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Release of acetylcholine at the motor endplate of the rat – evidence against a muscarinic acetylcholine autoreceptor

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1 The effect of some drugs on the release of endogenous acetylcholine from the phrenic nerve-hemidiaphragm preparation of the rat was measured. Muscarinic ligands had no effect. 8-Br-cyclic GMP, a penetrating analogue of cyclic guanosine 3′,5′-monophosphate (cyclic GMP) was also without effect. 8-Br-cyclic AMP somewhat enhanced the basal release while the potassium-induced release remained unaltered.

2 In supersensitivity experiments, no specific binding of ligand [^{3}H]-quinuclidinylbenzilate ([^{3}H]-QNB) was found in homogenates of the diaphragm, either before or after atropine treatment, while concomitant binding studies in the CNS demonstrated the expected increase in muscarinic binding sites after atropine.

3 Our conclusion is that muscarinic acetylcholine receptors are probably absent from the presynaptic motor endplate area of the rat. Certain preliminary results suggest that a presynaptic nicotinic mechanism might be involved in the release of acetylcholine.

Introduction

In the central nervous system and at certain places in the periphery, mascarinic autoreceptors seem involved in the regulation of acetylcholine (ACh) release. Evidence for a negative feedback inhibition involving presynaptic muscarinic receptors in the CNS was obtained by Bourdois, Mitchell, Somogyi & Szerb (1974), by Kato, Collier, Ilson & Wright (1975) and in the periphery at smooth muscle by Kilbinger (1977). In the hippocampus of the rat, muscarinic autoreceptors were shown to be localized at the nerve endings (Nordström & Bartfai, 1980).

Mechanistic studies were performed by Nordström & Bartfai (1981), by Alberts & Stjärne (1982) and others. In the hippocampus it was found that cyclic quanosine 3′,5′-monophosphate (cyclic GMP) acts as a second messenger to the muscarinic autoreceptor regulating ACh release. Hence its penetrating analogue 8-Br-cyclic GMP is able to mimic the action of agonists on this receptor. With the guinea-pig ileum preparation, on the other hand, addition of 8-Br-cyclic GMP was without effect while 8-Br-cyclic AMP enhanced the secretion of ACh. A muscarinic autoreceptor may also exist at the nerve ending of the electromotor neurone of *Torpedo marmorata*, often used as a model of the motor neurone of skeletal muscle. Thus Kloog, Michaelson & Sokolovsky (1978), Pickard & Strange (1978) and Strange, Dowdau, Golds & Pickard (1980) described the binding of muscarinic ligands to the nerve endings of this neurone. Michaelson, Avissar, Kloog & Sokolovsky (1979) localized it to the P_2 and $a_2 + a_3$ subcellular fractions of the electric organ. The authors suggested that this receptor might also be involved in a negative feedback regulation of ACh-release.

The present work addresses the question whether or not a muscarinic receptor (mAChR) exists at the presynapse of the motor endplate of skeletal muscle and is involved in the regulation of ACh released there. The literature offers only very conflicting information; results are both in favour of (Das, Ganguly & Vedasiromoni, 1978, Duncan & Publicover, 1979, Abbs & Joseph, 1981) and against (Gundersen & Jenden, 1980) the occurrence of such receptors in the motor nerve terminal region. Where the occurrence of a muscarinic presynaptic autoreceptor has been suggested, pharmacological evidence is quoted. Muscarinic ligands have been described to affect a positive feedback system (Das *et al.*, 1978; Ganguly & Das, 1979) as well as a negative feedback system

(Abbs & Joseph, 1981). In the present paper we describe experiments performed on the rat hemidiaphragm in order to elucidate further the possible involvement of a presynaptic mAChR in ACh release at the motor endplate.

We have studied the effects of a muscarinic agonist and an antagonist and of cyclic GMP and cyclic AMP analogue, on ACh release. Further, super-sensitivity experiments involving atropine treatment of rats *in vivo* were performed, followed by quantitation of muscarinic receptors and by measurements of ACh release. A chronic pharmacological block of muscarinic receptors causes supersensitivity (Herman & Slominska-Zurek, 1979) as shown by increase in the tremorogenic effects of oxotremorine. An increase in the number of muscarinic antagonist binding sites in rat hippocampus has been observed, accompanied by a decrease in the affinity for antagonists in a dose-dependent manner (Westlind Grynfarb, Hedlund, Bartfai & Fuxe, 1981). Similar observations have been made with scopolamine (Ben-Barak & Dudai, 1980).

