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Affinity and efficacy of racemic, (+)-, and (−)-methacholine in muscarinic inhibition of [^{3}H]-noradrenaline release

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1 The right postganglionic sympathetic nerves of rat isolated perfused hearts (previously loaded with [^{3}H]-noradrenaline) were stimulated electrically with 10 trains of 10 pulses at 10 Hz. The inhibition by methacholine of stimulation-evoked [^{3}H]-noradrenaline overflow into the perfusate (determined in the presence of corticosterone, desipramine, phentolamine, and propranolol) was taken as a measure for activation of presynaptic muscarinic receptors.

2 The evoked [^{3}H]-noradrenaline overflow was inhibited by (+)-, racemic, and (−)-methacholine in a reversible and concentration-dependent manner. The concentration causing 50% inhibition (IC_{50}) was 0.1, 0.26, and 65 μM, respectively, resulting in an isomeric potency ratio $IC_{50}(+)/IC_{50}(-)$ of 650.

3 The dissociation constant K_A of the (±)- or (+)-methacholine-presynaptic receptor complex was determined after fractional receptor inactivation according to Furchgott & Bursztyn (1967) with phenoxybenzamine or propylbenzilylcholine mustard as irreversible antagonists of muscarinic receptors. K_A for (−)-methacholine was estimated according to Mackay (1966). K_A of (+)-, (±)-, and (−)-methacholine were 2.5, 4 and 440 μM, resulting in an isomeric affinity ratio $K_A(+)/K_A(-)$ of 180. The discrepancy between the isomeric IC_{50} ratio and the isomeric K_A ratio is explained by a higher intrinsic efficacy of the (+)-enantiomer compared to the (−)-enantiomer. Thus, (+)-methacholine has to occupy fewer receptors to induce a given inhibition of release than its antipode as revealed by a plot of fractional receptor occupancy vs response.

4 The results show that, in the effector system of presynaptic muscarinic inhibition, methacholine enantiomers differ greatly not only in affinity for the receptor, but also to some extent in the efficiency of signal transmission, and both parameters contribute to the high isomeric potency ratio. The activity of the racemate is fully accounted for by the activity of the (+)-enantiomer.

Introduction

A tissue response upon addition of an agonist depends, in any effector system, on the concentration of receptors in the tissue, on the affinity for the receptor of the agonist used, and on the efficiency of signal transmission after formation of the agonist-receptor complex to induce the pharmacological response measured. A week stimulus followed by a weak response at a high agonist concentration may be related to a low receptor concentration in the tissue, a low affinity for the receptor of the agonist, or a low efficacy, i.e. the probability of receptor occupancy to be followed by a biological response. While, to some extent, the concentration of specific binding sites (receptors?) and affinity constants for agonist-binding sites can be estimated in binding studies, the determination of the efficacy remains the exclusive domain of functional experiments.

Methacholine, a selective muscarinic agonist, possesses a chiral centre at the ß-carbon atom and therefore exists as two stereoisomers ((+) and (−)). The isomeric potency ratio has been determined before in functional studies carried out in postsynaptic effector systems (for review see Triggle, 1984). To our knowledge, however, isomeric potency ratios in presynaptic effector systems, or isomeric affinity ratios for the enantiomer-presynaptic receptor complex, have not been investigated before. We therefore studied the inhibition of transmitter release from the rat heart sympathetic nerves by the racemate and the pure enantiomers of methacholine. We also attempted

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to determine in the same presynaptic model the contribution to the stereoisomer potency of affinity (as reflected by the dissociation constant of the agonist receptor complex according to Furchgott & Bursztyn, 1967 or Mackay, 1966) and of efficacy (as reflected by the relative receptor occupancy resulting in a given response; Besse & Furchgott, 1976). The results show that not only affinity but also efficacy is a decisive parameter in presynaptic mechanisms.

Methods

Hearts of male Wistar rats (160–350 g, i.p. injection of 1000 u heparin 15 min prior to killing) with the right postganglionic sympathetic nerves attached were perfused according to the Langendorff technique at a rate of 6 ml min^{-1} and at 34–35°C as described previously (Fuder *et al.*, 1982; 1983). The perfusion medium (Tyrode solution of the following 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, and (+)-ascorbic acid 0.057; gassed with 5% CO_2 in O_2) contained corticosterone 10, desipramine 0.1, phentolamine 1.0, and propranolol 0.1 μM (beginning 20 min before the first nerve stimulation until the end of an experiment).

The neuronal stores were labelled by an infusion (10 min) of (−)-[7,8−^{3}H]-noradrenaline (17.2 or 22.4 Ci $mmol^{-1}$, sp.act.) at a final concentration in the aorta of 1.2–3 nM (at a reduced $CaCl_2$ concentration of 0.45 mM). The radioactivity was washed out for either 60, 80 or 100 min (depending on the required washout time of the irreversible muscarinic receptor antagonists, see below). The sympathetic nerves were stimulated by platinum ring electrodes at a supramaximal current strength (30–40 mA; maintained throughout an experiment by a constant current unit and monitored on an oscilloscope). Square wave pulses were delivered by a Grass S6 stimulator (controlled by an electronic impulse generator) in 9 trains of 10 pulses (1 ms duration) at 10 Hz and at 5 min intervals (plus a 10th train at an interval of 15 min, S1–S10, to check the reversibility of presynaptic inhibition and the viability of exocytotic nerve function).

