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Quantitative effects of some muscarinic agonists on evoked surface-negative field potentials recorded from the guinea-pig olfactory cortex slice

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1 The effects of muscarinic receptor agonists on the electrically-evoked surface-negative field potential (N-wave) were measured in the guinea-pig olfactory cortex slice maintained *in vitro*.

2 Bath-superfusion of (±)-muscarine, acetylcholine (ACh), carbachol (CCh), or methacholine (MCh) (10–200 μM) produced reversible, dose-dependent depressions of the N-wave (ACh and MCh effects were observed in the presence of 10 μM neostigmine). The order of potencies (based on agonist dose causing 50% field depression: IC_{50}) was: ACh $\geqslant$ muscarine > CCh > MCh. All four agonists depressed the field potential by 100% at doses greater than 500 μM.

3 Pilocarpine and bethanechol were weak agonists and only produced measurable effects at high doses (1–2 mM). Neither agonist evoked a maximum response at doses up to 10 mM.

4 The muscarinic ganglion stimulant, McN-A-343 yielded inconsistent results, depressing the field potential in some slices, but having no effect in others. Pre-application of a conditioning dose (100 μM) of McN-A-343 reduced subsequent responses to CCh, suggesting possible partial agonist properties.

5 Oxotremorine (up to 100 μM) did not depress the field potential, but it reversibly antagonized the effects of CCh.

6 It is concluded that reproducible, quantifiable responses to muscarinic agonists can be evoked in the olfactory cortex slice. We suggest this preparation may be useful for conducting pharmacological studies of 'intact' central muscarinic receptors.

Introduction

The possibility that muscarinic receptors might form a heterogeneous population has been extensively investigated (Hammer *et al.*, 1980; Watson *et al.*, 1983; Kubo *et al.*, 1986). Evidence suggests that there are at least two and possibly three muscarinic receptor subtypes (for recent review, see Eglen & Whiting, 1986). Currently, one of the most selective agents able to distinguish between these subtypes is pirenzepine which has a high affinity for the M_1-receptor subtype ($K_D \sim$ 10–20 nM), but a lower affinity for M_2-receptors ($K_D \sim$ 200–800 nM) (Hammer *et al.*, 1980; Hammer & Giachetti, 1982). These receptors have a differential distribution: M_1-receptors are found in high density in the cerebral cortex, hippocampus and autonomic ganglia, while M_2-receptors are found predominantly in lower brain areas and in peripheral tissues, such as autonomic effector organs and the myocardium (Watson *et al.*, 1983; Mash & Potter, 1986). Other antagonists appear to be more selective towards M_2-receptors, e.g. gallamine and 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP; Barlow *et al.*, 1976; Clark & Mitchelson, 1976; Berrie *et al.*, 1983). While some agonists also appear to be selective, evidence for this is less well established because of the difficulties of quantifying agonist effects (see Barlow, 1980).

Many authors have speculated on the possibilities of pharmacological exploitation of the subtle differences between muscarinic receptor subtypes to develop a treatment for Alzheimer's disease, a degenerative condition in which the cholinergic innerva-

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tion to the higher brain areas is disrupted (Whitehouse *et al.*, 1981). A selective M_1-receptor agonist might alleviate some of the worst symptoms of Alzheimer's disease without the unpleasant and dangerous peripheral side effects usually associated with muscarinic cholinomimetics. In spite of the potential therapeutic interest in muscarinic receptor subtypes, very few studies have attempted to characterize pharmacologically the M_1-receptor in intact brain tissue. Although the muscarinic receptor in sympathetic neurones of the superior cervical ganglion has been shown to be highly sensitive to the selective M_1 antagonist pirenzepine (Brown *et al.*, 1980), several regions of the brain contain muscarinic receptors that are relatively insensitive to this antagonist, including locus coeruleus (Egan & North, 1985) and nucleus parabrachialis (Egan & North, 1986). Very little is known about the muscarinic receptors found in the higher brain areas, areas more likely to be important in cognitive deficits. For this reason we chose to study the properties of muscarinic receptors in an *in vitro* cortical preparation, the olfactory cortex slice. This structure has a muscarinic receptor that can be studied with simple electrophysiological recording techniques (Williams *et al.*, 1985). In this paper, the general properties of the muscarinic response were investigated in detail, while in the accompanying paper (Williams & Constanti, 1988) a quantitative assessment of the receptor subtype mediating the response was attempted. Some of the present results have previously appeared in a preliminary form (Williams & Constanti, 1986).

