*Br. J. Pharmacol.* (1988), **93**, 357–366

# Subtypes of muscarinic receptor on cholinergic nerves and atrial cells of chicken and guinea-pig hearts

D. Jeck, R. Lindmar, [1]K. Löffelholz & M. Wanke

Department of Pharmacology, University of Mainz, Obere Zahlbacher Str. 67, D-6500 Mainz, Federal Republic of Germany

**1** Electrically driven chicken and guinea-pig atria were used to investigate the negative inotropic effects of the muscarinic agonists methacholine and acetylcholine (ACh). The release of ACh from isolated hearts into the perfusate in response to (preganglionic) vagal or (pre- and postganglionic) field stimulation was bioassayed on the guinea-pig ileum or determined by labelling with [$^3$H]-choline.

**2** Concentration-response curves for the negative inotropic effect of methacholine were shifted to the right by pirenzepine in various concentrations (0.03 to 10 $\mu$mol l$^{-1}$). The $pA_2$ values were 7.76 in chicken atria and 6.53 in guinea-pig atria. Pirenzepine and atropine antagonized the negative inotropic response to 0.3 $\mu$mol l$^{-1}$ ACh. The half-maximally effective concentrations ($IC_{50}$) of pirenzepine (Pz) and atropine were 40 and 5.4 nmol l$^{-1}$ in chicken atria and 330 and 3.5 nmol l$^{-1}$, respectively, in guinea-pig atria. Thus, the respective potency ratios ($IC_{50Pz}/IC_{50atropine}$) were 7.4 and 94.3 in the two species.

**3** Pirenzepine in low concentrations increased the release of unlabelled and $^3$H-labelled ACh from isolated hearts evoked by vagal and field stimulation only in chicken, but not in guinea-pigs. The half-maximally-effective concentration of pirenzepine was about 30 nmol l$^{-1}$ in the chicken heart, whereas, in the guinea-pig heart, an increased release was observed at 300 nmol l$^{-1}$.

**4** (+)-Tubocurarine [(+)-Tc; 100 $\mu$mol l$^{-1}$] reduced the release of ACh evoked by (preganglionic) vagal stimulation to a (+)-Tc-resistant release of about 30%. The time-course of the neuronal release of [$^3$H]-ACh was markedly altered: the onset was delayed and the termination was extended beyond the period of stimulation (1 min or 5 s) by several seconds. The (+)-Tc-resistant release was nearly abolished by 30 nmol l$^{-1}$ pirenzepine.

**5** In conclusion, the pre- and post-synaptic muscarinic receptors of the parasympathetic neuroeffector junction of the heart both belong to the $M_1$-subtype in the chicken and to an $M_2$-subtype in the guinea-pig. Block of the nicotinic ganglionic transmission in the chicken heart by (+)-Tc unmasked a muscarinic transmission, which presumably was mediated through $M_1$-receptors stimulating a low and prolonged postganglionic release of ACh.

## Introduction

In general, muscarinic receptors may be involved in transmission along the peripheral parasympathetic nerves at four different levels. (1) Presynaptic inhibitory autoreceptors at the parasympathetic ganglion modulate the release of acetylcholine; (2) postsynaptic excitatory somadendritic receptors produce slow depolarizing currents (slow e.p.s.p.); (3) presynaptic inhibitory autoreceptors at the neuroeffector junction regulate the release of acetylcholine and finally, (4) activation of postsynaptic receptors trigger the response of the target organ. For the heart, clear evidence has been obtained only for the pre- and post-synaptic receptors of the neuroeffector junction (reviewed by Löffelholz & Pappano, 1985).

Muscarinic receptors comprise at least two subtypes, $M_1$ and $M_2$, distinguished by an approximate 30 fold difference in their affinities for the antagonist pirenzepine (Hammer & Giachetti, 1982; Eglen & Whiting, 1986). It is generally believed that the somadendritic receptor mediating the slow e.p.s.p. belongs to the $M_1$-subtype, whereas the presynaptic autoreceptor and the receptors on non-neuronal cells (e.g. those mediating chronotropic and inotropic effects in the heart) all belong to the (heterogeneous) $M_2$-subtype. The present study provides evidence for presynaptic $M_1$-receptors and confirms the presence

[1]Author for correspondence.

of myocardial $M_1$-receptors mediating functional responses of the heart (Lindmar & Löffelholz, 1987).

The aim of this study was to investigate the presence of muscarinic receptors at the four different locations (described above) within the parasympathetic innervation of the heart and to classify these receptors as $M_1$ or $M_2$ according to their affinities for pirenzepine. Unfortunately the methods used did not allow conclusions to be drawn about the presence of ganglionic presynaptic autoreceptors, but only of somadendritic receptors, presynaptic autoreceptors of the neuroeffector junction and myocardial receptors.

Preliminary results have been published elsewhere (Jeck *et al.*, 1987).

## Methods

Chickens and guinea-pigs were stunned by a blow on the head and bled from the neck vessels. The hearts with or without attached vagus nerves were quickly removed and perfused according to Langendorff with Tyrode solution at rates of 20 (chickens) or 7 (guinea-pigs) ml $min^{-1}$. In other experiments, atria (some with attached vagus nerves) were incubated in 60 ml of Tyrode solution. The solutions were gassed with 95% $O_2$ plus 5% $CO_2$ and adjusted to a final temperature of 36°C. The composition of the Tyrode solution was (in mmol $l^{-1}$): $Na^+$ 149.3, $K^+$ 2.7, $Ca^{2+}$ 1.8, $Mg^{2+}$ 1.05, $Cl^-$ 145.5, $HCO_3^-$ 11.9, $H_2PO_4^-$ 0.4, (+)-glucose 5.6; and choline 0.01 was present in the release experiments.

The cervical vagus nerves of isolated hearts or atria were stimulated electrically (40 V, 1 ms) for various times (5–60 s) and at various frequencies (1–30 Hz). 'Bilateral' and 'unilateral' vagal stimulation indicate simultaneous and consecutive stimulation, respectively, of the right and left vagus nerves (see below). In some release experiments, the hearts were stimulated by field electrodes (85 V, 1 ms) (Lindmar *et al.*, 1979) for 1 min at 3 or 20 Hz.

To determine affinities of the muscarinic antagonist pirenzepine ($pA_2$ values), the effect of methacholine was analysed using atria, which were electrically paced at 240 beats $min^{-1}$ (25 V, 1 ms). The mean basic force of contraction was 6.8 mN $g^{-1}$ (chicken) or 20.0 mN $g^{-1}$ (guinea-pig). Concentration-response curves for methacholine were obtained by cumulative addition of the agonist every 30 s. The concentration-response curves were repeated four times, followed by a washout period of 30 min. In the absence of pirenzepine, the concentration-response curves could be reproduced without significant change. For Schild analysis (Arunlakshana & Schild, 1959), pirenzepine was added after completion of the first concentration-response curve. The concentration was increased each time a concentration-response curve was obtained. Since the regression of log [pirenzepine] on log (dose ratio $-1$) was found to be linear with slopes not significantly different from unity, $pA_2$ values were calculated.

