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The determination of presynaptic K_A values of methacholine and pilocarpine and of a presynaptic receptor reserve in the rat perfused heart

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1 Rat isolated perfused hearts with the right sympathetic nerves attached were loaded with [^{3}H]-noradrenaline. The nerves were stimulated with up to 11 trains of 10 pulses at 0.1 Hz. The evoked increases of [^{3}H]-noradrenaline overflow into the perfusate were measured in the presence of cocaine, corticosterone and propranolol.

2 Activation of presynaptic muscarinic receptors by methacholine or pilocarpine inhibited the evoked transmitter release in a reversible and concentration-dependent manner.

3 Preperfusion with phenoxybenzamine (5 μM) for 15 min (followed by a washout of 35 min) changed neither resting nor evoked overflow of [^{3}H]-noradrenaline. The concentration-response curve of methacholine was shifted to the right after exposure of the hearts to phenoxybenzamine (1 μM) without depression of the maximum effect. Pretreatment with phenoxybenzamine (5 μM) reduced the maximum inhibition of release by about 50%. Analysis of the data gave a dissociation constant for the agonist-receptor complex (K_A) of 4.0 μM and a receptor reserve of roughly 70%. Half-maximal inhibition of [^{3}H]-noradrenaline release occurred when about 2% of the total receptor population was occupied.

4 Comparison of the concentration-response data for methacholine and pilocarpine revealed a relative efficacy (methacholine/pilocarpine) of 16, a K_A of 10 μM for pilocarpine and no receptor reserve for this agonist.

5 The results show that K_A values for methacholine and pilocarpine obtained at presynaptic receptors are similar to those obtained at postsynaptic muscarinic receptors. This is in agreement with the idea that muscarinic receptors located on postganglionic adrenergic nerves are not different from those located on effector sites of non-neuronal tissue.

Introduction

Receptors can be characterized by the dissociation constant (K_A) of the agonist-receptor complex. Various procedures can be used to determine agonist K_A values. Furchgott & Bursztyn (1967), for example, derived the K_A of muscarinic agonists from concentration-response curves obtained in tissue before and after fractional receptor inactivation by an irreversibly acting antagonist. Another procedure (Mackay, 1966) compares the effects of two agonists (of different efficacy) acting at the same receptor. From this method, the K_A of another agonist can be estimated, if the K_A of one agonist is known.

In the case of a large receptor reserve the concentration of half-maximal response (EC_{50}) of a given agonist is up to 1000 fold lower than the K_A, because activation of a small fraction of the total receptor population may already induce a maximum response. The EC_{50} depends, thus, on the number of receptors in the tissue and on the efficacy of the agonist used. The efficacy, on the other hand, reflects the probability of fractional receptor occupation to be followed by a pharmacological response. The question of a receptor reserve in presynaptic inhibitory mechanisms has been discussed before (Starke, 1981). However, K_A values of agonists at presynaptic receptors (modulating transmitter release) have not been determined previously. The purpose of the present paper was to characterize muscarinic receptors located at postganglionic sympathetic nerves by K_A values of selective agonists in the rat perfused heart. Therefore, a number of the presynaptic muscarinic receptors were irreversibly blocked by phenoxyben-

zamine. The inhibition of [^{3}H]-noradrenaline release was investigated as a function of fractional receptor occupation.

Preliminary results of some of this work have been published at the 5th Catecholamine Symposion in Gothenburg (Fuder & Fuchs, 1983) and at the Spring Meeting of the German Pharmacological Society in Mainz (1983).

Methods

Detailed descriptions of the preparation, the labelling procedure and the separation of [^{3}H]-noradrenaline according to Graefe *et al.* (1973) have been published previously (Fuder *et al.*, 1982; 1983). In brief, hearts of male Wistar rats (200–310 g, i.p. injection of 1000 u heparin 5 min before death) with the right postganglionic sympathetic nerves attached were perfused according to the Langendorff technique at a rate of 6 ml min^{-1}. The perfusion medium was Tyrode solution of the following composition (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHCO_3$ 11.9, NaH_2PO 0.42, D-glucose 5.6, and (+)-ascorbic acid 0.057; gassed with 5% CO_2 in O_2, temperature 34–35 °C. The hearts were labelled by an infusion (10 min) of (−)-[7-^{3}H]-noradrenaline (3.0 Ci mmol^{-1}, sp. act.) at a final concentration in the aorta of 17 nM (at a reduced $CaCl_2$ concentration of 0.45 mM in Tyrode solution). The radioactivity was washed out for 60 min before the first nerve stimulation was carried out. The sympathetic nerves were stimulated by platinum ring electrodes at supramaximal current strength (around 30 mA). Square wave pulses were delivered by a Grass S 6 stimulator (controlled by an electronic impulse generator) in 10 trains of 10 pulses at 0.1 Hz and at 5 min intervals (plus an 11th train at an interval of 15 min, SNS 1–11). These stimulation parameters were selected because preliminary experiments had established that α_2-adrenoceptor mediated autoinhibition was not activated under these conditions. The degree of autoinhibition of noradrenaline release was investigated by adding phentolamine 3 μM 3 min before SNS 3 for 20 or 40 min (see below for design of control experiments). The perfusate was collected continuously for 65 min in 2.5 min samples starting 2.5 min before SNS 1 (with a break of 7.5 min starting 10 min before SNS 11). Total tritium content of a given perfusate sample was determine in an aliquot of 1 ml, and [^{3}H]-noradrenaline (after separation from the ^{3}H-metabolites by column chromatography,