Methods

Male guinea-pigs (200–400 g) were decapitated and the brain quickly removed and placed into chilled Krebs solution (4°C). Surface slices of olfactory cortex ($\sim 500\,\mu$m thick) were cut manually with a bow cutter and recessed guide, as previously described (Harvey *et al.*, 1974). Slices were placed (pial surface uppermost) in a recording chamber, perfused at $\sim 5\,\text{ml}\,\text{min}^{-1}$ with oxygenated Krebs solution, and incubated for at least 2 h at 23–25°C before recording was started. Bipolar platinum stimulating electrodes were placed on the severed (rostral) end of the lateral olfactory tract (LOT) and single supramaximal stimuli applied at 0.2 Hz (0.02–0.2 ms duration, 2–20 V). Surface field potential responses were measured from the prepiriform region using a low resistance (1–3 MΩ) glass microelectrode (filled with 0.9% NaCl). The evoked field potential consisted of a large surface negative wave (N-wave) representing summed superficial excitatory postsynaptic potentials, sometimes with positive notches (population spikes) and occasionally a positive-going P-wave (Richards & Sercombe, 1968; Harvey *et al.*, 1974). Signals were displayed and captured by use of a digital oscilloscope (Gould OS1420), and selected records played out at reduced speed on a Bryans 28000 chart recorder. Field potential peak negativity was continuously monitored by the sample-and-hold circuit of a peak height detector (Courtice, 1977) and recorded on the chart recorder.

All drugs were freshly prepared in Krebs solution and bath-applied at room temperature (23–25°C). Agonists were applied for 2 min periods and antagonists were preincubated for at least 45 min before agonist testing. The Krebs solution had the following composition (mM): NaCl 118.0, KCl 3.0, $CaCl_2$ 1.5, $NaHCO_3$ 25, $NaH_2PO_4 \cdot 2H_2O$ 1.2, $MgCl_2 \cdot 6H_2O$ 1.0 and D-glucose 11; solutions were continually bubbled with 95% CO_2/5% O_2 (pH 7.4). Drugs were obtained from B.D.H. (Analar) or Sigma Ltd. (U.K.), except pirenzepine (Karl Thomae GmbH), methylfurmethide courtesy of Dr G.R. Martin (Wellcome Laboratories) and 4-(*m*-chlorophenylcarbamoxyl)-2-butynyltrimethylammonium chloride (McN-A-343; Research Biochemicals, U.S.A.). Oxotremorine was obtained from Sigma Ltd. as the sesquifumarate salt.

Results

Effects of muscarinic agonists

Bath-superfusion of (±)-muscarine or 'mixed' cholineoceptor agonists depressed the amplitude of the recorded field potential, an effect shown previously to be mediated probably through a presynaptic muscarinic receptor (Williams *et al.*, 1985). An example of this effect is shown in Figure 1 in which 100 μM carbachol (CCh) was applied for 2 min. The field potential was clearly depressed by CCh (Figure 1a, centre trace). Although this effect was reversible on washout of the agonist, recovery was typically slow as shown in the chart record of peak N-wave amplitude (Figure 1b). Responses to muscarinic agonists were also rather slow to develop compared with amino acid responses (Brown & Galvan, 1979) and usually the peak response to the agonists was seen after the 2 min superfusion period. Longer drug applications gave larger responses, but the effects were prolonged and often showed incomplete recovery. A 'compromise' dose contact time of 2 min was therefore regularly employed.