Methods

Release and measurement of acetylcholine

The phrenic nerve-left hemidiaphragm preparation of the rat was used. Male Sprague-Dawley rats (200–250 g) were decapitated and bled. Left hemidiaphragms were quickly removed and carefully dissected in an oxygenated medium (composition mM: NaCl 114, KCl 3.5, $CaCl_2$ 2.5, $MgCl_2$ 1.0, glucose 11 and HEPES 6, pH 7.4). Care was taken not to damage any fibres belonging to the hemidiaphragm while intercostal fibres and ribs were removed. Transmitter release studies were performed as previously described (Häggblad, Eriksson & Heilbronn, 1983). Release of ACh was followed in the presence of either low (3.5 mM, resting release) or high (50 mM, evoked release) K^+ concentration in the above mentioned medium. The increase in K^+ concentration was compensated for by an equivalent reduction in Na^+. Cholinesterases, present in the preparation, were inhibited by preincubation for 45 min, in low K^+ medium containing 10 μM of the organophosphate sarin and 1 μM tetrodotoxin (TTX), the latter to prevent backfiring and fibrillations. All media used for transmitter release studies contained sarin (1 μM) and TTX (1 μM). Fractions were collected in 15 or 30 min periods at 25 °C.

When effects of drugs acting on cholinoceptors were studied *in vitro*, the drug was present during the whole release experiment, starting during the basal release time-period. The amounts of ACh released were analysed by a chemiluminescence method using tritiated ACh as an internal standard (Häggblad *et al.*, 1983). Released ACh was purified and concentrated with precipitation by KI_3. The recovery of tritiated acetylcholine after precipitation ranged from 40–50% for biological samples.

In vivo *atropine treatment*

Male Sprague-Dawley rats weighing 110 g at the start of the experiment were injected intraperitonealy once a day for 21–24 days with atropine sulphate 20 mg kg^{-1}, dissolved in isotonic sodium chloride. Controls were injected with isotonic sodium chloride only.

Quantitation of muscarinic acetylcholine receptors

Right hemidiaphragms were first homogenized in a Virtis mixer then in a loose-fitting glass-Teflon homogenizer. Cerebral cortex and hippocampus samples were only homogenized in a glass-Teflon homogenizer, 20 strokes. The homogenisation medium was 0.1 M sodium phosphate buffer pH 7.4, containing 2 mM EDTA and 10 μM phenylmethylsulphonyl fluoride.

The homogenized tissues were centrifuged at 70,000 *g* for 20 min. The pellets were resuspended and recentrifuged. The final pellets were resuspended in phosphate buffer and assayed for the

Table 1 Effect of muscarinic drugs on release of acetylcholine (ACh) from rat left hemidiaphragm

	Low K^+ (3.5 mM)	*High K^+* (50 mM)	*Evoked release*	*Weight* (mg)
No drug	0.52 ± 0.08 (9)	1.83 ± 0.30 (7)	1.31 ± 0.28	303 ± 60 (9)
[^{3}H]-QNB 10^{-8} M	0.52 ± 0.06 (7)	1.80 ± 0.19 (5)	1.28 ± 0.14	271 ± 129 (7)
[^{3}H]-QNB 10^{-7} M	0.55 ± 0.06 (4)	1.99 ± 0.09 (4)	1.44 ± 0.07	236 ± 21 (4)
[^{3}H]-QNB 10^{-6} M	0.50 ± 0.03 (4)	1.96 ± 0.05 (4)	1.45 ± 0.09	270 ± 37 (4)
Oxo-T 10^{-6} M	0.57 ± 0.09 (4)	2.08 ± 0.29 (4)	1.51 ± 0.25	330 ± 33 (5)
Oxo-T 10^{-5} M	0.41 ± 0.05 (5)	1.67 ± 0.25 (5)	1.26 ± 0.24	285 ± 25 (5)

Values are expressed as pmol ACh released min^{-1} per hemidiaphragm, mean ± s.d. Evoked release was taken as the difference in release between high and low K^+ concentration. Figures in parentheses show number of observations.

content of mAChRs. Cerebral cortex and hippocampus samples were assayed for mAChRs by the filtration assay of Yamamura & Snyder (1974) using Whatman GF/C-filters. Diaphragm homogenates were analysed with a centrifugation assay, essentially as described for brain by Hedlund & Bartfai, (1981). Approximately 0.2 mg protein (brain) or 3.0 mg (diaphragm) were used per sample (0.5 ml). Cortex and hippocampus samples were assayed at [^{3}H]-quinuclidinylbenzilate ([^{3}H]-QNB) concentrations ranging from 1–20 nM, diaphragm homogenates at 5 nM. Specific binding of [^{3}H]-QNB was defined as the difference in the absence and presence of 100 μM atropine. Protein was determined according to Lowry, Rosebrough, Farr & Randall, (1951) using bovine serum albumin as standard.