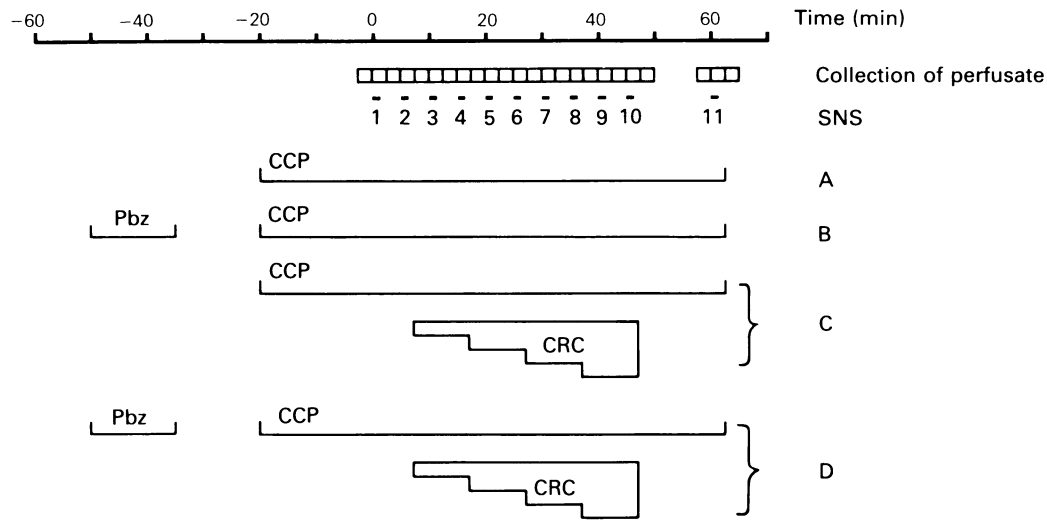

Figure 1 Protocol for perfusion of rat hearts prelabelled with [^{3}H]-noradrenaline and exposed to drugs in order to determine dissociation constants of the agonist-presynaptic receptor complex. Time (min) 0 corresponds to the beginning of the first sympathetic nerve stimulation (SNS, see Methods) 60 min after the end of the labelling procedure. The perfusate was collected every 2.5 min. (A) Perfusion of the heart with Tyrode solution containing cocaine 10, corticosterone 10, and propranolol 0.1 μM (CCP). (B) Pretreatment with phenoxybenzamine (5 μM, 15 min, Pbz) and washout of the irreversible antagonist for 15 min, then perfusion as described in (A). (C) Cumulative concentration-response curve (CRC) for the inhibition by methacholine or pilocarpine of stimulation-evoked [^{3}H]-noradrenaline overflow under condition (A). The agonists were preperfused for 3 or 8 min and were present during two stimulation trains. (D) concentration-response curve of agonists after partial irreversible muscarinic receptor inactivation by Pbz (1 or 5 μM).

Fuder *et al.*, 1983) in the remaining perfusate by liquid scintillation spectrometry. The overflow of total tritium and of [^{3}H]-noradrenaline was expressed as pmol referring to the specific activity of [^{3}H]-noradrenaline infused. We have shown before that in the rat heart [^{3}H]-noradrenaline accounts for most of the stimulation-evoked (1 Hz, 10 pulses) increase of tritium, even in the absence of inhibitors of neuronal and extraneuronal uptake, while the ^{3}H-metabolites are not measurably increased (Fuder *et al.*, 1983). Moreover, [^{3}H]-noradrenaline is concentrated about 14 fold by column chromatography and thus, represents a far more sensitive parameter of stimulation-evoked transmitter overflow than total tritium. Therefore, the changes in stimulation-evoked overflow of [^{3}H]-noradrenaline are presented as the relevant parameter for presynaptic mechanisms.

The mean value of [^{3}H]-noradrenaline overflow from the samples collected before and after a given SNS was subtracted from the overflow observed in the sample collected during SNS plus 60 s afterwards, and the difference between both values was considered as stimulation-evoked overflow.

The inhibition by the exogenous agonists methacholine or pilocarpine of the stimulation-evoked [^{3}H]-noradrenaline overflow was taken as a measure of activation of inhibitory presynaptic muscarinic receptors (Fuder *et al.*, 1982). The dissociation constant of the agonist-presynaptic receptor complex was assessed by adapting a procedure described by Furchgott & Bursztyn (1967) for postsynaptic receptors. The procedure was modified and a scheme of perfusion with different drugs is depicted in Figure 1.

At first the decline in the evoked [^{3}H]-noradrenaline overflow upon repeated stimulations was established (Figure 1, A, untreated hearts). Generally the overflow evoked by SNS 3–11 (S3–S11) was expressed as a percentage of the overflow evoked by SNS 2 (S2). When the decline in stimulation-evoked overflow in control groups was documented, the mean overflow of two successive SNS was calculated and expressed as a percentage of the mean overflow evoked by SNS 1 and SNS 2. In order to demonstrate effects of pretreatment of the hearts with phenoxybenzamine (5 μM, the highest concentration used in agonist studies) on basal and evoked [^{3}H]-noradrenaline overflow, and on the decline of evoked overflow upon repeated stimulation, a second control series (B) was carried out (pretreated hearts). In a third series, 4 concentrations of methacholine (increasing by a factor of 4, beginning either at 0.016 or at 0.063 μM) or of pilocarpine (increasing by a factor of 10,1–1000 μM) were added

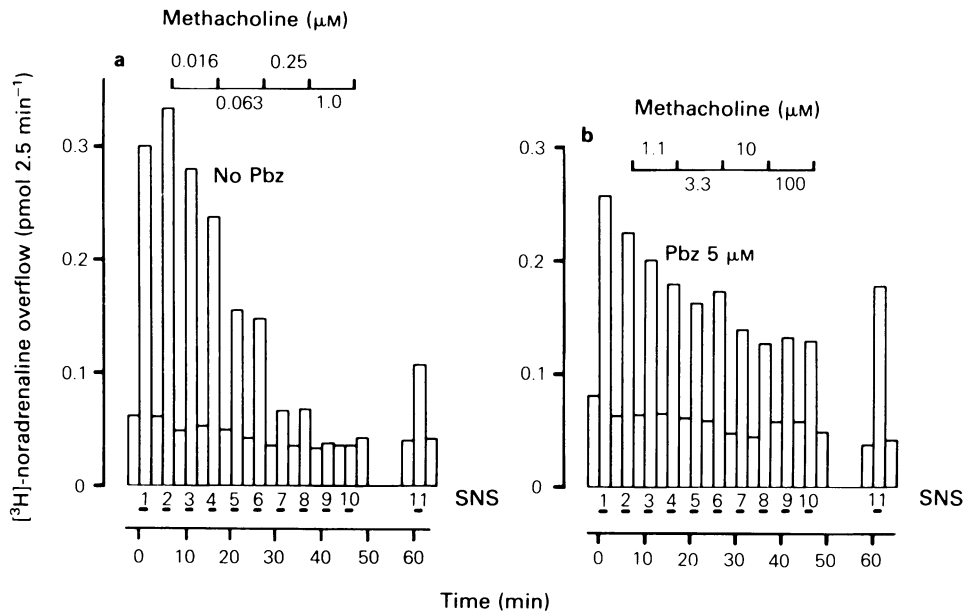

Figure 2 The effect of 11 successive periods (5 or 15 min intervals) of sympathetic nerve stimulations (SNS, 10 pulses, 0.1 Hz) on [^{3}H]-noradrenaline overflow (collected in 2.5 min samples; shown by vertical columns) from 2 rat hearts. The results shown in (a) were obtained in an untreated heart as described under (C) of Figure 1, those in (b) after exposure to and washout of phenoxybenzamine (Pbz) 5 μM (condition (D) of Figure 1). Note the differences between the methacholine concentrations, the maximum inhibition of evoked overflow, and the reversibility of inhibition after washout of methacholine.

