Journal of Physiology (1990), **422**, *pp.* 369–380
With 7 figures
Printed in Great Britain

MUSCARINE REDUCES INWARDLY RECTIFYING POTASSIUM CONDUCTANCE IN RAT NUCLEUS ACCUMBENS NEURONES

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(*Received 9 June 1989*)

SUMMARY

1. Intracellular recordings were made from neurones in the nucleus accumbens in slices from the rat brain maintained *in vitro*.

2. Muscarine (1–100 μM) depolarized 101 of 107 neurones; this was associated with an increase in the input resistance. The potential change reversed polarity with conditioning hyperpolarization and the reversal potential was linearly related to the logarithm of the extracellular potassium concentration.

3. The depolarization caused by muscarine was not changed by tetrodotoxin (1 μM) or by a solution that contained lower levels of calcium (0·24 instead of 2·4 mM), higher levels of magnesium (5 instead of 1·2 mM) and cobalt (2 mM).

4. Muscarine caused an inward current and a decrease in slope conductance when applied to neurones voltage clamped near their resting potential (−82 mV). The current caused by muscarine reversed polarity at the potassium equilibrium potential. The current–voltage relation of the neurones between −60 and −120 mV was well fitted by assuming a voltage-independent potassium conductance and an inward rectifier potassium conductance; muscarine reduced predominantly the inward rectifier conductance.

5. Phorbol-12,13-diacetate (3 μM) and 5-hydroxytryptamine mimicked the action of muscarine. The inward currents caused by muscarine or 5-hydroxytryptamine were occluded by the inward current evoked by the phorbol ester.

6. The depolarization caused by muscarine was competitively antagonized by pirenzepine; the dissociation constant of 11 nM suggested involvement of the M_1 receptor.

7. It is concluded that muscarine acts at M_1 receptors to reduce the membrane potassium conductance and that activation of protein kinase C may be an intermediate step.

INTRODUCTION

The nucleus accumbens (or ventral striatum), like the dorsally situated striatum proper, has one of the highest concentrations of acetylcholine (ACh) in the mammalian brain (Feldberg & Vogt, 1948; Hebb & Silver, 1956). The ACh is contained within a population of relatively large interneurones (McGeer, McGeer,

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Fibiger & Wickson, 1971; Herkenham, Moon Edley & Stuart, 1984; Mesulam, Mufson, Levey & Wainer, 1984), which are found particularly in the acetylcholinesterase-rich neurochemical compartment referred to as the matrix (Graybiel, Baughman & Eckenstein, 1986). The neurones also express high levels of muscarinic ligand binding sites (Cortes & Palacios, 1986; Nastuk & Graybiel, 1988), and together these findings imply that ACh plays a functional role within the nucleus, presumably in some aspect of the initiation of voluntary movement (Jones, Mogenson & Wu, 1981; Austin & Kalivas, 1988).

Neurones of the striatum are excited by application of exogenous ACh (Bloom, Costa & Salmoiraghi, 1965; Herz & Zieglgänsberger, 1968); more recently, Dodt & Misgeld (1986) showed that the excitation was accompanied by an increase in the apparent input resistance of the neurones and inferred that it was due to a reduction in potassium conductance. However, the details of the mechanism of excitation have been studied for other transmitters: substance P reduces an inwardly rectifying potassium conductance in rat striatal cells in culture (Stanfield, Nakajima & Yamaguchi, 1985) and 5-hydroxytryptamine (5-HT) has a similar action on rat nucleus accumbens neurones in tissue slices (North & Uchimura, 1989). The experiments described in the present paper had two primary purposes. The first was to determine whether ACh excited nucleus accumbens neurones by affecting primarily or exclusively the same inward rectifier potassium conductance as that reduced by 5-HT; the second was to determine the subtype of the muscarinic receptors involved in the excitation of the nucleus accumbens neurones. A preliminary report has been made to the Physiological Society (Uchimura, 1989).

METHODS

The forebrains of male rats (200–300 g) were removed under light ether anaesthesia. Frontal slices (300 μm) were cut with a vibratome; one was placed in a recording chamber (volume 500 μl) where it was submerged in a continuously flowing solution (3 ml min^{-1}) at 36 °C. The composition of the solution was (mM): NaCl, 126; KCl, 2·5; NaH_2PO_4, 1·2; $MgCl_2$, 1·3; $CaCl_2$, 2·4; glucose, 10; $NaHCO_3$, 26; gassed with 95% O_2 and 5% CO_2. Muscarine and other drugs were applied to the tissue by changing this solution to one that differed only in the content of the drug. The effect of muscarine reached a steady state within 2–3 min; the action of pirenzepine took 20–30 min to reach a steady state.

