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EVIDENCE FOR THE PHARMACOLOGICAL SIMILARITY BETWEEN THE CENTRAL PRESYNAPTIC MUSCARINIC AUTORECEPTOR AND POSTSYNAPTIC MUSCARINIC RECEPTORS

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1 Twenty antagonist substances with varying potencies for central and peripheral postsynaptic muscarinic receptors have been examined for effects on the central presynaptic muscarinic autoreceptor. This has been monitored by measuring the stimulating effects of the substances on acetylcholine synthesis by rat neocortical tissue prisms.

2 Dose-response curves for selected agents showed that maximal stimulation of synthesis was to 136–140% of the value without an antagonist.

3 At a concentration of 1 μM, 17 of the substances caused a significant increase in synthesis, whilst at 0.01 μM significant stimulation occurred with only atropine, dexetimide, N-methyl-piperdin-4-yl (R)-2-cyclohexyl-2-hydroxyl-2-phenylacetate, quinuclidinyl benzilate (QNB) and scopolamine.

4 Linear regression analysis between synthesis values obtained with the substances and published data for the effects on either cholinoceptor-agonist induced contraction of guinea-pig ileum or the binding of [^{3}H]-QNB to rat forebrain membranes gave correlation coefficients of $r = 0.84$ ($P < 0.01$), and $r = 0.75$ ($P < 0.02$) respectively.

5 The results provide no indication of a pharmacological difference between the central presynaptic muscarinic autoreceptor and central and peripheral postsynaptic muscarinic receptors.

Introduction

Pharmacological differences have been demonstrated between postsynaptic receptors for some neurotransmitters and presynaptic autoreceptors that modulate the release and synthesis of transmitter during nerve stimulation (Walters & Roth, 1976; Langer, 1977). Thus presynaptic receptors are now being considered as potential target receptors for the development of new drugs and as being involved in the production of side effects induced by certain drugs (Langer, 1977; Delina-Stula, Baumann & Buch, 1979; Bowen, 1981). Whether presynaptic receptors differ pharmacologically from the corresponding postsynaptic receptor is thus important but has not been determined for most putative neurotransmitters. We have investigated the cholinergic system because it has been implicated in side effects caused by certain antidepressants (Snyder & Yamamura, 1977) and in a wide variety of neurological and psychiatric disorders, especially those associated with ageing. A prominent example is the non-treatable dementing disorder of Alzheimer's disease in which there is a marked reduction, of clinical relevance, in the formation of acetylcholine (ACh) in the brain. The reduction in synthesis appears to be due to loss of presynaptic cholinergic nerve endings. The remaining presynaptic cholinergic terminals seem to retain normal function and postsynaptic muscarinic receptors are apparently not affected (Sims, Bowen, Smith, Flack, Davison, Snowden & Neary, 1980; Bowen, 1981).

Muscarinic receptor antagonists cause increased release and synthesis of acetylcholine (ACh) in slices and prisms of rat brain (Molenaar & Polak, 1970; Lefresne, Rospars, Beaujouan, Westfall & Glowinski, 1978). Acting even in the presence of tetrodotoxin (Molenaar & Polak, 1970), their effect is reversed by muscarinic agonists (Szerb, Hadhazy & Dudar, 1977). Morphological examination of tissue prisms reveals that these are primarily preparations of intact synaptic endings in the presence of disintegrated cell structures (Garthwaite, Woodhams, Collins & Balazs, 1979, 1980; Sims *et al.*, 1980; Sims, Bowen & Davison, 1981). As with conventional preparations of synaptosomes, prisms seem to provide a useful means of determining ACh synthesis, free from the influence of the cell body (Sims *et al.*, 1980; 1981). It is thought that there are muscarinic inhibitory autoreceptors located on the cholinergic nerve terminal (Iversen, 1974), in addition to the classical postsynaptic muscarinic receptor (Birdsall & Hulme, 1976). Thus, it is suggested that muscarinic antagonists act in prisms by blocking the autoreceptor-