cumulatively (C). In series (D), methacholine or pilocarpine was present in the medium perfused through hearts which had been exposed to phenoxybenzamine (either 1 or 5 μM, respectively). The agonist concentration was increased in steps of 3 to 10 fold to obtain many points in the lower range of the concentration-response curve. Typical experiments from series (C) and (D) show the reversibility of presynaptic inhibition (Figure 2). The concentration-response curves for the inhibition of [^{3}H]-noradrenaline overflow were constructed by dividing the individual evoked overflow (% of SNS 2) at a given SNS in the presence of agonist (e.g., values shown in Figure 2) by the mean relative evoked overflow in the respective sample of perfusate observed in controls (A plus B) in the absence of agonist. The decrease was expressed as a percentage of total suppression of release (= 100% inhibition). The individual concentration of half-maximal inhibition (IC_{50}) was estimated graphically from a semilogarithmic plot, and the geometric mean IC_{50} calculated. At lower agonist concentrations ($<0.1\,\mu M$) the preperfusion time of 3 min for the agonist appeared to be insufficient, as a slightly higher degree of presynaptic inhibition was observed after 8 min preperfusion time (not documented). Therefore, values obtained after a longer equilibration time were used for the concentration-response curve in the case of concentrations of agonist $<0.1\,\mu M$. Higher concentrations of agonist inhibited to a similar degree after either a short or long equilibration time (e.g., inhibition by methacholine 1 μM, 3 min, 96 ± 2%, 8 min, 99 ± 1%, $n = 5$, NS, paired t test) and therefore 3 min values could be used. A desensitization of muscarinic inhibition apparently does not occur within the time interval of 8 min.

The methacholine dissociation constant (K_A) was calculated by comparing equieffective concentrations of agonist in untreated hearts [A] with those observed in hearts pretreated with phenoxybenzamine [A'] in a double reciprocal plot (Furchgott & Bursztyn, 1967). The concentrations of agonists were estimated visually from semilogarithmic plots of the mean concentration-response curves (steps of 5–10% inhibition). According to Furchgott & Bursztyn (1967) the following relationship exists between the dose-response curves of an agonist before and after partial irreversible receptor inactivation with a haloalkylamine:

$$\frac{1}{[A]} = \frac{1-q}{q \cdot K_A} + \frac{1}{q \cdot [A']} \qquad (1)$$

where [A] and [A'] are corresponding equieffective agonist concentrations before and after partial receptor inactivation, respectively. The fraction of receptors not alkylated and still active after phenoxybenzamine exposure is q. The dissociation constant K_A can, thus, be obtained from the linear plot of 1/[A] vs. 1/[A'] by the following equation:

$$K_A = \frac{\text{slope} - 1}{\text{intercept}} \qquad (2)$$

The pilocarpine dissociation constant was calculated by comparing equieffective concentrations of pilocarpine with those of methacholine (in untreated hearts) in a double reciprocal plot (Mackay, 1966). According to this paper the following relationship exists between the effects of two agonists (A_1 and A_2) acting at the same receptor (symbols not from Mackay, 1966, but adapted to those used in equation (1) and (2) according to Furchgott & Bursztyn, 1967):

$$\frac{1}{[A_1]} = \frac{\frac{\varepsilon_1}{\varepsilon_2} - 1}{KA_1} + \frac{KA_2}{KA_1} \times \frac{\varepsilon_1}{\varepsilon_2} \times \frac{1}{[A_2]} \qquad (3)$$

Thus, if the K_A value of one agonist (KA_1) is known, the relative efficacies ($\varepsilon_1/\varepsilon_2$) can be calculated from the double reciprocal plot when the following equation is used:

$$\frac{\varepsilon_1}{\varepsilon_2} = (\text{intercept} \cdot KA_1) + 1 \qquad (4)$$

and KA_2 can be calculated from the relationship:

$$KA_2 = \frac{\text{slope} \cdot KA_1}{\frac{\varepsilon_1}{\varepsilon_2}} \qquad (5)$$

In our case, K_A for methacholine derived from equation (2) served as KA_1 in the analysis of K_A for pilocarpine (KA_2). The fractional presynaptic muscarinic receptor occupancy for the agonists at each concentration [A] was calculated according the Besse & Furchgott (1976), by applying the following equation on the control concentration-response curves from unpretreated hearts:

$$\frac{[RA]}{[R_t]} = \frac{[A]}{K_A + [A]} \qquad (6)$$

where [RA] is the concentration of receptor-agonist complex, [R_t] is the total presynaptic receptor concentration, and K_A is the dissociation constant of the agonist determined as described above.

The right atrial and ventricular tension development of the hearts used in the release studies was recorded transversely, and the ventricular beating frequency was recorded via a rate meter triggered from the ventricular action (Fuder *et al.*, 1982). The atrial and ventricular tension development and heart rate (in the absence of nerve stimulation) during the course of the experiment were followed in controls (no agonist present) and in the presence of agonist (i.e. in untreated hearts and hearts previously exposed to phenoxybenzamine). In the rat heart, however, the transverse recording method yields satisfactory tracings only in about 2/3 to 1/2 of the experi-

ments because the ventricular contraction often distorts the atrial tension recording. Therefore, results are given for only two treatment groups. The decrease by methacholine of atrial tension and heart rate was compared to control values and concentration-response curves were constructed for the postsynaptic effect (100% = total suppression of mechanical activity).

The tritium content of hearts (mean wet weight: 0.97 ± 0.01 g, $n = 47$) was determined after the end of the perfusion, as described previously (Fuder *et al.*, 1983). It amounted to 142 ± 5 pmol g^{-1} wet weight ($n = 47$).

The results are expressed as the mean ± s.e. mean, or as geometric mean with 95% confidence limits. Statistical differences between means ($P < 0.05$) were determined either by paired t test, or by Student's t test, and, if more than one treatment group was to be compared with one control group, by modified t test according to Bonferroni (Wallenstein *et al.*, 1980). All straight lines were drawn by linear regression.