The nucleus accumbens was identified with respect to the longitudinal cerebral fissure, the lateral ventricle and the anterior commissure. Intracellular recordings were made from a region about one-third of the distance along the rostral-caudal extent of the nucleus and within 200 μm ventral and/or medial to the anterior commissure. Glass microelectrodes were used; they contained potassium chloride (2 M) and had resistances of 40–80 MΩ. Signals were amplified with a single-electrode voltage clamp amplifier (Axoclamp 2A) and displayed on a chart recorder (Gould, pen response 0–300 Hz). Membrane currents were measured using a 2·5–3·5 kHz switching frequency. Current–voltage relations were plotted by two methods, which gave the same result. First, currents were measured at the end of voltage steps (0·5–5 s) from the holding potential (typically −80 mV) to various potentials. Second, current was plotted on an X–Y plotter in response to a slow depolarizing ramp command (1 mV s^{-1} from −130 to −55 mV). A computer program (Statistical Graphics Corporation) was used to fit current–voltage curves to the experimental observations by assuming that membrane current passed through three conductances: G_l (voltage-independent potassium conductance), G_{ir} (inward rectifier potassium conductance $[= G_{ir,max}/(1+\exp((V-V_{0.5})/k))]$) and G_{in} (all other conductances). The reversal potential for the potassium currents

(E_K) was taken as the potential at which muscarine caused no net current (E_{rev}). Currents were fitted to

$$I = \{G_l[V+E_K]\}+\{G_{ir,max}[V+E_K]/(1+\exp((V-V_{0.5})/k))\}+\{(G_{in}[40-V]\}$$

to provide the least-squares estimates of G_l, $G_{ir,max}$, $V_{0.5}$ (the potential at which $G_{ir} = 0.5\,G_{ir,max}$) and k (the slope factor (mV) of the inward rectifier conductance). In the fitting procedure, G_{in} was replaced by the solution of

$$\{G_l[V_r+E_K]\}+\{G_{ir,max}[V_r+E_K]/(1+\exp((V_r-V_{0.5})/k))\}+\{G_{in}[V_r-E_{in}]\} = 0$$

because membrane current is zero when $V = V_r$ (the experimentally measured resting potential in control solution or in muscarine). E_{in} (the reversal potential for membrane currents passing through G_{in}) was assumed to be 40 mV but the results were little different if this was taken as -20 mV; G_{in} was assumed to be unaffected by muscarine.

Drugs used were acetylcholine chloride, 5-hydroxytryptamine, muscarine chloride (batches used were reported to be 40% pure), pirenzepine, 4β-phorbol-12,13-diacetate, 4β-phorbol-12,13-didecanoate, 4α-phorbol-12,13-didecanoate and scopolamine. They were purchased from Sigma, with the exception of pirenzepine, which was a gift from Boehringer.

RESULTS

Intracellular recordings from 107 neurones are included. The cells had a resting potential of -82.1 ± 0.7 mV (mean $\pm$ S.E. of the mean).

Muscarine reduces potassium conductance

Muscarine depolarized 101 cells. The time course of the action of muscarine is shown in Fig. 1, from which it can be seen that the effect reversed quickly when the solution was changed back to one that did not contain muscarine. Acetylcholine (1 mM) also depolarized neurones, by 5.0 ± 0.7 mV ($n = 5$). Depolarizations caused by either ACh ($n = 2$) or muscarine (30 μM, $n = 2$) were completely blocked by scopolamine (1 μM).

Muscarine caused an inward current in cells voltage clamped at the resting potential (Fig. 1*B*). Higher concentrations of muscarine caused progressively greater effects, whether this was depolarization or inward current. Figure 2 illustrates such result from a single neurone (Fig. 2*A*) and from many neurones (Fig. 2*B*). Also illustrated in Fig. 1*C* and *D* are the observations that the depolarizing response to muscarine was unaffected by tetrodotoxin (1 μM, $n = 4$) and that it persisted in solutions having a reduced calcium concentration (calcium-free ($n = 2$), calcium-free with 2 mM-cobalt ($n = 3$), 0.24 mM-calcium with 5 mM-magnesium and 2 mM-cobalt ($n = 2$)).

The response to muscarine was dependent on the membrane potential. Figure 3*A* illustrates one of three experiments in which the membrane potential was allowed to change: the membrane potential was set to an initial value by passage of current and this current was then unaltered during the action of muscarine. The depolarization caused by muscarine at the resting potential (-80 mV in this case) became smaller with hyperpolarization and reversed polarity at about -105 mV. Most experiments were done by voltage clamp (Fig. 3*B*). Current–voltage relations for the neurones

generated before and after adding muscarine intersected at $-106{\cdot}6 \pm 1{\cdot}6$ mV ($n = 10$). This reversal potential (E_{rev}) became less negative when the potassium concentration ($[K_o^+]$) was raised (Fig. 3*C*) according to $E_{rev} = 57 \log([K_o^+]/185)$.