Chemicals

[^{3}H]-ACh and [^{3}H]-QNB was bought from the Radiochemical Centre, Amersham. Oxotremorine sesquioxalate (Oxo-T) was a kind gift from Prof. R. Dahlbom, Uppsala University, Sweden. All other chemicals used were of analytical grade and bought from Sigma, Fluka or Merck.

Results

Drugs in vitro

In control experiments it was found that basal (3.5 mM K^+) and evoked (50 mM K^+) ACh release were 0.52 ± 0.08 ($n = 9$) and 1.31 ± 0.28 ($n = 6$) pmol min^{-1} per hemidiaphragm, respectively. Evoked release was taken as the difference in release at high and low K^+-concentration. The values compare well with results obtained by others (e.g. Miledi, Molenaar & Polak, 1978). Table 1 shows that the muscarinic antagonist [^{3}H]-QNB and the muscarinic agonist oxotremorine have no significant effect on either basal or evoked release in concentrations which have effects on CNS and smooth muscle muscarinic AChRs. In another series of experiments, effects of the penetrating cyclic nucleotide analogues 8-Br-cyclic GMP and 8-Br-cyclic AMP were assayed. It was found that 8-Br-cyclic GMP did not significantly alter either basal or evoked release at 2×10^{-4} M (Table 2). 8-Br-cyclic AMP (2×10^{-4} M) somewhat enhanced the basal release ($P < 0.05$) while the potassium evoked release remained unchanged (Table 2).

In vivo *long term treatment with atropine sulphate*

Subchronic treatment of rats with atropine causes supersensitivity in several brain areas. In our experiments we chose the cerebral cortex and the hippocampus as a control of this atropine effect. The number of specific [^{3}H]-QNB binding sites (pmol mg^{-1} protein), under equilibrium conditions, in brain homogenates from atropine-treated animals was 30% higher ($P < 0.05$) than in NaCl-treated controls. In contrast phrenic nerve-hemidiaphragm preparations showed no detectable specific [^{3}H]-QNB binding in either controls or long-term atropine receiving animals. The release of ACh from left hemidiaphragm preparations of controls and experimental animals was unaltered (Table 3); samples were collected for either 15 or for 30 min. However, the amount of ACh released per minute as calculated from 15 min samples was higher than that calculated from 30 min samples, confirming earlier observations (Häggblad *et al.*, 1983).

Discussion

Little is known about the regulation of transmitter release, in terms of presynaptic modulating receptors, at the neuromuscular junction. Phenomena such as backfiring, in the presence of anti-cholinesterases, are inhibited by the nicotinic antagonist, tubocurarine (Masland & Wigton, 1940) and have been explained by the possible presence of a nicotinic ACh-receptor-like presynaptic activity. The effect of tubocurarine on transmitter release is, however, controversial (Miyamoto, 1978). We have observed that preincubation with α-bungarotoxin increases K^+-evoked release (unpublished observation) from the

Table 2 Effects of 8-Br-cyclic GMP and 8-Br-cyclic AMP

	Low K^+ (3.5 mM)	*High K^+* (50 mM)	*Evoked release*	*Weight* (mg)
No drug	0.43 ± 0.05 (5)	2.71 ± 0.23 (6)	2.27 ± 0.21	238 ± 57 (6)
8-Br-cyclic GMP 2×10^{-4} M	0.45 ± 0.06 (4)	2.61 ± 0.54 (4)	2.15 ± 0.48	226 ± 46 (4)
8-Br-cyclic AMP 2×10^{-4} M	0.55 ± 0.09 (4)*	2.78 ± 0.15 (4)	2.23 ± 0.20	273 ± 42 (4)

Values are expressed as pmol ACh released min^{-1} per hemidiaphragm, mean ± s.d. Evoked release was taken as the difference in release between high and low K^+ concentration. Figures in parentheses show number of observations.
*$P < 0.05$ as compared to control.