Concentration-response curves were obtained for atropine and pirenzepine in antagonizing the negative inotropic response of chicken and guinea-pig atria to $3 \times 10^{-7}$ mol $l^{-1}$ acetylcholine. The agonist was added for six periods of 30 s at intervals of 30 min. Atropine or pirenzepine was added after the first exposure to acetylcholine and was present throughout the experiment; the concentration of the antagonist was increased after each exposure to acetylcholine. In control experiments, acetylcholine was added in the same way, but in the absence of an antagonist.

In order to study the atrial response to vagal stimulation, chicken atria (right plus left) were electrically paced at 240 beats $min^{-1}$ (15 V, 1 ms). The attached cervical vagus nerves were stimulated for 15 s at frequencies of 1 to 30 Hz (40 V, 1 ms). Atropine or pirenzepine was added at least 15 min before the first stimulation period.

The release of acetylcholine from perfused chicken hearts expressed as pmol per g wet weight was evoked by 1 min periods of electrical stimulation (see above) and was determined in three perfusate samples. These were collected before (to determine 'basal release'), during and in the minutes following the stimulation. The 'evoked release' was obtained from the second and the third samples after subtraction of the basic release. In some experiments, the right and left vagus nerves were stimulated consecutively at an interval of 5 min. The amounts of acetylcholine released at these two periods were determined separately and were averaged so as to represent 'release evoked by unilateral vagal stimulation'. Acetylcholine was bioassayed on the guinea-pig ileum (Dieterich & Löffelholz, 1977). The presence of $3 \times 10^{-8}$ mol $l^{-1}$ pirenzepine and of $10^{-4}$ mol $l^{-1}$ (+)-tubocurarine did not interfere with the bioassay. The specificity of the determination was tested with atropine in selected samples. In addition, the release of acetylcholine was studied by labelling the transmitter with [$^3$H]-choline according to Lindmar *et al.* (1980). In this method, 10 $\mu$Ci of [$^3$H]-choline (specific activity 80 Ci $mmol^{-1}$) was infused for 1 min after a 1 min period of vagal or field stimulation ('activating stimulation'), i.e., at a time when the neuronal uptake of choline was maximally activated. The $^3$H-overflow into the perfusate was evoked by 1 min- or 5 s-periods of field or vagal stimulation (3, 5 or 20 Hz) 20, 30 and 40 min after the 'activating stimulation'. The evoked $^3$H-overflow, which was obtained after subtracting the basal release, was identical with the neuronal release of [$^3$H]-acetylcholine (Lindmar *et al.*, 1980; 1982).

Results are expressed as means ± s.e.mean of $n$ experiments. The half-times of mono-exponential

washout curves were calculated from the regression equation. The significance of drug effects ($P < 0.05$) was calculated by Student's $t$ test.

Drugs used: acetylcholine chloride, choline chloride, methacholine chloride (Sigma Chemie GmbH, Deisenhofen, FRG), atropine sulphate (Merck, Darmstadt, FRG), [methyl-$^3$H]-choline chloride (80 Ci mmol$^{-1}$) (NEN Chemicals GmbH, Dreieich, FRG), (+)-tubocurarine chloride (Fluka AG, Buchs, Switzerland).

## Results

### *Negative inotropic effect of methacholine and acetylcholine*

Methacholine reduced the force of contraction of electrically paced chicken and guinea-pig atria (240 beats min$^{-1}$) in a concentration-dependent manner (Figure 1). Four concentration-response curves were carried out in each preparation, each after a washout interval of 30 min. In the absence of pirenzepine, the four curves were not significantly different from each other; the $EC_{50}$ values (concentration at half-maximal response) ranged from 0.34 to 0.47 μmol l$^{-1}$ in chicken atria and from 0.18 to 0.22 μmol l$^{-1}$ in guinea-pig atria. Pirenzepine, which was added cumulatively after completion of each curve, caused parallel shifts of the curves without depression of maxima. For Schild analysis, $pA_2$ values were calculated, since the regression of log [pirenzepine] on log (dose-ratio − 1) was found to be linear with mean slopes (1.06 in chicken and 0.92 in guinea-pig atria) not significantly different from unity (Figure 2). The $pA_2$ values were 7.76 ± 0.18 ($n = 8$) in chicken and 6.53 ± 0.02 ($n = 4$) in guinea-pig atria. Thus, the negative inotropic effects of muscarinic agonists are mediated through $M_1$-receptors in the chicken and through $M_2$-receptors in the guinea-pig.

The described species-difference of the receptor subtypes was also evident from the potency ratios for pirenzepine versus atropine in antagonizing the negative inotropic effect of $3 \times 10^{-7}$ mol l$^{-1}$ acetylcholine (Figure 3). In controls, the agonist was added for six periods of 30 s at intervals of 30 min with almost the same inotropic response. The basal force of contraction (6.8 mN g$^{-1}$ in chicken and 20.0 mN g$^{-1}$ in

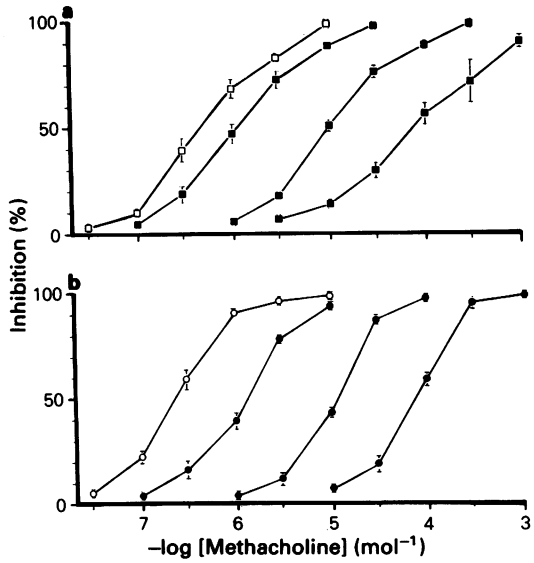



**Figure 1** Effect of pirenzepine on the concentration-response curves for methacholine in producing a negative inotropic effect on right atria of chickens (a) or guinea-pigs (b). Incubated atria, electrically paced at 240 beats min$^{-1}$, contracted with a mean force of 6.8 mN g$^{-1}$ (chicken) or 20 mN g$^{-1}$ (guinea-pig) under control conditions. Ordinate scale, inhibition by methacholine of force of contraction as % of control. The control curves (□, ○) were shifted in the presence of increasing concentrations of pirenzepine (■, ●: from left to right; $3 \times 10^{-8}$, $3 \times 10^{-7}$ and $3 \times 10^{-6}$ mol l$^{-1}$ in chicken atria and $10^{-7}$, $10^{-6}$ and $10^{-5}$ mol l$^{-1}$ in guinea-pig atria). The results represent the means of 8 (chicken) or 4 (guinea-pig) preparations; vertical lines indicate s.e.mean.