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mediated suppression of ACh release and synthesis, whereas in slices a disinhibition of cholinergic neurones through inhibitory neuronal circuits (Yonehara, Matsuda, Saito, Ishida & Yoshida, 1980), may also occur. The objective of the present work was to establish whether the auto- and postsynaptic receptors differ pharmacologically because one of the criteria for a muscarinic antagonist to be effective in dementia is that it must act preferentially on presynaptic rather than postsynaptic receptors. A variety of antimuscarinic agents, with varying postsynaptic potencies (Brimblecombe, Inch, Wetherell & Williams, 1971a; Brimblecombe, Green, Inch & Thompson, 1971b; Snyder & Yamamura, 1977) have been examined for their influence on ACh synthesis *in vitro*. Antimuscarinics act optimally in brain tissue preparations that have been depolarized (Szerb & Somgyi, 1973; Rospars, Lefresne, Beaujouan & Glowinski, 1977). Consequently, the presynaptic muscarinic receptor has been characterized by measuring the effects of agents on ACh synthesis in K^+-stimulated rat neocortical tissue prisms. The incorporation of [U-^{14}C]-glucose into [^{14}C]-ACh and $^{14}CO_2$ was measured to determine the amount of ACh synthesized and total glucose metabolized, respectively (Sims *et al.*, 1981).

Methods

Measurement of acetylcholine synthesis

Male Wistar rats (Porton strain, 6–9 months) were decapitated and the brains rapidly removed and placed in ice-cold, modified Krebs Ringer phosphate buffer (composition, mM: NaCl 141, KCl 5, $CaCl_2$ 1.3, $MgSO_4$ 1.3 and Na_2HPO_4 10, pH 7.4) containing 2.5 mM glucose and freshly gassed with 100% oxygen. Neocortex, including all cortical layers, was dissected. Tissue prisms were prepared, preincubated and assayed in medium containing 31 mM K^+ for incorporation of [U-^{14}C]-glucose into [^{14}C]-ACh and $^{14}CO_2$, using two slight modifications of the method of Sims *et al.* (1981). The incubation period with labelled glucose was for 30 min instead of 1 h and after the preincubation period the prisms were maintained at room temperature, instead of on ice, before assay. The first modification was made because in incubations terminated after 30 min, 1 μM atropine enhanced [^{14}C]-ACh formation by 39% ± 14% (mean ± s.d., $n = 4$), whereas the value was lower (31% ± 8%) for incubations of 1 h. The other modification was made because when preincubated prisms were maintained on ice before assay, 1 μM atropine enhanced [^{14}C]-ACh formation by only 18% ± 7% (mean ± s.d., $n = 4$) of the control value (prisms without the drug). The value was higher (42% ± 11%), with prisms maintained at room temperature. The control values were independent of the temperature at which the preincubated prisms were maintained. These results were consistent with those of El-Fakahany & Richeson (1980). To identify acetylcholine, the radioactive product was hydrolysed using acetylcholinesterase (Sims *et al.*, 1981). The radioactivity isolated as [^{14}C]-ACh from incubations with 1 μM quinuclidinyl benzilate (QNB) and without drug, was hydrolysed by acetylcholinesterase by 95 ± 2% and 95 ± 3% (mean ± s.d., $n = 3$) respectively. This indicated that essentially all of the radioactivity was attributable to labelled ACh.

