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Uptake of [^{3}H]-nicotine and [^{3}H]-noradrenaline by cultured chromaffin cells

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1 Three day-old cultured bovine adrenal chromaffin cells incubated at room temperature with Krebs-HEPES solution containing different concentrations of [^{3}H]-nicotine, took up and retained increasing amounts of the drug by a mechanism that did not saturate.

2 Concentrations of cold nicotine as high as 100 μM did not alter the amount of [^{3}H]-nicotine retained by cells.

3 Imipramine, cocaine, tetracaine or mecamylamine, at concentrations (10 μM) that blocked the catecholamine secretory effects of nicotine completely, did not modify the uptake of [^{3}H]-nicotine.

4 Both imipramine and cocaine drastically inhibited [^{3}H]-noradrenaline uptake by cells in a concentration-dependent manner (IC_{50}s of 0.08 and 1 μM, respectively).

5 These data indicate that the secretory effects of nicotine are not coupled to its previous uptake into cells, and are evidence in favour of a site of action for nicotine located in or at the surface of the chromaffin cell membrane.

Introduction

Nicotine and related drugs evoke the release of noradrenaline from sympathetically innervated organs through the activation of nicotinic receptors (Lindmar, Löffelholz & Muscholl, 1968; Su & Bevan, 1970; Furchgott, Stainsland & Wakade, 1975; Kirpekar, García & Prat, 1980). Although it had been suggested that these receptors might be located at noradrenergic nerve terminals, only recently has direct evidence from our laboratory strongly suggested that this is the case (Alonso, Ceña, García, Kirpekar & Sánchez-García, 1982).

It seems obvious that at neuroeffector junctions such as the motor endplate, acetylcholine (ACh) acts on specific nicotinic cholinoceptors whose nicotinic binding sites seem to be accesible only from the outside of the cell (Castillo & Katz, 1955). However, there is considerable controversy on the localization of the site on the nerve terminal through which nicotine exerts its noradrenaline secretory effects. Su & Bevan (1970) found that cocaine, phenoxybenzamine and desipramine blocked the nicotine-induced noradrenaline release from sympathetic nerves as well as the uptake of [^{3}H]-nicotine into the adventitial layer of rabbit aorta (Bevan & Su, 1972). These authors suggested that nicotine needed to enter the nerve terminal in order to release the transmitter through the same axolemmal carrier system used by noradrenaline. However, Westfall & Brasted (1972) concluded that nicotine caused release of [^{3}H]-noradrenaline from perfused guinea-pig heart by stimulation of a nicotinic receptor located at the adrenergic nerve terminal and that the effect was independent of an intact noradrenaline uptake system.

[^{3}H]-nicotine is taken up into nerve tissue but also accumulates at extraneuronal sites (Bevan & Su, 1972; Nedergaard & Schrold, 1977). In order to solve this problem, it seems desirable to perform these studies in catecholaminergic systems free of effector cells. The adrenal medullary chromaffin cells maintained in monolayer primary cultures are a suitable system for such studies because (a) they derive embryologically from the neural crest, sharing a common origin with sympathetic neurones; (b) while in culture, they grow lateral branches and varicosities with cone-like structures (Bader, Ciesielski-Treska, Hesketh & Aunis, 1981); (c) they have many properties in common with noradrenergic neurones as far as uptake and secretory mechanisms are concerned, and (d) they are free of non-neural tissue.

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In this paper we have studied the characteristics of the accumulation of [^{3}H]-nicotine and [^{3}H]-noradrenaline by cultured bovine adrenal chromaffin cells and the effects of cocaine, imipramine, tetracaine and mecamylamine on such uptake processes. Since these four drugs potently inhibit the release of [^{3}H]-noradrenaline evoked by nicotine or acetylcholine from cultured bovine chromaffin cells (Ceña, Nicolás, Sánchez-García, Kirpekar & García, 1983), differences betweeen their effects on uptake and release processes will possibly allow the dissociation of the two mechanisms, as well as determining the location of the site of action of nicotine as a secretagogue. A preliminary account of some of these results has been given (Ceña, Nicolás, Sánchez-García & García, 1982).

Methods

Preparation of cells

Cells were prepared from bovine adrenal medulla according to the procedure of Fenwick, Fajdiga, Howe & Livett (1978) with some modifications (Ceña *et al.*, 1983). Cells were plated on uncoated plastic culture wells, 16 mm in diameter (5×10^5 cells per well) and maintained at 37°C in a humidified incubator (Heraeus) under an atmosphere of 5% CO_2:95% air. Dulbecco's modified Eagle's medium (DMEM) was changed 24 h later and every 2–3 days.

