ^{3}H-metabolites (not shown) but significantly enhanced that of [^{3}H]-noradrenaline (Figure 2).

The [^{3}H]-noradrenaline efflux in response to 1200 pulses at 10 Hz was abolished in the presence of tetrodotoxin 0.3 μM ($n = 3$, not shown). Thus, opening of sodium channels precedes transmitter release.

The fractional rate of [^{3}H]-noradrenaline release per pulse during S1 in the absence of any drug varied depending on the number of pulses applied and on the frequency at which the pulses were delivered (Table 1). Upon stimulation with 1200 pulses at 10 Hz the amounts of [^{3}H]-noradrenaline released per pulse were significantly greater than those evoked by each of 360 pulses delivered at the same stimulation frequency (10 Hz). Upon stimulation with 360 pulses at 3 Hz, the fractional rate of

Table 1 The fractional rate of evoked [^{3}H]-noradrenaline ([^{3}H]-NA) release per pulse from superfused rabbit isolated irides during the first (S1) or the seventh (S7) stimulation period at the specified conditions

Stimulation conditions		*Fractional rate of [^{3}H]-NA release*	
No. pulses	*Frequency* (Hz)	*S1* (10^{-6})	*S7* (10^{-6})
360	3	3.48 ± 0.25 (21)[b]	7.05 ± 0.85 (6)
360	10	5.62 ± 0.49 (24)[a]	6.85 ± 1.69 (4)
1200	10	11.27 ± 1.55 (19)[a, b]	5.93 ± 1.55 (3)

Each value is the mean ± s.e. mean with the number of experiments in parentheses. The values for S1 were pooled from all experimental groups in which no drug was present during this stimulation period. The fractional rate of release during S7 was calculated only from the control experiments (no drug was present throughout). Statistical comparison of differences (analysis of variance followed by Newman-Keuls test): [a] vs [a] and [b] vs [b], $P < 0.01$.

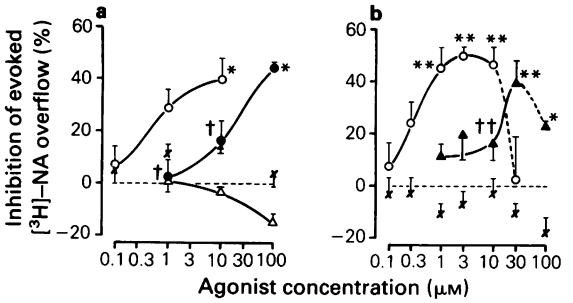

Figure 4 The muscarinic inhibition of the stimulation-evoked (10 Hz; (a) 1200 pulses, (b) 360 pulses) [^{3}H]-noradrenaline ([^{3}H]-NA) overflow from superfused rabbit isolated irides. Concentration-inhibition curves for methacholine were obtained in the absence (○), and in the presence of atropine 0.1 μM (●) or of pilocarpine 50 μM (▲). Pilocarpine alone (1 to 100 μM) failed to affect the [^{3}H]-noradrenaline overflow evoked by either 1200 pulses (△, a) or 360 pulses (not shown). Controls (X), predicted individual control values from experiments carried out in the absence of drugs. Each value is the mean of 3–9 individual observations; vertical lines indicate s.e. mean. For further explanations of symbols indicating statistical differences see legend to Figure 3.

[^{3}H]-noradrenaline release per pulse tended to be (but was not significantly) lower than when the same number of pulses was delivered at 10 Hz.

In the seventh stimulation period the fractional rate of release per pulse no longer differed between the various stimulation conditions (Table 1). This appears to result from the different kinetic behaviour of the evoked overflow observed in the three groups. The [^{3}H]-noradrenaline overflow in response to 360 pulses at 3 Hz at S7 was larger by 113.3 ± 46.7% (n = 6) than that at S1 (Figure 2a). In contrast, the [^{3}H]-noradrenaline overflow evoked by 7 successive periods of 360 pulses at 10 Hz remained fairly constant (Figure 2b), and the [^{3}H]-noradrenaline efflux induced by 1200 pulses at 10 Hz progressively declined with repetitive stimulation (Figure 1) to 52.3 ± 14.1% (S7, n = 4) of that caused by S1.