Materials

[U-^{14}C]-glucose (230 Ci/mol) was from The Radiochemical Centre, Amersham, and was diluted appropriately with glucose (BDH Chemicals, Poole, Dorset). Paraoxon, ACh bromide, choline chloride, acetylcholinesterase (Type VI-S from electric eel), atropine and scopolamine were from the Sigma Chemical Co., Poole, Dorset. The gifts of clozapine and thioridazine HCl (Sandoz Products), amitriptyline (Roche Products), trihexylphenidyl HCl (Lederle Labs), maprotiline HCl (CIBA Labs), benztropine mesylate (Merck Sharp and Dohme), and nomifensine maleate (Hoechst) are gratefully acknowledged. Oxotremorine was from the Aldrich Chemical Co., Ltd, Gillingham. All other agents listed in Table 1 were generous gifts from Dr T.D. Inch and Dr D.M. Green.

Results

Effect of potent muscarinic agents on [^{14}C]-acetylcholine synthesis

The compounds were incubated with prisms for 30 min in medium containing 31 mM K^+. Oxotremorine, a muscarinic agonist, caused a dose-dependent decrease in incorporation of [U-^{14}C]-glucose into [^{14}C]-ACh, over a dose range of 5–500 μM (Figure 1a). The maximal inhibition was to 45% of the control value and occurred at 500 μM.

The effect of three antimuscarinic agents, atropine, dexetimide and QNB was measured over a dose range of 0.001–10 μM. The results show (Figure 1a, b) that each drug increased [^{14}C]-ACh formation in a dose-dependent manner, with maximal stimulation occurring between 0.1 μM–1 μM. The maximal increase over the control value in the formation of the labelled ester was 36% for atropine, 34% for dexetimide and 40% for QNB. The maximal effect of only dexetimide and QNB persisted to a concentration as low as 0.01 μM, indicating that these drugs are

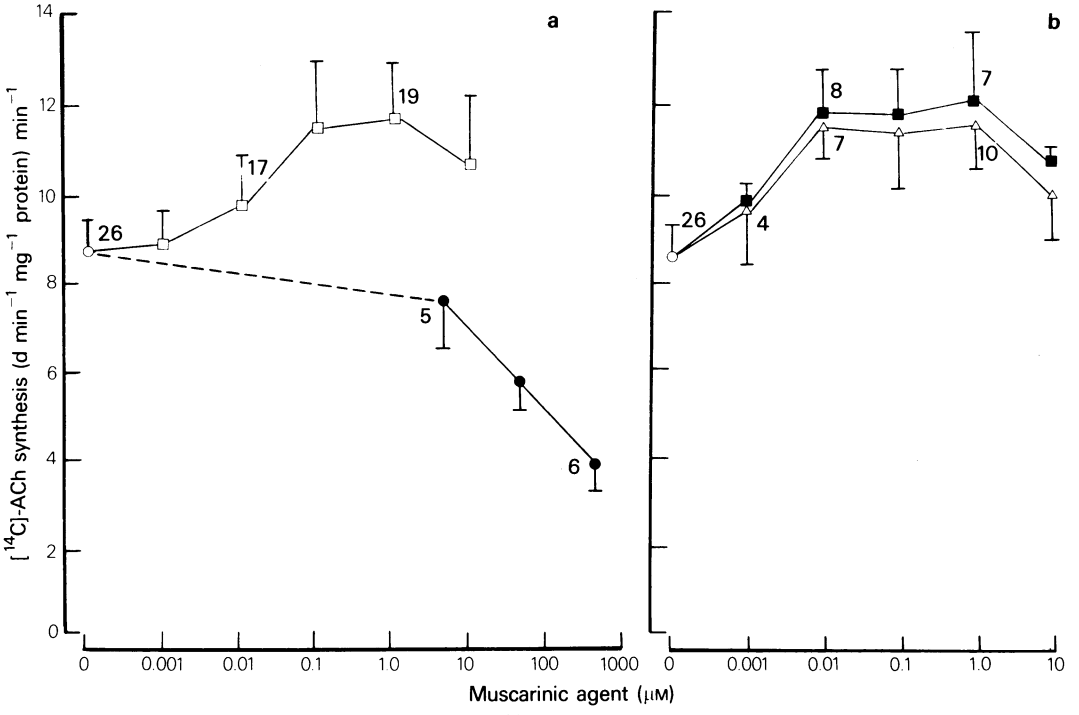

Figure 1 Muscarinic agent dose-response curves for [^{14}C]-acetylcholine synthesis by rat neocortical tissue prisms: (○) no drug; (●) oxotremorine; (□) atropine; (■) quinuclidinyl benzilate and (△) dexetimide. The points represent the mean from three (unnumbered) to 26 experiments, vertical lines show s.d.

