Nicotinic excitation of rat ventral tegmental neurones *in vitro* studied by intracellular recording

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- 1 Intracellular recordings were made from presumed dopamine-containing neurones in the ventral tegmental area (VTA) in rat brain slices.
- 2 Nicotine (10–100 μ M) and acetylcholine (ACh) depolarized the neurones. The depolarization caused by ACh was typically biphasic; both components were increased by neostigmine (0.1–10 μ M), but only the slower component was blocked by scopolamine (1–10 μ M).
- 3 The nicotinic action of ACh, studied in the presence of neostigmine and scopolamine, persisted in the presence of tetrodotoxin (1 μ M) and cobalt (2–5 mM).
- 4 ACh or carbachol (30 μ M) caused inward currents in neurones voltage-clamped near the resting potential. These currents reversed polarity at around –4 mV, were blocked by hexamethonium (1–100 μ M) in a voltage-dependent manner, and showed desensitization with prolonged or repeated agonist applications.
- 5 Depolarizations caused by ACh and carbachol were reduced in slices pretreated with κ -bungarotoxin, but were not changed by α -bungarotoxin.
- 6 These responses to ACh and nicotine resemble those previously described on autonomic ganglion cells. The direct action on VTA neurones may contribute to the positive reinforcement associated with nicotine consumption.

Introduction

Acetylcholine (ACh) applied to presumed dopaminergic neurones during electrophysiological studies *in vivo* has been found to be either ineffective (Collingridge & Davies, 1981; Pinnock & Dray, 1982) or excitatory (Dray & Straughan, 1976; Lichtensteiger *et al.*, 1982; Scarnati *et al.*, 1986). Nicotine, applied systemically or by iontophoresis, excites neurones in both the substantia nigra (Lichtensteiger *et al.*, 1982; Clarke *et al.*, 1985) and ventral tegmental area (VTA) (Grenhoff *et al.*, 1986; Mereu *et al.*, 1987) of rats. Nicotine also increases dopamine concentrations in terminal regions of these cells, as do cocaine, amphetamine and morphine (DiChiara & Imperato, 1988). These findings are consistent with the idea that the excitation of VTA neurones by nicotine contributes to the reinforcement associated with tobacco

consumption (Grenhoff *et al.*, 1986; Mereu *et al.*, 1987).

Although autoradiographic studies suggest that [³H]-nicotine binding sites are on dopamine-containing neurones themselves (Clarke & Pert, 1985), electrophysiological experiments using extracellular recording *in vivo* are generally not able to resolve whether the nicotinic excitation is taking place on these cells or on other neurones afferent to them. Nor are those methods well suited to determine the type of nicotinic receptor involved. The purpose of the present experiments was to examine the actions of ACh and nicotine, by using intracellular recording from rat VTA neurones *in vitro*.

Methods

Intracellular recordings were made from cells in the VTA as previously described in detail by Lacey *et al.* (1987, 1989). In brief, coronal slices (300 μ m) of rat ventral mesencephalon were maintained in a chamber completely submerged in a continuously

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Figure 1 The effect of nicotine and acetylcholine (ACh) on ventral tegmental (VTA) neurones. (a) Nicotine (100 μM) depolarized the membrane and caused action potentials (full amplitude not shown). The open bar indicates the period during which the superfusing solution contained nicotine. Cell membrane was held at -57 mV (by passing -70 pA current) before the application of nicotine to prevent spontaneous action potentials; this current was constant throughout. (b) Nicotinic and muscarinic actions of ACh on a VTA neurone. ACh was applied from a blunt pipette (triangles) during spontaneous firing (no applied current, i and iii) and during passage of hyperpolarizing current to hold the membrane potential at -68 mV (ii and iv). (i) and (ii) Control. ACh caused a biphasic increase in firing rate (i) and, at -68 mV , a depolarization with early and late components (open arrows) (ii). (iii) and (iv) Muscarinic receptor antagonist scopolamine (3 μM) blocked the late but not the early component.