Effect of methacholine

Under all stimulation conditions (±)-methacholine decreased the evoked [^{3}H]-noradrenaline overflow in a concentration-dependent manner (Figures 3 and 4). The EC_{50} values and the maximal inhibitions under the various conditions are summarized in Table 2. The transmitter efflux evoked by 360 pulses at 3 Hz was nearly abolished by methacholine 10 μM (Figure 3). In contrast, the maximal inhibition of the [^{3}H]-noradrenaline overflow evoked by either 360 or 1200 pulses at 10 Hz was significantly lower (approximately 50%, Figure 4). However, the EC_{50} values under the three conditions were similar (Table 2). A bell-shaped concentration-inhibition curve was observed in the experiments with 360 pulses at 10 Hz (Figure 4b).

The inhibitory action of methacholine was fully reversible. The amount of [^{3}H]-noradrenaline recovered after S7 (i.e. 30 min after starting superfusion with drug-free solution), expressed as a percentage of the release during S1, was not significantly different from that obtained under control conditions.

Table 2 Maximum inhibition (I_{max}), concentration of half-maximum reduction of evoked [^{3}H]-noradrenaline overflow (EC_{50}) for (±)-methacholine or pilocarpine, and apparent dissociation constant of pilocarpine (as presynaptic antagonist against methacholine, K_B) on the rabbit isolated iris

Stimulation conditions		I_{max}*	EC_{50}†	K_B‡
No. pulses	*Frequency* (Hz)	(%)	(μM)	(μM)
		(±)-Methacholine		
3	360	92.5 ± 3.3 (3)[a, b]	0.29	—
10	360	49.6 ± 4.0 (5)[a]	0.32	—
10	1200	40.5 ± 8.3 (5)[b]	0.42	—
		Pilocarpine		
3	360	63.4 ± 6.2 (4)[c]	0.85	—
10	360	—	—	0.95

* I_{max} expressed as a percentage of total suppression of the evoked [^{3}H]-noradrenaline release and given as the mean ± s.e. mean with the number of experiments in parentheses.
† EC_{50} values estimated from the mean concentration-inhibition cuves in Figures 3 and 4 as described in Methods.
‡ K_B of pilocarpine acting as an antagonist against methacholine calculated according to Furchgott (1972).
Statistical comparison of differences (analysis of variance followed by Newman-Keuls test): [a] vs [a] and [b] vs [b], $P < 0.01$. [c] Denotes statistical significance (two-tailed Student's t test, $P < 0.01$) with respect to methacholine (3 Hz, 360 pulses).

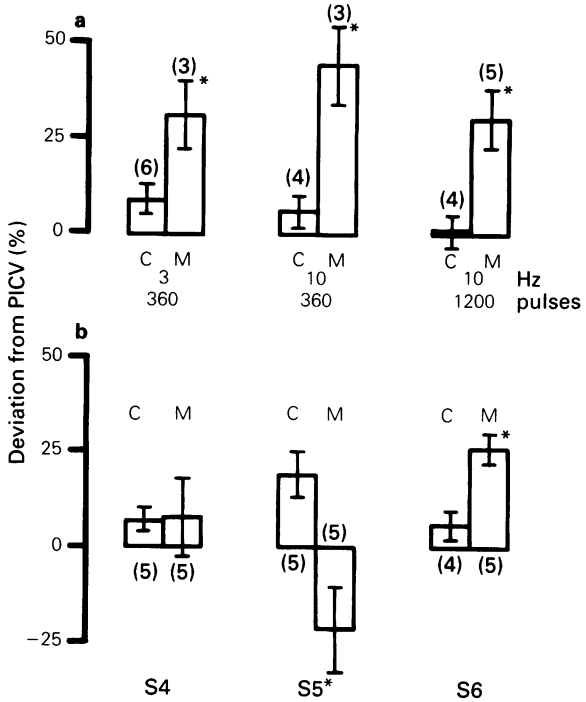

Figure 5 Reversal of the methacholine-induced inhibition of the evoked [^{3}H]-noradrenaline overflow from rabbit isolated irides by superfusion with agonist-free solution. (a) Percentage deviation from the predicted individual control values (PICV) of the [^{3}H]-noradrenaline overflow evoked by S6 carried out at the specified conditions in control tissues (C, no drug present throughout) or 10 min after the end of superfusion with methacholine 10 μM (M). (b) Percentage deviation from the predicted individual control values of the [^{3}H]-noradrenaline overflow evoked by 360 pulses at 10 Hz in the presence of methacholine 30 μM (S4, left), and 10 or 30 min (S5 and S6, middle and right, respectively) after returning to agonist-free solution (columns designated by M). The values of the corresponding stimulation periods in control tissues (C, no agonist contact) are included for comparison. Values are means of the number of experiments in parentheses; vertical lines indicate s.e. mean. Under all stimulation conditions, the amounts of [^{3}H]-noradrenaline released at S7 (expressed as % of the release at S1) in the methacholine-treated tissues did not differ significantly from the controls (not shown). Asterisks indicate significant differences from controls (Student's t test for unpaired observations: * $P < 0.05$).

