Br. J. Pharmac. (1985), **85**, 733–735

Acetylcholine acts on m_2-muscarinic receptors to excite rat locus coeruleus neurones

T.M. Egan & R.A. North

Neuropharmacology Laboratory, 56–245, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, U.S.A.

Intracellular recordings were made from neurones of the rat locus coeruleus *in vitro*. Acetylcholine increased the firing rate by depolarizing the membrane; both muscarinic and nicotinic antagonists partially reduced this effect. In hexamethonium, pirenzepine shifted the acetylcholine dose-response curve to the right with an equilibrium dissociation constant of 200 nM, indicating an interaction with a m_2-type of muscarinic receptor.

Introduction The existence of subtypes of muscarinic receptors was first predicted from functional studies of peripheral organ systems (Burgen & Spero, 1968; Barlow *et al.*, 1976; Goyal & Rattan, 1978) and subsequently from agonist and antagonist binding experiments (Birdsall *et al.*, 1976; Hammer *et al.*, 1980). These receptors have been divided into two classes based on their affinity for the antagonist pirenzepine (Hammer & Giachetti, 1982). m_1-Muscarinic receptors, found in brain and autonomic ganglia, have a high affinity for pirenzepine (K_D 10–20 nM); m_2-receptors, present on smooth and cardiac muscle, have a lower affinity (K_D 200–500 nM).

Acetylcholine (ACh) can excite or inhibit neurones through actions on muscarinic receptors. The m_1-subtype is generally assumed to be the receptor involved in postsynaptic excitations, as has been shown in peripheral autonomic ganglia (Brown *et al.*, 1980). However, ligand binding studies indicate that the brainstem contains both high and low affinity binding sites (Potter *et al.*, 1984). In the present study, the actions of muscarinic agonists on the membrane properties of single neurones were investigated, and pharmacological null methods were used to characterize the muscarinic receptor subtypes involved.

Methods Intracellular recordings were made from locus coeruleus (LC) neurones contained in slices (300 μm) cut from rat pons (for methods, see Henderson *et al.*, 1982). Submerged slices were superfused with warmed (37°C) Krebs solution that contained (mM): NaCl 126, KCl 2.5, $CaCl_2$ 2.4, $NaHPO_4$ 1.2, $MgCl_2$ 1.3, $NaHCO_3$ 25, glucose 11, saturated with 5% CO_2/95% O_2. Microelectrodes contained 2M KCl and had resistances of 30–80MΩ. The effect of agonists on the rate of discharge of spontaneously occurring action potentials was measured directly; or agonist-induced depolarizations were measured in neurones held at −75 mV by passing steady current. Drugs were applied by changing the superfusion solution; agonist effects reached steady state within 2 min. Antagonists were applied for 20 min prior to, and then throughout, the period of agonist application. Drugs used were: ACh chloride, atropine sulphate, gallamine triethiodide, hexamethonium bromide (Sigma), methylfurmethide iodide (gift of N. Birdsall), (±)-muscarine chloride (Sigma, Boehringer), neostigmine methylsulphate (Calbiochem), nicotine sulphate (Sigma), physostigmine salicylate (Sigma), pirenzepine (Boehringer), propylbenzilylcholine mustard (New England Nuclear) and (−)-scopolamine hydrochloride (Sigma).

Results ACh increased the rate of action potential discharge (Figure 1a). In cells held at −75 mV, ACh caused concentration-dependent depolarizations (Figure 1b). These effects were also observed in superfusing solutions which contained no calcium ions. Effective ACh concentrations were 30–100 times lower in the presence of physostigmine or neostigmine (0.1–10 μM). Muscarinic agonists (methylfurmethide (1–60 μM), muscarine (1–60 μM) and oxotremorine (0.1–10 μM), as well as nicotine (5–100 μM), increased the firing rate. Hexamethonium (100–400 μM) completely blocked depolarizations caused by nicotine and was present in all experiments in which ACh was used as the agonist to study muscarinic effects; hexamethonium did not change responses to any muscarinic agonists.

ACh and muscarinic agonists were less effective in increasing the firing rate during superfusion with muscarinic antagonists: atropine (1–10 nM), (−)-scopolamine (1–10 nM) and pirenzepine (0.2–30 μM). Each antagonist caused a parallel shift to the right in the agonist dose-response curve (dose-ratios up to 100) (see Figure 1c). Gallamine (30–100 μM) also antagonized the muscarinic actions of ACh, apparently in a competitive manner (dose-ratio up to 10). Propylbenzilylcholine mustard (100–600 nM) caused