Table 3 Acetylcholine release from rat left hemidiaphragm preparations of long term *in vivo* atropine-treated animals

	Low K^+ (3.5 mM)	*High K^+* (50 mM)	*Evoked release*	*Weight* (mg)
NaCl-treated controls (Low and high K^+ release 30 min/fraction)	0.52±0.13 (10)	1.87±0.29 (8)	1.33±0.30	248±38 (10)
Atropine-SO_4-treated rats (Low and high K^+ release 30 min/fraction)	0.58±0.15 (17)	1.83±0.34 (16)	1.24±0.33	260±39 (17)
NaCl-treated controls‡ (High K^+ release 15 min/fraction)				
0–15 min	†	2.36±0.29 (6)**	1.83±0.31*	263±18 (6)
15–30 min	†	3.19±0.32 (6)***	2.66±0.33***	–"–
Atropine-SO_4-treated rats‡ (High K^+ release 15 min/fraction)				
0–15 min	†	2.71±0.42 (5)***	2.10±0.44***	332±58 (5)
15–30 min	†	3.55±0.94 (5)***	2.94±0.95***	–"–

Values are expressed as picomol ACh released min^{-1} per hemidiaphragm, mean ± s.d. Evoked release was taken as the difference in release between high and low K^+ concentration. Figures in parentheses show number of observations.

†Basal release values as calculated per minute during a 30 min collection period were: 0.53±0.11(5) (NaCl-controls) and 0.68±0.21(6) (atropine-treated).

‡Evoked release was followed during two consecutive 15 min periods.

*$P<0.05$; **$P<0.01$; ***$P<0.001$ as compared to the corresponding stimulated 30 min/fraction samples.

hemidiaphragm of the rat. This effect has also been reported by Miledi *et al.*, (1978). Muscarinic interactions at the skeletal neuromuscular junction have been described, but the evidence is very confusing. It may be appropriate to mention that the revelant studies of various authors on mammals have been carried out using a number of different techniques, as this may be crucial. Thus some authors describe the release of endogenous ACh, while others describe that of radiolabelled ACh, formed after loading of the tissue with labelled choline, a notoriously difficult method for the rat diaphragm (see e.g. Potter, 1970). Released endogenous ACh has been measured using g.c.m.s., but also with the leech dorsal muscle assay, highly sensitive to the simultaneous presence of any other drug. Further, type and concentration of anticholinesterases has varied. These compounds can have other pharmacological effects than those resulting from the inhibition of acetylcholinesterase. Finally, the oxotremorine preparation used could be the cause of divergent results. Among our preparations we have found one with very small amounts of impurities (<2%) possibly formed on storage, that gave abnormal release values.

The aim of this study was to discover whether or not a presynaptic muscarinic autoreceptor exists at the motor nerve endings of skeletal muscle and is involved in the release of ACh from motor neurones. The results presented here provide evidence against the occurrence of such a muscarinic receptor at the neuromuscular junction of the rat, i.e. if one accepts a standard deviation of 5–20%. In studies of muscarinic autoreceptors both in the CNS and in the periphery (smooth muscle) done by others, muscarinic antagonists enhance evoked release with a factor 1.3–1.8 (CNS, Nordström & Bartfai, 1980; Bartfai, Nordström & Tjörnhammar, 1980) to approximately 3.1 (guinea-pig ileum, Alberts & Stjärne, 1982). Our release experiments were restricted to events at the neuromuscular junction by the use of TTX, which excluded backfiring and recruitment of possible regulative neural loops.

The released endogenous ACh was measured in the presence of an organophosphate, thus preventing AChE-catalyzed hydrolysis. ACh therefore accumulated in the bath during collection time. More ACh per min was found when samples were collected for 15 min than for 30 min suggesting (re)uptake of ACh in either nerve or muscle. It could be argued that the ACh present in the bath activated a (hypothetical) AChR, thus interfering with the action of added agonists or antagonists. However, as these were,

present from the beginning of the experiment and in concentrations much higher than the released ACh, we believe that the design of our experiments was reasonable.

In the supersensitivity experiments, the effects of a presumptive muscarinic autoreceptor would have been amplified. However, the number of [^{3}H]-QNB-binding sites at the neuromuscular junction remained zero and the evoked ACh release was unchanged. In contrast muscarinic supersensitivity in the CNS (no efforts were made to separate pre- and postsynaptic events) was observed, as judged from the binding experiments.