The properties of the nucleus accumbens neurones in the voltage range -60 to

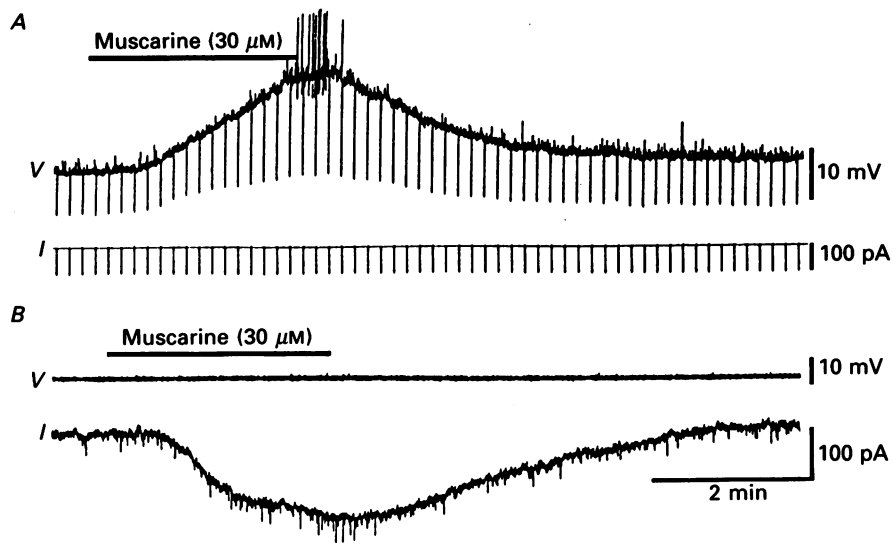

Fig. 1. Muscarine depolarizes a nucleus accumbens neurone. *A*, recording of membrane potential (*V*) and membrane current (*I*). Resting potential -82 mV. Muscarine was applied during the period indicated by the bar. In this and other figures the apparent delay in onset and offset of action is in part due to the time required for the superfusing solution to pass through a heater before reaching the chamber that contained the tissue. The depolarization caused by muscarine initiated action potentials (full amplitude not resolved by pen recorder) and was associated with an increase in the input resistance (downward deflections are electrotonic potentials resulting from rectangular (500 ms) current pulses of 100 pA). *B*, a similar experiment under voltage clamp. Holding potential -75 mV. (Resting potential was -82 mV.)

-120 mV can be described by assuming that there are two potassium conductances (Uchimura, Cherubini & North, 1989). The first (G_l) is independent of membrane potential and unaffected by low concentrations (< 100 μM) of barium. The second (G_{ir}) changes with membrane potential (V) according to $G_{ir,max}/(1+\exp((V-V_{0{\cdot}5})/k))$ (see Methods). The average values ($n = 8$) of the membrane current (I) at different potentials (V) were fitted to the function $I = (G_l[V-E_K]) + (G_{ir}[V-E_K]) + (G_{in}[V-E_{in}])$ (see Methods). In the absence of muscarine the values were $G_l = 9{\cdot}2 \pm 0{\cdot}2$ nS, $G_{ir,max} = 33{\cdot}2 \pm 0{\cdot}8$ nS, $V_{0{\cdot}5} = -102{\cdot}5 \pm 0{\cdot}7$ mV and $k = 16{\cdot}4 \pm 0{\cdot}5$ mV. In muscarine (30 μM), the values were $7{\cdot}8 \pm 0{\cdot}2$ nS, $25{\cdot}9 \pm 0{\cdot}9$ nS, $-99{\cdot}3 \pm 0{\cdot}9$ mV and $14{\cdot}8 \pm 0{\cdot}7$ mV. According to this analysis, muscarine reduced both the inward rectifier and the linear (or leakage) conductance to potassium ions, but the predominant effect was on the inward rectifier.

Another way to separate the potassium conductances is into barium-sensitive (inward rectifier) and barium-insensitive (linear) components (Uchimura *et al.* 1989). Figure 4 shows that muscarine evoked no membrane current in the presence of

barium (100 μM, $n = 4$), implying that the residual (barium-insensitive) conductance is not affected by muscarine.