Reproducibility of agonist responses

In view of the prolonged recovery time necessary for agonist responses, it was important to check that reproducible responses could be evoked over many hours. In the experiment shown in Figure 2, 100 μM

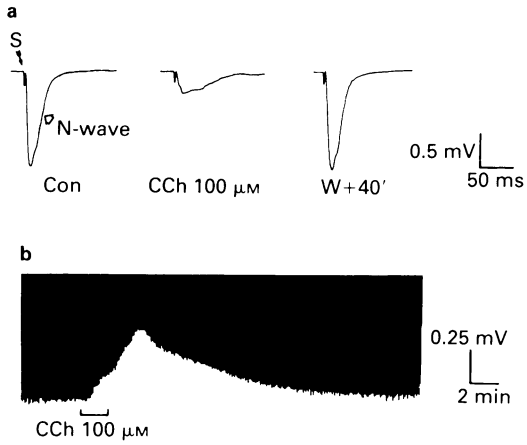


Figure 1 (a) Typical surface-negative field potentials recorded from the prepiriform region of an olfactory cortex in response to supramaximal stimulation of the LOT. Left: the downward deflection, labelled N-wave, is the monosynaptic population e.p.s.p. measured in control solution. S indicates the stimulus artifact. Middle: a 2 min application of 100 μM carbachol (CCh) decreased the peak amplitude of the N-wave and slightly increased its duration. The peak of the CCh response was recorded 4 min after the start of the CCh application. The right hand panel shows full recovery of the N-wave obtained after a 40 min wash period (W). (b) Continuous chart record showing the effects of CCh (100 μM) over a longer time period, using the output of the peak height detector. Each downward deflection represents the peak negative amplitude of a single N-wave evoked by 0.2 Hz stimulation of the LOT, although single deflections cannot be distinguished due to the slow chart speed. A 2 min application of 100 μM CCh markedly depressed the field potential amplitude. Note the slow onset and particularly prolonged recovery time of the CCh response.

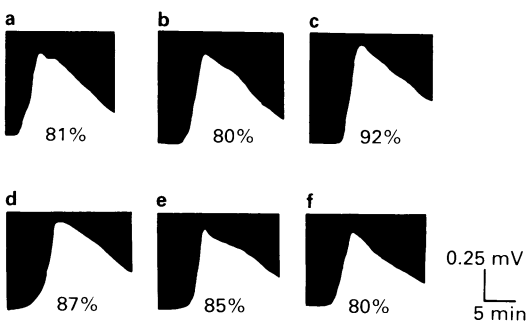
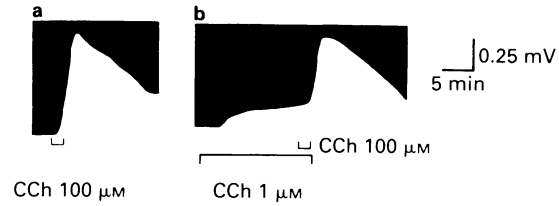


Figure 2 (a–f) Peak height detector records showing the reproducibility of successive responses to CCh in a single experiment. Two minute applications of CCh (100 μM) were given with dose intervals of 45 min. Values beneath each trace correspond to the maximal percentage reduction in peak N-wave amplitude for each application.



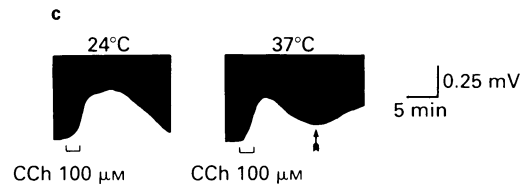


Figure 3 Test for desensitization of the muscarinic response. (a) Control response to 100 μM carbachol (CCh). (b) A 1 μM dose of CCh was pre-applied for 15 min and then the 100 μM dose repeated. Note that the peak depression of the field potential was unaffected by the small conditioning dose of CCh. (c) Temperature-sensitivity of the muscarinic response observed in a different slice. Records show that the action of 100 μM CCh at 24°C was quite comparable to that recorded at 37°C. However, although full N-wave recovery was seen at the lower temperature, at 37°C there was a sudden irreversible decrement in the field potential amplitude (arrow) during drug washout.

doses of CCh were applied to the slice every 45 min. Over a time period of more than 5 h there was clearly little variation in the amplitude of the response. Similar results were seen with lower CCh concentrations, but with doses exceeding 200 μM a loss of sensitivity was often observed.

