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Nicotine washout rates from isolated rat ganglia in relation to recovery from nicotine depolarization

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Summary

1. Isolated rat superior cervical ganglia recovered more slowly from the depolarizing action of nicotine than from that of carbachol or acetylcholine. This was due to sustained high nicotine concentrations in the vicinity of the receptors, since recovery was hastened by adding hexamethonium to the washout fluid.

2. Ganglia incubated for 4 min in 80 μM ^{3}H-nicotine accumulated nicotine to a level exceeding the extracellular space, as judged from the uptake of ^{3}H-mannitol.

3. The subsequent efflux of ^{3}H-nicotine into non-radioactive solution could be largely resolved into two exponential components, with rate constants of $0{\cdot}55 \pm 0{\cdot}04$ and $0{\cdot}094 \pm 0{\cdot}007$ min^{-1}. The former was similar to that for total mannitol efflux, and so might be largely ascribed to clearance of extracellular nicotine. The slower efflux might be due to clearance from intracellular compartments. Nicotine efflux rates were not affected by hexamethonium indicating that receptor-activation did not modify the slow efflux.

4. Efflux of choline compounds (^{3}H-acetylcholine, ^{3}H-choline and ^{3}H-carbachol) showed an additional, very slow component (rate constant 0·001 to 0·002 min^{-1}).

5. It was suggested that slow efflux of intracellular nicotine might sustain depolarization on washing by maintaining high perineuronal concentrations of nicotine. With choline compounds the efflux rate from such sources may be too slow to affect perineuronal concentrations.

Introduction

Isolated sympathetic ganglia recover more slowly from the depolarizing action of nicotine than from that of acetylcholine or carbachol when the bath fluid is changed (Brown, Brownstein & Scholfield, 1972). This suggests that nicotine might be washed out of the ganglion rather slowly, perhaps because of intracellular accumulation (cf. Brown, Halliwell & Scholfield, 1971). In the present experiments we have tried to obtain further information on the rate of nicotine clearance, firstly by observing the effect of hexamethonium on recovery from nicotine depolarization, and secondly by measuring the rate of washout of radioactively labelled nicotine from isolated ganglia.

Methods

Superior cervical sympathetic ganglia with attached pre- and postganglionic nerve

trunks were isolated from rats of approximately 200 g weight anaesthetized with urethane (1·5 g/kg, i.p.). The connective tissue sheaths were removed from ganglia and nerve trunks and the ganglia were maintained in Krebs solution at room temperature (22°–27° C) bubbled with a mixture of 95% oxygen and 5% carbon dioxide.

Nicotine-depolarization was recorded as previously described (Brown *et al.*, 1972). To observe the efflux of nicotine, the ganglion preparation was soaked for 4 min in Krebs solution containing ^{3}H-nicotine (usually 20 μCi/ml, 80 μM) blotted briefly to remove some of the adhering radioactivity, and then immersed for successive periods of 1 to 5 min in a series of vials containing 0·5 or 1 ml of non-radioactive Krebs solution previously bubbled with the oxygenating gas mixture and agitated gently. (Initially the ganglion was agitated by hand in the collecting vials, but this did not make an obvious difference to the rate at which the radioactivity was leached out and so was discontinued.) Washout was followed for 30 to 60 min then the ganglion was blotted and weighed, and the radioactivity in the vials and in the ganglion measured as described previously (Brown *et al.*, 1971). To allow for the radioactivity in the suspending thread (unspun ligature silk), a corresponding length of the thread was carried through the same procedure of soaking and washing, and a correction applied. The same method was used to measure the efflux of other labelled substances.