In collaboration with Dr L. Eriksson, Dept. of Anatomy, University of Göteborg, Sweden, preliminary attempts were made to localize muscarinic receptors by autoradiography with [^{3}H]-QNB at the light and electronmicroscopic levels. No silver grains were observed at skeletal muscle endplates, while blood vessels were readily stained.

In conclusion, our search for a presynaptic mAChR of the motor nerve terminal suggests absence of such a receptor. In contrast, other studies by us of rat or rabbit endplate regions could definitely suggest the occurrence of a non-clustered α-toxin binding material at the presynapse of the motor endplate. Staining the postsynaptic nicotinic acetylcholine receptor (nAChR) with ^{3}H-labelled α-bungarotoxin always results in an admittedly very thin, dark line even at the presynapse. Lentz, Mazurkiewicz & Rosenthal, (1977) and Bender, Ringel & Engel, (1979) describe similar results using horse radish peroxidase labelled α-bungarotoxin. This line could be an artifact but there is no staining seen inside the synaptic cleft. nAChR may be present in low concentrations and without the formation of clusters. The properties of such a presumptive α-toxin binding material and/or its location in the presynaptic membrane would in certain ways have to differ from those of a traditional nAChR. In Myasthenia gravis or experimental Myasthenia gravis where nAChR antibodies are known to attack postsynaptic nAChR and where the postsynaptic membrane area is known to be lesioned, probably by antibody-coated macrophages and cytotoxic cells, lesions of the presynaptic membrane are hardly ever seen. Thus, if presynaptic nAChR-like receptors exist, antibodies are either not able to crosslink them because their immunological properties differ from the postsynaptic ones or because their location in the membrane is not suitable. A discussion of possible biochemical differences between pre- and post-synaptic nicotinic cholinoceptor sites at skeletal neuromuscular junction is, however, premature.

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References

ABBS, E.T. & JOSEPH, D.N. (1981). The effects of atropine and oxotremorin on acetylcholine release in rat phrenic nerve-diaphragm preparations. *Br. J. Pharmac.*, **73**, 481–483.

ALBERTS, P. & STJÄRNE, L. (1982). Secretion of ^{3}H-acetylcholine from guinea-pig ileum myenteric plexus is enhanced by 8-Br-adenosine-3,5′-cyclic monophosphate but not changed by 8-Br-guanosine-3′,5′-cyclic monophosphate. *Acta physiol. scand.*, **115**, 269–272.

BARTFAI, T., NORDSTRÖM, Ö. & TJÖRNHAMMAR M.-L. (1980). Cyclic guanosine 3′,5′-monophosphate in the nervous system. Pre-, post- and transsynaptic effects. *Progress in Pharmacology*, **4**, 151–157.

BEN-BARAK, J. & DUDAI, Y. (1980). Scopolamine induces an increase in muscarinic receptor level in rat hippocampus. *Brain Res.*, **193**, 309–313.

BENDER, A. N., RINGEL, S.P. & ENGEL, W.K. (1976). The acetylcholine receptor in normal and pathologic states. *Neurology*, **26**, 477–483.

BOURDOIS, P.S., MITCHELL, J.F., SOMOGYI, G.T. & SZERB, J.C. (1974). The output per stimulus of acetylcholine from cerebral cortical slices in the presence or in the absence of cholinesterase inhibition. *Br. J. Pharmac.*, **52**, 509–517.

DAS, M., GANGULY, D.K. & VEDASIROMONI, J.R. (1978). Enhancement by oxotremorin of acetylcholine release from the rat phrenic nerve. *Br. J. Pharmac.*, **62**, 195–198.

DUNCAN, C.J. & PUBLICOVER, S.J. (1979). Inhibitory effects of cholinergic agents on the release of transmitter at the frog neuromuscular junction. *J. Physiol.*, **294**, 91–103.

GANGULY, D.K. & DAS, M. (1979). Effects of oxotremorine demonstrate presynaptic muscarinic and dopaminergic receptors on motor nerve terminal. *Nature*, **278**, 645–646.

GUNDERSEN, C.B. & JENDEN, D.J. (1980). Oxotremorine does not enhance acetylcholine release from rat diaphragm preparations. *Br. J. Pharmac.*, **70**, 8–10.