The following drugs (dissolved in distilled water, saline or propylene glycol) were used: (−)-cocaine hydrochloride (Merck), corticosterone (Sigma, stock solution 60 mM in propylene glycol), (±)-methacholine chloride (Sigma), (−)-[7-^{3}H]-noradrenaline (NEN, dissolved in 0.9% w/v NaCl solution), phenoxybenzamine hydrochloride (Röhm Pharma, stock solution 7 mM in propylene glycol), phentolamine mesylate (Ciba-Geigy), pilocarpine hydrochloride (Boehringer Ingelheim), (±)-propranolol hydrochloride (ICI).

In agreement with a previous study (Weitzel *et al.*, 1979), propylene glycol (up to 25 mM) affected neither the resting nor the stimulation-evoked [^{3}H]-noradrenaline overflow (unpublished observations).

Results

The overflow of [^{3}H]-noradrenaline (Table 1) from hearts not exposed to phenoxybenzamine represented $15.3 \pm 1.5\%$ ($n = 7$) of total tritium overflow in the sample collected before SNS 1, and $19.1 \pm 5.9\%$ in the sample collected before SNS 11. Pretreatment with phenoxybenzamine (5 μM) affected neither the contribution of [^{3}H]-noradrenaline to total tritium (before SNS 1, $14.3 \pm 1.4\%$; before SNS 11, $14.2 \pm 2.1\%$, for each $n = 5$), nor the absolute values of resting [^{3}H]-noradrenaline overflow (Table 1), nor the degree of stimulation-evoked [^{3}H]-noradrenaline overflow (Table 1). The decline in evoked overflow between SNS 1 and SNS 11 observed in untreated hearts was not significantly different from that observed in hearts previously exposed to phenoxybenzamine 5 μM (Table 2). Addition of phentolamine before SNS 3 failed to affect the decline in evoked [^{3}H]-noradrenaline overflow (Table 2) indicating a lack of α-adrenoceptor mediated feedback inhibition by endogenous (plus tritiated) noradrenaline released.

Table 1 The effect of phenoxybenzamine on resting oveflow and stimulation-evoked overflow of [^{3}H]-noradrenaline (NA) from the rat perfused heart

	Resting overflow of [^{3}H]-NA before SNS 1 (pmol 2.5 min^{-1})	*Stimulation-evoked increase in [^{3}H]-NA overflow above resting overflow at SNS 1* (multiple of resting overflow)	n	*Perfusion with additional drugs after SNS 2*
No phenoxybenzamine	0.055 ± 0.012	1.25 ± 0.22	7	–
	0.062 ± 0.028	0.63 ± 0.09	8	Phentolamine
	0.092 ± 0.011	1.39 ± 0.63	6	Methacholine
	0.098 ± 0.016	0.89 ± 0.34	5	Pilocarpine
Preperfusion (15 min) with Phenoxybenzamine				
1 μM	0.106 ± 0.027	0.85 ± 0.174	3	Methacholine
5 μM	0.065 ± 0.008	1.77 ± 0.88	5	–
5 μM	0.088 ± 0.009	1.12 ± 0.183	9	Methacholine
5 μM	0.071 ± 0.005	0.90 ± 0.17	4	Pilocarpine

The right sympathetic nerves were stimulated with 10 pulses of 1 ms duration at 0.1 Hz (SNS). The hearts were perfused for 15 min with Tyrode solution containing phenoxybenzamine beginning 10 min after the end of the labelling procedure, i.e. 50 min before SNS 1. Phenoxybenzamine-free solution was perfused for 15 min, before cocaine 10, corticosterone 10, and propranolol 0.1 μM were added to the perfusion medium (starting 20 min before SNS 1). The values represent means with s.e. mean of n hearts and stem from treatment groups as indicated in the last column. Phenoxybenzamine pretreatment affected neither resting nor stimulation–evoked overflow of [^{3}H]-NA.

Table 2 The effect of exposure to phenoxybenzamine and perfusion with phentolamine on the stimulation-evoked [^{3}H]-noradrenaline (NA) overflow (S) from the rat perfused heart

Stimulation-evoked [^{3}H]-NA overflow as % of (S 1 + S 2)/2	*S 2*	*(S 3 + S 4)/2*	*(S 5 + S 6)/2*	*(S 7 + S 8)/2*	*(S 9 + S 10)/2*	*S 11*
Controls	93 ± 4.6 (7)	75 ± 8.5 (7)	70 ± 5.7 (7)	70 ± 7.4 (7)	56 ± 5.2 (7)	57 ± 7.2 (6)
Preperfusion with phenoxybenzamine 5 μM for 15 min	83 ± 7.4 (5)	81 ± 5.1 (5)	69 ± 3.9 (5)	64 ± 3.5 (5)	59 ± 4.7 (5)	67 ± 2.2 (5)
Phentolamine 3 μM (S – S 10)	98 ± 6.9 (8)	85* ± 10.2 (8)	81* ± 6.0 (8)	81* ± 9.4 (6)	54* ± 6.4 (6)	52 ± 16.1 (3)

Conditions as described in Methods and Table 1. The sympathetic nerves were stimulated with 11 trains of 10 pulses (0.1 Hz) at 5 min intervals (except S 11 which was carried out after an interval of 15 min). The means of pre- and post-stimulation values of resting [^{3}H]-NA overflow were subtracted to calculate evoked release. Neuronal and extraneuronal uptake, and β-adrenoceptors were blocked in all groups including controls (no additional drugs). Values represent means with s.e. mean of the evoked overflow expressed as % of (S 1 + S2)/2. Except at S 2 and S 11, the mean evoked release of two subsequent stimulation periods was used for the calculations. In the group with phenoxybenzamine preperfusion, phenoxybenzamine-free solution was perfused starting 35 min before S 1. Phentolamine was added to the perfusion medium 3 min before S 3 and was present until the end of S 10 (as indicated by asterisks). Figures in parentheses are the number of hearts. The ratios of stimulation-evoked release at a given stimulation period did not differ between groups.

The resting overflow in the presence of the highest methacholine or pilocarpine concentrations (in untreated and pretreated hearts) was not different from controls (not shown). Since untreated and pretreated hearts showed a similar decline in evoked overflow, the values of both groups were combined and their means for the individual stimulation periods (S 3 – S 10) served as reference values for the calculation of agonist concentration-response curves in untreated and pretreated hearts. The control values for, e.g., S 4 (control for stimulation after a long equilibration time in low agonist concentrations), S 5, 7, and 9 (short equilibration time) were 85, 77, 73 and 63% of the overflow evoked by SNS 2 (see also Table 2).