The perfusate (2.5 min samples) was collected continuously for 50 min starting 2.5 min before S1 and, after a break of 7.5 min for another 7.5 min. The total tritium content of each perfusate sample was determined in an aliquot of 1 ml by liquid scintillation spectrometry. [^{3}H]-noradrenaline was determined in the remaining perfusate after separation of ^{3}H-metabolites from [^{3}H]-noradrenaline by column chromatography according to Graefe *et al.* (1973) and with the modification described by Fuder *et al.* (1983). The overflow of ^{3}H and of [^{3}H]-noradrenaline was expressed as fmol 2.5 min^{-1} referring to the specific activity of [^{3}H]-noradrenaline infused. The evoked overflow of ^{3}H and of [^{3}H]-noradrenaline was calculated by subtracting the mean value of overflow from the samples before and after nerve stimulation from the overflow observed in the sample collected during nerve stimulation plus 2 min 29 s afterwards. The stimulation-evoked increase in [^{3}H]-noradrenaline overflow under control conditions (no exposure to irreversible antagonists) in the stimulation period S2 before addition of agonists (24 ± 4 fmol per 10 pulses, $n = 25$) accounted for most of the evoked increase in total tritium overflow (25 ± 4 fmol per 10 pulses, $n = 25$). In addition, [^{3}H]-noradrenaline represents the more sensitive parameter (Fuder *et al.*, 1983) of evoked release in the rat heart. We therefore used the modulation of evoked [^{3}H]-noradrenaline release for the determination of presynaptic agonist affinity constants.

The inhibition by (±)-, (+)-, and (−)-methacholine of the evoked [^{3}H]-noradrenaline overflow was taken as a measure of presynaptic muscarinic receptor activation (Fuder *et al.*, 1982; Fuchs & Fuder, 1985). We have previously described a modification of the method proposed by Furchgott & Bursztyn (1967) that allows the determination of the dissociation constant of the agonist-presynaptic receptor complex (Fuchs & Fuder, 1985). In the present paper, basically the same procedure was applied except that non-cumulative concentration-response curves were established. The determination of dissociation constants is probably independent of the type of concentration-response curve as the degree of inhibition of transmitter release observed upon cumulative drug addition ($n = 2$) was very similar to that observed in the non-cumulative procedure. These two experiments showed that the exposure time (2.5 vs 7.5 min) to any concentration of (±)-methacholine failed to affect the extent of inhibition.

Control experiments were carried out on (i) hearts perfused with the solution described above (naive hearts), (ii) hearts exposed to phenoxybenzamine 5 μM for 25 min (starting 10 min after the end of the labelling procedure; washout of the alkylating drug for 25 min), (iii) hearts exposed to propylbenzilylcholine mustard 0.2 μM for 10 min (starting 10 min after labelling; washout for 80 min).

Ascending agonist concentration-response curves (one in each heart) were obtained in naive hearts (S1 starting 60 min after the end of labelling) by addition of 4 concentrations for 5 min beginning at 2.5 min before S3, S5, S7, and S9 (5 min, washout of agonist before addition of the higher concentration). The agonist concentrations were increased in steps of either 10 fold (0.01, 0.1, 1, 10 μM, (±)-methacholine) or 4 fold (0.05, 0.2, 0.8, 3.2 μM, (+)-methacholine; 5, 20, 80, 320 μM, (−)-methacholine). The concentra-

tion-effect relationship for (±)-methacholine appeared unaffected by prolonged perfusion before S1 started as the effects observed after short (S1, 60 min after the end of labelling) or prolonged perfusion time (S1, 100 min after the end of labelling, $n = 2$) were very similar (not shown).

In a separate group of naive hearts, the enantiomer interactions were studied. Concentration-response curves for (+)-methacholine were determined in the presence of (−)-methachlorine 3 μM, a concentration without an effect on its own (present from 20 min before S1 throughout).

The effect of the acetylcholinesterase inhibitor, physostigmine, on the muscarinic inhibition by the degradable (+)-enantiomer was investigated. (+)-Methacholine (0.02 and 0.2 μM) was present for 5 min each in the absence (S3 and S5, respectively) and in the presence of physostigmine 1 μM (S7 and S9, respectively) which was added to the perfusion medium starting 2.5 min before S6 until the end of the perfusion.

The effect of (±)-methacholine was then assessed in hearts exposed (beginning 10 min after the end of labelling) to either phenoxybenzamine 5 μM (for 15 or 25 min) or propylbenzilylcholine mustard 0.2 μM (for 10 min). While the concentration-response curve for (±)-methacholine was not affected when the washout time for phenoxybenzamine was prolonged (S1 25 vs 45 min after the end of exposure to phenoxybenzamine, see Results), propylbenzilylcholine mustard had to be washed out for 80 min. After washout periods of 40 or 60 min, the slope of the concentration-response curves was similar to control curves and the maximum was depressed, but the concentration of half maximal response IC_{50} was shifted to the right > 100 fold. However, after prolonged washout lower (±)-methacholine concentrations regained effectiveness while the decrease in maximum effect persisted. This observation is compatible with the view that low concentrations of the alkylating derivative still present in the biophase will compete with the agonist in occupying the receptors. On the other hand, binding to the receptor of propylbenzilylcholine mustard may be reversible at first, and may become irreversible after some time as shown for other irreversible antagonists (Triggle, 1971). The latter possibility appears less likely, because in that case the maximum agonist effect should not have decreased after short washout periods. However, independent of the molecular mechanism, a reliable agonist dissociation constant can, at least in the rat perfused heart, only be derived after prolonged washout of the highly potent propylbenzilylcholine mustard: a short washout of the less potent phenoxybenzamine suffices.

Concentration-response curves for (+)-methacholine were determined in hearts pretreated for 25 min with phenoxybenzamine 5 μM (washout 25 min).