Radioactive compounds were obtained from the Radiochemical Centre. Those used were (with specific activities): ^{3}H-nicotine, 260 mCi/mmol ; ^{3}H-l-D-mannitol, 500 mCi/mmol ; ^{3}H-acetylcholine, 500 mCi/mmol ; ^{3}H-carbachol, 500 mCi/mmol ; ^{3}H-choline, 15 Ci/mmol. The compounds were added to the solution in the same molar concentration as that of nicotine, by diluting if necessary with unlabelled material. The radiochemical purities of ^{3}H-nicotine and ^{3}H-mannitol were checked as described previously (Brown *et al.*, 1971), and were as stated therein. Purities of the labelled choline compounds were determined by paper electrophoresis (Potter & Murphy, 1967). With ^{3}H-carbachol and ^{3}H-choline, more than 99% of the label was associated with unchanged parent compound ; ^{3}H-acetylcholine, checked after incubation, contained 8 to 15% ^{3}H-choline. Tritium in ganglia incubated in ^{3}H-acetylcholine was extracted in 1 ml of 0·3 N HCl and freeze-dried, and the residues dissolved in 0·2 ml ethanol and subjected to paper electrophoresis in the same manner.

Results

Recovery from nicotine-depolarization

When acetylcholine or carbachol is washed out of an isolated rat ganglion the depolarization produced by the drug rapidly reverses to a hyperpolarization. This after-hyperpolarization is due to the electrogenic extrusion of accumulated Na^+ ions from the ganglion cells (Brown, Brownstein & Scholfield, 1969, 1972 ; Kosterlitz, Lees & Wallis, 1968, 1970). The hyperpolarization produced by the electrogenic Na^+ current is dependent upon the membrane conductance. This is rather high immediately after washing out the carbachol, because sufficient carbachol remains in the interstitial spaces to activate the receptors, but declines rapidly thereafter. Consequently the electrogenic hyperpolarization is somewhat attenuated on washing with normal Krebs solution, and is increased when hexamethonium is

added to the washout fluid to inhibit receptor stimulation (Brown *et al.*, 1972). Thus, the effect of hexamethonium on the after-hyperpolarization provides some indication of the rate of decline of receptor-activation on washing.

When nicotine is washed out with hexamethonium solution an after-hyperpolarization is observed of similar magnitude and time-course to that seen after carbachol. However, in the absence of hexamethonium, no after-hyperpolarization occurs (Fig. 1a). It would seem from this that, when washing with normal Krebs solution, enough nicotine remains in the vicinity of the receptors to increase the membrane conductance sufficiently to annul completely the electrogenic effect of Na^+ extrusion. This persistent effect can also be seen from Fig. 1b, where hexamethonium was added to the washout fluid for only a short time ; on washing out the hexamethonium, the hyperpolarization reversed to a depolarization before regaining isopotential, indicating the presence of residual nicotine after 10 min washing. A corollary to this is that hexamethonium washes out of the ganglion much more rapidly than nicotine. Thus, when hexamethonium was added to the bath fluid before nicotine and the two compounds were washed out together, the small depolarization produced by nicotine in the presence of hexamethonium increased on washing.

The threshold concentration of nicotine required to depolarize the ganglion is about 0·3 μM (Brown *et al.*, 1971) i.e., about one-hundredth of the concentration applied in Figure 1. It would appear from these experiments that the concentration of nicotine in the vicinity of the receptors remained considerably above this level for at least 15 min after washing. Since the membrane potential in the absence of such residual receptor-stimulation would be dependent upon at least two factors, the electrogenic Na^+ current and the concentrations of Na^+ and K^+