However, the ^{3}H-transmitter released by the first stimulation immediately after returning to superfusion with Tyrode solution was significantly greater compared to the predicted individual control values (Figure 5a). In the experiments with methacholine 30 μM (10 Hz, 360 pulses) in which no inhibition was seen (Figures 4b and 5b), an inhibition of [^{3}H]-noradrenaline overflow appeared 10 min after the onset of the washout phase (S5), and an enhancement in the following stimulation period was observed (S6, Figure 5b).

Addition of atropine 0.1 μM to the superfusion solution did not affect the spontaneous [^{3}H]-noradrenaline overflow but significantly increased by 25.7 $\pm$ 6.6 (n = 3) and 27.9 $\pm$ 10.4% (n = 5) the ^{3}H-transmitter release evoked by stimulation with either 360 or 1200 pulses at 10 Hz, respectively. However, at 3 Hz stimulation atropine failed to increase the evoked release significantly (21.4 $\pm$ 28.3%, n = 5), although the greater variation of the individual values may have masked any significant difference. The ratio S7/S1 (90 min superfusion with atropine) of evoked [^{3}H]-noradrenaline overflow did not differ from that of the controls (atropine not present).

The concentration-inhibition curves of methacholine determined at either 3 or 10 Hz were displaced to the right in a nearly parallel manner by atropine 0.1 μM (Figures 3 and 4). The maximal inhibitions in the presence of the antagonist were not significantly different from those observed in its absence. An apparent $-\log K_B$ value of 8.5 or 9 (10 or 3 Hz, respectively) was determined, thus confirming the muscarinic nature of the receptors stimulated by methacholine.

When methacholine was washed out in experiments carried out in the continuous presence of atropine 0.1 μM the facilitation of evoked [^{3}H]-noradrenaline release (360 pulses at 3 Hz or 1200 pulses at 10 Hz) was not observed (S7 not different from S7 of controls, not shown).

Effect of pilocarpine

Addition of pilocarpine (1 to 100 μM) to the superfusion solution failed to affect the spontaneous [^{3}H]-noradrenaline overflow. The transmitter release evoked by 360 pulses at 3 Hz was reduced by pilocarpine in a concentration-dependent manner (Figure 3). The maximum inhibition was significantly lower compared to that of methacholine (Table 2). When release was evoked by stimulation at 10 Hz, pilocarpine up to 100 μM failed to inhibit the evoked [^{3}H]-noradrenaline overflow (1200 pulses, Figure 4a; 360 pulses, not shown). Pilocarpine competitively antagonized the methacholine-induced inhibition of [^{3}H]-noradrenaline overflow evoked by 360 pulses at 10 Hz (Figure 4b). The apparent K_B value of 0.95 μM estimated for pilocarpine under these conditions closely agrees with the EC_{50} of 0.85 μM obtained for the inhibition of the evoked [^{3}H]-noradrenaline release at 3 Hz (Table 2).

Discussion

The purpose of the present paper was to find out whether the noradrenaline release from the rabbit iris can be inhibited by pilocarpine via activation of presynaptic muscarinic receptors. A negative answer would not have easily been reconciled with the idea that in the rabbit under *in vivo* conditions pilocarpine acts indirectly by inhibiting the dilator sympathetic tone. At first, release conditions were established that should provide an exocytotic transmitter release sensitive to presynaptic modulation.

Release of [^{3}H]-noradrenaline from rabbit isolated irides

Field stimulation of the rabbit isolated irides caused a marked increase of tritium overflow which consisted mainly of unmetabolized [^{3}H]-noradrenaline, even in the absence of inhibitors of neuronal and extraneuronal uptake. Apparently the released transmitter reaches the superfusion medium quickly and is not extensively metabolized.