The non-cumulative concentration-response curves for the inhibition of [^{3}H]-noradrenaline overflow were constructed by subtracting the individual evoked overflow values (fmol per 10 pulses) at a given S3, S5, S7, and S9 in the presence of agonist from the mean of the evoked overflow before and after that. The decrease observed in the presence of agonist compared to the individual expected control values was expressed as a percentage of total suppression of release. The control values for calculation of cumulative concentration-response curves were taken from the combined control stimulations in the absence of agonists. The agonist concentrations resulting in a given degree of inhibition (steps of 5–10%) were estimated graphically from a semilogarithmic plot for the mean value curves (naive hearts) and for the individual curves obtained in pretreated hearts.

The (±)- and (+)-methacholine dissociation constants (K_A) and the fraction of intact receptors (q) after washout of the irreversible antagonist were calculated by comparing the mean of equieffective concentrations of agonist in naive hearts [A] with the individual values observed in the hearts pretreated with the alkylating compounds [A'] in double reciprocal plots (Furchgott & Bursztyn, 1967). A precise description of the mathematical procedures used here has been given elsewhere (Fuchs & Fuder, 1985).

The amount of (−)-methacholine available was not sufficient to allow the K_A determination according to Furchgott & Bursztyn (1967). Therefore K_A was calculated by comparing individual concentrations of (−)-methacholine with the mean of (+)-methacholine concentrations (in naive hearts) that resulted in equal effects in double reciprocal plots (Mackay, 1966). This procedure allows the estimation of K_{A_2} of one agonist when the K_{A_1} of another agonist (i.e., (+)-methacholine) acting at the same receptor is known. In addition, the relative efficacy ($\varepsilon_1/\varepsilon_2$) of the two agonists can be derived from the relationship between equieffective concentrations [A_1] and [A_2] (Mackay, 1966; modified according to Furchgott & Bursztyn, 1967; for details see Fuchs & Fuder, 1985).

When the K_A of an agonist-receptor complex is known and the agonist-receptor interaction obeys the law of simple mass action, the original agonist concentration-response curves can be analysed and replotted with respect to the fraction of total receptor population which has to be occupied to induce a given response (Besse & Furchgott, 1976; Fuchs & Fuder, 1985.

Occasionally the ^{3}H-content of hearts was determined as described previously (Fuder *et al.*, 1983) after the end of the perfusion. It was 14.3 ± 1 pmol g^{-1} wet weight ($n = 12$). In these hearts a fractional rate of [^{3}H]-noradrenaline overflow per pulse at S10 of $1.5 \pm 0.26 \times 10^{-4}$ ($n = 12$) was calculated.

The results are expressed as the mean ± s.e.mean.

Statistical differences between two means ($P < 0.05$) were determined either by paired t test, Student's t test, and, if more than one group of treatment was compared with one control group, by Dunnett's test following analysis of variance (Dunnett, 1964). All straight lines were drawn by linear regression.

Drugs

The following drugs dissolved in distilled water, saline, propylene glycol, or ethanol were used: corticosterone (Sigma; 50 mM, stock solution in propylene glycol), desipramine hydrochloride (Ciba-Geigy), (±)-methacholine chloride (Sigma), (+)- and (−)-methacholine iodide (synthesized by Dr Scott and kindly provided by Dr J.M. Young, Cambridge), (−)-[7,8−^{3}H]-noradrenaline (NEN, dissolved in 0.9% w/v NaCl solution), phenoxybenzamine hydrochloride (Röhm Pharma; 1 mM, stock solution in propylene glycol), phentolamine mesylate (Ciba-Geigy), physostigmine salicylate (Merck), (±)-propranolol (ICI), propylbenzilylcholine mustard (Amersham; 1 mM, dissolved in ethanol. Cyclization to form the active aziridinium ion was carried out at 22°C for 25 min plus for 5 min at 45°C).

Results

The [^{3}H]-noradrenaline overflow in the sample collected before S1 represented 25 ± 2 (n = 25), 22 ± 3 (n = 15) and 24 ± 3 (n = 9) % of total [^{3}H] in naive, phenoxybenzamine-pretreated, and propylbenzilylcholine mustard-pretreated hearts, respectively. S1 increased the overflow 3.1 fold from 11 ± 1.3 to 34 ± 6.4 fmol per 2.5 min (n = 25) in naive hearts. After exposure to phenoxybenzamine (Fuder & Fuchs, 1985) or propylbenzilylcholine mustard the resting overflow was not different from that in naive hearts, and the increase by S1 was 3.1 fold or 2.9 fold, respectively (NS, analysis of variance, not shown). The evoked [^{3}H]-noradrenaline overflow varied depending on the loading concentration and was similar in naive and pretreated hearts when expressed as a percentage of S1 in the course of the stimulation periods (S2, 4, 6, 8, 10). The evoked overflow observed after washout of agonist did not differ from that observed in hearts not exposed to agonist (Table 1).

The resting overflow was unaffected by (±)-methacholine in naive, in phenoxybenzamine- (Figure 1) or propylbenzilylcholine mustard-pretreated hearts, and by the pure enantiomers under the conditions tested (not shown). The evoked [^{3}H]-noradrenaline overflow was depressed by (+)-, (±)- or (−)-methacholine in a concentration-dependent manner (Figure 2). The IC_{50} values were 0.1, 0.26, and 65 μM, respectively. All three agonists abolished the evoked release completely. The potency of (+)-methacholine was not affected by the presence of (−)-methacholine 3 μM, indicating that this concentration of the less potent isomer cannot compete with (+)-methacholine at the presynaptic receptor. This excludes the possibility that the (−)-form has a high affinity but a low efficacy.