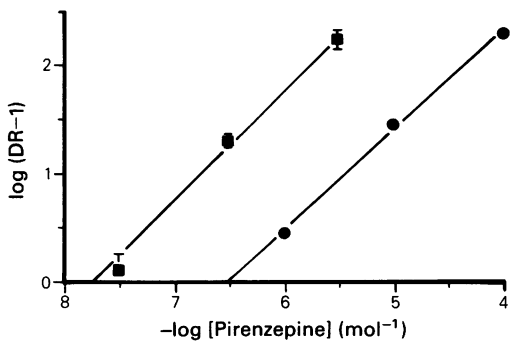



**Figure 2** Schild analysis of antagonism of methacholine-evoked negative inotropic effect by pirenzepine in chicken and guinea-pig atria. The analysis is based on data shown in Figure 1. Ordinate scale, log of the dose-ratio (ratio of $IC_{50}$ values of methacholine in the presence of pirenzepine to $IC_{50}$ values in the absence of pirenzepine) minus 1. Regression analysis of the plots yielded straight lines with slopes not significantly different from unity. The $pA_2$ values as determined from the extrapolated × intercept are 7.76 ± 0.18 (chicken; ■) and 6.53 ± 0.02 (guinea-pig; ●). The data presented are means and vertical lines indicate s.e.mean (in some instances, very small s.e.mean values are not shown as they were masked by the symbols).

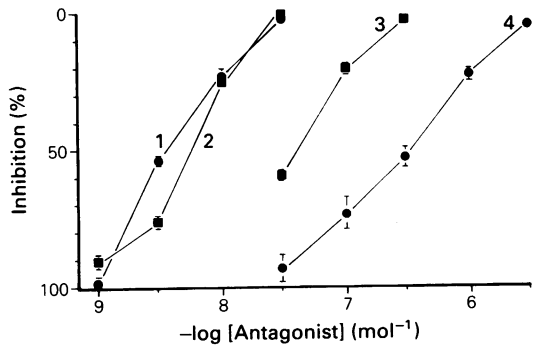



**Figure 3** Concentration-response curves for pirenzepine and atropine in antagonizing the negative inotropic effect of $3 \times 10^{-7}$ mol $l^{-1}$ acetylcholine in chicken and guinea-pig atria. Incubated right atria of chickens (■) and of guinea-pigs (●), electrically paced at 240 beats $min^{-1}$, contracted with a mean force of 6.8 mN $g^{-1}$ (chicken) and 20 mN $g^{-1}$ (guinea-pig). Acetylcholine was added for 30 s repeatedly at intervals of 30 min. Atropine or pirenzepine was added after the first exposure to the agonist; the antagonist concentration was increased after each addition of acetylcholine. Ordinate scale, inhibition by atropine or pirenzepine of the response to acetylcholine as % of the respective control. In separate control experiments (absence of antagonists), acetylcholine was also added six times (for details, see text). Abscissa scale, negative log concentration of atropine (1 and 2) or pirenzepine (3 and 4). Data presented are means of 4 to 8 experiments and vertical lines indicate s.e.mean (in some instances, very small s.e.mean values are not shown as they were masked by the symbols).

guinea-pig atria) was reduced by 32.6 ± 1.4% ($n = 6$) in chicken atria and by 75.1 ± 2.5% ($n = 6$) in guinea-pig atria. Both atropine and pirenzepine antagonized this effect. For guinea-pig atria, the half-maximally effective concentrations ($IC_{50}$) of atropine and pirenzepine were 3.5 and 330 nmol $l^{-1}$, respectively, whereas the corresponding $IC_{50}$ values for the chicken atria were 5.4 and 40 nmol $l^{-1}$, respectively. Thus the potency ratios $IC_{50\,pirenzepine}/IC_{50\,atropine}$ were 94.3 in guinea-pig atria and 7.4 in chicken atria.

*Release of unlabelled acetylcholine*

The release of acetylcholine into the perfusate was studied in chicken isolated hearts (Figure 4a and b). The release was evoked by field (pre- and postganglionic) or by vagal (preganglionic) stimulation for 1 min at 3 or 20 Hz.

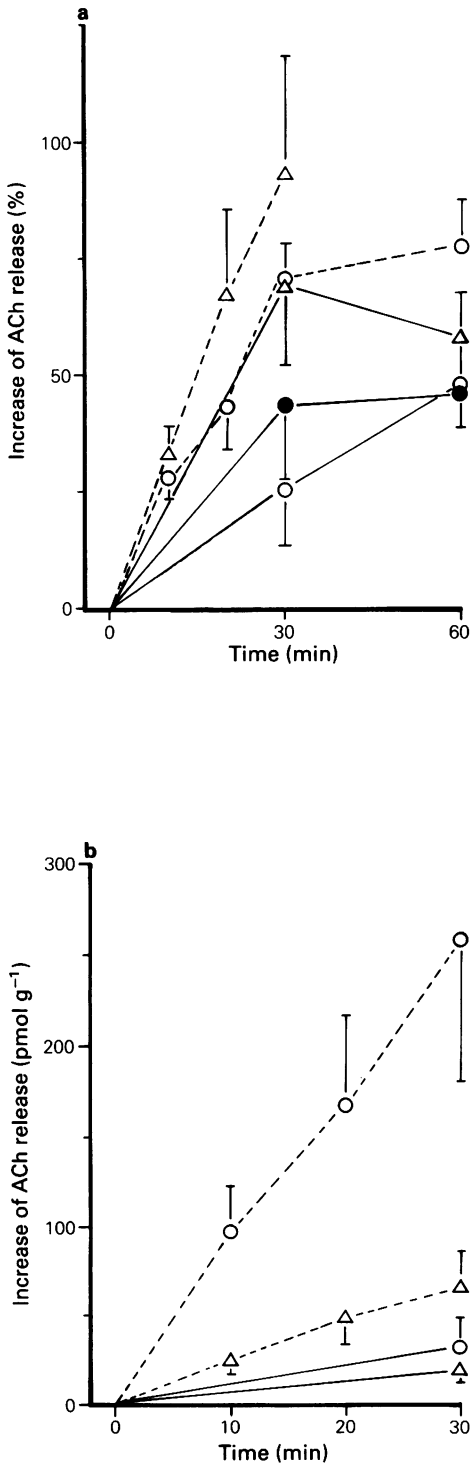
Initially the rate of basal release of acetylcholine was 19.0 ± 1.4 pmol $g^{-1}$ $min^{-1}$ ($n = 21$), which gradually declined to reach a rate of 13.4 ± 1.0 pmol $g^{-1}$ $min^{-1}$ after 90 min. Pirenzepine ($3 \times 10^{-8}$ mol $l^{-1}$) did not alter the basal release (19.0 ± 0.3 pmol $g^{-1}$ $min^{-1}$, $n = 7$) and (+)-tubocurarine ($10^{-4}$ mol $l^{-1}$) caused a small reduction (8.6 ± 0.7 pmol $g^{-1}$ $min^{-1}$, $n = 9$) at the end of the experiment.