The slowness of development of muscarinic responses made it difficult to determine if there was any covert desensitization, while the prolonged period required for field potential recovery made it impossible to apply agonists in quick succession. To test for desensitization, a just suprathreshold dose of agonist (e.g. CCh) was applied for 15 min, followed by a higher agonist dose for 2 min. The response evoked by the latter dose was unaffected by pre-application of the lower dose (Figure 3), suggesting that prolonged application of at least low agonist doses did not induce desensitization. However, it was possible that larger doses might induce some desensitization. Indeed, the experiments performed to determine the optimal contact time showed that long application times of large agonist doses did reduce the sensitivity of the slice to later doses; this could however, reflect a fairly long-term loss of sensitivity, rather than a classical desensitization.

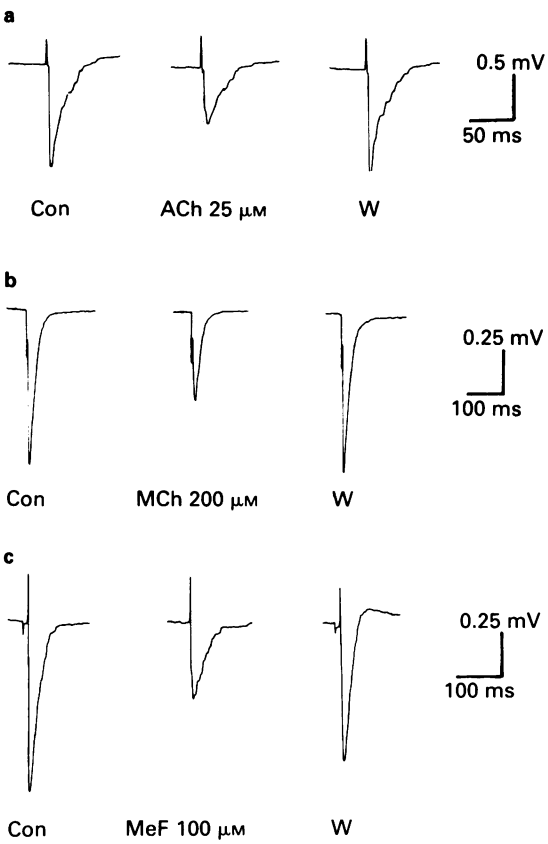


Figure 4 Reversible depressions of the N-wave field potential produced by various muscarinic agonists. Each panel shows an N-wave recorded in control solution (Con), after a 2 min application of agonist, then after a 40 min wash (W). (a, b) Effects of acetylcholine (ACh, 25 μM) or methacholine (MCh, 200 μM), recorded in the presence of 1 μM neostigmine; (c) Effect of methylfurmethide (MeF, 100 μM). Each agonist was tested on a different slice.

Effects of temperature

Brain slice studies are often conducted at temperatures lower than 37°C, so that oxygen tension is maintained in all parts of the slice (Harvey *et al.*, 1974). To check that the muscarinic response was not substantially different at the lower temperature used in this study, responses to CCh were compared at 24°C and 37°C (Figure 3c). It was noted that CCh was slightly less potent at the higher temperature and that the rates of onset and washout of responses were more rapid. However, at 37°C, field potential amplitudes often showed a sudden irreversible decline during the experiment, probably due to progressive anoxia in the slice (Fujii *et al.*, 1982). This phenomenon was also seen in slices that had not received any drug applications. In view of the poor long-term survivability of slices at physiological temperatures we chose to conduct the present experiments at 23–25°C.