Table 1 The [^{3}H]-noradrenaline overflow (expressed as % of S1) evoked by the pulse train series (S2–S10) in hearts not exposed or exposed to phenoxybenzamine (Pbz) 5 μM (15–25 min), propylbenzilylcholine mustard (PrBCM) 0.2 μM (10 min) or methacholine (MCh; racemate, (+)- or (−)-enantiomer)

	S2	*S4*	*S6*	*S8*	*S10*
Controls, without and after Pbz or PrBCM, no MCh, n = 12	97 ± 5.3	100 ± 7.1	88 ± 6.2	100 ± 11.7	81 ± 8.2
Before and after MCh, no Pbz or PrBCM, n = 11	99 ± 3.3	96 ± 7.9	101 ± 9.1	93 ± 5.2	82 ± 7.3
Before and after MCh, after Pbz n = 15	108 ± 8.0	104 ± 7.4	98 ± 6.0	93 ± 7.8	85 ± 9.4
Before and after MCh, after PrBCM n = 4	97 ± 4.7	100 ± 3.8	92 ± 11.6	92 ± 9.3	77 ± 8.1

Conditions as described in Methods and Figure 1. Table contains combined values from hearts not exposed to agonists (line 1), from hearts exposed to agonist at S3, 5, 7, 9, (agonist was washed out before S2, 4, 6, 8, 10) but not to alkylating compounds (line 2), from hearts exposed to agonist after Pbz (line 3) or PrBCM (line 4). No significant differences between groups were observed. The results show that the agonist effect was quickly reversible and the course of evoked overflow after washout of drugs was affected neither by agonist nor by irreversible antagonist exposure.

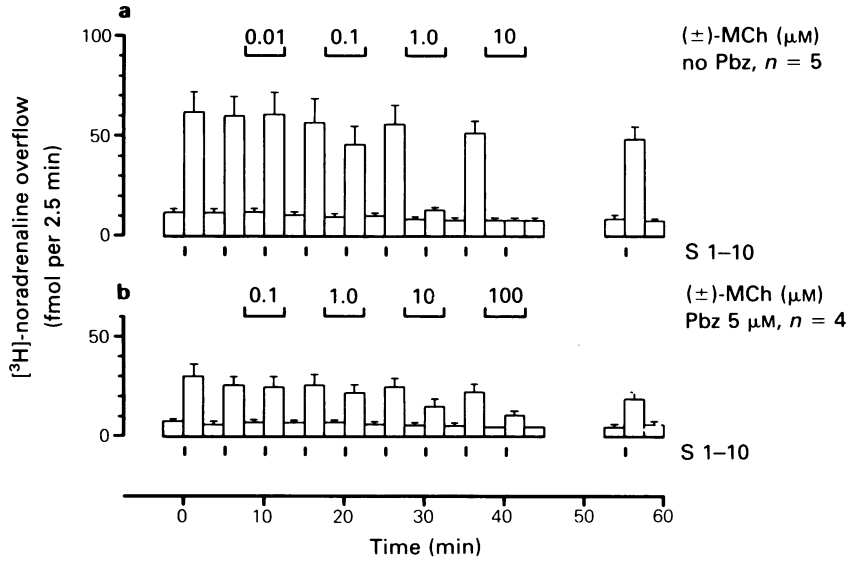

Figure 1 The overflow of [^{3}H]-noradrenaline from rat isolated hearts perfused with Tyrode solution in the presence of corticosterone 10, desipramine 0.1, phentolamine 1, and propranolol 0.1 μM. The mean overflow from 4–5 hearts (*n*) collected in 2.5 min samples with s.e.mean (columns, vertical bars) is expressed as fmol per 2.5 min referring to the specific activity of [^{3}H]-noradrenaline infused previously. Time 0 corresponds to 60 min after the end of the labelling procedure. The right postganglionic sympathetic nerves were stimulated with 10 trains of 10 pulses at 10 Hz (1 ms) when indicated by vertical bars below the columns (S1–10). The overflow shown in (a) was obtained from otherwise unpretreated hearts in which (±)-methacholine ((±)-MCh) (0.01–10 μM) was intermittently present in the perfusion medium as indicated by horizontal brackets. In (b) is shown the overflow from hearts exposed to phenoxybenzamine (Pbz) 5 μM for 25 min (25 min, washout of phenoxybenzamine before S1) in the absence and presence of (±)-methacholine (0.1–100 μM). The absolute values of evoked overflow at S1 did not differ significantly between the groups, as confirmed when all values observed in naive hearts were compared with hearts pretreated with phenoxybenzamine.