In unpretreated hearts, methacholine decreased the evoked [^{3}H]-noradrenaline overflow in a concentration-dependent manner (Figures 2 and 3). The maximum inhibition (96 ± 1.4%) was observed at a concentration of 1 μM, and the geometric mean IC_{50} was 0.072 μM (0.05–0.1, $n = 6$). Exposure of the hearts to phenoxybenzamine 1 μM for 15 min before nerve stimulation caused a parallel shift (roughly 3 fold) in the methacholine concentration-response curve to the right with a maximum inhibition (91 ± 10.7% at 5 μM and 100 ± 0% at 50 μM, $n = 3$) which was not different from that of untreated hearts. Exposure of the hearts to phenoxybenzamine 5 μM for 15 min, however, resulted in a significant depression of individual maximum inhibition by methacholine (51 ± 5.7%, $n = 9$, $P < 0.0001$, compared to individual maxima in untreated hearts). Half

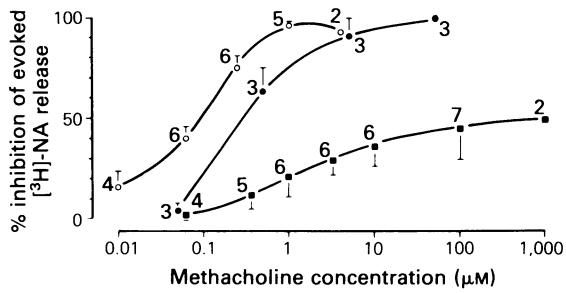

Figure 3 The inhibition by methacholine of the stimulation-evoked (10 pulses, 0.1 Hz) [^{3}H]-noradrenaline (NA) overflow without or after fractional irreversible receptor inactivation of the rat perfused heart. From any one heart, 4 points of an ascending cumulative concentration-response curve were obtained either in untreated (○) or in phenoxybenzamine (Pbz)-pretreated (●, 1 μM; ■, 5 μM; 15 min each) hearts. The evoked overflow (expressed as % of SNS 2) induced by a given pulse train in the presence of agonist was divided by the mean evoked overflow of the corresponding stimulation period in the absence of the agonist (combined controls from untreated hearts and those preperfused with Pbz 5μM, see Results). The inhibition was expressed as a percentage of total suppression of evoked transmitter release. The symbols are means of n (number above each point) determinations with vertical lines showing s.e. mean. Preperfusion with the agonist before nerve stimulation ensured equilibrium conditions for the agonist. The maximum inhibition in untreated hearts was larger than in Pbz-pretreated (5 μM) hearts ($P < 0.01$).

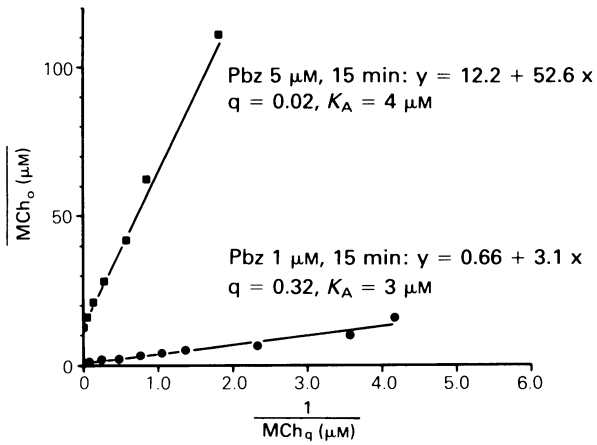

Figure 4 Determination of the dissociation constant K_A (Furchgott & Bursztyn, 1967) for methacholine (MCh) at the presynaptic muscarinic receptor. The data are taken from the log concentration-response curves shown in Figure 3 obtained with the inhibition of the [^{3}H]-noradrenaline overflow before (MCh_o) and after fractional irreversible receptor inactivation (MCh_q) by phenoxybenzamine (Pbz, ●, 1 μM; ■, 5 μM). The double reciprocal plot for equieffective concentrations was constructed by graphical estimation of the agonist concentrations inducing a given degree of inhibition (steps of 5–10% of the curves on Figure 3) in untreated hearts and in hearts preperfused with Pbz. Linear regression lines were calculated (correlation coefficients, $P<0.001$) and are inserted. The fraction of total receptor population (q) remaining intact after irreversible inactivation was derived from 1/ slope. K_A values were calculated using equation (2) as described in Methods.

maximal inhibition was observed at 2.9 μM (1–7.8, $n=9$). When the reciprocals of equieffective concentrations obtained in untreated and pretreated (phenoxybenzamine 1 or 5 μM) hearts were compared (Figure 4), their relationships were described by straight lines (correlation co-efficients, $P<0.001$). Exposure of the heart to phenoxybenzamine 1 or 5 μM eliminated 68 or 98%, respectively, of the total presynaptic muscarinic receptor population (fractions of intact receptors, 0.32 or 0.02). Nevertheless 100% or 51% of the maximum inhibition was observed, respectively. Both lines gave similar estimations for the methacholine K_A value (3 and 4 μM, respectively), independent of the fraction of intact receptors and the concentration of phenoxybenzamine used. Because the concentration-response curve obtained after phenoxybenzamine 5 μM was better defined than that obtained after 1 μM, the methacholine K_A of 4 μM was used for further calculations.

Pilocarpine reduced the evoked [^{3}H]-noradrenaline overflow by 86 ± 5.8%, and the IC_{50} in untreated hearts was 6.2 μM (3.1–12.5, $n=5$, Figure 5). Pretreatment of the hearts with phenoxybenzamine 5 μM nearly totally abolished the inhibition by pilocarpine (Figure 5). The pilocarpine K_A was therefore estimated according to Mackay (1966) using methacholine as the reference agonist (4 μM, K_A for methacholine). The double reciprocal plot of equieffective methacholine and pilocarpine concentrations resulted in a straight line (correlation coefficient, $P>0.001$, Figure 6). The ratio (methacholine/pilocarpine) of intrinsic efficacies (ε) was 16. Since the data points deviated from the calculated straight line in part, the efficacy ratio (and the K_A for pilocarpine) could be in error by a factor of 2. The K_A value for pilocarpine, 10 μM, was not much higher than the corresponding IC_{50} value, in contrast to results with methacholine. The K_A value for methacholine was 48 fold higher than the IC_{50} for methacholine in untreated hearts. The methacholine IC_{50} (2.9 μM) observed after pretreatment with phenoxybenzamine 5 μM was, however, close to the K_A value.