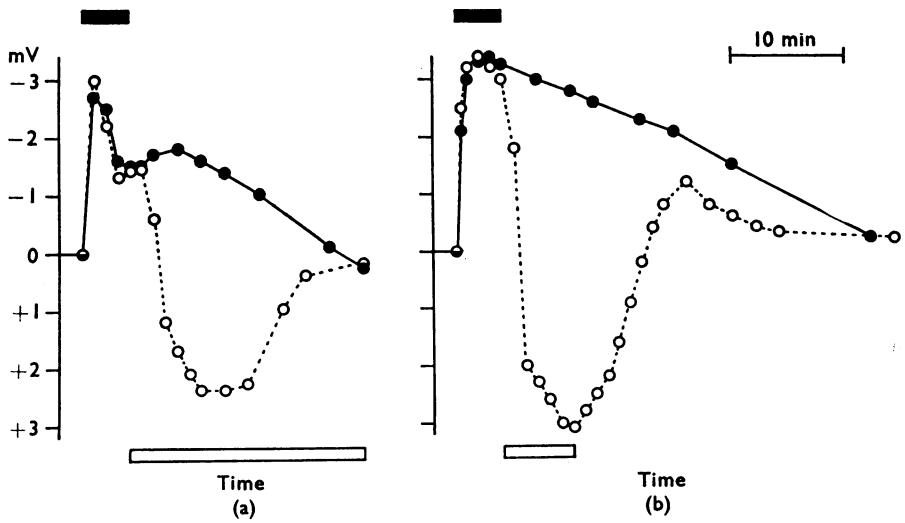

FIG. 1. Effect of adding hexamethonium (2·5 mM) to the washout fluid on the recovery of isolated rat ganglia from the depolarization produced by 60 μM nicotine. Records show changes in ganglionic surface potential (mV, ganglionic negativity upwards) with time (scale: 10 min). Nicotine was added to the bath for 4 min (filled bar above) and then washed out with normal Krebs solution (●——●) or with Krebs solution containing hexamethonium (○- - -○). Hexamethonium was in the bath for 20 min in (a), and for 6 min in (b) (duration indicated by open bars below).

in the cell, it is extremely difficult to calculate the time-course of nicotine washout from the potential recovery curves alone. For this reason, the washout of labelled nicotine was measured directly.

Efflux of ^{3}H-nicotine

An average curve for the efflux of ^{3}H-nicotine from 6 isolated ganglia soaked for 4 min in 80 μM ^{3}H-nicotine is shown in Fig. 2; numerical data for the individual efflux rates are given in Table 1. The efflux curve could be largely described as the sum of two exponential components—a fast efflux component (dashed line in Fig. 2) with an average rate constant (k_1) of $0{\cdot}55 \pm 0{\cdot}035$ min^{-1}, and slower component (solid line in Fig. 2) with rate constant (k_2) of $0{\cdot}094 \pm 0{\cdot}007$ min^{-1}. These two components accounted for 85–90% of the nicotine accumulated. Most of the remaining nicotine was that washed out in the first min, and was probably

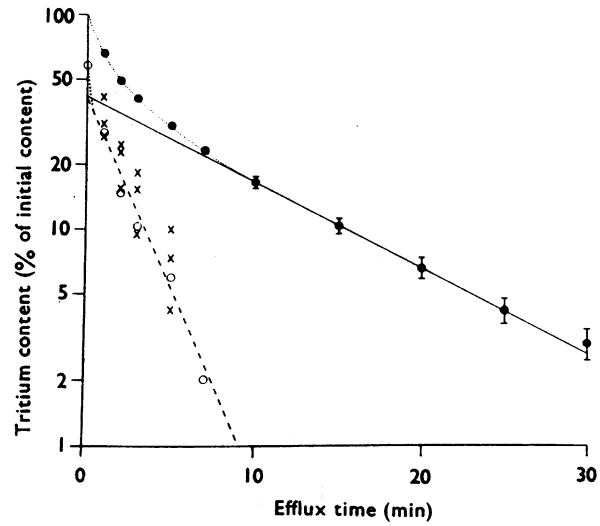

FIG. 2. Efflux of radioactivity from isolated rat ganglia previously soaked for 4 min in ~80 μM ^{3}H-nicotine and then rinsed in non-radioactive Krebs solution. Ordinates: ganglion content of tritium, expressed as % of pre-rinse content, and calculated from summed efflux counts. Abscissae: time after loading. Each point (○) gives the mean of 6 determinations on separate ganglia. The extrapolated line (mean rate constant 0·09 min^{-1}, Table 1) was subtracted from the experimental curve to yield the fast efflux component indicated ○- - -○ (mean rate constant 0·55 min^{-1}). The crosses (×) show individual measurements of ^{3}H-mannitol content in 3 ganglia after 4 min loading in 100 μM ^{3}H-mannitol and subsequent rinsing.