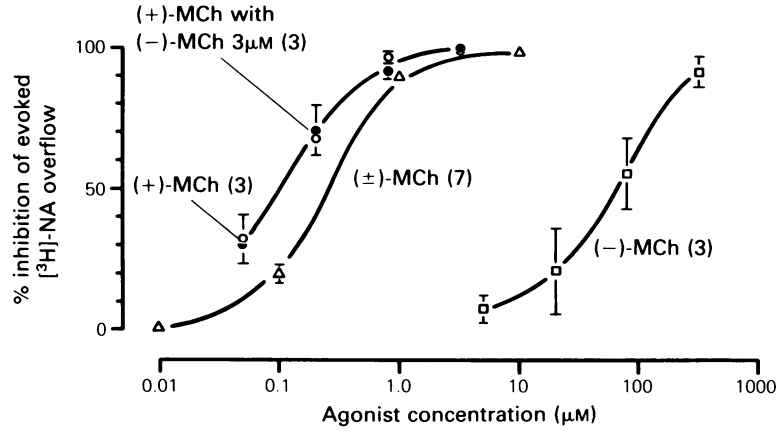

Figure 2 The inhibition of the stimulation-evoked (10 pulses, 10 Hz) [^{3}H]-noradrenaline ([^{3}H]-NA) overflow (determined in the presence of corticosterone, desipramine, phentolamine, and propranolol) by (+)-methacholine (MCh) in the absence (○) and presence (●) of (−)-methacholine 3 μM, by (±)-methacholine (△) and by (−)-methacholine (□) in the rat isolated heart. Non-cumulative concentration-response curves were constructed (except in 2 out of 7 hearts with (±)-methacholine, in which a cumulative procedure was carried out) as described in Methods. The inhibition was expressed as % of total suppression of [^{3}H]-noradrenaline release. Symbols are means of 3–7 hearts (4 points on a concentration-response curve each) with s.e.mean as vertical lines.

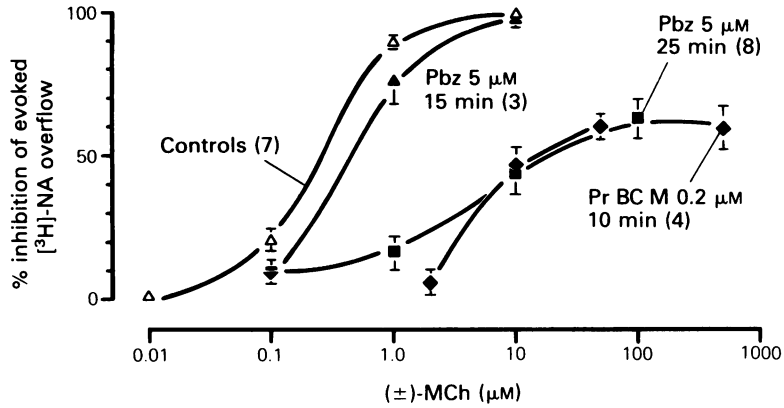

Figure 3 The inhibition by (±)-methacholine of the evoked [^{3}H]-noradrenaline overflow (conditions as under Figure 1, 2, and Methods) in naive hearts (Controls, △) and in hearts exposed to phenoxybenzamine (Pbz) 5 μM for 15 min (▲) or 25 min (■), and to propylbenzilylcholine mustard (PrBCM) 0.2 μM for 10 min (◆). Means with s.e.mean of 3–8 hearts.

Moreover, in 4 separate hearts the inhibition by (+)-methacholine (0.02 and 0.2 μM, present at S3 or S7 and S5 or S9, respectively) of evoked [^{3}H]-noradrenaline overflow was similar in the absence (22 ± 13 and 60 ± 8%, S3 and S5) and in the presence of physostigmine 1 μM (21 ± 7 and 69 ± 8%, S7 and S9), a concentration that failed to affect the evoked release (when present from S6 to S10) in the absence of (+)-methacholine. This concentration is known to inhibit acetylcholinesterase in guinea-pig ileum (Kilbinger & Wessler, 1980) and rabbit atria (Muscholl & Muth, 1982) by 80%. It is therefore unlikely that acetylcholinesterase activity falsifies the determination of the K_A of the (+)-enantiomer or racemate. (+)-Methacholine can be hydrolysed by the acetylcholinesterase, but (−)-methacholine is a weak inhibitor of the enzyme (Beckett *et al.*, 1963).

Exposure of the hearts to phenoxybenzamine 5 μM for 15 min shifted the (±)-methacholine concentration-response curve 2 fold to the right (Figure 3). After an exposure time of 25 min (and independent of the washout time of phenoxybenzamine, 25 or 45 min, each $n = 4$) both, slopes and maxima of the curves were decreased (Figure 3). Pretreatment with propylbenzilylcholine mustard 0.2 μM for 10 min resulted in a similar reduction of the maximum (about 40%), although the effect of the lowest (±)-methacholine

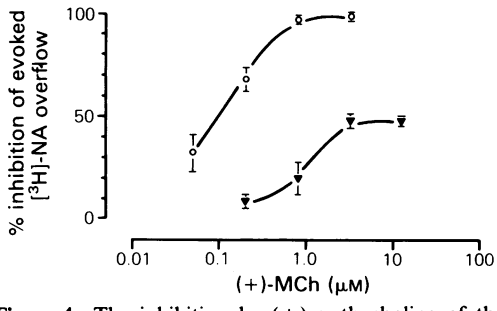

Figure 4 The inhibition by (+)-methacholine of the evoked [^{3}H]-noradrenaline overflow (conditions as under Figure 1, 2, and Methods) in naive (○) and phenoxybenzamine-pretreated (Pbz 5 μM, 25 min, ▼) hearts ($n = 3$–4).

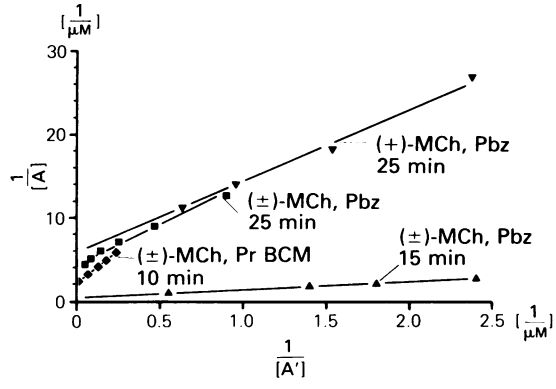

Figure 5 Double reciprocal plots of equieffective agonist concentrations before ([A]) or after fractional irreversible muscarinic receptor inactivation ([A']) by phenoxzybenzamine (Pbz) 5 μM or propylbenzilylcholine mustard (PrBCM) 0.2 μM (see Figures 1–4). Symbols represent single observations for (±)- and (+)-methacholine (MCh) from different groups of experiments: (▲) (±)-MCh, Pbz 15 min, $K_A = 2.4$ μM; (■) (±)-MCh, Pbz 25 min (short washout), $K_A = 1.1$ μM; (◆) (±)-MCh, PrBCM 10 min, $K_A = 5.8$ μM; (▼) (+)-MCh, Pbz 25 min, $K_A = 2.4$ μM. The reciprocal of a slope represents the fraction of intact receptors after Pbz or PrBCM exposure.