Muscarine acts at M_1 receptors

The antagonism between pirenzepine and muscarine was studied; concentration–response curves for muscarine were constructed in the absence and then

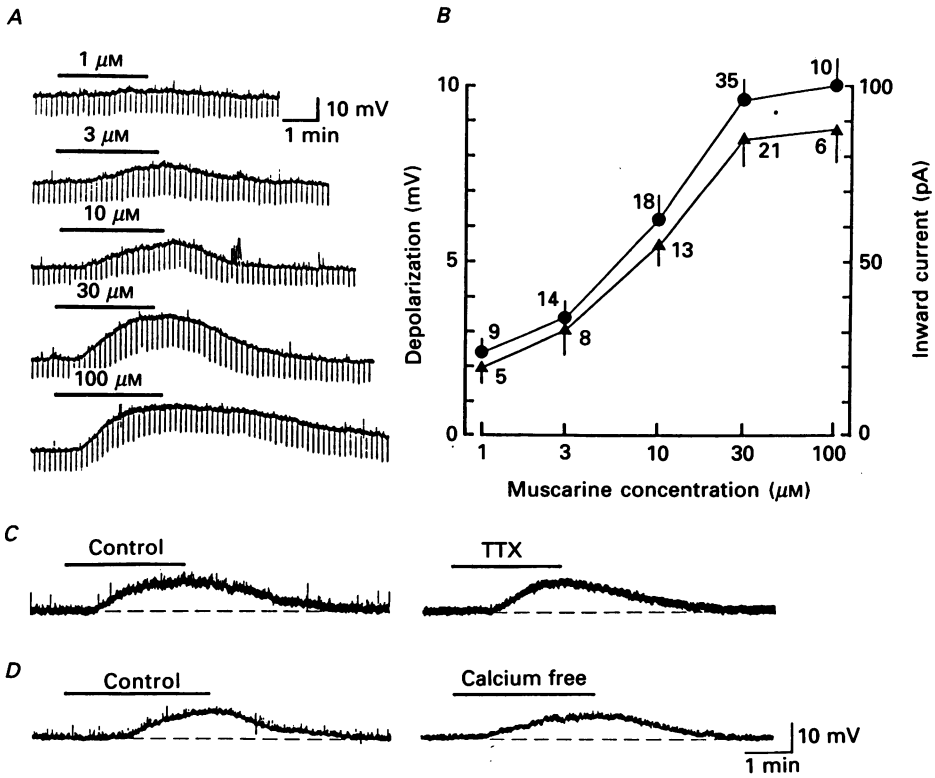

Fig. 2. *A* and *B*, muscarine action depends on concentration. *A* shows responses of one neurone to five applications of muscarine. Initial membrane potential was set to −75 mV; electrotonic potentials were evoked by 100 pA pulses of 500 ms duration. *B*, effects of muscarine on membrane potential (●) and current (▲) in several neurones (bars indicate S.E.M.; number of cells beside each point). Holding potentials were between −75 and −77 mV. *C* and *D*, action of muscarine is unaffected by tetrodotoxin (1 μM, *C*) or by calcium-free solution (also containing 2 mM-cobalt, *D*). In each case, the bar indicates the application of muscarine (30 μM). Potential set to −75 mV; resting potentials were −83 mV (*C*) and −85 mV (*D*).

in the presence of one ($n = 3$), two ($n = 2$) or three ($n = 2$) concentrations of pirenzepine. Figure 5 illustrates a result from the most complete such experiment. Pirenzepine acted as a competitive antagonist of muscarine, for the Schild plot was straight with unit slope (Arunlakshana & Schild, 1959). For the four cells in which two or three pirenzepine concentrations were used, the dissociation equilibrium constant was 11·5±2·6 nM. Inclusion of the three estimates from experiments with single pirenzepine concentrations gave a value of 11·7±2·6 ($n = 7$). The 4-chloro-