more potent than atropine. The stimulation of synthesis declined slightly at the highest concentration tested. This is unlikely to be due to a generalized inhibition of glycolysis and respiration (O'Neill, Simon & Cummins, 1962) for none of the drugs, even at high concentration, significantly affected the production of $^{14}CO_2$.

Relationship between antimuscarinic potency and stimulation of [^{14}C]-acetylcholine synthesis

Antimuscarinic agents of varying structure (Table 1) were compared for their effect on [^{14}C]-ACh formation. At a concentration of 1 μM most of the substances caused a significant increase in synthesis of between 20% – 40% of the control value, QNB being the most effective agent. Measurements at lower concentrations indicated (Figure 1b) that 0.01 μM was the lowest concentration at which QNB caused a maximal stimulation of [^{14}C]-ACh synthesis. This was also the concentration at which differential response to QNB and atropine was detected, which led us to test all the other compounds at a concentration of 0.01 μM. The results at this concentration show (Table 1) the most potent agents to be QNB, dexetimide, N-methyl-piperdin-4-yl(R)-2-cyclohexyl-2-hydroxy-2-phenyl-acetate and scopolamine, which increased [^{14}C]-ACh formation by 38%, 24%, 23% and 22% of the control value, respectively.

Amitryptiline tends to be more effective than maprotoline and nomifensine in stimulating synthesis (Table 1), producing anticholinergic side-effects and displacing [^{3}H]-atropine bound to postsynaptic muscarinic receptors (Gold, Przyslo & Strange, 1980). In Figure 2 other indices of the potency of substances towards the postsynaptic receptor are compared with the effect on [^{14}C]-ACh synthesis. The results show that significant positive linear correlations exist between the effects on synthesis and the postsynaptic receptor.

None of the substances had a significant effect on the production of $^{14}CO_2$ (mean value ± s.d., for control incubations was 817 ± 110 (d min^{-1} mg^{-1} protein) min^{-1}, $n = 18$).

Discussion

Oxotremorine inhibited ACh synthesis by the prisms (Figure 1a) as has also been shown from the release of ACh from rat neocortical slices (Hadhazy & Szerb, 1977). As estimates of synthesis of ACh have been made using a radioactive tracer, apparent declines in synthesis may result from dilutions of the label by

Table 1 Effects of antimuscarinic substances on the incorporation of [U-^{14}C]-glucose into [^{14}C]-acetylcholine by rat neocortical prisms