Table 2 Dissociation constants K_A of the methacholine (MCh) enantiomer-presynaptic receptor complex in the rat isolated heart

Agonist	*Antagonist*	*Exposure/Washout of antagonist* (min)		q°	K_A (μM)	n
(±)-MCh*	Pbz 5 μM	15	35	$0.56^a \pm 0.13$	$1.8^c \pm 0.6$	3
		25	25	$0.17^b \pm 0.02$	$1.2^d \pm 0.1$	4
		25	45	$0.13^b \pm 0.08$	4.8 ± 1.3	4
	PrBCM 0.2 μM	10	80	$0.05^b \pm 0.01$	$7.5^{cd} \pm 1.7$	4
	Pbz or PrBCM				$4.0^e \pm 0.9$	15
(+)-MCh*	Pbz 5 μM	25	25	0.09 ± 0.01	$2.5^f \pm 0.3$	4
(−)-MCh†					$440^{ef} \pm 200$	3

*K_A determined according to Furchgott & Bursztyn (1967) after fractional irreversible receptor inactivation.
†determined according to Mackay (1966) with K_A of (+)-MCh (2.5 μM) as reference value; relative efficacy (+)/(−), 4.4 ± 0.3 ($n = 3$).
°fraction of intact receptors, the reciprocal of the slope of straight lines in double reciprocal plots of equieffective agonist concentrations [A] vs [A'].
a vs b, c vs c, d vs d, f vs f, $P < 0.05$; e vs e, $P < 0.001$

concentration tended to be slightly less pronounced than presumed from the curves observed after phenoxybenzamine. Both, slope and maximum of the (+)-methacholine effect curve were depressed after exposure to phenoxybenzamine 5 μM for 25 min (Figure 4).

Double reciprocal plots of equieffective agonist concentrations (individual values from curves observed in pretreated hearts vs mean values from control hearts) resulted in straight lines ($P < 0.001$, for representative examples of plots see Figure 5). The mean reciprocal of the slopes of the straight lines are listed in Table 2 and represent the fraction of intact receptor population after fractional receptor inactivation (q). The mean K_A value was derived from the individual slopes and intercepts of the straight lines (Table 2).

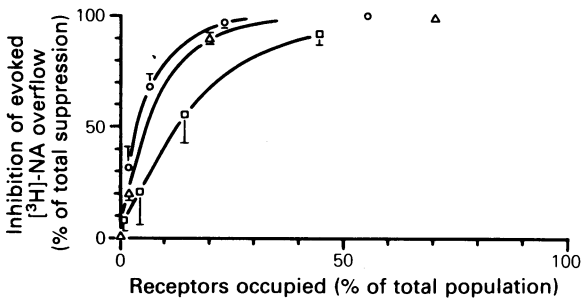

Figure 6 The presynaptic inhibition of [^{3}H]-noradrenaline overflow (% of total suppression) by (+)-(○), (±)-(△), and (−)-methacholine, (□) and the relative occupancy of presynaptic muscarinic receptors. The curves represent the original concentration-response curves in naive hearts transformed according to the simple mass action law of a bimolecular reaction with respect to K_A of agonists as summarized in Table 2. The curves of (+)- and (±)-methacholine should be superimposible, because only (+)-methacholine is responsible for the effect of the racemate. The slight deviation between the curves may reflect the experimental variation rather than different receptor reserves.

The K_A of (±)-methacholine after prolonged washout time for phenoxybenzamine tended to be higher and was certainly not lower than after short washout (as would be expected if the alkylating drug would still have competed with the agonist after incomplete clearance of reversibly bound phenoxybenzamine). The K_A determined after propylbenzilylcholine mustard (washout of 80 min) was not different from K_A after phenoxybenzamine and prolonged washout (washout of 45 min, Table 2). Hence, all four values were combined. The mean K_A of (+)-methacholine (2.5 μM) was slightly, but not significantly lower than the combined K_A of (±)-methacholine (4 μM).

The K_A of (−)-methacholine was derived from double reciprocal plots of equieffective concentrations of (+)-methacholine vs (−)-methacholine. The plots resulted in straight lines (0.99–0.94, correlation coefficients; n of 10% steps on concentration-inhibition curves = 5–8; $P < 0.001$). The mean relative efficacy (+)/(−) was 4.4 ± 0.3 ($n = 3$), and the mean K_A of (−)-methacholine (calculated with 2.5 μM, K_A of (+)-

methacholine as K_{A_1}) was 440 ± 200 μM (n = 3, Table 2).

The isomeric K_A ratio ((+)/(−)) of 180 accounts only in part for the 650 fold potency difference as reflected by the isomeric ratio of IC_{50} values. In addition to the agonist affinity ratio, a 4 fold 'isomeric efficacy ratio' contributes to the isomeric IC_{50} ratio. This finding is illustrated by the plot of relative receptor occupancy vs relative inhibition of [^{3}H]-noradrenaline overflow (Figure 6). In order to inhibit the release to the same extent, (−)-methacholine has to occupy more receptors than (+)-methacholine. Thus, 50 and 90% inhibition were obtained when 4 and 16% of the receptors were occupied by (+)-methacholine, or 13 and 41% by (−)-methacholine.