The fractional rate of release per pulse during S1 increased with the number of pulses applied (Table 1). A tendency to a higher fractional rate of release per pulse with increasing frequency at 360 pulse trains did not reach the level of statistical significance. Facilitation of transmitter release by increasing the length of the stimulus train or the stimulation frequency has been shown to occur in a great variety of tissues and may be explained by the residual 'CaX hypothesis' (for review see Starke, 1977).

When measured at S7 the fractional rate of [^{3}H]-noradrenaline release per pulse no longer differed between the various conditions (Table 1). A reduction of a fractional rate of radioactivity release within a series of pulse trains, such as that observed under the 10 Hz stimulation condition (1200 pulses, Figure 1), may be partly due to isotope dilution (though other factors such as exhaustion of highly active transmitter pools cannot be excluded). Tritiated transmitter stores within the nerve terminals are expected to become diluted by unlabelled and probably newly synthesized noradrenaline with successive stimulation. Isotope dilution may have also occurred under the two other conditions (360 pulses, 3 or 10 Hz). Nevertheless, the fractional rate of [^{3}H]-noradrenaline release remained constant (10 Hz) or was doubled (3 Hz) indicating that the release of endogenous transmitter probably also remained constant or, more likely, increased with the application of pulse trains. Apparently, under the latter conditions (360 pulses, 3 or 10 Hz) a facilitation of neurotransmitter release occurred which, depending on the stimulation conditions, compensated or even overrode the reduction of fractional release due to dilution of the labelled transmitter. We cannot exclude that a similar facilitation is responsible for the high fractional rate of release seen in the first period of stimulation with 1200 pulses at 10 Hz, since a conditioning stimulation preceded that period. However, since stimulation periods were placed 20 min apart from each other (and facilitation according to the CaX hypothesis generally comes into play within seconds), it seems rather unlikely that the 'long-term' facilitation we observed may have been due to an augmented calcium availability. These results seem to indicate that the noradrenaline release from the rabbit iris may be regulated not only in an immediate, 'short-term' way (within the first pulses or action-potentials) but also in a 'long term' manner, the first stimulation periods being able to modify the effects of subsequent stimulation periods.

Inhibition of release by muscarinic agonists

The results of the present study show that [^{3}H]-noradrenaline release from sympathetic nerve terminals in the rabbit iris dilator and sphincter muscles can be inhibited by activation of presynaptic muscarinic receptors, in agreement with results obtained in the rabbit ciliary body-iris preparation in which [^{3}H]-noradrenaline release was evoked by field stimulation (300 pulses, 10 Hz; Jumblatt & North, 1986). Methacholine reduced the noradrenaline release in response to field-stimulation in a concentration-dependent manner, with EC_{50} values (0.29 to 0.42 μM) similar to those obtained for the presynaptic muscarinic receptors in other test systems, such as the rat heart (0.19 μM; Fuder *et al.*, 1982) and the perfused rabbit atria (0.27 μM; Muscholl & Muth, 1982). The effects of methacholine were competitively antagonized by atropine and thus characterized as muscarinic. The apparent $-\log K_B$ values (as approximate estimates of an affinity constant) were 8.5 and 9, and closely agree with presynaptic values determined or quoted in a previous paper (Fuder *et al.*, 1981).

Albino and pigmented irides were used for our experiments. In the incubated rabbit iris sphincter, Salazar *et al.* (1976) observed a linear Schild plot for atropine in the albino tissues ($pA_2 = 8.8$) but a non-linear plot in the pigmented iris sphincters. This deviation from linearity may be accounted for by the binding of atropine to a low affinity site on the pigment melanin (Salazar *et al.*, 1976; Akesson *et al.*, 1983). Our apparent $-\log K_B$ of 9 (obtained when atropine failed to enhance the stimulation-evoked [^{3}H]-noradrenaline overflow) was obviously not affected by binding of the antagonist to the pigment. This may be due to the superfusion technique we used. Even if the drug concentration had been

decreased in the biophase by binding to melanin, the constant drug supply would have compensated for the loss and allowed a steady-state on a level higher than in an incubation system without replacement by new drug. The same would hold true for other drugs tested in our superfusion system.