TABLE 1. *Efflux rates for ^{3}H-nicotine* (T/M = *tissue/medium concentration ratio;* k = *rate constant*)

Expt.	Initial	Fast efflux component (Phase 1)			Slow efflux component (Phase 2)		
	T/M	% content*	T/M_1*	k_1	% content	T/M_2*	k_2
1	2·32	35	0·79	0·63	54	1·25	0·092
2	2·01	44	0·89	1·50	46	0·92	0·119
3	1·57	48	0·76	0·63	46	0·72	0·098
4	1·93	49	0·97	0·63	45	0·89	0·075
5	1·66	38	0·63	0·50	40	0·67	0·074
6	1·27	41	0·53	0·43	38	0·48	0·104
mean	1·96	42·5	0·76	0·55	44·8	0·82	0·094
±S.E.	±0·13	2·26	0·066	0·035	2·29	0·11	0·007

*By extrapolation to zero time.

adsorbed on the surface of the preparation. Less than 1% of the initial radioactivity remained in the ganglion after 40 min washing and less than 0·5% after 60 minutes. Efflux rates at such times could not be measured at all accurately because the count rate in the collecting fluid was too low.

The ^{3}H-nicotine accumulated in the ganglion during the 4 min incubation period was estimated by adding the radioactivity released to that remaining in the ganglion after completing the efflux measurements. The ganglion nicotine concentration at the end of the incubation was 1·3–2·3 times that in the incubation medium (mean tissue: medium concentration ratio, T/M, $1{\cdot}96 \pm 0{\cdot}13$). Thus, an accumulation of nicotine occurred in the ganglion against a concentration gradient, as described previously (Brown *et al.*, 1971). The fast and slow efflux components extrapolated to mean initial tissue: medium ratios of $0{\cdot}76 \pm 0{\cdot}11$ and $0{\cdot}82 \pm 0{\cdot}11$ respectively.

Effect of hexamethonium. Addition of 2·5 mM hexamethonium did not alter the rate of efflux of ^{3}H-nicotine (Fig. 3).

Efflux of ^{3}H-mannitol

Efflux of radioactivity following 4 min incubation in ^{3}H-mannitol (50 μCi/ml, 100 μM) was measured in 3 ganglia. Such measurements were not very accurate, because the efflux was so rapid that the count rate in the collecting vials fell below detectable limits within 5–7 minutes. Also the tissue: medium ratios were low (see below) so errors due to surface absorption were correspondingly greater than those obtaining with nicotine. Measurements over the first 5 min of washing indicated

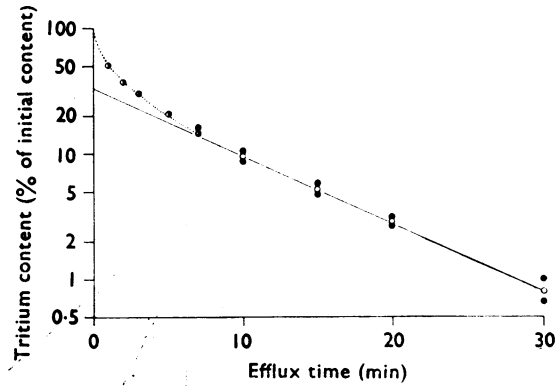

FIG. 3. Effect of hexamethonium (2·5 mM) on the efflux of ^{3}H-nicotine from an isolated rat ganglion expressed as in Fig. 2. The ganglion was soaked repeatedly in 80 μM ^{3}H-nicotine solution for 4 min at intervals of 90 min, and efflux of radioactivity into normal Krebs solution (●) or Krebs solution containing hexamethonium (○), measured in the following sequence: normal Krebs—hexamethonium—normal Krebs.