Discussion

The significance and the theoretical background of the determinations of agonist affinity constants and the estimation of parameters describing the processes subsequent to receptor activation in terms of efficacy or transducer function are subjects of comprehensive recent reviews (Ruffolo, 1982; Black & Leff, 1983). The validity of the determination of agonist-receptor dissociation constants after fractional irreversible receptor inactivation has been questioned (Triggle, 1971; El Fakahany & Richelson, 1981). A possible reason to doubt the validity of the use of alkylating agents is that these drugs, at high concentrations and when not removed from the biophase, may not only reduce the concentration of active receptors, but also in some way disturb the normal receptor-effector coupling mechanisms. However, the agonists affinity constants determined after fractional receptor inactivation (Furchgott, 1966) are very close to those determined according to a method not involving the use of alkylating agents (Waud, 1969) as confirmed by Parker (1972) and by Ringdahl (1984a) for muscarinic receptors in the intestinal smooth muscle, and by Ruffolo *et al.* (1979) for α-adrenoceptors in rat aorta.

We have shown before that phenoxybenzamine in the concentration used here and under similar conditions failed to affect the resting or evoked [^{3}H]-noradrenaline release from the rat heart, and that the modification of the muscarinic inhibition by methacholine is compatible with the concept of fractional inactivation rather than with unspecific effects (Fuchs & Fuder, 1985). The present results confirm the previous observation with phenoxybenzamine and extend it to propylbenzilylcholine mustard. The reported findings are unlikely to be affected by the well known phenoxybenzamine effects such as inhibition of neuronal and extraneuronal uptake and irreversible blockade of presynaptic α_2-adrenoceptors as the experiments were carried out in the presence of desipramine, corticosterone, and phentolamine. The prerequisites that have to be met before such a determination of presynaptic K_A values can be carried out have been discussed elsewhere (Fuchs & Fuder, 1985) and are mentioned only briefly here with special respect to the use of stereoisomers: (i) Methacholine stereoisomers were investigated separately and as a racemic mixture (with increasing concentrations of both antipodes). In addition, the (+)-enantiomer was studied in the presence of a fixed concentration of the (−)-form (Figure 2). The results showed that (+)-methacholine is approximately twice as potent as the racemic mixture. This is expected when the racemate consists of equal parts of both compounds and the (−)-form does not behave as a partial agonist (with high afinity but low efficacy) acting as an antagonist towards an agonist of higher intrinsic efficacy. The latter possibility can be excluded (at least for the (−)-methacholine concentration 3 μM) because the potency of the (+)-enantiomer was not affected by the presence of (−)-methacholine. This finding is important in the interpretation of the 'apparent K_A' of (±)-methacholine. When both antipodes in a racemate interact with the receptor either as two agonists of different affinity but similar efficacy, or as agonists of equal affinity but different efficacy, or as agonist and antagonist, in no case can the simple mass action law of a bimolecular interaction be applied as first discussed with respect to the K_A of racemic methacholine by Furchgott & Bursztyn (1967). Our results emphasize that the K_A of the racemate roughly corresponds to the K_A of the (+)-enantiomer after correction for the concentration which is probably only half of the racemate concentration. A contribution of the (−)-form can apparently be neglected. (ii) K_A values were determined with selective muscarinic agonists under equilibrium conditions at an apparently homogeneous presynaptic muscarinic receptor population (Muscholl, 1980; Fuder, 1982; Fuchs & Fuder, 1985). Moreover the effector system fails to exhibit signs of desensitization within the time interval of measurements. (iii) The concentration of available receptors is not allowed to vary in time within a given experiment, i.e., the inactivation of receptors should be irreversible, at least over the time of S2–S10. We have shown (Table 2) that the K_A of (±)-methacholine was not greatly affected by prolonged washout of phenoxybenzamine. Moreover the maximum inhibition did not increase with prolonged washout (compare Figure 1 and 3) as would be expected if new receptors had appeared or the inactivation were quickly reversible. It has been shown before that the half time of reappearance of receptor function after propylbenzilylcholine mustard exposure was 32 h at 37°C (Young *et al.*, 1972). (iv) The K_A value of (±)-methacholine determined after receptor inactivation by phenoxybenzamine was not different from that

observed after propylbenzilylcholine mustard although in the latter case a tendency to a slightly higher value existed. This may be due to a low concentration of the aziridinium ion still present in the biophase and competing with the lower agonist concentrations. The affinity of propylbenzilylcholine mustard for muscarinic receptors is high ($-\log K_B$, 8.0, quoted from Hulme *et al.*, 1978). On the other hand it may reflect a variation in sensitivity between different animals which was also observed in the three different groups after exposure to phenoxybenzamine. Despite the use of different irreversible antagonists, the rather non-selective phenoxybenzamine at a high concentration and at a shorter or longer exposure time, and the selective drug propylbenzilylcholine mustard at a low concentration and a short exposure time, the K_A values did not differ to an appreciable extent that might suggest inactivation of different receptor subclasses under different conditions. On the other hand, the similarity in K_A values argues for the assumption (which cannot be verified) that changes in the concentration-response relationships by the alkylating compounds are due rather to *receptor inactivation* than to changes of the reactions subsequent to receptor activation. It appears unlikely that phenoxybenzamine 5 μM and propylbenzilylcholine mustard 0.2μM modulate the effector system in such a strikingly similar way.