	Antimuscarinic agent	*[^{14}C]-acetylcholine synthesis (d min^{-1} mg^{-1} protein) min^{-1}*	
		Medium containing 0.01 μM antimuscarinic	*Medium containing 1 μM antimuscarinic*
1	Quinuclidinyl benzilate	11.9 ± 0.9 (8)*	12.1 ± 1.5 (7)*
2	Dexetimide	11.6 ± 0.7 (7)*	11.6 ± 1.0 (10)*
3	N-methyl-piperdin-4-yl-(R)-2-cyclohexyl-2-hydroxy-2-phenylacetate HCl	10.7 ± 1.2 (4)*	11.3 ± 0.9 (4)*
4	Scopolamine HBr	10.5 ± 1.1 (4)*	11.7 ± 0.6 (4)*
5	Atropine	9.8 ± 1.0 (17)*	11.7 ± 1.2 (19)*
6	Trihexyphenidyl HCl	9.7 ± 0.8 (4)	11.8 ± 1.7 (4)*
7	N-methyl-piperidin-4-yl-2-cyclohexyl-2-hydroxy-2-phenylacetate HCl	9.6 ± 1.3 (4)	11.8 ± 1.7 (6)*
8	N-ethyl-3-piperidyl benzilate HCl	9.5 ± 0.9 (4)	10.8 ± 0.8 (5)*
9	Benztropine mesylate	9.5 ± 1.5 (4)	11.1 ± 1.1 (4)*
10	Amitryptiline HCl	9.5 ± 0.6 (3)	10.9 ± 1.1 (4)*
11	Benactyzine HCl	9.4 ± 1.0 (4)	11.2 ± 1.2 (6)*
19	Maprotoline	9.2 ± 0.6 (3)	9.5 ± 1.6 (3)
12	N-methyl-3-piperidyl benzilate HCl	9.2 ± 0.9 (4)	10.5 ± 1.3 (5)*
13	Clozapine	9.2 ± 0.9 (3)	10.1 ± 1.6 (4)*
14	Aprophen HCl	9.2 ± 0.7 (3)	10.7 ± 0.7 (6)*
20	Nomifensine maleate	9.0 ± 0.9 (3)	10.2 ± 1.1 (3)*
15	Dimethylaminoethyl-2-cyclohexyl-2-hydroxy-2-phenylacetate HCl	9.0 ± 1.3 (4)	10.4 ± 0.6 (4)*
16	D-*trans*-4-S-dimethyl-aminoethyl-2-S-(S-1-cyclohexyl-1-hydroxy-1-pheny) methyl-1-. 3-dioxolan	8.7 ± 1.0 (4)	8.4 ± 0.8 (5)
17	Thioridazine HCl	8.6 ± 1.5 (3)	10.3 ± 1.6 (3)*
18	D-*cis*-4-S-dimethylaminoethyl-2-R-(R-1-cyclohexyl-1-hydroxy-1-phenyl) methyl-1, 3-dioxolan	8.1 ± 0.9 (3)	8.8 ± 0.9 (4)
	No antimuscarinic agent	8.6 ± 0.8 (26)	

Values are mean ± s.d. of the number of experiments indicated in parentheses. Asterisk denotes a statistical significant difference ($P<0.01$, Student's t test) from the value with no antimuscarinic agent.

pools of intermediate compounds in the synthetic pathway. Investigations with this (Sims *et al.*, 1981) and related preparations (Browning & Schulman, 1968; Lefresne, Guyenet & Glowinski, 1973; Gibson & Blass, 1976), indicate that label from glucose is not diluted in synthesis of ACh in control incubations. The appearance of such diluting pools may conceivably explain the decrease in synthesis with oxotremorine. Thus, in contrast to Szerb *et al.* (1977), we did not use an agonist to widen the range of graded response to muscarinic antagonists. A much higher concentration of oxotremorine than atropine was needed to produce an effect on synthesis (Figure 1a), which is in agreement with results obtained by Szerb *et al.* (1977), studying ACh release from hippocampal slices.

The data in Figure 1 confirm those of Lefresne *et al.* (1978) in showing that atropine increases the K^+-stimulated incorporation of [U-^{14}C]-glucose into ACh by rat neocortical tissue prisms. A similar concentration range of atropine also enhanced ACh release from synaptosomes (Nordstrom & Bartfai, 1979). This is expected for synthesis and release of ACh are clearly interdependent (Tucek, 1978), and the prisms are essentially a preparation of nerve endings. Atropine increased glucose incorporation into ACh by the prisms in a dose-dependent manner as has been reported for the release of ACh from rat