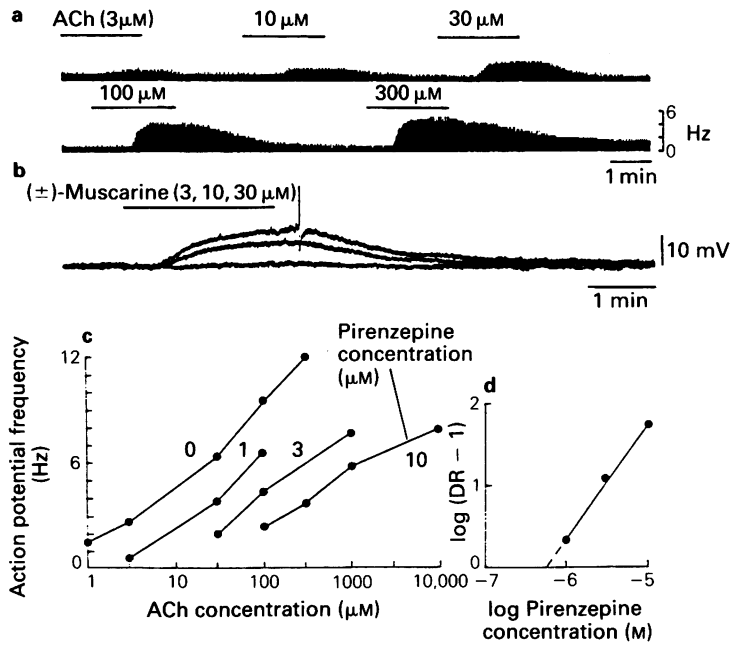

Figure 1 (a) Acetylcholine increases the firing rate of a locus coeruleus neurone. Neostigmine (10 μM) and hexamethonium (400 μM) were also present. (b) (±)-Muscarine depolarizes a neurone in which spontaneous firing was prevented by current injection (holding potential − 70 mV). Three records of membrane potential are photographically superimposed. The highest concentration applied (30 μM) depolarized the cell sufficiently to fire a single action potential (full amplitude not shown). (c) Dose-response relation for the increase in firing rate of a single neurone (peak firing rate minus control firing rate) resulting from superfusion with acetylcholine; the effects of acetylcholine were determined in the presence of various concentrations of pirenzepine (indicated beside each curve, μM). (d) Schild plot of results shown in (c); line is fitted by least squares and has a slope of 1.1. The pirenzepine K_D is 288 nM.

a non-parallel shift to the right and also decreased the maximum excitation obtainable. Schild plots (Arunlakshana & Schild, 1959) were made from the ACh dose-response curves in increasing concentrations of pirenzipine, measuring either the increase in firing rate (4 neurones, see Figure 1d) or the membrane depolarization (2 neurones). Schild plots had slopes of 0.8 to 1.2 (95% confidence limits in each experiment embraced unity). The pirenzepine K_D was 233 ± 30 nM (mean ± s.e.mean, 6 neurones). The scopolamine K_D determined in similar experiments was 680 ± 230 pM (4 neurones).

Discussion The results indicate that the excitation of LC neurones by muscarinic agonists is caused by an action on a population of postsynaptic muscarinic receptors having a low affinity for pirenzepine. Pirenzepine binding sites with similar affinities (200 nM to 1 μM, i.e. m_2-receptors) have been described in medulla-pons (Hammer *et al.*, 1980), but it is generally thought that these receptors are presynaptically located (see Potter *et al.*, 1984). This belief arises largely by analogy with results on peripheral tissues, in which m_2-receptors are responsible for presynaptic inhibition whereas m_1-receptors are involved in postsynaptic excitation (Brown *et al.*, 1980; Kilbinger & Nafziger, 1985; North & Surprenant, 1985). The present experiments are the first in which the muscarinic receptor subtypes have been characterized on individual central neurones; they indicate that direct postsynaptic excitatory effects of ACh can be brought about by m_2-receptor activation.

Supported by U.S. Department of Health and Human Services grants DA03161 and AM32979. The advice of John Williams and Annmarie Surprenant was greatly appreciated. We thank Drs N. Birdsall and R. Hammer (Boehringer) for gifts of compounds.

References

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

BARLOW, R.B., BERRY, K.J., GLENTON, P.A.M., NIKOLAOU, N.M. & SOH, K.S. (1976). A comparison of affinity constants for muscarine-sensitive acetylcholine receptors in guinea-pig atrial pacemaker cell at 29°C and in ileum at 29°C and 37°C. *Br. J. Pharmac.*, **58**, 613–620.

BIRDSALL, N.J.M., BURGEN, A.S.V., HILEY, C.R. & HULME, E.C. (1976). Binding of agonists and antagonists to muscarinic receptors. *J. supramol. Struct.*, **4**, 367–371.

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

BURGEN, A.S.V. & SPERO, L. (1968). The action of acetylcholine and other drugs on the efflux of potassium and rubidium from smooth muscle of the guinea-pig intestine. *Br. J. Pharmac.*, **34**, 99–115.

GOYAL, R.K. & RATTAN, S. (1978). Neurohumoral, hormonal and drug receptors for the lower esophageal sphincter. *Gastroenterology*, **74**, 598–516.

HAMMER, R., BERRIE, E.P., BIRDSALL, N.J.M., BURGEN, A.S.V. & HULME, E.C. (1980). Pirenzepine distinguishes between different subclasses of muscarinic receptors. *Nature*, **283**, 90–92.

HAMMER, R. & GIACHETTI, A. (1982). Muscarinic receptor subtypes: m_1 and m_2 biochemical and functional characterization. *Life Sci.*, **31**, 2991–2998.

HENDERSON, G., PEPPER, C.M. & SHEFNER, S.A. (1982). Electrophysiological properties of neurones contained within the locus coeruleus and mesencephalic nucleus of the trigeminal nerve *in vitro*. *Exp. Brain Res.* **45**, 29–37.

KILBINGER, H. & NAFZIGER, M. (1985). Two types of neuronal muscarinic receptors modulating acetylcholine release from the guinea-pig myenteric plexus. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **328**, 304–309.

NORTH, R.A. & SURPRENANT, A. (1985). Muscarine m_1 and m_2 receptors mediate depolarization and presynaptic inhibition in guinea-pig enteric neurones. *J. Physiol.*, (in press).

POTTER, L.T., FLYNN, D.D., HANCHETT, H.E., KALINOSKI, D.L., LUBER-NAROD, J. & MASH, D.C. (1984). Independent m_1 and m_2 receptors; ligands, autoradiography and functions. *Trends Pharmac. Sci* (suppl.), 22–31.

(Received May 8, 1985.
Accepted May 23, 1985.)