The isomeric potency ratio of methacholine stereoisomers reported in the literature varies considerably (Triggle, 1984). Thus, Beckett *et al.* (1963) found a ratio of 240 in the guinea-pig ileum, Ellenbroeck *et al.* (1965) of 320 in rat isolated jejunum, and Ward & Young (1977) and Chang & Triggle (1973) ratios of 470 and 1000, respectively, in the guinea-pig ileum isolated longitudinal muscle. The differences may arise from differences in purity of the methacholine stereoisomer preparations. Our presynaptic isomeric ratio of 650 comes close to that observed in the guinea-pig ileum (470) for the compound of identical origin (Ward & Young, 1977). However, we are aware that the null method used for the determination of the K_A value for (−)-methacholine (Mackay, 1968) may be subject to a certain error because the ratio of intrinsic efficacies of the two agonists compared is not very high (Mackay, 1966; Black & Leff, 1983). Such an error could affect our isomeric K_A ratio.

The isomeric ratios of binding constants from binding studies also vary greatly (Triggle, 1984). An isomeric IC_{50} ratio of 60 for methacholine enantiomers in inhibiting specific (±)-[^{3}H]-3-quinuclidinyl benzilate binding to rat cortex membranes (Ikeda *et al.*, 1980) contrasts with a ratio of 520 found in inhibiting [^{3}H]-*cis*-methyldioxolane binding to rat forebrain membranes (Ehlert *et al.*, 1980). A ratio of approximately 200 was found for the enantiomers of the same origin as our compounds when the inhibition of [^{3}H]-propylbenzilylcholine mustard binding to intact muscle strips of guinea-pig ileum was investigated (Ward & Young, 1977). Apparently the isomeric ratio of binding constants was smaller than the isomeric potency ratio when determined in the same organ with the same enantiomers by the same authors (200 vs 470, Ward & Young, 1977 280 vs 1000, Aronstam *et al.*, 1979).

A ratio of 230 was found for the inhibition of [^{3}H]-propylbenzilylcholine mustard to the high affinity binding site in rat brain membranes (Birdsall *et al.*, 1978), but it was only 31 at a low affinity binding site. In a later study in which displacement of labelled agonists was investigated, three agonist binding sites could be detected, and the affinity constants for the methacholine stereoisomers were determined (Birdsall *et al.*, 1980). At the site of super high, high, and low affinity, the K_A of (+)-methacholine was 0.13, 1.7, and 50 μM, and of (−)-methacholine 25, 170, and 1000 μM, respectively. The corresponding isomeric ratios were 190, 100, and 20, respectively. Among the three binding sites, only that of high affinity displays methacholine binding constants that correspond reasonably well to our functional affinity constants ((+), 2.5; (−), 440 μM). The isomeric K_A ratio (180) in our functional studies, however, is close to that found for the binding site of super high affinity (190). It is at present not easy to reconcile these divergent findings with a concept that combines results from functional experiments and biochemical binding studies.

Nevertheless our results show that differences in agonist affinity constants alone may not sufficiently explain high potency differences of enantiomers. In addition, differences in efficacy can affect the potency ratio. In the case of the methacholine stereoisomers, the potency difference was enhanced by a difference in efficacy. This may not always be the case and a decrease could be expected when the isomer of lower affinity has a higher efficacy. The structural requirements for high affinity and high efficacy of agonists can differ considerably (Ruffolo *et al.*, 1979; Ringdahl, 1984b). The isomeric affinity ratio of enantiomers of a certain oxotremorine analogue was 17 and the efficacy was similar; but the enantiomers of another analogue differed only 8 fold in affinity, and the compound of higher affinity also had a 4 fold higher efficacy (Ringdahl, 1984b). The potency differences of the latter pair was 33 fold, while that of the former was roughly 20 fold. Thus, a difference in efficacy can cause a greater potency difference (at a smaller isomeric affinity ratio) than a larger affinity difference at a similar efficacy.

When stereoisomers with a large difference in affinity are investigated, the question of even a small contamination of the less active compound by the isomer of higher affinity becomes crucial. Although we cannot totally exclude the possibility that (−)-

methacholine is virtually inactive and its apparent potency is due to traces of (+)-methacholine, the differences in efficacy between the stereoisomers argue for a distinct pattern of interaction of the two isomers with the receptor. When contaminating (+)-methacholine would contribute to the activity of (−)-methacholine, no differences in efficacy were to be expected.

The K_A of (±)-methacholine is similar to that found in the rat heart when the nerves were stimulated at a lower frequency (0.1 vs 10 Hz, Fuchs & Fuder, 1985; present paper) but under otherwise very similar conditions. Compared to the previous experiments, the IC_{50} of (±)-methacholine in naive hearts was higher at the 10 Hz frequency of stimulation. When the sympathetic nerves were stimulated at a frequency of 0.1 Hz, approximately 20% of the receptors were needed for 96 ± 2% inhibition of the [^{3}H]-noradrenaline release (Fuchs & Fuder, 1985). At a frequency of 10 Hz, 90 ± 2% or 99 ± 1% inhibition was observed when about 20 or 71% of the receptors were occupied (Figure 6). The spareness of presynaptic muscarinic receptors is thus still present at a high stimulation frequency although perhaps to a lesser extent. When potent agonists like methacholine are used, the receptor reserve at presynaptic sites is apparently high enough to overcome totally even the strongest stimuli of release in the peripheral sympathetic nervous system as evident from total suppression of the 140 mM potassium-evoked release (for review see Muscholl, 1980).

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