The maximum effect of methacholine depended on the stimulation conditions (Table 2). Presynaptic muscarinic inhibition of noradrenaline release has been shown to be inversely related to the stimulation frequency (Steinsland *et al.*, 1973; Kirpekar *et al.*, 1975). Under our experimental conditions, the presynaptic inhibitory effect of methacholine was more pronounced at low (3 Hz) than at high (10 Hz) frequency (Table 2). Pilocarpine behaved as a partial agonist (relative to methacholine) at 3 Hz. At 10 Hz, in contrast, pilocarpine was inactive as an agonist and acted as a competitive antagonist against methacholine. The close agreement of the EC_{50} value estimated for pilocarpine at 3 Hz with the K_B determined at 10 Hz (Table 2) indicates that the affinity of the receptors for pilocarpine was not changed by varying the stimulation conditions. If one assumes that the receptor affinity and concentration are unchanged by a manipulation of an effector system, the simplest explanation for the decrease in agonist effectiveness remains a reduction in the effective receptor reserve (Kenakin, 1986). The effective receptor reserve for a given agonist is determined by the receptor concentration in the tissue and by the number of receptors to be activated for a given response which, in turn, depends on the intrinsic efficacy of the agonist and the efficiency of coupling between the receptor activation and response. The precise molecular mechanism of the presynaptic inhibitory muscarinic effector system is not known, but it may involve the reduction of calcium availability for exocytosis achieved either directly (Dubey *et al.*, 1975), or indirectly by altering the electrical properties of the sympathetic nerves and consequently the impulse propagation (Wakade & Wakade, 1982). Since the fractional rate of release per pulse upon stimulation with 1200 pulses at 10 Hz was higher compared to that upon 360 pulses at 3 Hz, the calcium influx is likely to be larger at 10 Hz (1200 pulses) than at 3 Hz (360 pulses). Hence, under the conditions of increased calcium influx and at an unchanged receptor concentration and coupling between receptor activation and stimulus-generation, an equal degree of presynaptic inhibition can be achieved only by formation of a higher number of agonist-receptor complexes. The observation that the EC_{50} values of methacholine under the various conditions were similar (but that maxima decreased with increasing stimulation parameters) points towards a low density of muscarinic receptors in the iris. When a receptor reserve is lacking a decrease in the effective receptor reserve will result in a reduction of the maximum agonist response. In the case of the partial agonist pilocarpine which is known to possess a very low efficacy on muscarinic receptors (relative to methacholine: 1/14 at the postsynaptic muscarinic receptors in rabbit stomach, Furchgott & Bursztyn, 1967; or 1/16 at the presynaptic muscarinic effector system of rat heart noradrenergic nerves, Fuchs & Fuder, 1985), the modification of the stimulation procedure resulted in a complete loss of presynaptic inhibitory activity. Hence, the presynaptic muscarinic inhibition of noradrenaline release from the rabbit iris appears to fit into the concept of dynamic changes of the effective receptor reserve determined by the particular conditions of the experiment (Yoshida *et al.*, 1979; Kenakin, 1986).

Besides modification of the effective receptor reserve, differences in the biophase concentration of released endogenous acetylcholine may also explain the variation of the activity of exogenous agonists between different stimulation conditions. Atropine (0.1 μM) slightly but significantly increased the [^{3}H]-noradrenaline overflow evoked at 10 Hz (360 or 1200 pulses) but failed to affect the release induced at 3 Hz. This observation clearly indicates that the noradrenaline release, evoked by field stimulation at the higher frequency, was already inhibited to some extent by acetylcholine secreted from simultaneously stimulated cholinergic fibres. In the presence of endogenous acetylcholine, any exogenous agonist would cause further inhibition of a magnitude that would originate from a higher 'baseline' and presumably be lower than that observed in the absence of the endogenous agonist.

The inhibitory activity of methacholine was completely reversible. After 10 min of superfusion with drug-free solution the evoked release of [^{3}H]-noradrenaline was not only restored but even greater than that expected if no drug were present (Figure 5). Conceivably, the prolonged inhibition of release by the agonist may have resulted in an increased availability of transmitter for secretion by further stimuli and, thus, in an 'apparent rebound' phenomenon. When the highest concentration of methacholine (30 μM) was superfused in the absence of antagonist, no transmitter could have been saved because no inhibition occurred. Decreasing the concentration of agonist in the biophase by washout resulted initially in a minute inhibition (not induced by the presence of this high concentration, see Figure 4); thereafter, the facilitation of release became apparent (Figure 5). We cannot exclude a desensitization of presynaptic inhibitory muscarinic receptors (Fuder *et al.*, 1985) as a reason for the decreasing part of the bell-shaped concentration-response curve. However, it is possible that the high concentrations of muscarinic agonists