In the absence of pirenzepine, the release expressed as pmol $g^{-1}$ evoked by the first stimulation period was as follows (number of experiments in parentheses): unilateral vagal stimulation at 3 Hz, 28 ± 5 (15) and 20 Hz, 122 ± 18 (9); bilateral vagal stimulation at 20 Hz, 191 ± 49 (7) and at 20 Hz in the presence of (+)-tubocurarine, 68 ± 3 (7); field stimulation at 3 Hz, 61 ± 6 (12) and 20 Hz, 395 ± 41 (12). These data suggest that the evoked release was frequency-dependent and was stronger with bilateral than with unilateral stimulation. The release evoked by (preganglionic) vagal stimulation was reduced by (+)-tubocurarine to about one third. In contrast, (+)-tubocurarine failed to alter the release caused by field stimulation (Lindmar *et al.*, 1979).

The values obtained in the first stimulation period served as controls for evaluating the responses to pirenzepine, which was added after the first stimulation (Figure 4). For all of the above listed conditions, separate experiments with repeated stimulations were carried out in the absence of pirenzepine. In these experiments, the amounts of acetylcholine released by the last stimulation were equal to the initial release. The 'greatest decrease' was observed with field stimulation at 3 Hz, which was only 10 ± 7% ($n = 4$) of the initial release. Therefore no corrections of the facilitatory effects of pirenzepine (Figure 4a and b) were necessary.

The effects of pirenzepine on the release of acetylcholine from chicken isolated hearts were investigated in the absence and presence of (+)-tubocurarine. Pirenzepine in a low concentration ($3 \times 10^{-8}$ mol $l^{-1}$) markedly increased the release of acetylcholine evoked by vagal or field stimulation in the absence of (+)-tubocurarine. The increase is obvious whether expressed as % (Figure 4a) or in absolute terms (Figure 4b). The maximum increase, which was reached between 30 and 60 min, ranged from 50 to 100%. There was no difference between the effects of pirenzepine on the release caused by uni- and bilateral vagal stimulation. In contrast, pirenzepine was less effective during vagal stimulation than during field stimulation (Figure 4a and b). It follows that pirenzepine must have increased the release of acetylcholine by blocking a postganglionic rather than a ganglionic autoreceptor. The absolute increase by pirenzepine of the transmitter release was greater at 20 Hz than at 3 Hz, an observation that was based on the experiments with field and also with vagal stimulation (Figure 4b). This result suggests that the autoinhibition increased with increasing synaptic concentrations of the transmitter.

(+)-Tubocurarine ($10^{-4}$ mol $l^{-1}$) reduced the release




of acetylcholine evoked by bilateral vagal stimulation at 20 Hz to about one third of the control (see above). This remaining release may be called 'tubocurarine-resistant', because $10^{-4}$ mol $l^{-1}$ (+)-tubocurarine was found to be maximally effective (not documented). It was found (Figure 5) that this tubocurarine-resistant release was markedly reduced by $3 \times 10^{-8}$ mol $l^{-1}$ pirenzepine. Again the effect of pirenzepine developed gradually just as the (enhancing) effects observed without (+)-tubocurarine (see above). This result is compatible with the somadendritic excitatory muscarinic receptors frequently characterized as $M_1$-receptors (Kilbinger, 1984; Brown *et al.*, 1986; Eglen & Whiting, 1986).

*Release of [$^3$H]-acetylcholine*

The release of [$^3$H]-acetylcholine was studied in perfused hearts of the chicken and guinea-pig (Figures 6 and 7). The method of labelling acetylcholine in the perfused heart and its release were described and analysed previously (Lindmar *et al.*, 1980).

In a first experimental series, the effect of (+)-tubocurarine on the time-course of the evoked release of [$^3$H]-acetylcholine was studied in chicken perfused hearts (Figure 6). The release was caused by (preganglionic) bilateral vagal stimulation for 1 min or 5 s at 20 Hz. In the experiments with 1 min periods of stimulation, atropine ($3 \times 10^{-6}$ mol $l^{-1}$) was present in all experiments, i.e., in the absence and presence of (+)-tubocurarine. Analysis of the time-course of the $^3$H-overflow into the perfusate revealed that (+)-tubocurarine did not affect the monoexponentially declining rate of $^3$H-washout from the extra-cellular space. The half-times ranged from 12.1 to 16.0 s (Figure 6) and were not different from a mean value (14.2 s) that had been found previously in a detailed investigation on the kinetics of acetylcholine in the same preparation (Lindmar *et al.*, 1982). In contrast, the onset of the neuronal $^3$H-release was delayed and

**Figure 4** The increase induced by $3 \times 10^{-8}$ mol $l^{-1}$ pirenzepine of the evoked release of acetylcholine (ACh) in the perfused chicken heart. ACh was released by field stimulation (broken lines) or unilateral vagal stimulation (solid lines) at 3 Hz (△) or 20 Hz (○), or (a) bilaterial vagal stimulation (●; 20 Hz). The stimulation periods lasted for one min and were carried out at various times. (a) Ordinate scale, increase of ACh release as % of the release evoked at zero-time (control effect), i.e., just before addition of pirenzepine. (b) Ordinate scale, increase of ACh release expressed as pmol $g^{-1}$ above the control release (stimulation at zero-time). Abscissa (scales), time after the control stimulation (min). For the absolute amounts of control release, see text. The data presented are means of 4 to 9 experiments and vertical lines indicate s.e.mean.

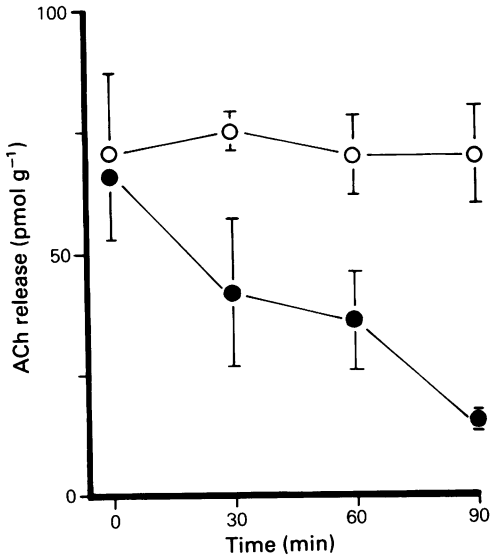



**Figure 5** Effect of $3 \times 10^{-8}$ mol l$^{-1}$ pirenzepine on the evoked release of acetylcholine (ACh) in the presence of (+)-tubocurarine. The release of ACh was evoked by (preganglionic) bilateral vagal stimulation at 20 Hz in perfused chicken hearts. The nerves were stimulated for periods of one min at 0, 30, 60 and 90 min in the presence of $10^{-4}$ mol l$^{-1}$ (+)-tubocurarine. (○) Absence of pirenzepine, (●) pirenzepine was added after the first stimulation period. Abscissa scale, time after the first stimulation period. Data presented are means of 4 experiments and vertical lines indicate s.e.mean.