HÄGGBLAD, J., ERIKSSON, H. & HEILBRONN, E. (1983). Microanalysis of endogenous acetylcholine released from the hemidiaphragm of the rat. *J. Neurochem.*, **40**, 1581–1587.

HEDLUND, B. & BARTFAI, T. (1981). Binding of ^{3}H-pilocarpine to membranes from rat cerebral cortex. *Naunyn-Schmiedebergs Arch. Pharmac.*, **317**, 126–130.

HERMAN, Z.S. & SLOMINSKA-ZUREK, J. (1979). Central cholinergic supersensitivity after long term atropine administration. *Psychopharmac.*, **64**, 337–340.

KATO, A.C., COLLIER, B., ILSON, D. & WRIGHT, J.M.

(1975). The effect of atropine upon acetylcholine release from cat superior cervical ganglia and rat cortical slices: measurement by a radioenzymatic method. *Can. J. Physiol. Pharmac.*, **53**, 1050–1057.

KILBINGER, H. (1977). Modulation by oxotremorine and atropine of acetylcholine release evoked by electrical stimulation of the myenteric plexus of guinea pig ileum. *Naunyn-Schmiedebergs Arch. Pharmac.*, **300**, 145–151.

KLOOG, Y., MICHAELSON, D.M. & SOKOLOVSKY, M. (1978). Identification of muscarinic receptors in the Torpedo electric organ. Evidence for their presynaptic localization. *FEBS Letters*, **95**, 331–334.

LENTZ, T.L., MAZURKIEWICZ, J.E. & ROSENTHAL, J. (1977). Cytochemical localization of acetylcholine receptors at the neuromuscular junction by means of horseradish peroxidase-labeled α-bungarotoxin. *Brain Res.*, **132**, 423–442.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L., & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

MASLAND, R.L. & WIGTON, R.S. (1940). Nerve activity accompanying fasciculation produced by prostigmin. *J. Neurophysiol.*, **3**, 269–375.

MICHAELSON, D.M., AVISSAR, S., KLOOG, Y.B. & SOKOLOVSKY, M. (1979). Mechanism of acetylcholine release: Possible involvement of presynaptic muscarinic receptors in the regulation of acetylcholine release and protein phosphorylation. *Proc. natn. Acad. Sci. U.S.A.*, **76**, 6336–6340.

MILEDI, R., MOLENAAR, P.C. & POLAK, R. (1978). α-Bungarotoxin enhances transmitter "released" at the neuromuscular junction. *Nature*, **272**, 641–643.

MIYAMOTO, M.D. (1978). The actions of cholinergic drugs on motor nerve terminals. *Pharmac. Rev.*, **29**, 221–247.

NORDSTRÖM, Ö. & BARTFAI, T. (1980). Muscarinic autoreceptor regulates acetylcholine release in rat hippocampus: in vitro evidence. *Acta physiol. scand.*, **108**, 347–353.

NORDSTRÖM, Ö. & BARTFAI, T. (1981). 8-Br-cyclic GMP mimics activation of muscarinic autoreceptor and inhibits acetylcholine release from rat hippocampal slices. *Brain Res.*, **213**, 467–471.

PICKARD, M.R. & STRANGE, P.G. (1978). The binding of the muscarinic receptor antagonist [3–^{3}H] Quinuclidinyl Benzilate to Torpedo marmorata membrane fragments. *Biochem. Trans.*, **6**, 129–131.

POTTER, L.T. (1970). Synthesis, storage and release of [^{14}C]-acetylcholine in isolated rat diaphragm muscles. *J. Physiol.*, **206**, 145–166.

STRANGE, P.G., DOWDALL, M.J., GOLDS, P.R. & PICKARD, M.K. (1980). Ligand-binding properties of a muscarinic acetylcholinereceptor from Torpedo electric organ. *FEBS Letters.*, **122**, 293–296.

WESTLIND, A., GRYNFARB, M., HEDLUND, B., BARTFAI, T. & FUXE, K. (1981). Muscarinic supersensitivity induced by septal lesion or chronic atropine treatment. *Brain Res.*, **225**, 131–141.

YAMAMURA, H.I. & SNYDER, S.H. (1974). Muscarinic cholinergic receptor binding in the longitudinal muscle of the guinea pig ileum with ^{3}H-quinuclidinyl benzilate. *Mol. Pharmac.*, **10**, 861–867.

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