flowing (1.5 ml min^{-1}) solution containing (mM): NaCl 126, KCl 2.5, NaH_2PO_4 1.2, MgCl_2 1.3, CaCl_2 2.4, glucose 10 and NaHCO_3 26, saturated with 95% $\text{O}_2/5\%$ CO_2 and heated to 36°C . NaH_2PO_4 was omitted when solutions contained cobalt chloride (2–5 mM). Intracellular recordings were made from VTA neurones by means of glass microelectrodes, of resistance 40–80 M Ω , filled with either KCl or CsCl (2 M). Signals were amplified with an Axoclamp 2A single electrode voltage-clamp amplifier and displayed on a Gould 2400 chart recorder. Most voltage-clamp recordings were made with electrodes coated with Sylgard (Dow-Corning) to reduce their capacitance; headstage voltage was continuously monitored.

With the exception of ACh, drugs were applied by changing the superfusion solution to one containing known concentrations of drug; new solutions entered the recording chamber within 30 s. The drugs used were: nicotine sulphate, carbamylcholine chloride (carbachol), (–)-scopolamine hydrochloride, hexamethonium chloride (Sigma), tetrodotoxin (TTX) and neostigmine methylsulphate (Calbiochem). ACh (Sigma) was applied by ejecting a few nl of a 100 mM solution from the tip of a blunt pipette positioned beneath the surface of the superfusing solution and just above the tissue slice; a pressure of 50–70 kPa for 50–500 ms was typically used.

Experiments with α -bungarotoxin and κ -bungarotoxin were carried out as follows. Slices were kept for 2 h at 36°C in 3–6 ml of continuously gassed superfusion medium, sometimes with added α - (1 μM) or κ - (0.5 μM) bungarotoxin. They were then transferred to the recording chamber and intracellular recordings obtained. Lyophilized purified bungarotoxins were a gift from Dr V.A. Chiappinelli, St. Louis, U.S.A.

Numerical data are expressed as mean $\pm$ s.e.mean.

Results

Effects of nicotine and acetylcholine

Fifty-nine cells were studied; all had properties similar to those of principal dopamine-containing neurones in the adjacent substantia nigra zona compacta, including hyperpolarization by dopamine (Lacey *et al.*, 1987; 1989). Nicotine (10–100 μM) depolarized the neurones and increased their firing rate, or caused an inward current in cells held at the resting potential ($n = 5$) (Figures 1a and 2c).

ACh caused a depolarization and an increase in cell firing rate in 27 of 35 cells. This depolarization often comprised a transient phase lasting for a few seconds, followed by a slower component lasting

tens of seconds. The slower component was reduced or abolished by scopolamine ($1\text{--}10\text{ }\mu\text{M}$; Figure 1b), indicating that a late part of the response to ACh was mediated by a muscarinic receptor. The effect of ACh was enhanced by the acetylcholinesterase inhibitor neostigmine ($100\text{ nM--}10\text{ }\mu\text{M}$); in the presence of neostigmine, ACh depolarized all 26 cells tested. Neostigmine itself had no effect on membrane potential or membrane current ($n = 28$). All subsequent experiments with ACh and carbachol were carried out in the presence of scopolamine and neostigmine.

Responses to ACh or carbachol persisted in the presence of tetrodotoxin ($1\text{ }\mu\text{M}$; 10 cells; Figures 2c and 3) or cobalt ($2\text{--}5\text{ mM}$; 4 cells; Figure 3a). Either treatment blocked spontaneous action potentials and action potentials evoked by the agonists: therefore, we expected that they would block release of transmitter resulting from the excitation of any adjacent neurones, making it likely that the nicotinic effect was due to a direct action of the agonists on the impaled neurone.

ACh (in the presence of scopolamine) was also applied to seven neurones in the substantia nigra zona compacta; six were depolarized.