TABLE 2. *Efflux rates for ^{3}H-acetylcholine, ^{3}H-choline and ^{3}H-carbachol*

Compound	Expt.	Initial	Fast efflux component (a)			Slow efflux component (b)		
		T/M	% content	T/M_a	k_a	% content	T/M_b	k_b
^{3}H-acetylcholine	1	0·74	25	0·18	0·122	27	0·19	0·0022
	2	0·71	13	0·09	0·174	21	0·15	0·0013
	3	1·29	13	0·17	0·145	27	0·35	0·0017
^{3}H-choline	1	0·98	26	0·25	0·184	54	0·52	0·0023
	2	1·13	17	0·18	0·248	67	0·75	0·0025
^{3}H-carbachol	1	1·08	37	0·37	0·128	33	0·34	0·0023
	2	1·44	36	0·52	0·090	52	0·75	0·0025
	3	1·51	45	0·68	0·110	48	0·72	0·0012

that the rate of washout of mannitol was quite similar to that of the fast component of nicotine efflux (see Fig. 2). Cumulative counts gave initial tissue:medium ratios of 0·43 to 0·75 of which an uncertain but probably substantial fraction would be surface adsorption.

Efflux of choline compounds

Washout curves for radioactivity were obtained following 4 min loading in 100 μM solutions of ^{3}H-carbachol (3 experiments), ^{3}H-acetylcholine in the presence of 10 μM physostigmine (3 experiments) and ^{3}H-choline (2 experiments) (Table 2). In some of these experiments a direct comparison with nicotine efflux was obtained by first measuring nicotine efflux and then 90 min later (when efflux of nicotine was complete) reloading the ganglion with the choline compound (Fig. 4).

Washout curves for these choline compounds were similar to each other but collectively differed from that for nicotine through the appearance of a very slow phase of efflux (solid line in Fig. 4) with a rate constant of 0·001–0·002 min^{-1}, i.e., 50–100 times slower than the slowest phase of nicotine efflux. This slow efflux applied to a substantial proportion of the accumulated material (up to 65%), so that a considerable amount of radioactivity remained in the ganglion after 60 min washing. By subtracting this slow efflux phase from the overall washout curve, the more rapidly washed-out material could be resolved into at least two further efflux components—an exponential component of rate constant 0·1–0·2 (i.e., slightly faster than the slower phase of nicotine efflux), accounting for 13–45% of the initial radioactivity (open triangles in Fig. 4); and a very fast component