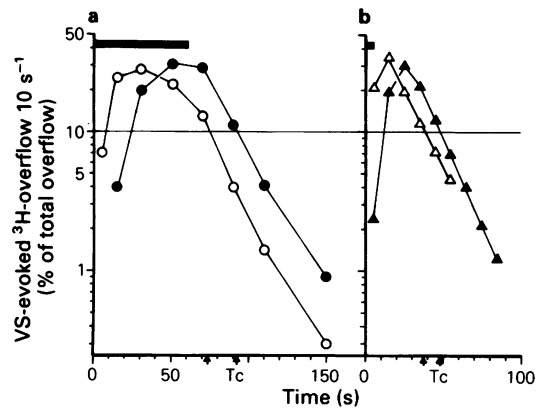



**Figure 6** The effect of (+)-tubocurarine on the time-course of the $^3$H-overflow into the perfusate of the chicken heart evoked by bilateral vagal stimulation (VS). Both vagus nerves were stimulated at 20 Hz for 1 min (a) or 5 s (b) (horizontal bars) to release [$^3$H]-acetylcholine. (a) Overflow in the presence of $3 \times 10^{-6}$ mol l$^{-1}$ atropine (○) or atropine plus $10^{-4}$ mol l$^{-1}$ (+)-tubocurarine (Tc; ●). (b) Overflow in drug-free solution (△) or presence of (+)-Tc (▲). The perfusate was collected for periods of 10 or 20 s. Ordinate scales (log scales), $^3$H-overflow 10 s$^{-1}$ expressed as % of the total overflow (100% = area under the curve) after subtraction of the basal $^3$H-overflow. Abscissa scales, time after beginning of stimulation (s); the arrows indicate the time when the descending release curves reached the 10% level. Note that the $^3$H washout into the perfusate followed first order kinetics with half-times (from left to right) of 14.9 ± 0.3 s, 16.0 ± 0.6 s, 13.8 ± 1.0 s and 12.1 ± 0.2 s. Symbols represent means of 3 to 5 experiments.

its termination was postponed by about 10 or 20 s following vagal stimulation for 5 s or 1 min, respectively. The time at which the $^3$H-overflow was reduced to the 10% level was shifted by (+)-tubocurarine from 73.8 ± 1.2 s to 92.4 ± 1.3 s (Figure 6a) and from 38.2 ± 1.6 s to 48.0 ± 0.5 s (Figure 6b).

In a second series, the effect of atropine and pirenzepine on the $^3$H-release evoked by field stimulation was studied comparatively in chicken and guinea-pig hearts. The release was evoked by three consecutive periods of field stimulation for 1 min at 3 Hz (chicken) and 5 Hz (guinea-pig) (40 V, 1 ms) and at intervals of 10 min. The perfusate samples were collected in the same way as described for the determination of unlabelled acetylcholine (see Methods). The total amount of evoked $^3$H-release (c.p.m. g$^{-1}$) was obtained after subtraction of the basal release. It has been shown in previous studies on the same preparation (Lindmar *et al.*, 1980; 1982), that the evoked $^3$H-release was due to neuronal release of [$^3$H]-acetylcholine and that about two thirds of this acetylcholine was hydrolyzed to [$^3$H]-choline.

Figure 7 shows that, under control conditions, the evoked release of [$^3$H]-acetylcholine declined from the first to the second and the third period of stimulation, which were carried out at 0, 10 and 20 min. Atropine ($3 \times 10^{-7}$ mol l$^{-1}$) caused a 2 to 3 fold increase in the release of [$^3$H]-acetylcholine in chicken and guinea-pig hearts, an effect which may be considered maximal (total inhibition of autoreceptors). In the presence of atropine, the decline of $^3$H-release from the second to the third stimulation period was relatively steep, probably due to a marked depletion of the [$^3$H]-acetylcholine store at the second stimulation. Therefore, quantitative evaluation of the drug effects was preferentially based on the data observed at the second period of stimulation ('10 min' in Figure 7). It was found that pirenzepine in a concentration as low as $10^{-8}$ mol l$^{-1}$ caused a small, but significant increase of [$^3$H]-acetylcholine release in the chicken (Figure 7), but not in the guinea-pig heart (not documented). The half-maximal increase was seen with $3 \times 10^{-8}$ mol l$^{-1}$

pirenzepine in the chicken heart, whereas the guinea-pig heart did not respond at this concentration. Only $3 \times 10^{-7}$ mol $l^{-1}$ pirenzepine increased the release of [$^3$H]-acetylcholine in the guinea-pig heart.

The potency of pirenzepine in antagonizing the muscarinic autoinhibition of the release of [$^3$H]-acetylcholine (Figure 7) was compatible with the results obtained by determination of unlabelled acetylcholine (Figure 4a and b) and did not differ from that found for the antagonism against the negative inotropic response to exogenous acetylcholine (Figure 3). Thus the presynaptic autoreceptor at the neuroeffector junction belongs to the $M_1$-subtype in the chicken heart and to the $M_2$-subtype in the guinea-pig heart. The results yielded the same species-difference as that found for the myocardial muscarinic receptor mediating negative inotropic responses.

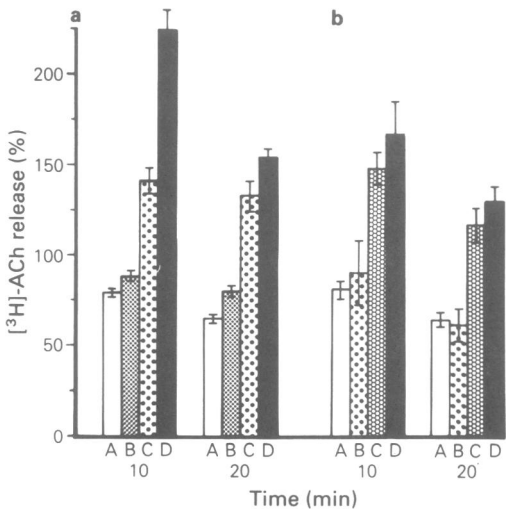



**Figure 7** Increase by pirenzepine and atropine of the evoked release of [$^3$H]-acetylcholine ([$^3$H]-ACh) in chicken (a) and guinea-pig (b) hearts. ACh was labelled according to Lindmar *et al.* (1979). [$^3$H]-ACh was released by field stimulation (1 min periods; 3 Hz for chicken and 5 Hz for guinea-pig hearts) at zero-time (control 100%), at 10 and 20 min. Pirenzepine or atropine was added after the first stimulation period. In (a), the columns indicate [$^3$H]-ACh release under control conditions (100% = 1493 c.p.m./heart) (A), in the presence of $10^{-8}$ mol $l^{-1}$ pirenzepine (B), $3 \times 10^{-8}$ mol $l^{-1}$ pirenzepine (C) and $3 \times 10^{-7}$ mol $l^{-1}$ atropine (D). In (b), the columns indicate [$^3$H]-ACh release under control conditions (100% = 996 c.p.m./heart (A), in the presence of $3 \times 10^{-8}$ mol $l^{-1}$ pirenzepine (B), $3 \times 10^{-7}$ mol $l^{-1}$ pirenzepine (C) and $3 \times 10^{-7}$ mol $l^{-1}$ atropine (D). Data presented are means of 4 experiments and vertical lines indicate s.e.mean.