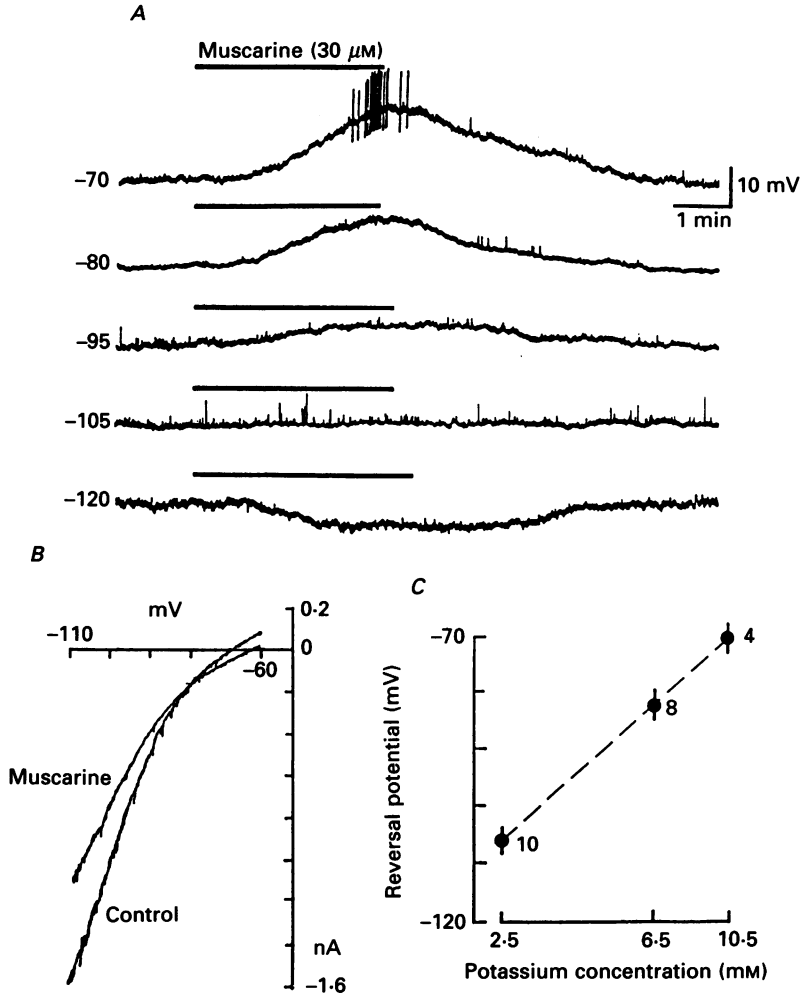

Fig. 3. Muscarine action reverses at potassium equilibrium potential. *A*, potential changes evoked by muscarine at five holding potentials (indicated beside each trace, mV). *B*, membrane currents elicited by a ramp depolarizing voltage command, in the absence and presence of muscarine (30 μM). Potassium concentration 6·5 mM. *C*, reversal potential of current caused by muscarine plotted as a function of the potassium concentration (from experiments such as the one illustrated in *B*). Points are mean ± S.E.M. for number of cells indicated beside each point.

phenyl analogue of McN-A-434, which is a somewhat selective M_1 receptor agonist in some tissues (Eltze, Gmelin, Wess, Strohmann, Tacke, Mutschler & Lambrecht, 1988) depolarized the neurones ($n = 7$). The amplitudes of the depolarization were not different from those caused by the same concentrations of muscarine (Fig. 2*B*).

5-hydroxytryptamine and phorbol esters occlude the effect of muscarine

It was found recently (North & Uchimura, 1989) that 5-HT acts at 5-HT_2 receptors on nucleus accumbens neurones to reduce the membrane potassium conductance. Ketanserin (1 μM), which blocks the action of 5-HT (North &

Uchimura, 1989), did not affect the response to muscarine ($n = 2$). The properties of the potassium conductance reduced by 5-HT are very similar to those described above for muscarine, suggesting that they may be the same set of channels. Figure 6 shows the result of an experiment in which the current caused by muscarine occluded the action of 5-HT. Similar findings were obtained in three other cells.