Desensitization

The nicotinic response desensitized markedly: the response amplitude declined during $1\text{--}2\text{ min}$ superfusion with nicotine and carbachol (Figures 1a, 2b and c). The desensitization was also apparent when ACh was applied; reproducible responses were obtained only when the interval between applications of ACh was at least 30 s, and sometimes 2 min or more (Figure 2a). The depolarizations (or inward currents) caused by carbachol ($30\text{ }\mu\text{M}$) and nicotine ($100\text{ }\mu\text{M}$) showed cross-desensitization with ACh (Figure 2b and c).

Reversal potential

The depolarization or inward current caused by carbachol or ACh became larger with membrane hyperpolarization (11 cells) (Figure 3). The response was studied at potentials less negative than the resting potential, after reducing outward potassium currents with intracellular caesium (2 M in the electrode, 2 cells) or with extracellular tetraethylammonium (30 mM) plus barium (1 mM ; 2 cells). TTX ($1\text{ }\mu\text{M}$) and cobalt ($2\text{--}5\text{ mM}$) were also present to block voltage-activated inward currents. Under these conditions the effect of ACh or carbachol reversed polarity at $-3.7 \pm 2.4\text{ mV}$ ($n = 4$).

Effects of antagonists

The response to ACh was reduced by hexamethonium ($1\text{--}100\text{ }\mu\text{M}$). The block by hexa-

Figure 2 Desensitization of nicotinic responses. (a) Depolarizations caused by acetylcholine (ACh) became smaller when the interval between applications was reduced to less than 1 min . Membrane potential held at -72 mV by constant current injection. ACh was applied from a blunt pipette (triangles). Traces are continuous from top to bottom. Carbachol ($30\text{ }\mu\text{M}$, solid bar) caused an inward current that partially desensitized during the application. Responses to ACh were completely occluded by carbachol. (c) A similar experiment on a different neurone. Currents in response to ACh were occluded by nicotine ($100\text{ }\mu\text{M}$, open bar), which itself caused an inward current that desensitized. The response to ACh recovered only partially 80 min after washing out nicotine. Tetrodotoxin (TTX, $1\text{ }\mu\text{M}$) was present in (c); scopolamine ($3\text{ }\mu\text{M}$) and neostigmine ($100\text{ nM--}3\text{ }\mu\text{M}$) were present in (a), (b) and (c).

methonium was greater at more negative potentials (6 cells; Figure 3b) and reversed on washing for $10\text{--}15\text{ min}$. (+)-Tubocurarine ($10\text{ }\mu\text{M}$; 4 cells) also reduced the response by about 50%.

We tested the effectiveness of ACh and carbachol (in the presence of scopolamine ($3\text{ }\mu\text{M}$) and neostigmine (100 nM)) to depolarize a further 37 VTA neurones in tissues pretreated with either α - or κ -bungarotoxin (or controls). Neurones were held at $-70 \pm 0.7\text{ mV}$ ($n = 37$) by passage of constant hyperpolarizing current. The maximum depolarization elicited by ACh was $6.7 \pm 1.3\text{ mV}$ (9 cells)

Figure 3 Voltage-dependence of the nicotinic current, and the inhibitory effect of hexamethonium. (a) Current/voltage relation before and after carbachol ($30 \mu\text{M}$). Membrane current recorded in response to slow ramp voltage command from -120 to 0 mV. Rectification was reduced by adding tetrodotoxin (TTX, $1 \mu\text{M}$), cobalt (2 mM), barium (1 mM), caesium (2 mM) and tetraethylammonium (30 mM). Scopolamine ($3 \mu\text{M}$) was also present. The inward current caused by carbachol became zero at about -10 mV. (b) Membrane currents evoked by acetylcholine (ACh) in a single cell at holding potentials indicated before (left column) and after (middle and right columns) adding hexamethonium (3 and $30 \mu\text{M}$). The effect of hexamethonium on the response to ACh is greater at more negative membrane potentials. Scopolamine ($3 \mu\text{M}$), neostigmine (100 nM) and TTX ($1 \mu\text{M}$) were present.