*Negative inotropic effect of vagal stimulation*

In incubated chicken atria, which were electrically paced at 240 beats $min^{-1}$, bilateral vagal stimulation (1–30 Hz, 15 s) caused a frequency-dependent negative inotropic response (Figure 8). Pirenzepine at a low concentration of $3 \times 10^{-8}$ mol $l^{-1}$ did not alter the vagal response. In contrast, it was blocked by atropine at a maximally effective concentration of $3 \times 10^{-7}$ mol $l^{-1}$.

## Discussion

The heart is innervated by preganglionic parasympathetic fibres running in the vagus nerves and their cardiac ramifications. Intrinsic ganglia give rise to postganglionic varicose fibres forming the parasympathetic neuroeffector junctions (reviewed by Löffelholz & Pappano, 1985). In mammalian ventricles, the

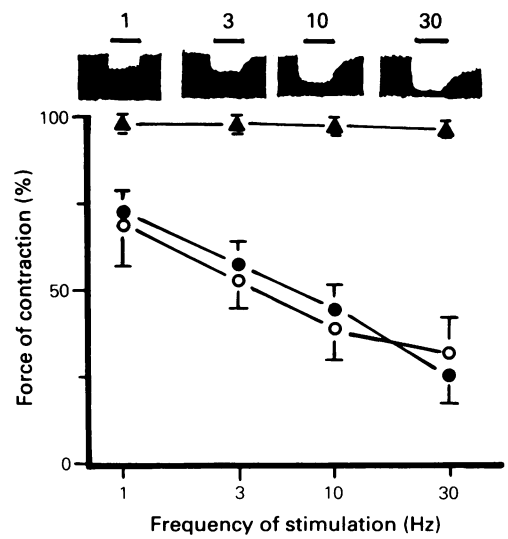



**Figure 8** Effect of $3 \times 10^{-8}$ mol $l^{-1}$ pirenzepine and of $3 \times 10^{-7}$ mol $l^{-1}$ atropine on the negative inotropic responses of chicken atria to vagal stimulation. The vagus nerves of incubated atria (paced at 240 beats $min^{-1}$) were stimulated at increasing frequencies of 1, 3, 10 or 30 Hz for 15 s at intervals of 5 min. Ordinate scale, force of contraction as % of the basal force (about 6 mN $g^{-1}$) in the absence of drugs (●) or in the presence of pirenzepine (○) or of atropine (▲) (both added at least 15 min before the first period of nerve stimulation). An original record of a control experiment is shown on the top. Data presented are means of 3 to 4 experiments and vertical lines indicate s.e.mean.

parasympathetic innervation is sparse, whereas the ventricles of amphibia and birds are densely innervated by cholinergic neurones. Thus the perfused heart of the chicken proved to be a valuable tool for studying cholinergic mechanisms (Löffelholz *et al.*, 1982).

When, in studies on the release of acetylcholine, vagal (preganglionic) stimulation and field stimulation, which activates directly postganglionic fibres, are combined, drug-induced effects on the ganglionic transmission can be distinguished from those localized at the postganglionic terminal fibres (Lindmar *et al.*, 1979). In the present study, these methods were further combined with experiments on the negative inotropic effect of a muscarinic agonist in incubated atria. The study was aimed at a comparative analysis of the muscarinic receptors distinguished by their location within the parasympathetic innervation of the heart. In the following sections, our data on myocardial receptors, presynaptic autoreceptors of the neuroeffector junction and somadendritic ganglionic receptors, and their affinities for pirenzepine (see Introduction) are discussed.

*Myocardial receptors*

The negative inotropic effect of acetylcholine is mediated by activation of muscarinic receptors (reviewed by Löffelholz & Pappano, 1985). Until recently, the high affinity pirenzepine receptors ($M_1$) were found exclusively in neuronal tissue, such as ganglia and brain, but not on muscular cells. Thus pirenzepine antagonized the muscarinic depolarization in ganglia or the muscarinic facilitation of transmitter release in the nucleus accumbens ($pA_2$ 7.5–8.4), whereas lower affinities ($pA_2$ 6.2–7.0) were found in atrial and smooth muscle (reviewed by Eglen & Whiting, 1986). While the present study was in progress, O'Rourke & VanHoutte (1987) published evidence for $M_1$-receptors mediating vasoconstriction of the canine saphenous vein ($pA_2$ 8.1). Moreover, in dissociated embryonic chicken heart cells, pirenzepine antagonized the carbachol-evoked inhibition of the formation of cyclic AMP with a (moderately) high affinity ($K_i$ 48 nmol $l^{-1}$), whereas the carbachol-evoked phosphoinositide hydrolysis was antagonized by pirenzepine with low affinity ($K_i$ 255 nmol $l^{-1}$) (Brown *et al.*, 1985; Brown & Jones, 1986). These authors concluded that the population of myocardial muscarinic receptors mediating biochemical responses in these cells is heterogeneously composed of $M_1$- and $M_2$-receptors (Brown *et al.*, 1985; Brown & Jones, 1986).

We found here that the negative inotropic effect of the muscarinic agonist methacholine was mediated by a high affinity pirenzepine receptor ($M_1$; $pA_2$ 7.76) in chicken atria and by an $M_2$-receptor ($pA_2$ 6.53) in guinea-pig atria; a similar $pA_2$ value of 6.71 had been found in guinea-pig atria by Barlow *et al.* (1981). This species difference was not due to a difference in the hydrolysis of methacholine. Firstly, the potency values ($EC_{50}$) for methacholine were similar in guinea-pig and chicken atria (Figure 1) and the Schild plots were linear with slopes of almost 1 in both species (Figure 2). Secondly, the cholinesterase activities of chicken, guinea-pig, cat and rabbit hearts were in the same range (Dieterich *et al.*, 1978). Moreover, the species difference in the $pA_2$ values was reflected by the concentration-response curves for pirenzepine as antagonist against the negative inotropic effect of acetylcholine. The $IC_{50}$ values of pirenzepine were almost 10 fold lower in chicken atria (40 nmol $l^{-1}$) than in guinea-pig atria (330 nmol $l^{-1}$), whereas those of atropine were identical for both species being about 4.5 nmol $l^{-1}$. Thus the potency ratios $IC_{50\,pirenzepine}/IC_{50\,atropine}$ were 7.4 and 94.3 in chicken and guinea-pig atria, respectively. The present study on atrial muscle and the recent results (O'Rourke & VanHoutte, 1987) on vascular smooth muscle clearly indicate that muscarinic $M_1$-receptors are not confined to neuronal tissue, but mediate functional responses of cardiac and smooth muscle cells. It was perhaps predictable from analogous experiences with other receptors that the labelling of pharmacologically defined muscarinic receptor subtypes ($M_1$ and $M_2$) according to their localization (e.g. 'cardiac' for an $M_2$-subtype) would be shortlived.