The concentration-response data for methacholine and pilocarpine from Figures 3 and 5 (no phenoxybenzamine exposure) were plotted to show inhibition of [^{3}H]-noradrenaline overflow as a function of the receptor occupation by the agonists (Figure 7). The curve for methacholine indicates that half maximal inhibition of [^{3}H]-noradrenaline overflow was obtained when roughly 2% of the total receptor population was occupied by methacholine, and 90% of the maximum response resulted from occupation of 10–20% of the receptors. On the other hand, half maximal inhibition was observed when about 40–60% of the receptors were occupied by pilocarpine. However, even when virtually all the receptors

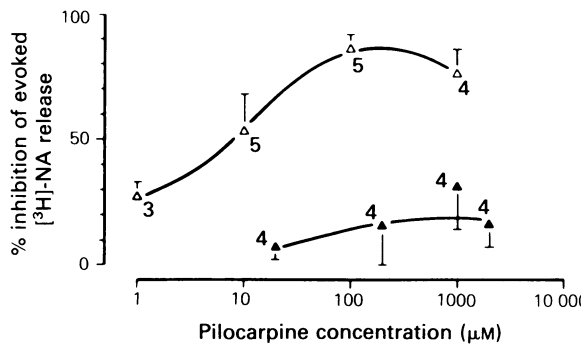

Figure 5 The inhibition by pilocarpine (μM) of the stimulation-evoked [^{3}H]-noradrenaline (NA) overflow. Symbols: (△) muscarinic receptors intact; (▲) after preperfusion with phenoxybenzamine (Pbz) 5 μM for 15 min. For further explanations see legend to Figure 3. After exposure to Pbz, no significant inhibition by pilocarpine was observed.

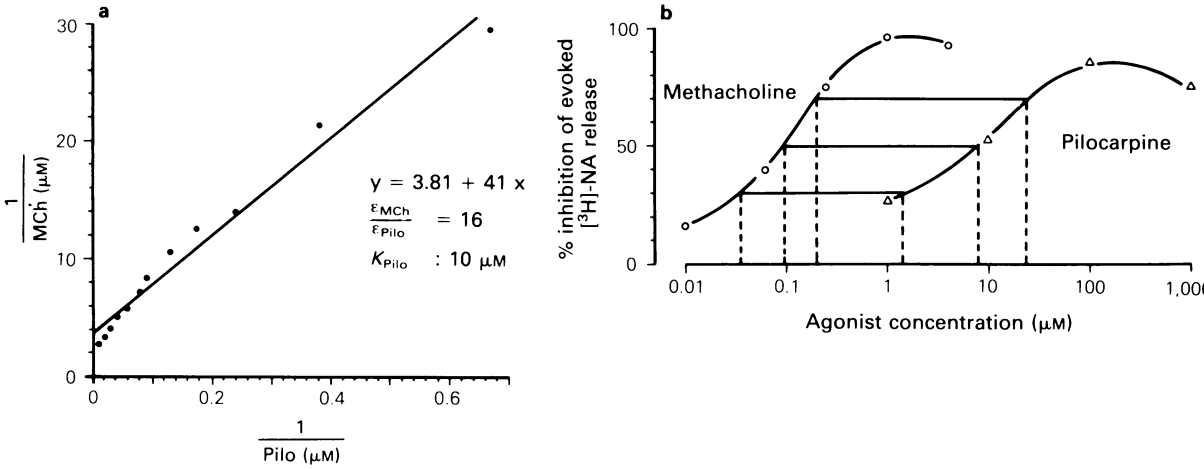

Figure 6 Determination of the dissociation constant K_A for pilocarpine (Pilo) at the presynaptic muscarinic receptor. The procedure for comparing equieffective agonist concentrations is illustrated in (b). The data shown in (b) are taken from Figures 3 and 5. The double reciprocal plot (a) of equieffective concentrations (pilocarpine vs. methacholine, (MCh)) is based on equation (3), see (Mackay, 1966). The value of the K_A for methacholine (4 μM, Figure 4) was used to calculate the relative efficacy ($\varepsilon_{MCh}/\varepsilon_{Pilo}$, equation (4) Methods) and the K_A value for pilocarpine (equation (5) Methods).

were occupied at the highest pilocarpine concentration, the evoked overflow was not totally inhibited.

Comparison of the presynaptic potency of methacholine (Figure 3) with that at postsynaptic sites (Figure 8) shows that the stimulation-evoked [^{3}H]-noradrenaline release is inhibited by lower methacholine concentrations than resting atrial tension development or beating frequency under the present conditions. Half maximal inhibition of transmitter release occurred at methacholine concentrations <0.1 μM, while half maximal inhibition of resting atrial tension development or heart rate was observed at concentrations around 0.5 or 1.5 μM. Phenoxybenzamine pretreatment rendered the agonist less potent; this result confirms the observations on presynaptic sites. Postsynaptic parameters, however, vary even more than presynaptic inhibition with respect to sensitivity towards the irreversible antagonist. The large inter-individual scatter of phenoxybenzamine effects, thus, prevented a meaningful analysis of postsynaptic K_A values under the present conditions.

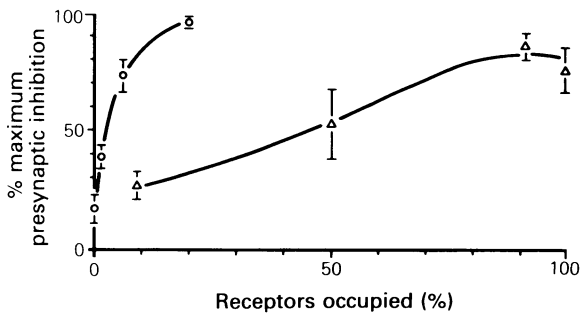

Figure 7 Presynaptic inhibition of [^{3}H]-noradrenaline release (% of maximum) by methacholine (○) and pilocarpine (△) as a function of receptors occupied (% of total population). The curves were calculated according to the law of mass action (equation (6) Methods; Besse & Furchgott, 1976) from the mean concentration-response curves in untreated hearts with K_A values for methacholine of 4 μM, and pilocarpine 10 μM (Figures 4 and 6). Each point represents the mean value and vertical lines show s.e. mean.