Experimental designs

Uptake of [^{3}H]-nicotine

Three day-old cells were taken from the incubator and maintained at room temperature (22 ± 2°C) during 1 h in 0.5 ml oxygenated Krebs-HEPES solution with continuous shaking; the medium was replaced at 10 min intervals. At the end of this pre-incubation period, increasing concentrations of (±)-[^{3}H]-nicotine (New England Nuclear; sp. act. 35 Ci mmol^{-1}) were added. Thirty minutes later, cells were washed 3 times with fresh solution, and finally collected in 0.5 ml of 10% trichloracetic acid. The extract was then added to a minivial containing 2.5 ml Instagel (Packard), counted in a liquid scintillation counter ISOCAP 300 and quenching corrected with an automatic external standard. [^{3}H]-nicotine taken up and retained by cells was expressed as d.p.m. per 5×10^5 cells.

To test the effects of drugs on [^{3}H]-nicotine uptake, cells were first incubated in the presence of 10 μM imipramine, cocaine, tetracaine or mecamylamine for 5 min and then, still in the presence of each drug, with [^{3}H]-nicotine (125 nM) as above.

Uptake of [^{3}H]-noradrenaline

The uptake of [^{3}H]-noradrenaline by cells was tested in an identical manner as that for [^{3}H]-nicotine, but here (±)-[^{3}H]-noradrenaline (Amersham, sp. act. 27 Ci mmol^{-1}) was used.

The effects of increasing concentrations (10^{-9}–10^{-5} M) of imipramine and cocaine on the uptake of [^{3}H]-noradrenaline were tested. The drugs were present 5 min before and during the 30 min period of incubation with [^{3}H]-noradrenaline. The assay of the radioactivity present in the cells was performed as with [^{3}H]-nicotine, and the results expressed as d.p.m. per 5×10^5 cells or as % of controls.

Results

Uptake of [^{3}H]-nicotine by cultured chromaffin cells

Three day-old cells incubated in the presence of different concentrations of [^{3}H]-nicotine (ranging from 4 to 125 nM) for 30 min at room temperature, retained increasing amounts of nicotine by a mechanism that did not saturate (Figure 1). In fact, in 8 experiments the amount of radioactivity retained by cells was directly proportional to the concentration of tritiated nicotine present during the incubation period. In one experiment, addition of up to 100 μM cold nicotine before the incubation with a fixed amount of [^{3}H]-nicotine (125 nM) did not alter the radioactivity retained by cells.

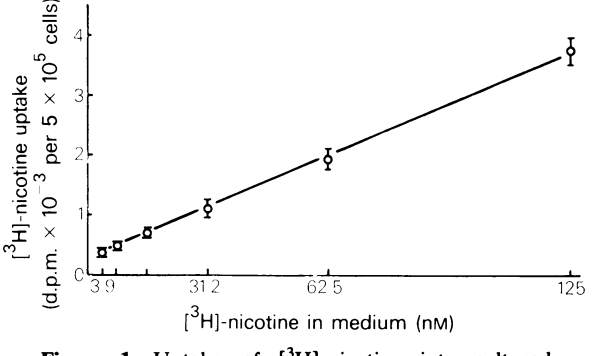

Figure 1 Uptake of [^{3}H]-nicotine into cultured chromaffin cells. Three day-old cells were incubated for 30 min at room temperature with 0.5 ml Krebs-HEPES solution containing (±)-[^{3}H]-nicotine at the concentrations shown on the abscissa scale. After a 10 min washout period, cells were collected and their radioactivity content measured (ordinate scale). Data are means of 8 different wells; vertical lines are s.e.mean.

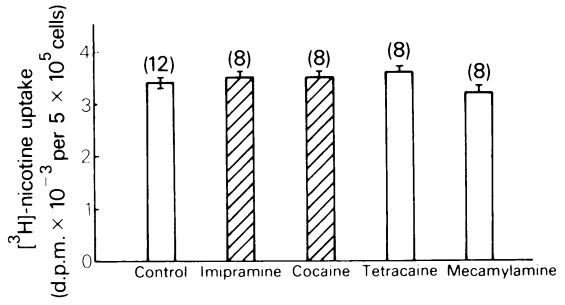

Figure 2 Effects of different drugs on the uptake of [^{3}H]-nicotine by cultured chromaffin cells. Control cells were incubated for 30 min in the presence of [^{3}H]-nicotine (125 nM). The other columns represent cells maintained in the presence of 10 μM imipramine, 10 μM cocaine, 10 μM tetracaine and 10 μM mecamylamine 5 min before and during incubation with [^{3}H]-nicotine. The radioactivity present in the cells (ordinate scale) is expressed as means of the number of wells shown in parentheses; vertical lines are s.e.mean.