*Presynaptic autoreceptor of neuroeffector junction*

This receptor, which has been detected in previous studies (Löffelholz *et al.*, 1984), was re-investigated by measuring the release of unlabelled and $^3$H-labelled acetylcholine into the perfusate of isolated hearts. In the chicken heart, pirenzepine at a low concentration ($3 \times 10^{-8}$ mol $l^{-1}$) markedly enhanced the release of acetylcholine evoked by uni- and bilateral vagal stimulation and by field stimulation (Figure 4a and b). Comparative analysis led to the following conclusions: (1) the increase of the transmitter release expressed in absolute terms (pmol $g^{-1}$) was greater at 20 Hz than at 3 Hz. The results indicate a positive relationship between the synaptic transmitter concentration and the magnitude of the response to pirenzepine, a result which is characteristic for autoinhibition of transmitter release. (2) The increase by pirenzepine of the release of acetylcholine was greater at field stimulation than at (preganglionic) vagal stimulation and therefore was due to an effect on the presynaptic autoreceptors of the neuroeffector junction, rather than on autoreceptors of the ganglionic synapse.

The high potency of pirenzepine in facilitating the evoked release of acetylcholine in chicken heart was confirmed by experiments on the release of $^3$H-labelled

acetylcholine (Figure 7). An approximately half-maximal increase was found at a concentration of 30 nmol $l^{-1}$. This value is close to the $IC_{50}$ value of pirenzepine as antagonist of the negative inotropic effect of acetylcholine in chicken atria (40 nmol $l^{-1}$), but much lower than the corresponding $IC_{50}$ value found in guinea-pig atria (330 nmol $l^{-1}$) (Figure 3). The high potency of pirenzepine in facilitating the release of acetylcholine was reflected by the observation, that even $10^{-8}$ mol $l^{-1}$ pirenzepine was effective in chicken heart, whereas the guinea-pig heart did not respond even to $3 \times 10^{-8}$ mol $l^{-1}$. Only a ten fold higher concentration ($3 \times 10^{-7}$ mol $l^{-1}$), which also blocks the $M_2$-receptor subtype (Figure 3), markedly augmented the release of [$^3$H]-acetylcholine in this species.

In conclusion, the presynaptic autoreceptors of the parasympathetic neuroeffector junctions of the chicken and guinea-pig heart yielded the same species-difference as was found for the myocardial muscarinic receptors (see above): $M_1$-receptors are present in chicken atria and $M_2$-receptors in guinea-pig atria. The few $pA_2$ values of pirenzepine that have been obtained for muscarinic presynaptic autoreceptors in guinea-pig ileum (Kilbinger, 1984; North *et al.*, 1985), in rabbit striatum (James & Cubeddu, 1987) and rat cerebral cortex (Roberts & Tutty, 1986), are all in a narrow range between 6.9 to 7.1. These affinities are compatible with the autoreceptor being a low affinity pirenzepine receptor ($pA_2$ 6.0 to 7.0; Eglen & Whiting, 1986). The present data represent the first evidence for a presynaptic autoreceptor belonging to the $M_1$-receptor subtype.

*The somadendritic receptor of the parasympathetic ganglion*

It is well known that, in autonomic ganglia, block of the nicotinic ganglionic transmission unmasks a muscarinic postsynaptic potential (Trendelenburg, 1966) which is slow in onset and prolonged in duration (reviewed by Brown *et al.*, 1986). This slow excitatory postsynaptic potential (slow e.p.s.p.) was readily antagonized by pirenzepine ($pA_2$ 8.4) in the superior cervical ganglion (Brown *et al.*, 1980) and in the ileum (Kilbinger, 1984; North *et al.*, 1985). The primary targets for the muscarinic excitation in, for example, autonomic ganglia, hippocampus and cortex appear to be $K^+$-currents (Cassell & McLachlan, 1987), whose normal functions are to stabilize the neurone and limit the output firing frequency (Brown *et al.*, 1986).

In the chicken heart, block of the nicotinic receptor of the parasympathetic ganglion by $10^{-4}$ mol $l^{-1}$ (+)-tubocurarine reduced the release of acetylcholine evoked by (preganglionic) vagal stimulation to a (+)-tubocurarine-resistant release of about 30% of the control release, whereas the release by field stimulation was unchanged (present study; Lindmar *et al.*, 1979). Moreover, in the presence of (+)-tubocurarine, the time-course of the neuronal release of [$^3$H]-acetylcholine evoked by (preganglionic) vagal stimulation for 5 s or 1 min was markedly altered: the onset was delayed and the termination of the release was postponed by 10 to 20 s (Figure 6a and b); the rate of $^3$H-washout from the extracellular space into the perfusate (half-time about 14 s) remained virtually unaltered in the presence of (+)-tubocurarine. Brown *et al.* (1986) recorded slow e.p.s.ps in rat superior cervical ganglion cells after preganglionic nerve stimulation in the presence of $10^{-4}$ mol $l^{-1}$ (+)-tubocurarine (to block the fast e.p.s.p.). The slow e.p.s.p. lasted for about 20 s after preganglionic stimulation for 5 s at 20 Hz or even after single preganglionic shocks. In our experiments, the prolonged (+)-tubocurarine-resistant release of acetylcholine extending beyond the period of stimulation by several seconds was a first indication that most of the acetylcholine, released under this condition, probably originated from postganglionic neurones. Preganglionic nerves being a source for the major amount of acetylcholine released seemed less likely but cannot be excluded.

The presumed existence of a muscarinic ganglionic transmission leading to postganglionic activation was verified by experiments with $3 \times 10^{-8}$ mol $l^{-1}$ pirenzepine; it nearly blocked the (+)-tubocurarine-resistant release of acetylcholine (Figure 5). Theoretically this effect could have been caused by inhibition of a presynaptic excitatory $M_1$-autoreceptor. Such a mechanism has not been found in autonomic ganglia. In contrast, North *et al.* (1985) detected presynaptic inhibitory $M_2$-receptors at the ganglia of the guinea-pig myenteric and submucous plexus.

In summary, the present release experiments suggest that ganglionic transmission in the parasympathetic ganglion of the chicken heart is maintained, after block of the nicotinic receptors, through somadendritic $M_1$-receptors analogous to those found in other ganglia. These receptors mediate a postganglionic transmitter release that is slow in onset but terminates several seconds beyond the period of stimulation.