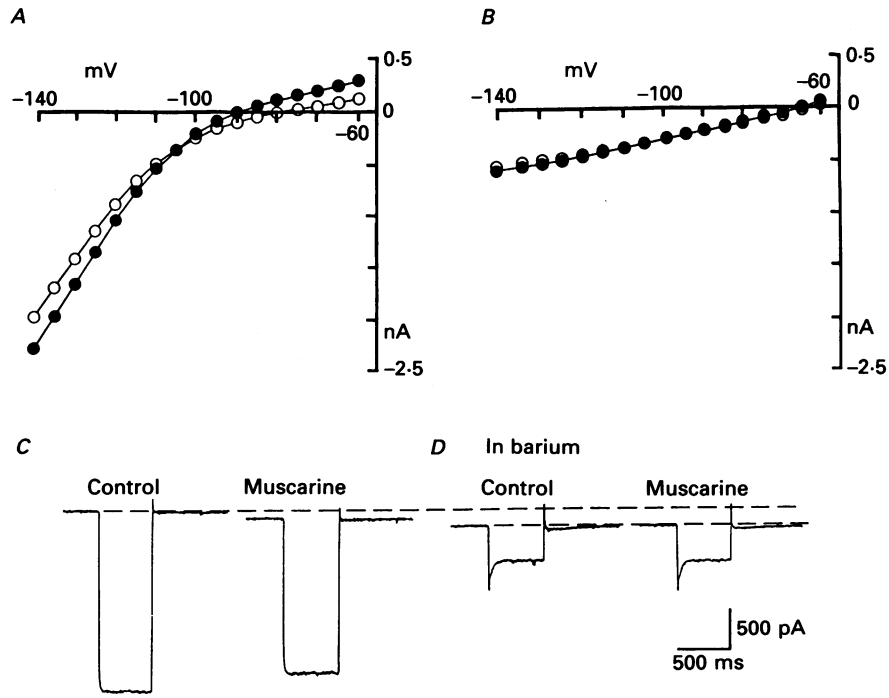

Fig. 4. Muscarine-sensitive current is blocked by barium. *A*, current–voltage relation before (●) and after adding muscarine (○, 30 μM). Points were measured from termination of currents such as those shown in *C*. Holding potential was −90 mV. *B*, barium (100 μM) depolarized the cell by about 25 mV and completely blocked the inward rectification. Muscarine (○) had no effect in the presence of barium (●, control). *C*, typical membrane currents in response to voltage clamp steps from −90 to −140 mV, before and after adding muscarine (30 μM). *D*, a similar experiment in the presence of barium (100 μM).

4β-Phorbol-12,13-diacetate also depolarized neurones (Fig. 7*A*). Under voltage clamp, phorbol esters caused a membrane current that was inward at the resting potential and reversed to outward at the potassium equilibrium potential ($n = 3$). 4β-Phorbol-12,13-diacetate (3 μM) depolarized by 5·0 ± 0·4 mV ($n = 3$) and caused an inward current at −76 mV of 62·5 ± 11·5 pA; at 10 μM these effects were 7·3 ± 0·9 mV ($n = 4$) and 82·5 ± 12·4 pA ($n = 7$). The inward current evoked by the phorbol ester (3 or 10 μM) completely occluded the action of both muscarine (30 μM, $n = 5$) and 5-HT (100 μM, $n = 5$, Fig. 7*B*). 4β-Phorbol-12,13-didecanoate (10 μM) had the same effect as the diacetate ($n = 4$) but 4α-phorbol-12,13-didecanoate was inactive ($n = 2$).

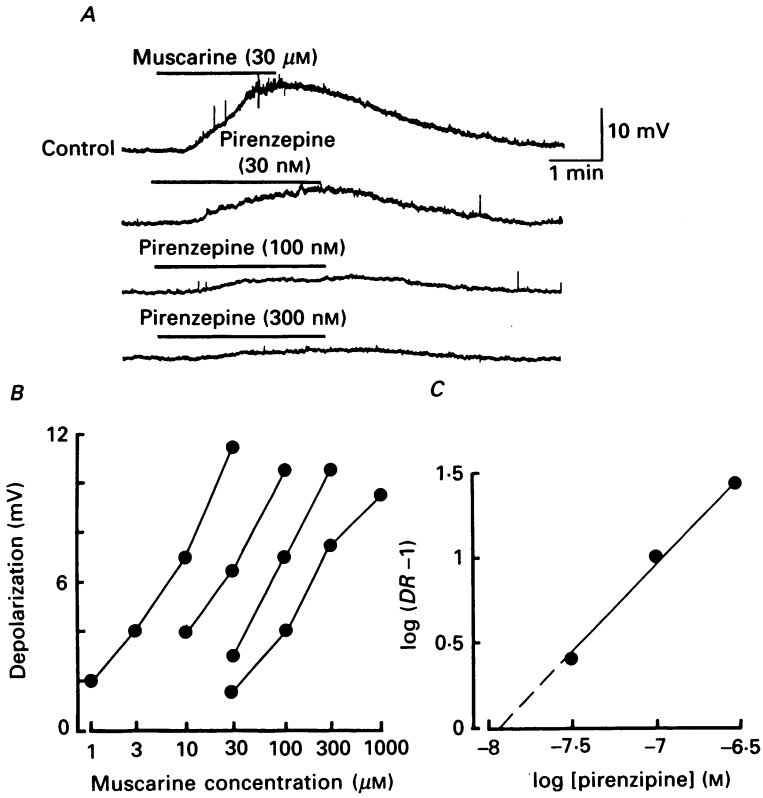

Fig. 5. Muscarine acts at M_1 receptors. *A*, depolarizations caused by muscarine in the absence and in the presence of 30, 100 and 300 nM-pirenzepine. Resting potential −79 mV. *B*, depolarizations caused by different concentrations of muscarine, with and without pirenzepine. *C*, Schild plot (Arunlakshana & Schild, 1959) was straight with unit slope. Line is drawn by eye. Estimated dissociation equilibrium constant in this cell is 11 nM. *DR* is the ratio of the concentrations of muscarine that caused the same depolarization in the presence and absence of pirenzepine.

DISCUSSION

Almost all the neurones were depolarized by muscarine. The results with pirenzepine suggest that this involves the M_1 muscarinic receptor, rather than the cardiac M_2 or glandular M_2 receptors (also known as m2 and m3 receptors: Doods, Mathy, Davidesko, van Charldorp, de Jonge & van Zwieten, 1987; Bonner, 1989) although experiments with other antagonists would help to characterize the receptor more fully.