exert a facilitatory effect on release which may counteract the inhibition prevailing at low agonist concentrations. High concentrations of muscarinic agonists are known to activate the breakdown of polyphosphoinositides in the iris (reviewed by Abdel-Latif, 1986). As a result inositolphosphates (IP) or diacylglycerol could be formed; these are known to activate a series of intracellular events leading ultimately to an increase in the availability of calcium ions for the cellular responses. In the rabbit iris sphincter muscle, it has been suggested that IP_3 is involved in the phasic component, and diacylglycerol-induced protein kinase C activation in the tonic component of contraction (Howe *et al.*, 1986). In our case an increase in the intraneuronal calcium availability should result in enhanced exocytosis.

The miotic effect of pilocarpine

Pilocarpine effectively reduced the noradrenaline release from rabbit isolated irides evoked by stimulation at 3 Hz (Figure 3). In contrast, the agonist had a very low intrinsic activity in contracting the rabbit iris sphincter isometrically (Akesson *et al.*, 1983) and failed to contract it isotonically (Bognar *et al.*, 1987), thus casting doubts about a direct postsynaptic mechanism underlying the miotic effect observed *in vivo*. It cannot be excluded that disruption of tissue connections or loss of essential blood factors (required for optimum function of postsynaptic muscarinic mechanisms), in connection with the isolation procedure, reduce the efficiency of coupling mechanisms or receptor number. In that case it would be surprising that the presynaptic efficacy of pilocarpine would be better preserved than the postsynaptic efficacy. Nevertheless, the present results support the hypothesis of an indirect mechanism of action of pilocarpine in the rabbit eye as advanced by Akesson *et al.* (1983).

Pilocarpine may activate the inhibitory presynaptic muscarinic receptors on the noradrenergic nerve terminals, thereby reducing the sympathetic dilator tone and leading to a miosis. The low postsynaptic intrinsic activity of pilocarpine may be ascribed to a low postsynaptic effective receptor reserve. The density of the postsynaptic muscarinic receptors in the bovine iris, for instance, has been shown to be significantly lower than in other peripheral tissues (Yoshida *et al.*, 1979). Due to a low receptor concentration (and a threshold phenomenon) in the rabbit ciliary body, theoretical occupancy-response curves indicated that the postsynaptic stimulus generated by pilocarpine cannot reach the threshold level, even if the compound occupies all the muscarinic receptors in the tissue (Konno & Takayanagi, 1986). In contrast, receptor occlusion experiments with phenoxybenzamine indicated that some spare receptor capacity may be present in the human iris hemisphincter for carbachol (Kaumann & Hennekes, 1979). There are a few early studies indicating that, in contrast to the rabbit sphincter, the human muscle contracts in response to pilocarpine (Swan & Gehrsitz, 1951; Takáts, 1964), although a high pilocarpine concentration has been shown to reduce the extent of acetylcholine-induced contraction of human isolated iris sphincter *in vitro* (Boros & Takáts, 1961). Furthermore, preliminary experiments in our laboratory confirmed these observations, showing that the intrinsic activity of pilocarpine in the human iris sphincter may be as high as that of methacholine. Hence, caution should be exercised in extrapolation of results obtained with rabbit iris *in vitro* to other species. Further work is needed to find an animal species that may be characterized as similar to human iris tissue.

This work was supported by a grant of the Deutsche Forschungsgemeinschaft. The results are part of the Dr med. Thesis of S.P., Fachbereich Medizin der Universität Mainz. I.T.B. was supported in this study by a grant of the 'Konrad-Adenauer-Stiftung'. We are grateful to Ms A. Habermeier and Ms B. Hering for excellent technical assistance.

References

ABDEL-LATIF, A.A. (1986). Calcium-mobilizing receptors, polyphosphoinositides, and the generation of second messengers. *Pharmacol. Rev.*, **38**, 227–272.

AKESSON, C., SWANSON, C. & PATIL, P.N. (1983). Muscarinic receptors of rabbit irides. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **322**, 104–110.