Discussion

The validity of the determination of K_A values carried out here depends on the acceptance of the theoretical assumptions of receptor-agonist interactions and fractional irreversible receptor inactivation as outlined by Furchgott (1966). In addition several basic experimental assumptions have to be met (Furchgott & Bursztyn, 1967), and will be discussed with respect to presynaptic muscarinic receptors: (a) The agonist used has to inhibit noradrenaline release (and not to stimulate at any concentration range) via muscarinic receptors (and not via another mechanism). The selectivity of methacholine in presynaptic muscarinic inhibition has been demonstrated previ-

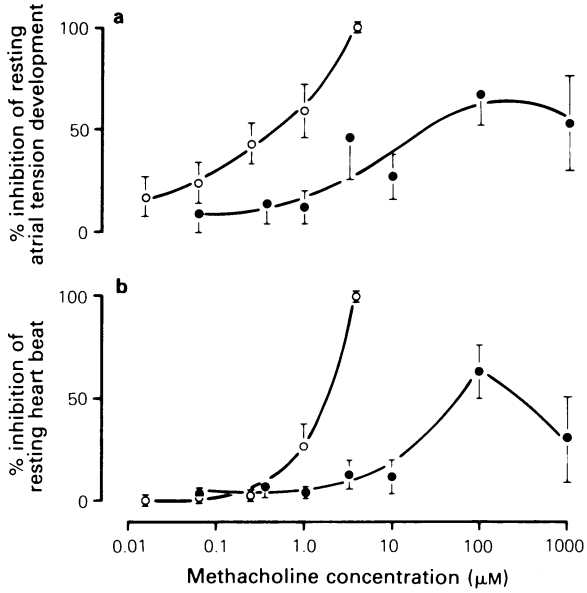

Figure 8 The inhibition by methacholine of resting atrial tension development (a) and ventricular rate (b) in the rat perfused heart. The inhibition is expressed as a percentage of total suppression of mechanical activity compared to resting parameters at the same time period in control hearts. Open symbols, no phenoxybenzamine pretreatment. Closed symbols, pretreatment with phenoxybenzamine 5 μM for 15 min. Each point represents a mean of 4–7 observations (except at 1000 μM, $n = 2$) and the vertical lines show s.e. mean. In control experiments the resting atrial tension development (100%) was 72 ± 9 mg ($n = 8$) and the resting beating frequency 143 ± 13 beats min^{-1} ($n = 8$). The absolute values (resulting in 100%) for the two groups shown did not differ significantly from the controls.

ously (Fuder *et al.*, 1982). Presynaptic nicotinic effects of methacholine and pilocarpine were not observed under the present conditions. The IC_{50} of pilocarpine in the presence of phentolamine 3 μM was very similar to the control IC_{50} (2 unpublished observations) indicating that presynaptic α_2-adrenoceptor activation was not involved in the action of pilocarpine.

(b) The equilibration times of agonists assume that a steady state exists, in which the concentration of agonist in the perfusion medium essentially reflects the concentration of drug in the biophase. It should be noted that agonist taken up by the tissue or perhaps metabolized was continuously replaced by new drug via the perfusion stream. When a given degree of inhibition is measured under equilibrium conditions the response is described by the mass action law which relates fractional receptor occupation to agonist concentration and K_A.

(c) The presynaptic muscarinic receptor population has to be uniform with respect to K_A. All the evidence accumulated so far points to homogeneity of the presynaptic muscarinic receptors with respect to dissociation constants (K_B) for a variety of selective antagonists including pirenzepine and gallamine in rat and rabbit heart (Fuder, 1982). The linearity of double reciprocal plots presented here is compatible with homogeneity with respect to K_A.

(d) Densensitization of the presynaptic muscarinic receptors appears unlikely because the inhibition by agonists observed after exposure times of 3 or 8 min were very similar, even at high concentrations, unless a very rapid desensitization (occurring within 3 min) took place. Desensitization of postsynaptic muscarinic receptors in guinea-pig atria has also been shown not to occur within 2.25 min of addition of acetylcholine (Furchgott *et al.*, 1960). If presynaptic muscarinic receptors behave like postsynaptic ones, a rapid or transient inactivation of presynaptic receptors upon formation of the receptor-agonist complex or afterwards would be unlikely.

(e) Both before and after the transient exposure to phenoxybenzamine, the response should remain the same function of the number of receptor-agonist complexes and of the intrinsic efficacy of the agonist, i.e., the efficacy must stay the same. On the other hand, phenoxybenzamine effects unrelated to irreversible muscarinic receptor inactivation have to be excluded. We chose phenoxybenzamine as irreversible receptor antagonist in these studies, be-

cause no other alkylating compound exists that has been as well characterized in studies on presynaptic mechanisms (for review see Furchgott, 1966; Gillespie, 1980). Inhibitors of neuronal and extraneuronal uptake were present throughout in all experiments. Thus, irreversible uptake blockade by phenoxybenzamine after washout can be neglected. In addition, the presence of cocaine and corticosterone ensured that essentially the total amount of [^{3}H]-noradrenaline released appeared in the perfusate as authentic [^{3}H]-noradrenaline. The stimulation conditions were carefully selected to prevent activation of the α_2-adrenoceptor-mediated autoinhibition (Table 2). A possible variation in the extent of presynaptic β-adrenoceptor mediated facilitation of release was excluded by the addition of propranolol. Taken together, the overflow of [^{3}H]-noradrenaline (independent of exposure to phenoxybenzamine) reflected the release as the functional parameter located as close to presynaptic receptor activation as possible (or at least closer than postsynaptic responses following transmitter release).

Phenoxybenzamine should not alter the sensitivity of the electrosecretary coupling mechanism leading to exocytotic noradrenaline release, except by reducing the concentration of active receptors to be occupied by the muscarinic agonist. The evoked release of noradrenaline was independent of phenoxybenzamine exposure (Table 1), and this suggests that the inhibition by phenoxybenzamine of calcium influx leading to release of transmitter is negligible under the present conditions. In mouse neuroblastoma cells, phenoxybenzamine 0.2 μM and dibenamine 5 μM appear to inactivate the muscarinic receptors and, in addition, to depress the calcium channel effectors of the muscarinic receptors (El-Fakahany & Richelson, 1981).