Several findings indicate that the depolarization from the resting potential results from a reduction in a standing outward potassium current; the most persuasive is the reversal of the evoked current at a potential which shifted according to the logarithm of the potassium concentration. Excitation by muscarinic agonists resulting from reduction in potassium conductance was first proposed by Weight & Votava (1970) and by Krnjević, Pumain & Renaud (1971) and has since been described in many

neurones (reviewed by North, 1989). The properties of the potassium conductance reduced by muscarine vary considerably from cell to cell. In many instances, the potassium conductance affected is not demonstrably voltage or time dependent (the background, or leakage, potassium conductance), whereas in other cells, M-currents, A-currents and two different calcium-activated currents are reduced (see North, 1989). Muscarine often reduces two or more distinct potassium conductances in the same cells (e.g. rat hippocampal pyramidal cells: Dutar & Nicoll, 1988; guinea-pig sympathetic ganglion cells: Cassell & McLachlan, 1987).

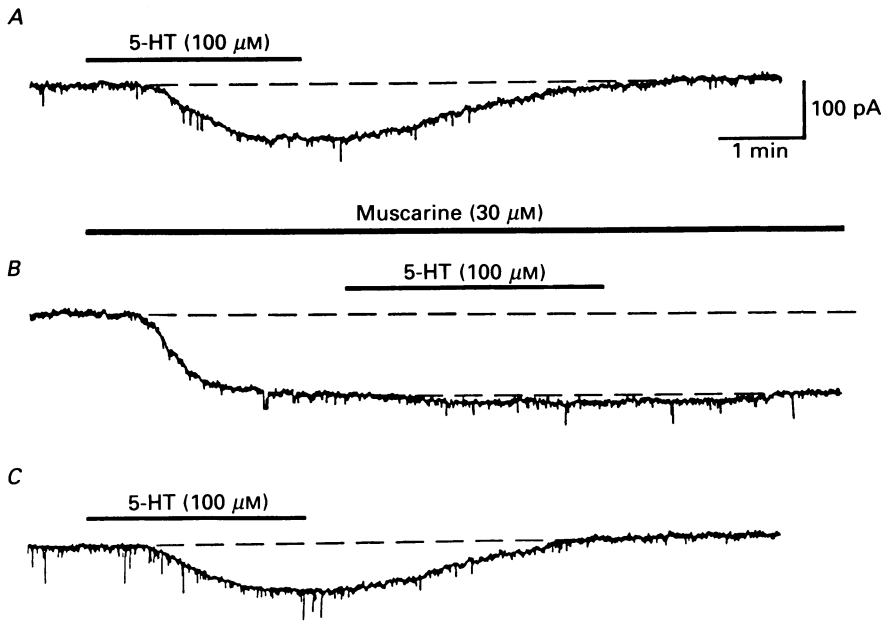

Fig. 6. Current caused by 5-HT is occluded by current caused by muscarine. 5-HT (100 μM) was applied before (*A*), during (*B*) and 20 min after (*C*) muscarine (30 μM). Both of these are maximal concentrations. Resting potential was −78 mV; initial holding potential was −70 mV.

Which conductance is the principal target of muscarine action in the nucleus accumbens depends on the manner in which the conductances are defined. If the inward rectifier conductance is defined pharmacologically as that which is sensitive to barium (100 μM), then muscarine affects only the inward rectifier and has no effect on the linear component (Fig. 4*B*). The modelling of the I–V curves on the assumption that they can be accounted for by the sum of two potassium conductances (linear and inward rectifier components) indicated that there was a reduction in both the inward rectifier and the linear component. In any event, the maximum action of muscarine was rather small (Fig. 2*B*), often insufficient to bring the neurones to threshold for action potential generation under our experimental conditions (although see Fig. 1*A*). The maximum effect at the resting potential (−82 mV) was to reduce the conductance by 2·7 nS, which is only 16% of the cell conductance at this potential. At the mechanistic level, this implies that there is a large population of potassium channels, particularly of inward rectifiers, which can not be closed by

muscarine. These channels are, however, readily blocked by quite low concentrations of barium; a concentration of 3–10 μM-barium had about the same effect on the I–V relation as did a maximal concentration of agonist (see Fig. 4; for details of barium action see Uchimura *et al.* 1989).