and by carbachol ($30 \mu\text{M}$) was 3.8 ± 0.5 mV (12 cells) in six slices incubated in control medium. In three slices preincubated with α -bungarotoxin ($1 \mu\text{M}$), ACh depolarized eight cells by 5.3 ± 1.1 mV and carbachol depolarized nine cells by 3.8 ± 0.7 mV. In four slices pretreated with κ -bungarotoxin ($0.5 \mu\text{M}$),

the ACh depolarization was only 2.5 ± 0.6 mV ($n = 14$) and the depolarization caused by carbachol was 1.4 ± 0.3 mV ($n = 14$). κ -Bungarotoxin reduced the nicotinic depolarizations by more than half ($P < 0.01$, Dunnett's t test modification) whereas α -bungarotoxin pretreatment had no effect ($P > 0.1$)

Discussion

The action of acetylcholine on VTA neurones resembles that at nicotinic receptors on other cells. Thus the value of the reversal potential (-4 mV, Figure 3a) is close to that estimated for the reversal of current flow through nicotinic receptor channels in many tissues (Takeuchi & Takeuchi, 1960; Nishi & Koketsu, 1960; Dennis *et al.*, 1971; Egan & North, 1986). Desensitization is also characteristic of nicotinic responses (Katz & Thesleff, 1957). Block by hexamethonium is typical of nicotinic actions in ganglia (Paton & Zaimis, 1949) and brain (Egan & North, 1986; McCormick & Prince, 1987): its voltage-dependence (Figure 3b) has previously been shown in ganglion cells (Rang, 1982). The finding of voltage-dependent block by hexamethonium, along with the insensitivity of the response to TTX and cobalt, indicate that the response was due to direct activation of nicotinic receptors on VTA neurones. The cross-desensitization with nicotine and carbachol (Figure 2b and c) suggests that all three agents act upon the same receptors on these cells.

κ -Bungarotoxin blocks nicotinic receptors on ganglion cells, but not at the neuromuscular junction, in contradistinction to α -bungarotoxin (Chiappinelli, 1983; Loring *et al.*, 1985; Loring & Zigmond, 1988). It also blocks the nicotinic receptor expressed in the membrane of oocytes injected with mRNA directing the synthesis of α_3 and α_4 agonist-binding nicotinic receptor subunits (Boulter *et al.*, 1987), but not α_2 (Wada *et al.*, 1988). *In situ* hybridization experiments have demonstrated the presence of mRNA encoding the synthesis of α_3 and α_4 (but not α_2 : Wada *et al.*, 1988) subunits in the VTA (Goldman *et al.*, 1986; 1987). Thus it may be that the nicotinic depolarization is mediated by receptors comprising either α_3 or α_4 subunits (or both). However, the failure to block completely the response with κ -bungarotoxin must necessarily temper this conclusion. It is possible that a mixed population of κ -bungarotoxin-sensitive and -insensitive receptors is present, or that in our experiments the binding of κ -bungarotoxin may have been incomplete at the time during which recordings were made.

The gratifying constituent of tobacco is nicotine, withdrawal from which can result in craving and physical distress (Johnston, 1949); it is therefore considered a drug of dependence (U.S. Surgeon

General's Report, 1988). Like other drugs of dependence, nicotine is rewarding; it is self-administered by animals (Goldberg *et al.*, 1981; Cox *et al.*, 1984) and by man (Henningfield *et al.*, 1983). Increased activity in the mesolimbic dopamine system, which arises predominantly from the ventral tegmental area (VTA), has been associated with a variety of rewarding behaviours including intracranial self-stimulation and self-administration of cocaine, amphetamine and heroin (Bozarth, 1983; Wise, 1984; Fibiger & Phillips, 1986). It appears likely that tobacco ingestion will activate nicotinic receptors of several different molecular species in the central and peripheral

nervous systems. Thus, in view of the evidence implicating the dopamine system arising from the VTA in behavioural reinforcement and the rewarding properties of addictive drugs, further characterization of the nicotinic receptor type(s) present on these neurones may have a significant impact in the search for novel therapeutic strategies to reduce the consumption of tobacco products.

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