In the rat perfused heart, inactivation of receptors probably does not vary within different regions of the heart, because the effective perfusion presumably exposes all regions to phenoxybenzamine to a similar extent. The diffusion barriers are as few as possible in a perfused organ, in contrast to incubated or superfused tissue where some but not all cells may be exposed to the irreversible antagonist (Waud, 1968).

Two major drawbacks of the model presented here should be pointed out: (i) due to a limited number of successfully applicable nerve stimulations, IC_{50} values for agonists cannot be determined in one and the same heart before and after irreversible receptor inactivation. Therefore, mean concentration-response curves were compared. This procedure appeared feasible as control responses showed very small inter-individual variations. (ii) The response measured is an inhibition of a function (and not a stimulation, e.g., like a contraction). However, the K_A values for carbamylcholine have been found to be very similar and independent of whether an inhibitory (decrease in atrial tension development of reserpinized guinea-pigs) or excitatory (contraction of rabbit stomach fundal or aortic smooth muscle) response was measured (Furchgott, 1966).

The main outcome of the present paper is that the muscarinic inhibition of [^{3}H]-noradrenaline release is an effector system that displays a receptor reserve in case of activation by an agonist of high intrinsic efficacy like methacholine, but not pilocarpine, an agonist with an affinity not much lower than that of methacholine (3 fold difference), but lower intrinsic efficacy (16 fold difference). The IC_{50} value for methacholine, thus, does not reflect the affinity of agonist for the receptor. On the other hand, the IC_{50} for pilocarpine was close to the K_A value. To our knowledge, this is the first direct functional evidence for spare receptors in a presynaptic effector system.

The relationship between the fractional receptor occupancy and response implies that low concentrations of a strong agonist like methacholine (or presumably acetylcholine, the natural ligand) induce a large response by occupying a small fraction of the total presynaptic receptor population. Acetylcholine has been shown to possess a higher efficacy than methacholine (Furchgott & Bursztyn, 1967). We do not know much about the physiological function of the presynaptic muscarinic receptors, but the evoked release of [^{14}C]-acetylcholine upon vagal stimulation has been shown to decrease the release of [^{3}H]-noradrenaline from rabbit isolated atria (Muscholl & Muth, 1982). It is tempting to speculate that, in the heart, acetylcholine released upon vagal activity has to be present in the biophase in a very low concentration to induce the muscarinic inhibition of noradrenaline release. Thus, vagal activity could not only effectively depress myocardial activity at a postsynaptic level, but also decrease the sympathetic nerve activity at the presynaptic postganglionic level with a high efficiency, though perhaps only in parts of the heart where reciprocal innervation exists (for review see Muscholl, 1980).

In the case of methacholine a steep increase in the response (up to 70% of the maximum) was observed at a low receptor occupancy. The increase became less steep and approached 100% response when about 30% of the receptors were occupied. This theoretical calculation of a receptor reserve of 70% was closely correlated to the experimental finding that full response was still observed after phenoxybenzamine (1 μM) pretreatment which left 32% of the receptors intact. The relationship between receptor occupancy and response for pilocarpine was less steep than that for methacholine, and a receptor reserve was lacking. Therefore, pilocarpine failed to inhibit the noradrenaline release after exposure to phenoxybenzamine 5 μM. This procedure left 2% of

the receptors intact, a fraction not sufficient to induce a presynaptic inhibition that can be detected at the present sensitivity limit of the method. The findings can be explained by the classical concept of 'spare receptors', and there is no need to postulate different types of interaction between the two different agonists and the receptor. The probability for a given agonist to induce a response depends not only on its affinity for the receptor but also on the ability to initiate reactions subsequent to receptor occupation. Apparently the coupling process between occupation and response is less stringent for pilocarpine. In an effector system with a limited number of receptors or an even weaker coupling process, a partial agonist like pilocarpine may behave as an antagonist by occupying all the receptors and thereby reducing the probability for a stronger agonist to induce a response. Pilocarpine has been shown to act as an antagonist in the fundus strip of the rabbit stomach after fractional receptor inactivation and its K_A value was found to be similar to its K_B value (Furchgott & Bursztyn, 1967. Similar results were observed for pilocarpine in the guinea-pig ileum (Waud, 1969). Pilocarpine acted as an antagonist against carbachol in the rabbit iris (Akesson *et al.*, 1983). Recently a compound (N-methyl-N-(1- methyl -4- pyrrolidino -2- butyl) acetamide) was investigated that behaved as a presynaptic antagonist (or agonist under certain conditions) and as a postsynaptic agonist (Nordström *et al.*, 1983). The differences in drug behaviour may arise from differences in receptor number or in the efficiencies of signal transmission from the activated receptor to the final end organ response.

The presynaptic muscarinic receptor structure as reflected by the determined K_A values is similar to that of postsynaptic muscarinic receptors investigated in various organs. The K_A values for methacholine (4.0 μM) and pilocarpine (10 μM) are close to those measured in rabbit stomach smooth muscle (2.5 μM and 7 μM, respectively; Furchgott & Bursztyn, 1967).

The K_A value for pilocarpine in the guinea-pig ileum is 9 μM (Waud, 1969) and the K_B against carbachol in the iris of albino rabbits, 4 μM (Akesson *et al.*, 1983). Unfortunately, our method did not allow the simultaneous determination of presynaptic and postsynaptic K_A values for the agonists. The postsynaptic data, however, qualitatively confirm the inactivation of muscarinic receptors by phenoxybenzamine at a myocardial level. Hence, the inhibitory presynaptic muscarinic receptors behave like postsynaptic receptors with respect to antagonists (Muscholl, 1980; Fuder, 1982) and two agonists (present paper). The functionally determined K_A value for pilocarpine is in agreement with the affinity constant determined in binding studies (6 μM; Birdsall *et al.*, 1978). In these binding studies, stereoisomers of methacholine were investigated and found to exhibit biphasic competition curves with [^{3}H]-propylbenzilylcholine as ligand. The K_A at the high-affinity and low-affinity sites for (+)-methacholine was 0.4 or 32 μM. If we assume that the racemate used in our studies consists of 50% (+)-methacholine (the more active isomer), we would expect a functional K_A for (+)-methacholine of 2 μM, and, on the other hand, in binding studies for the racemate K_A-high of 0.8 and K_A-low of 64 μM. Thus, our functional K_A value probably corresponds neither to the high- nor to the low-affinity binding site. Currently studies are being carried out in our laboratory to resolve whether methacholine stereoisomers differ not only in affinity but also in efficacy.

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