Agonist dose-response relationships

Acetylcholine (ACh) and methacholine (MCh) produced identical effects to CCh (Figure 4), but only in the presence of an anticholinesterase (the olfactory cortex is rich in acetylcholinesterase; Wenk *et al.*, 1977). When applied alone, neither ACh or MCh had any effect on the field potential at up to 500 μM. Responses to ACh and MCh were therefore measured in the presence of 1 μM neostigmine. No increase in the potency of ACh was observed when the concentration of neostigmine was raised from 1 to 10 μM, indicating that a 1 μM dose was sufficient to block totally cholinesterase activity in the slice. Neostigmine had no effect on responses to CCh, and in no case was any effect observed when neostigmine was applied alone. Application of (±)-muscarine produced similar effects to the choline esters.

The amplitude of the evoked field potential in the olfactory slice is fairly constant in individual slices but shows considerable variation between slices. Agonist responses were therefore expressed as a percentage depression of the control field potential peak negativity, measured just before drug application. In a few experiments the effects of agonists on both peak negativity and also the area of the field potential beneath the baseline (time integral) were measured. However, both techniques yielded very similar estimates of response size. Similarly, the effects of agonists on the rate of rise of the N-wave were identical in magnitude to the effects on peak negativity. In a small but significant number of cases, measurements were complicated by the presence of a large population spike that obscured the peak of the N-wave. These results were not included in quantitative analyses.

Log-dose response relationships for CCh, ACh, MCh and muscarine were constructed by pooling data from 5 to 18 slices (Figure 5) (experiments involving ACh or MCh were performed in 1 μM neostigmine). The slopes of the dose-response lines determined by linear regression did not differ significantly from each other (t test, $P < 0.05$). All four agonists shown were capable of evoking a maximal effect, i.e. 100% field potential depression in individual experiments, although this is not apparent on the pooled dose-response relations since mean values were plotted. Agonist potency was expressed numerically in the form of IC_{50} measurements; i.e. the mean agonist dose required to depress the field potential by 50% (Table 1). On this basis, the order of

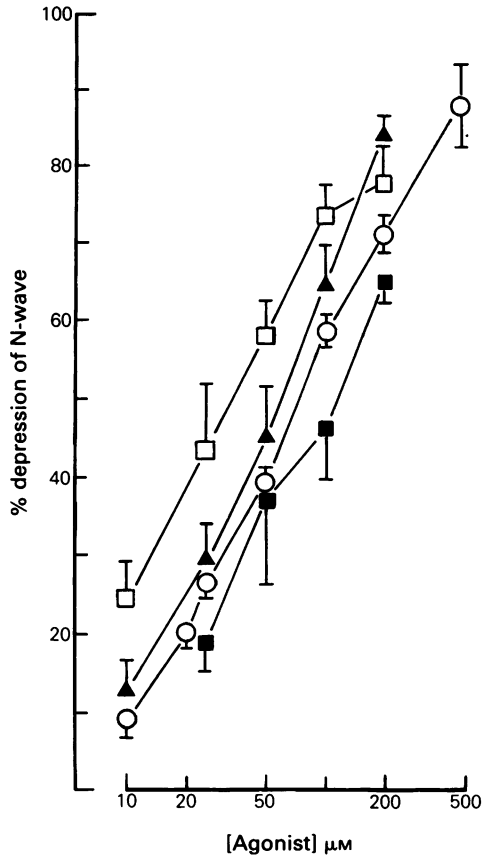


Figure 5 Log dose-response relationships showing the relation between percentage depression of peak N-wave amplitude (ordinate scale) and applied agonist concentration (abscissa scale) for various muscarinic agonists. Different symbols represent the four agonists used: acetylcholine (ACh, □), methacholine (MCh, ■), muscarine (▲) and carbachol (○). The points represent mean values pooled from 5–18 slices; vertical lines show s.e. mean. Data for ACh and MCh were obtained in the presence of 1 μM neostigmine.