BOGNAR, I., PALLAS, S. & FUDER, H. (1987). Is the pilocarpine-induced miosis due to inhibition of noradrenaline release on the rabbit iris? *Naunyn Schmiedebergs Arch. Pharmacol.*, **335**, (Suppl.), R 83.

BOROS, B. & TAKÁTS, I. (1961). Pharmakologische Wechselwirkungen am isolierten Irismuskelpräparat. *Albrecht v. Graefes Arch. Ophthal.*, **163**, 382–388.

BYNKE, G., WAHLESTEDT, C., BEDING, B. & HÅKANSON, R. (1985). Pupillary supersensitivity to substance P following prolonged treatment with tetrodotoxin or substance P antagonists. *Eur. J. Pharmacol.*, **108**, 217–223.

DUBEY, M.P., MUSCHOLL, E. & PFEIFFER, A. (1975). Muscarinic inhibition of potassium-induced noradrenaline release and its dependence on the calcium concentration. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **291**, 1–15.

EHINGER, B. (1966). Ocular and orbital vegetative nerves. *Acta Physiol. Scand.*, **67** (Suppl. 268), 1–35.

EHINGER, B., FALCK, B. & SPORRONG, B. (1970). Possible axo-axonal synapses between peripheral adrenergic and cholinergic nerve terminals. *Z. Zellforsch.*, **107**, 508–521.

FUCHS, P. & FUDER, H. (1985). The determination of presy-

naptic K_A values of methacholine and pilocarpine and of a presynaptic receptor reserve in the rat perfused heart. *Br. J. Pharmacol.*, **84**, 35–46.

FUDER, H., MEISER, C., WORMSTALL, H. & MUSCHOLL, E. (1981). The effects of several muscarinic antagonists on pre- and postsynaptic receptors in the isolated rabbit heart. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **316**, 31–37.

FUDER, H., MUSCHOLL, E. & WOLF, K. (1985). Cholinesterase activity and exposure time to acetylcholine as factors influencing the muscarinic inhibition of [^{3}H]-noradrenaline overflow from guinea-pig isolated atria. *Br. J. Pharmacol.*, **86**, 905–914.

FUDER, H., PALLAS, S. & MUSCHOLL, E. (1986). Muscarinic inhibition of [^{3}H]-norepinephrine (NE) release from isolated rabbit irides by pilocarpine. *Pharmacologist*, **28**, 178.

FUDER, H., RINK, D. & MUSCHOLL, E. (1982). Sympathetic nerve stimulation on the perfused rat heart. Affinities of N-methylatropine and pirenzepine at pre- and postsynaptic muscarine receptors. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **318**, 210–219.

FURCHGOTT, R.F. (1972). The classification of adrenoceptors (adrenergic receptors). An evaluation from the standpoint of receptor theory. In *Handb. Exp. Pharmacol.*, Vol. 33, *Catecholamines*. ed. Blaschko, H. & Muscholl, E. pp. 283–335. Berlin: Springer-Verlag.

FURCHGOTT, R.F. & BURSZTYN, P. (1967). Comparison of dissociation constants and of relative efficacies of selected agonists acting on parasympathetic receptors. *Ann. New York Acad. Sci.*, **144**, 882–899.

GRAEFE, K.H., STEFANO, F.J.E. & LANGER, S.Z. (1973). Preferential metabolism of (−)-[^{3}H]-norepinephrine through the deaminated glycol in the rat vas deferens. *Biochem. Pharmacol.*, **22**, 1147–1160.

HÖKFELT, T. & NILSSON, O. (1965). The relationship between nerves and smooth muscle cells in the rat iris. II. The sphincter muscle. *Z. Zellforsch.*, **66**, 848–853.

HOWE, P.H., AKHTAR, R.A., NADERI, S. & ABDEL-LATIF, A.A. (1986). Correlative studies on the effect of carbachol on myo-inositol triphosphate accumulation, myosin light chain phosphorylation and contraction in sphincter smooth muscle of rabbit iris. *J. Pharmacol. Exp. Ther.*, **239**, 574–583.

JONSSON, G., HAMBERGER, B., MALMFORS, T. & SACHS, C. (1969). Uptake and accumulation of [^{3}H]-noradrenaline in adrenergic nerves of rat iris. Effect of reserpine, monoamine oxidase and tyrosine hydroxylase inhibition. *Eur. J. Pharmacol.*, **8**, 58–72.