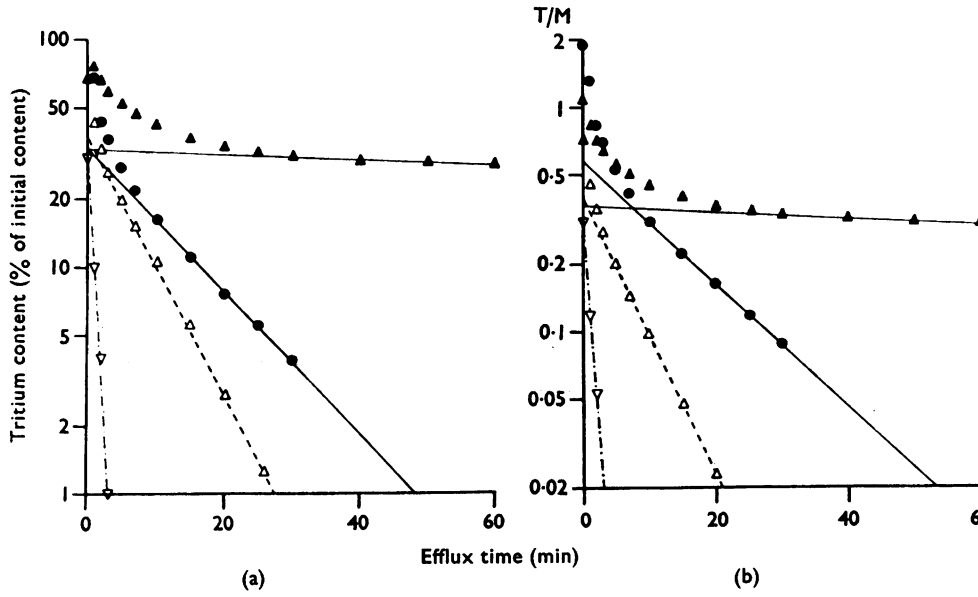

FIG. 4. Comparative efflux rates of ^{3}H-nicotine (●) and ^{3}H-carbachol (▲) measured sequentially in a single ganglion after immersion in 100 μM solutions of (first) ^{3}H-nicotine and (90 min later) ^{3}H-carbachol. Efflux is expressed (a) as % of total ganglionic content, as in Fig. 2, and (b) as the decline in the initial tissue:medium concentration ratio (T/M). △– – –△, Carbachol content calculated after substracting the expolated show exponential component (solid line) from the total content. ▽–·–·–▽, Results of a second subtraction of the extrapolated exponential component of the curve △– – –△.

of indeterminate kinetics but essentially complete within 2–3 min washing and accounting for up to half the total radioactivity in the ganglion (inverted triangles in Fig. 4). Total tissue uptake generally exceeded that of mannitol, the initial tissue : medium concentration ratios lying between 0·7 and 1·5. Attempts to determine the proportions of ^{3}H-acetylcholine and ^{3}H-choline in ganglia incubated in ^{3}H-acetylcholine solution did not yield very consistent results, but suggested that up to 20–30% of the acetylcholine was hydrolyzed to choline ; there seemed to be no differential release of the two during washing.

Discussion

The effect of hexamethonium on the recovery of the ganglion from nicotine-depolarization suggested that the concentration of nicotine in the vicinity of the receptors remained above 1% of that originally applied for at least 15 minutes.

A correspondingly slow-clearing fraction of nicotine was detected when the rate at which labelled nicotine was washed out of the ganglion was measured. About half of the nicotine accumulated after 4 min cleared with a half-time of 7·7 min, giving a rate constant (0·09 min^{-1}) some six times slower than that expected for clearance from extracellular space (as judged from the washout of labelled mannitol). This may most reasonably be attributed to the exit of nicotine accumulated intracellularly. Such intracellular accumulation has been demonstrated previously in ganglia (Appelgren, Hansson & Schmiterlöw, 1963 ; Brown, Hoffmann & Roth, 1969 ; Brown *et al.*, 1971), and the high ganglionic concentration observed in the present experiments (up to 2·3 times that in the bath fluid) accord with this. A component with a very similar rate constant has been noted in nicotine washout curves from skeletal muscle and salivary glands, and likewise attributed to clearance of intracellular nicotine (Weiss, 1968 ; Putney & Borzelleca, 1971). Receptor activation did not affect the washout of the slow-clearing fraction, since its rate constant was not altered by adding hexamethonium to the washing fluid.

Retention of nicotine inside cells would not itself cause depolarization. However, on diffusing out of the cells, it might sustain the perineuronal concentration at a sufficient level to stimulate the receptors. This might be analogous to the outward diffusion of K^+ ions from nerves or ganglia placed in K^+-free solution, which may be sufficiently rapid to partly activate the Na^+-pump externally (Hodgkin & Keynes, 1955 ; Brown *et al.*, 1972).

Since the ganglion recovers more rapidly from the depolarizing actions of acetylcholine or carbachol, these agents would be expected to show a more rapid washout than nicotine. This was only true in part, for a substantial fraction of accumulated choline or choline esters washed out even more slowly than nicotine (rate constant 0·001–0·002 min^{-1}). If due to intracellular retention, it might be that its outward movement was too slow to materially affect the perineuronal concentration.

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