Table 1 Agonist potency (expressed as IC_{50} measurements) of muscarinic receptor agonists

Agonists	IC_{50} μM (± s.e. mean)	*No. of experiments*
*Acetylcholine	40 ± 7	5
Carbachol	76 ± 8	18
* Methacholine	87 ± 20	6
Muscarine	49 ± 4	5

* Tested in the presence of 1 μM neostigmine.

potencies found was ACh ≥ muscarine > CCh > MCh. Methylfurmethide (1–100 μM), another muscarinic receptor agonist, also potently depressed the field potential with a similar potency to CCh (Figure 4c), but full quantification was not attempted because of the limited availability of this compound.

Low potency agonists

Both bethanechol, a choline ester that is relatively selective for muscarinic receptors (see Gilman *et al.*, 1985), and pilocarpine were found to have a low potency when applied to the olfactory slice and were only significantly active at mM concentrations (Figure 6). Neither agonist evoked a maximum response at doses up to 10 mM. Some experiments were also attempted with McN-A-343, a muscarinic ganglion stimulant with a suggested selectivity towards M_1-receptors (Hammer & Giachetti, 1982). Although in several slices McN-A-343 (100 μM) did reduce the field potential, its effects were very variable, even in the same slice. In some slices McN-A-343 was ineffective even though these slices showed normal responses to CCh. Interestingly however, responses to CCh could be reduced by a continuous preapplication of McN-A-343 in slices unresponsive to this agent alone. These low potency agonists might therefore act as partial agonists at this receptor.

Oxotremorine has been shown to be a very potent muscarinic agonist in a number of preparations including guinea-pig ileum (Takeyasu *et al.*, 1979), myenteric plexus (Morita *et al.*, 1982) and against the slow calcium-dependent afterhyperpolarization in the olfactory cortex (Constanti & Sim, 1987b). In the present study however, oxotremorine (from the same batch as that used by Constanti & Sim) was found to have very little effect on the field potential (Figure 6c). A range of concentrations was tested from 1 nM up to 100 μM but in no case was a clear depression of the N-wave observed. In ~10% of experiments an apparent 5–10% depression was observed for concentrations between 0.1 and 10 μM, although the action was so slow in onset as to make it difficult to distinguish from background variation. It was noted, however, that in experiments where a high dose of oxotremorine had been applied, subsequent responses to other agonists, such as CCh, appeared depressed. The possibility that oxotremorine had an antagonist action was therefore investigated. In the experiment shown in Figure 7, control responses were first obtained to 50 μM CCh and the doses then repeated in the presence of 10 μM oxotremorine. Oxotremorine was applied for 15 min before the CCh dose and had no effect itself on the field potential. Clearly, in the presence of oxotremorine the response to CCh was diminished (Figure 7b). On