JUMBLATT, J.E. & NORTH, G.T. (1986). Potentiation of sympathetic neurosecretion by forskolin and cyclic AMP in the rabbit iris-ciliary body. *Curr. Eye Res.*, **5**, 495–502.

KAUMANN, A.J. & HENNEKES, R. (1979). The affinity of atropine for muscarine receptors in human sphincter pupillae. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **306**, 209–211.

KENAKIN, T.P. (1986). Receptor reserve as a tissue misnomer. *Trends Pharmacol. Sci.*, **7**, 93–95.

KIRPEKAR, S.M., PRAT, J.C. & WAKADE, A.R. (1975). Effect of calcium on the relationship between frequency of stimulation and release of noradrenaline from the perfused spleen of the cat. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **287**, 205–212.

KONNO, F. & TAKAYANAGI, I. (1986). Comparison of the muscarinic cholinoceptors in the rabbit ciliary body and the guinea-pig ileum. *Eur. J. Pharmacol.*, **132**, 171–178.

LATIES, A.M. & JACOBOWITZ, D. (1964). A histochemical study of the adrenergic and cholinergic innervation of the anterior segment of the rabbit eye. *Invest. Ophthalmol.*, **3**, 592–600.

MUSCHOLL, E. (1980). Peripheral muscarinic control of norepinephrine release in the cardiovascular system. *Am. J. Physiol.*, **239**, H713–H720.

MUSCHOLL, E. & MUTH, A. (1982). The effect of physostigmine on the vagally induced muscarinic inhibition of noradrenaline release from the isolated perfused rabbit atria. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **320**, 160–169.

NILSSON, O. (1964). The relationship between nerves and smooth muscle cells in the rat iris. I. The dilator muscle. *Z. Zellforsch.*, **64**, 166–171.

NISHIDA, S. & SEARS, M. (1969a). Dual innervation of the iris sphincter muscle of the albino guinea pig. *Exp. Eye Res.*, **8**, 467–469.

NISHIDA, S. & SEARS, M. (1969b). Fine structural innervation of the dilator muscle of the iris of the albino guinea pig studied with permanganate fixation. *Exp. Eye Res.*, **8**, 292–296.

RICHARDSON, K.C. (1964). The fine structure of the albino rabbit iris with special reference to the identification of adrenergic and cholinergic nerves and nerve endings in its intrinsic muscles. *Am. J. Anat.*, **114**, 173–206.

SALAZAR, M., SHIMADA, K. & PATIL, P.N. (1976). Iris pigmentation and atropine mydriasis. *J. Pharmacol. Exp. Ther.*, **197**, 79–88.

STARKE, K. (1977). Regulation of noradrenaline release by presynaptic receptor systems. *Rev. Physiol. Biochem. Pharmacol.*, **77**, 1–124.

STEINSLAND, O.S., FURCHGOTT, R.F. & KIRPEKAR, S.M. (1973). Inhibition of adrenergic neurotransmission by parasympathomimetics in the rabbit ear artery. *J. Pharmacol. Exp. Ther.*, **184**, 346–356.

SWAN, K.C. & GEHRSITZ, L. (1951). Competitive action of miotics on the iris sphincter. *Arch. Ophthalmol.*, **46**, 477–481.

TAKÁTS, I. (1964). Pharmakologiai vizsgálatok isolált emberi musculus sphincter iridisen. *Szemészet*, **101**, 19–25.

WAKADE, A.R. & WAKADE, T.D. (1982). Mechanism of presynaptic actions of adenosine and acetylcholine in the guinea-pig heart. *Neurosci.*, **7**, 2267–2276.

WALLENSTEIN, S., ZUCKER, C.L. & FLEISS, J.L. (1980). Some statistical methods useful in circulation research. *Circ. Res.*, **47**, 1–9.

YAMAUCHI, D.N., DESANTIS, L. & PATIL, P.N. (1973). Relative potency of cholinomimetic drugs on the bovine iris sphincter strips. *Invest. Ophthalmol.*, **12**, 80–82.

YOSHIDA, H., TAKEYASU, K. & UCHIDA, S. (1979). Muscarinic acetylcholine receptor and smooth muscle contraction. *Trends Pharmacol. Sci.*, **1**, 34–36.

(Received October 10, 1987
Revised January 11, 1988
Accepted January 25, 1988)