Effects of different drugs on the uptake of [^{3}H]-nicotine

Although [^{3}H]-nicotine uptake did not show a saturation curve, it might take place through the carrier system used by noradrenaline in noradrenergic nerve terminals and demonstrated in culture bovine chromaffin cells by Kenigsberg & Trifaró (1980). We, therefore, tested whether drugs such as imipramine or cocaine, which are known to block the uptake of noradrenaline efficiently, altered the uptake of [^{3}H]-nicotine into cultured chromaffin cells.

Cells incubated in the presence of 125 nM [^{3}H]-nicotine for 30 min retained 3.427 ± 71 d.p.m. per 5×10^5 cells ($n = 12$). When imipramine (10 μM) or cocaine (10 μM) were present 5 min before and during the [^{3}H]-nicotine incubation period, the amounts of tritium present in the cells were identical to those found in control cells (Figure 2). In addition, neither the local anaesthetic agent, tetracaine (10 μM), nor the ganglionic blocking agent, mecamylamine (at 10 μM), affected the uptake of [^{3}H]-nicotine.

Uptake of [^{3}H]-noradrenaline by cultured chromaffin cells and its inhibition by cocaine and imipramine

When three day-old cells were incubated in the presence of [^{3}H]-noradrenaline (125 nM) for 30 min at room temperature, the accumulation of 74.089 ± 3.346 d.p.m. per 5×10^5 cells of radioactivity was recorded. This retention was drastically reduced in a dose-dependent manner when the cells were exposed to increasing concentrations of imipramine or cocaine 5 min before and during the [^{3}H]-noradrenaline incubation period (Figure 3). The IC_{50} for imipramine was 0.08 μM and that for cocaine 1 μM.

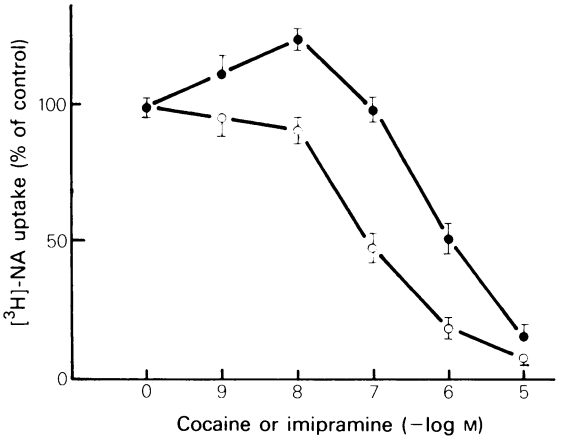

Figure 3 Uptake of [^{3}H]-noradrenaline ([^{3}H]-NA) by cultured chromaffin cells and its inhibition by cocaine (●) and imipramine (○). Three-day-old cells were incubated in the presence of 125 nM (±)-[^{3}H]-NA in Krebs-HEPES solution at room temperature for 30 min. After the washing period, cells were harvested in trichloroacetic acid to measure their radioactivity content. When used, cocaine or imipramine was present 5 min before and during the 30 min of incubation with [^{3}H]-NA at the concentrations shown on the abscissa scale. The uptake of [^{3}H]-NA is expressed as % of the amount retained by cells in the absence of drugs (control = 100%; ordinate scale). Values are means of 4 experiments; vertical lines are s.e.mean.

Discussion

In a previous paper (Ceña *et al.*, 1983), we have shown that cocaine and imipramine strongly inhibit the release of [^{3}H]-noradrenaline from cultured bovine chromaffin cells normally evoked by nicotine. Since these drugs are potent inhibitors of the uptake of noradrenaline into sympathetic nerve terminals (Iversen, 1965), it seems plausible that the secretory effects of nicotine might depend upon a functional noradrenaline uptake system, through which nicotine must first be taken up into the chromaffin cell as a pre-requisite to releasing noradrenaline.