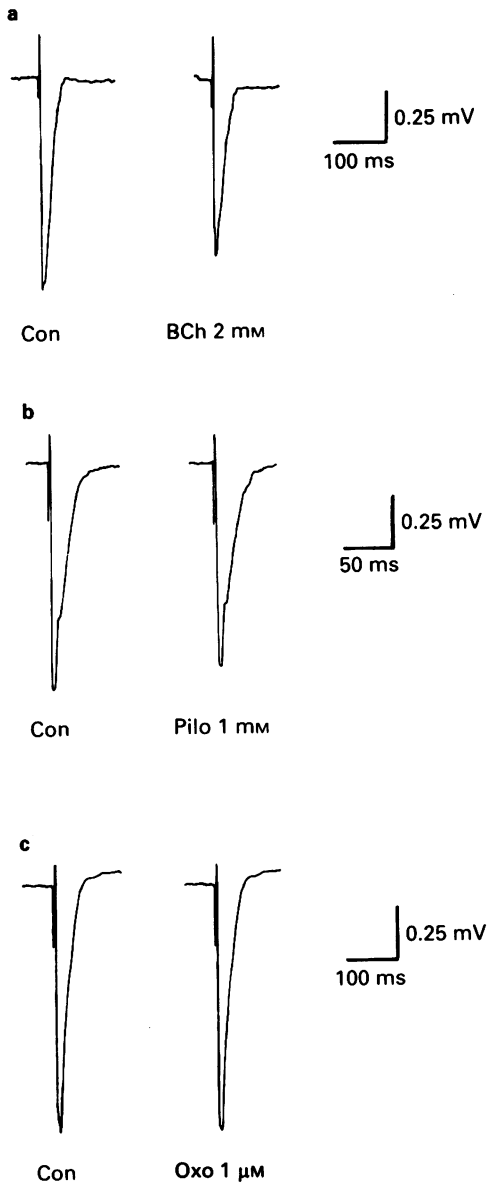


Figure 6 Effects of low potency muscarinic agonists on the N-wave field potential. Each panel shows N-wave recorded in control solution (Con), then after a 2 min application of agonist (recoveries not shown). Note weak depressions of N-wave produced by (a) bethanechol (BCh, 2 mM) or (b) pilocarpine (Pilo, 1 mM), whereas oxotremorine (Oxo, 1 μM) was ineffective (c). Each agonist was tested on a different slice.

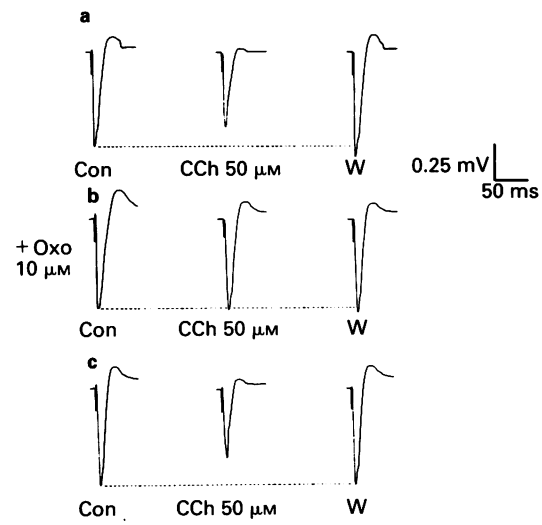


Figure 7 Antagonistic effect of oxotremorine on carbachol responses. (a) Control depression of the field potential evoked by 50 μM carbachol (CCh) dose. (b) A 15 min application of 10 μM oxotremorine (Oxo) alone did not affect the field potential. However, the response to 50 μM CCh applied during the presence of oxotremorine was greatly reduced. (c) Following a 2 h wash period, the response to CCh was re-established.

returning to control solution this reduction in the CCh effect remained, but after 2 h of washing, CCh responses had returned to their control value (Figure 7c). The threshold oxotremorine dose at which antagonism became detectable was around 1 μM. The actions of oxotremorine therefore appeared to mimic those of a reversible competitive antagonist and these properties were further investigated in the accompanying paper (Williams & Constanti, 1988).

Discussion

The present results show that clearly reproducible and dose-dependent responses to muscarinic agonists can be measured in the olfactory cortex slice, provided an adequate dose interval (at least 45 min) is employed. Agonist potencies for muscarine and active choline esters were low compared with values measured on smooth muscle and autonomic ganglia (Furchgott & Bursztyn, 1967; Burgen & Spero, 1968; Brown *et al.*, 1980) but similar to those found in other brain areas (Halliwell & Adams, 1982; Dodt & Misgeld, 1986) and in a previous intracellular study of olfactory neuronal responses to muscarinic agonists (Constanti & Galvan, 1983). This slightly lower potency of agonists in the olfactory slice may be a reflection of poor drug access, a particular problem in this preparation (Galvan, 1979). Interestingly, the IC_{50} values found in our study are similar to those quoted for the low affinity agonist binding site in

cerebral cortex, thought to correspond to the M_1-receptor (Birdsall *et al.*, 1978) and the values for stimulation of phospholipid turnover in the cerebral cortex (Fisher *et al.*, 1983; Jacobson *et al.*, 1985); such comparisons should however, be treated with caution, since there is no evidence to suggest that the muscarinic response in this study is mediated by phospholipid turnover. Another difficulty with these comparisons is that agonist responses are often complex and potency measurements may vary according to which component of the response is measured e.g. binding and efficacy are not equivalent (Stephenson, 1956).