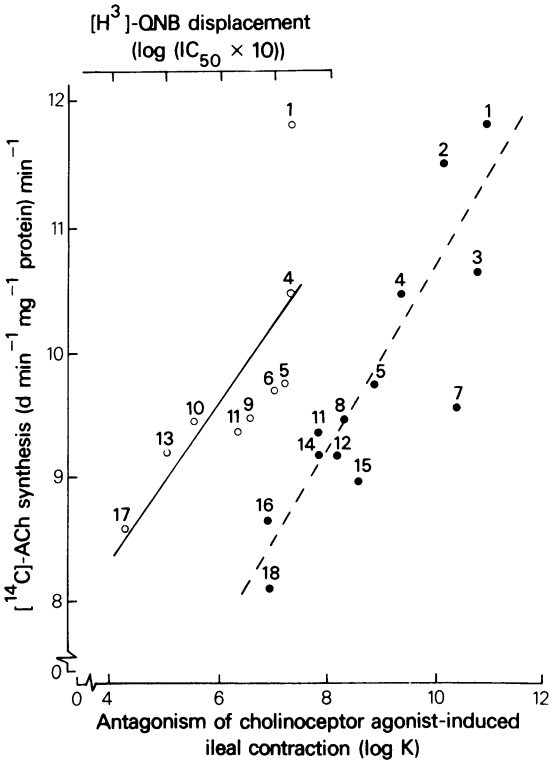

Figure 2 Comparison of the effect of antimuscarinic substances on [^{14}C]-acetylcholine ([^{14}C]-ACh) synthesis (data from Table 1, substances at 0.01 μM) and either the displacement of [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) from rat forebrain membranes (○, data from Snyder & Yamamura, 1977) or the antagonism of cholinoceptor agonist-induced contraction of guinea-pig ileum (●, data from Brimblecombe *et al.*, 1971 a,b, except for QNB which was from Dr D.M. Green, personal communication). The substances tested are identified by the numbers (see Table 1). The relation between synthesis and either displacement of [^{3}H]-QNB (unbroken line) or antagonism of ileal contraction (broken line) by linear regression analysis gives correlation coefficients of $r=0.75$ ($P<0.02$) and $r=0.84$ ($P<0.01$), respectively.

neocortical slices (Yonehara *et al.*, 1980). The effect on incorporation was maximal at concentrations of 0.1–1.0 μM, while that for maximal release from slices seems to be slightly higher (Yonehara *et al.*, 1980), suggesting that atropine penetrates prisms more readily that it does slices. These results indicate that the prism system provides a useful means of investigating the effect of antimuscarinic agents on the presynaptic muscarinic autoreceptor.

The presynaptic receptor has been the subject of various investigations, but until the present study there was no information on the relationship between antimuscarinic potency and the stimulation of ACh synthesis. Our data show that the antimuscarinics have a specific effect on [^{14}C]-ACh formation for the production of $^{14}CO_2$ was not affected. Atropine has less effect on [^{14}C]-ACh synthesis than QNB, which is in agreement with binding data obtained with synaptosomes from *Torpedo* electric organ (Kloog, Michaelson & Sokolvosky, 1980) where the presynaptic receptor is directly determined. The potencies of antimuscarinics, including antidepressant drugs with anticholinergic side effects, towards the postsynaptic receptor have been assessed in other laboratories by using either a radioactive ligand and brain membranes or isolated ileum. Comparison of these data with the present data for ACh synthesis show that a striking similarity exists in the relative effects of the drugs on the presynaptic muscarinic autoreceptor and central and peripheral postsynaptic muscarinic receptors. Furthermore, studies on ACh release (Szerb *et al.*, 1977) indicate that the affinity of the presynaptic receptor in mammals for QNB, atropine and scopolamine is approximately ten times less than the affinity for the postsynaptic receptor. There is, therefore, no indication that any antimuscarinic acts preferentially on the presynaptic receptor. Thus no specific class of compound has been identified which would be potentially useful for stimulating ACh synthesis in Alzheimer's disease. This study also indicates that none of the antidepressant drugs tested are likely to have a selective action on the presynaptic muscarinic receptor.

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