Uptake of [^{3}H]-nicotine into chromaffin cells

Being a weak base which at pH 7.4 is as much as 40% in the non-ionized form (Barlow & Hamilton, 1962), it is not surprising that [^{3}H]-nicotine penetrates cells not only through the noradrenaline uptake system, but also by passive diffusion. That the second

mechanism is the main route by which nicotine enters the cell is suggested in the present study by two facts: (a) the uptake of [^{3}H]-nicotine did not saturate and was unaffected in the presence of concentrations of cold nicotine as high as 100 μM; and (b) neither cocaine nor imipramine altered the amounts of [^{3}H]-nicotine retained by the cells, in spite of the fact that they both inhibited completely the uptake of [^{3}H]-noradrenaline by these cells.

Westfall & Brasted (1972) observed that [^{14}C]-nicotine accumulated in the isolated perfused heart of the guinea-pig by a non-saturating mechanism. However, pretreatment of the animals with 6-hydroxydopamine did not alter the uptake of labelled nicotine by the heart, nor did nicotine modify the uptake of [^{3}H]-noradrenaline. Nedergaard & Schrold (1977) also demonstrated that the uptake of [^{3}H]-nicotine into rabbit aorta was concentration-independent, suggesting that the drug is penetrating the cell membrane by a non-specific process. These latter experiments however had the limitations that [^{3}H]-nicotine was being taken up by neural as well as extraneuronal tissues. In pure catecholaminergic cultured cells of the experiments described here, however, nicotine uptake seems to follow a pattern similar to that seen in sympathetically innervated tissues.

The site of action of nicotine as a secretagogue

At noradrenergic nerve terminals it seems that nicotine releases noradrenaline through the activation of nicotinic cholinoceptors (Lindmar *et al.*, 1968; Kirpekar *et al.*, 1980) and direct evidence for the neural location of such receptors has been recently provided using neurosomes (ligated sympathetic nerve trunks as models of noradrenergic neurones free of effector cells; Alonso *et al.*, 1982). In view of the fact that cocaine, phenoxybenzamine and desipramine blocked nicotine-induced noradrenaline release from sympathetic nerve terminals as well as [^{3}H]-nicotine uptake into the adventitial layer of rabbit aorta, it was initially suggested that to evoke the release of the transmitter, nicotine needed to enter the nerve terminal through the axolemmal carrier system for noradrenaline (Su & Bevan, 1970; Bevan & Su, 1972).

That this is not the case in cultured chromaffin cells is indicated by the fact that in the present experiments there is a clear cut dissociation between the potent inhibition of nicotine-evoked noradrenaline release caused by imipramine and cocaine, and the lack of effect of these two drugs on the uptake of nicotine by the cells. In addition, neither the ganglionic blocking drug, mecamylamine, nor the local anaesthetic, tetracaine, which potently inhibit nicotine-evoked catecholamine release (Ceña *et al.*, 1983), affected [^{3}H]-nicotine uptake by cells.

A second argument which favours the concept of two independent mechanisms is the fact that the IC_{50} for the inhibition by imipramine of [^{3}H]-noradrenaline release evoked by nicotine was 2 μM, while the IC_{50} for inhibition of [^{3}H]-noradrenaline uptake was only 0.08 μM, 25 times lower. If the release of the amine evoked by nicotine were associated with its previous uptake inside the chromaffin cell through the plasma membrane carrier, then a closer relationship between the IC_{50}s for both parameters would be expected.

Finally, additional evidence favouring an action of nicotine on surface receptors comes from the fact that cocaine or imipramine block equally ACh- or nicotine-evoked release (Ceña *et al.*, 1983), in spite of the strong pharmacokinetic differences between the two secretagogues. While nicotine is mostly in its non-ionized form, ACh is a highly polar compound with a quaternary ammonium group; therefore, it should not be taken up by cells. Once more, the lack of parallelism between the kinetic properties of both secretagogues and the equal blockade of their secretory effects by cocaine and imipramine speak against an intracellular site of action for nicotine.

In conclusion, we have shown that the characteristics of [^{3}H]-nicotine uptake into cultured bovine adrenal chromaffin cells clearly differs from the uptake of [^{3}H]-noradrenaline. In addition, there is a dissociation between the blockade by drugs of [^{3}H]-noradrenaline uptake into and its release from cells, indicating that the secretory effects of nicotine are not coupled to its previous uptake by cells. These data are in favour of a site of action for the secretory effects of nicotine locate in or at the surface of the chromaffin cell membrane.

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