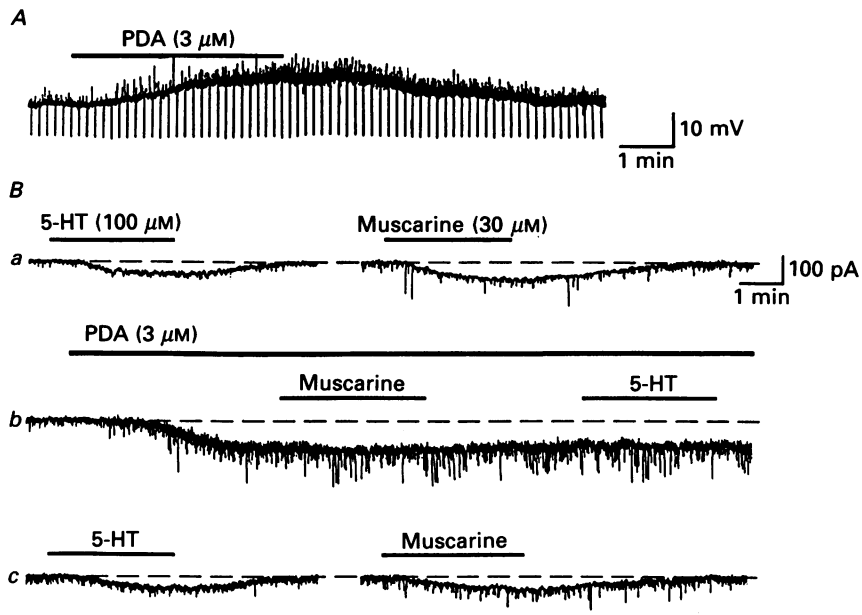

Fig. 7. Phorbol esters mimic and block the effects of muscarine and 5-HT. *A*, depolarization caused by 4β-phorbol-12,13-diacetate (PDA). Membrane potential −80 mV. Electrotonic potentials evoked by 200 pA current pulses of 500 ms. *B*, responses to 5-HT (100 μM) and muscarine (30 μM) before (*a*), during (*b*) and 30 min after perfusion (*c*) with 4β-phorbol-12,13-diacetate. Resting potential of cell in *B* was −83 mV; held at −75 mV.

Two findings suggest that 5-HT and phorbol esters reduced the same population of potassium channels that are affected by muscarine. First, the properties of the conductance are the same, and the maximal current caused by muscarine was not different from that previously reported for 5-HT (North & Uchimura, 1989); it was also the same as that caused by 10 μM-phorbol esters, which was the highest concentration tested. Second, muscarine caused no inward current when potassium channels were already closed by 5-HT or phorbol esters. Muscarine receptor activation is well known to stimulate the hydrolysis of phosphatidyl inositol in brain and other tissues (Fisher, Klinger & Agranoff, 1983; see Bonner, 1989) and one interpretation of the results is that activation of C kinase is an intermediate step in the pathway from muscarinic receptor to potassium channel: if this is true, then only a subset of inward rectifier potassium channels can be closed by C kinase (and hence by agonists using this pathway) whereas all inward rectifier channels can be closed by barium. However, it might be borne in mind that in frog sympathetic ganglia muscarinic agonists activate phosphatidyl inositol metabolism and reduce a potassium conductance but these two effects seem not be causally related (Pfaffinger, Leibowitz, Subers, Nathanson, Almers & Hille, 1988).

The functional role of ACh within the nucleus accumbens remains little understood.

Administration of dopamine into the nucleus accumbens, or more usually amphetamine which exerts its effects by releasing dopamine, stimulates locomotor activity (Kelly, Seviour & Iversen, 1975). Conversely, muscarinic agonists inhibit and muscarinic antagonists facilitate locomotor activity (Fibiger, Lytle & Campbell, 1970), leading to the generally accepted notion that the actions of dopamine in some way 'balance' those of ACh (see Lehmann & Langer, 1983). The predominant action of dopamine in the nucleus accumbens is inhibition (Woodruff, McCarty & Walker, 1976); this results from an increase in potassium conductance, consequent to activation of D_1 receptors (Uchimura, Higashi & Nishi, 1986; Higashi, Inanaga, Nishi & Uchimura, 1989). In the present study it was found that almost all cells are excited by muscarine; thus the 'balance' could arise from the directly opposing actions of the two transmitters on the same projection cells of the nucleus accumbens – dopamine to open potassium channels and ACh to close them. Agonists at D_1 dopamine receptors appear to exert their effects through an increase in cellular levels of cyclic adenosine 5′-monophosphate (Uchimura *et al.* 1986; Higashi *et al.* 1989) whereas the present results can be interpreted to suggest that muscarine acts through activation of C kinase. It will be of interest to determine whether the same population of potassium channels are the final targets for these two important striatal neurotransmitters.

This work was supported by grants from the US Department of Health and Human Services. (DA03160, DA03161 and MH40416).

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