*Functional effects of pirenzepine on the neuroeffector transmission in chicken atria*

Preganglionic vagal stimulation caused a frequency-dependent negative inotropic response of chicken atria electrically driven at 240 beats $min^{-1}$. Atropine, in a concentration ($3 \times 10^{-7}$ mol $l^{-1}$) that caused maximal postsynaptic inhibition, blocked this effect. In contrast, pirenzepine in a low concentration ($3 \times 10^{-8}$ mol $l^{-1}$) lacked a significant effect on the response (Figure 8), although $3 \times 10^{-8}$ mol $l^{-1}$ pirenzepine had been shown to cause an almost half-

maximal reduction of the negative inotropic effect of exogenous acetylcholine (Figure 3). The apparent discrepancy is easily explained by the fact that pirenzepine in the same concentration doubled the evoked release of acetylcholine (Figure 4a and b). Thus the postsynaptic inhibition and the presynaptic facilitation of neuroeffector transmission caused by pirenzepine must have cancelled each other out.

This work was supported by the Deutsche Forschungsgemeinschaft. Some of the results are part of the M.D. theses of D.J. and M.W. The technical assistance of Mrs M. Campanini and Mrs I. Schaperdoth is gratefully acknowledged. We thank Dr Ziegler from Thomae, Biberach FRG, for pirenzepine.

## References

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BARLOW, R.B., CAULFIELD, M.P., KITCHEN, R., ROBERTS, P.M. & STUBLEY, J.K. (1981). The affinities of pirenzepine and atropine for functional muscarinic receptors in guinea-pig atria and ileum. *Br. J. Pharmacol.*, **73**, 183P–184P.

BROWN, D.A., FORWARD, A. & MARSH, S. (1980). Antagonist discrimination between ganglionic and ileal muscarinic receptors. *Br. J. Pharmacol.*, **71**, 362–364.

BROWN, D.A., GÄHWILER, B.H., MARSH, S.J. & SELYANKO, A.A. (1986). Mechanisms of muscarinic excitatory synaptic transmission in ganglia and brain. *Trends Pharmacol. Sci.*, **7** (Suppl.), 66–71.

BROWN, J.H., GOLDSTEIN, D. & MASTERS, S.B. (1985). The putative $M_1$ muscarinic receptor does not regulate phosphoinositide hydrolysis. *Mol. Pharmacol.*, **27**, 525–531.

BROWN, J.H. & JONES, L.G. (1986). Phosphoinositide metabolism in the heart. In *Phosphoinositides and Receptor Mechanism*, ed. J.W. Putney, pp. 245–270. New York: Alan Liss.

CASSELL, J.F. & McLACHLAN, E.M. (1987). Muscarinic agonists block five different potassium conductances in guinea-pig sympathetic neurones. *Br. J. Pharmacol.*, **91**, 259–261.

DIETERICH, H.A. & LÖFFELHOLZ, K. (1977). Effect of coronary perfusion rate on the hydrolysis of exogenous and endogenous acetylcholine in the isolated heart. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **296**, 143–148.

DIETERICH, H.A., LINDMAR, R. & LÖFFELHOLZ, K. (1978). The role of choline in the release of acetylcholine in isolated hearts. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **301**, 207–215.

EGLEN, R.M. & WHITING, R.L. (1986). Muscarinic receptor subtypes: a critique of the current classification and a proposal for a working nomenclature. *J. Auton. Pharmacol.*, **5**, 323–346.

HAMMER, R. & GIACHETTI, A. (1982). Muscarinic receptor subtypes: $M_1$ and $M_2$ biochemical and functional characterization. *Life Sci.*, **31**, 2991–2998.

JAMES, M.K. & CUBEDDU, L.X. (1987). Pharmacologic characterization and functional role of muscarinic autoreceptors in the rabbit striatum. *J. Pharmacol. Exp. Ther.*, **240**, 203–215.

JECK, D., LINDMAR, R., LÖFFELHOLZ, K. & WANKE, M. (1987). The effect of pirenzepine (Pz) and (+)-tubocurarine (Tc) on the release of acetylcholine (ACh) from the perfused chicken heart. *Naunyn-Schmiedebergs Arch. Pharmacol.* **335** (Suppl.), R77.

KILBINGER, H. (1984). Facilitation and inhibition by muscarinic agonists of acetylcholine release from guinea pig myenteric neurones: mediation through different types of neuronal muscarinic receptors. *Trends Pharmacol. Sci.*, **5** (Suppl.), 49–52.

LINDMAR, R., LÖFFELHOLZ, K. & WEIDE, W. (1979). Inhibition by pentobarbital of the acetylcholine release from the postganglionic parasympathetic neuron of the heart. *J. Pharmacol. Exp. Ther.*, **210**, 166–173.

LINDMAR, R., LÖFFELHOLZ, K., WEIDE, W. & WITZKE, J. (1980). Neuronal uptake of choline following release of acetylcholine in the perfused heart. *J. Pharmacol. Exp. Ther.*, **215**, 710–715.

LINDMAR, R., LÖFFELHOLZ, K. & WEIDE, W. (1982). Interstitial washout and hydrolysis of acetylcholine in the perfused heart. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **318**, 295–300.

LINDMAR, R. & LÖFFELHOLZ, K. (1987). Muscarinic $M_1$ receptors mediate the negative inotropic effect of methacholine in chicken, but not in guinea-pig atria. *Eur. J. Pharmacol.*, **139**, 359–360.

LÖFFELHOLZ, K., LINDMAR, R. & WEIDE, W. (1982). The isolated perfused heart and its parasympathetic neuroeffector junction. In *Model Cholinergic Synapsis.*, Vol. 1, ed. Goldberg, A.M. & Hanin, I., pp. 95–111. New York: Raven Press.

LÖFFELHOLZ, K., BREHM, R. & LINDMAR, R. (1984). Hydrolysis, synthesis, and release of acetylcholine in the isolated heart. *Fed. Proc.*, **43**, 2603–2606.

LÖFFELHOLZ, K. & PAPPANO, A.J. (1985). The parasympathetic neuroeffector junction of the heart. *Pharmacol. Rev.*, **37**, 1–24.

NORTH, R.A., SLACK, B.E. & SURPRENANT, A. (1985). Muscarinic $M_1$ and $M_2$ receptors mediate depolarization and presynaptic inhibition in guinea-pig enteric nervous system. *J. Physiol.*, **368**, 435–452.

O'ROURKE, S.T. & VANHOUTTE, P.M. (1987). Subtypes of muscarinic receptors on adrenergic nerves and vascular smooth muscle of the canine saphenous vein. *J. Pharmacol. Exp. Ther.*, **241**, 64–67.

ROBERTS, F. & TUTTY, C. (1986). Quantification of the effects of muscarinic antagonists on the release of [$^3$H]-acetylcholine from rat cerebral cortex slices. *Br. J. Pharmacol.*, **88**, 358P.

TRENDELENBURG, U. (1966). Transmission of preganglionic impulses through the muscarinic receptors of the superior cervical ganglion of the cat. *J. Pharmacol. Exp. Ther.*, **154**, 426–440.

*(Received July 10, 1987.*
*Revised September 22, 1987.*
*Accepted September 24, 1987.)*