No evidence was found to suggest that the muscarinic depression of the field potential underwent a 'fade' or tachyphylaxis. This observation agrees with that of Brown *et al.*, (1980) in the rat superior cervical ganglion where no overt desensitization to muscarinic agonists was seen. The slow nature of the responses however, made it difficult to determine if high agonist doses would elicit rapid desensitization. In contrast to the rat ganglion, a long-term loss of sensitivity was observed for prolonged application of higher agonist doses.

The poor efficacy of pilocarpine and bethanechol may reflect a partial agonist action as seen in sympathetic ganglia (Burgen & Spero, 1968; Brown *et al.*, 1980), parasympathetic postganglionic neurones (Goyal & Rattan, 1978), embryonic chick limb bud cells (Schmidt *et al.*, 1984), the phosphoinositide response in the brain (Fisher *et al.*, 1983) and the inhibition of stimulated cyclic AMP formation in neuroblastoma cells (McKinney *et al.*, 1985). These agonists are generally considered to show little or no activity at nicotinic receptors in ganglia (Caulfield & Stubley, 1982; Gilman *et al.*, 1985) or skeletal muscle (Clague *et al.*, 1985). The likelihood that pilocarpine and bethanechol were partly acting on cortical nicotinic receptors in our experiments, therefore seems remote. Indeed, millimolar concentrations of nicotinic agonists have little effect on the olfactory cortical N-wave (Williams *et al.*, 1985). A possible effect of pilocarpine exerted via histamine receptors also seems unlikely, since histamine (up to 1 mM) does not influence N-wave amplitude (Galvan, 1979).

The finding that oxotremorine behaved as an antagonist (see Williams & Constanti, 1987) was rather unexpected in view of its reported high agonist potency in evoking other types of muscarinic responses (Takeyasu *et al.*, 1979; Morita *et al.*, 1982; Ringdahl, 1984). For example, oxotremorine (0.5–10 μM) is a potent inhibitor of the outward membrane current underlying the slow afterhyperpolarization in olfactory neurones, acting via a postsynaptic M_2-type muscarinic receptor (Constanti & Sim, 1987b). On some tissues however, oxotremorine can show little or no agonist effectiveness; e.g. it acts as a partial agonist on sympathetic ganglia (Brown *et al.*, 1980), a competitive antagonist of the muscarinic rise in cyclic GMP levels in neuroblastoma cells (McKinney *et al.*, 1985) and a blocker of carbachol-induced phospholipid turnover in brain synaptosomes (Fisher *et al.*, 1983).

In a previous paper (Williams *et al.*, 1985), we showed that the muscarinic depression of the field potential was not apparently mediated by a decrease in presynaptic LOT fibre tract excitability, nor via a synaptically released inhibitory neurotransmitter such as γ-aminobutyric acid (GABA) or adenosine. The action of muscarinic agonists could also not be explained by a reduction in postsynaptic M-current (I_M; Constanti & Galvan, 1983), since barium, a known blocker of this current, did not depress the N-wave potential (Williams, 1986). Indeed, muscarinic suppression of I_M appears to involve an M_2-type receptor, showing only a low sensitivity to pirenzepine (Constanti & Sim, 1987a). It therefore seems likely that the responses observed in the present study were mediated via a distinct presynaptic muscarinic receptor.

Although the muscarinic ganglion stimulant McN-A-343 is claimed to be a selective M_1-receptor agonist (Hammer & Giachetti, 1982; Birdsall *et al.*, 1983), several studies show that it is either not very selective towards M_1- and M_2-receptors or that it appears not to act exclusively through muscarinic receptors (Murayama & Unna, 1963; Brown *et al.*, 1980; North *et al.*, 1985). Our own results, although somewhat limited, tend to support the idea that McN-A-343 is not a very selective muscarinic agonist.

In conclusion, we have shown that quantifiable responses to muscarinic agonists can be elicited in the *in vitro* olfactory cortex slice, although agonist potencies may be somewhat underestimated. A quantitative investigation of muscarinic antagonist actions on this slice therefore appeared to be quite feasible (Williams & Constanti, 1988).

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