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Effect of chemical destruction of adrenergic neurones on some cholinergic mechanisms in adult rat sympathetic ganglia

B. Collier, G. Johnson, M. Quik & S. Welner

Department of Pharmacology, McGill University, 3655 Drummond Street, Montreal, P.Q., H3G 1Y6, Canada

1 Rats were treated for 2–6 weeks with guanethidine after which their superior cervical ganglia were removed.

2 Ganglionic tyrosine hydroxylase and α-bungarotoxin binding sites were reduced by the guanethidine treatment indicating adrenergic cell body destruction.

3 Choline acetyltransferase activity and acetylcholine content of ganglia were not clearly changed by the guanethidine treatment, indicating that the drug does not destroy presynaptic terminals and that these presynaptic indicators do not adapt markedly to postsynaptic loss.

4 The cholinesterase in the ganglia was reduced by guanethidine treatment, but such ganglia retained their ability to accumulate surplus acetylcholine when they were incubated with physostigmine. This is interpreted as indicating surplus acetylcholine accumulation is a presynaptic phenomenon.

5 Choline uptake by resting ganglia was not reduced as a result of guanethidine treatment nor was it affected by preganglionic denervation. This is interpreted as indicating that during rest, choline uptake is into supporting cells or intraganglionic cells rather than cholinergic nerve terminals or adrenergic cell bodies.

Introduction

The chronic administration of guanethidine to adult rats causes destruction of adrenergic cell bodies in sympathetic ganglia (Jensen-Holm & Juul, 1970; 1971; Burnstock *et al.*, 1971; Erankö & Erankö, 1971; Johnson & O'Brien, 1976: Bittiger, *et al.*, 1977). These studies provided morphological evidence for this destruction caused by guanethidine and, in some cases, biochemical measures showing loss of tyrosine hydroxylase, but they provided little, if any, information about the integrity of cholinergic nerve terminals in ganglia of the guanethidine-treated animals. If guanethidine is administered to new-born rats, cholinergic nerve terminals appear deficient up to 10 weeks later (Johnson *et al.*, 1977). But this might indicate failure of presynaptic development rather than being a direct consequence of guanethidine on presynaptic function, because the development of sympathetic ganglia continues after birth, and during this time, the establishment of cholinergic synapses depends upon the presence of target cells (Black *et al.*, 1972). However, it is not known whether the target cell plays any role in the maintenance of presynaptic function in the adult animal.

The objective of the present study was to provide some information about cholinergic mechanisms in superior cervical ganglia of adult rats treated with guanethidine.

Methods

The animals were male Sprague-Dawley rats which weighed 150–200 g at the start of treatment. They were injected (s.c.) with guanethidine (40 mg kg^{-1}) or with an equivalent volume of saline on the schedule described by Johnson & O'Brien (1976): i.e. 5 days treatment and 2 days rest each week. The rats were dosed for 2–6 weeks, after which treatment was withheld for 2 days, and then the animals were killed and their superior cervical ganglia were removed. All rats survived treatment, and there was no obvious untoward effects of the guanethidine treatment other than a small deficit in body weight gain,

an effect also noticed by others (e.g., Jensen-Holm & Juul, 1970; Johnson & O'Brien, 1976).

Enzyme assays

Tyrosine hydroxylase was measured as nmol DOPA formed from tyrosine by the method of Nagatsu *et al.*, (1964) with minor modifications. For this, the ganglia were homogenized in 350 μl of 5 mM Tris HCl (pH 7.4) containing 0.1% Triton X-100. The incubation mixture (200 μl), contained: 120 μl tissue homogenate; 40 μl 0.5 M Tris acetate (pH 6.2) containing 500 units catalase and 200 nmol brocresine; L-tyrosine-3, 5-[^{3}H] (final concentration 10 μM); and 6,7-dimethyl-5, 6, 7, 8-tetrahydropteridine HCl (final concentration 100 μM) dissolved in 25 mM ascorbic acid. The product (^{3}HOH) was separated from the precursor by chromatography on a Dowex 50WX-8 column.

Choline acetyltransferase was measured as nmol ACh formed from choline and acetyl-CoA by a method similar to that of Fonnum (1969). For this, ganglia were homogenized in 200 μl of 40 mM phosphate buffer (pH 7.4) containing 200 mM NaCl, 10 μM $MgCl_2$ and 0.5% Triton X-100; 30 μl of the homogenate was incubated with choline (12 mM), [^{14}C]-acetyl-CoA (0.25 mM) and physostigmine (0.2 mM); [^{14}C]-ACh was recovered by extraction into heptanone containing tetraphenylboron (25 mg ml^{-1}).

Cholinesterase activity was measured as μmol substrate hydrolysed by the method of Ellman *et al.*, (1961). For this, ganglia were homogenized in 200 μl phosphate buffer (0.1 M, pH 8.0); 75 μl of the homogenate was incubated with acetylthiocholine (0.5 mM) and dithiobisnitrobenzoate (0.32 mM) and the increase in coloured product formed was measured with a spectrophotometer. Esterase sensitive to inhibition by physostigmine (1.5×10^{-5} M) was measured because this parameter was used as the basis for studying physostigmine-induced alteration of ACh content.

α-Bungarotoxin binding

Binding of [^{125}I]-α-bungarotoxin to intact desheathed ganglia was carried out essentially as described by Brown & Fumagalli (1977) and Fumagalli & DeRenzis (1980). Ganglia were placed in 80 μl Krebs containing bovine serum albumin 0.1 mg ml^{-1} in the absence or presence of (+)-tubocurarine (final concentration 10^{-3} M). They were preincubated for 30 min at 22°C and subsequently incubated with 20 nM [^{125}I]-α-bungarotoxin for 1 h at 22°C; these parameters were found to be saturating for [^{125}I]-α-bungarotoxin binding to the ganglia. At the end of this time, the ganglia were washed in 4 changes of 10 ml Krebs containing albumin over a 2 h period. The ganglia were blotted dry and their radioactivity was determined. The non-specific binding (presence of tubocurarine) was 20% of the total binding.

Determination of acetylcholine content

The method used was based on the procedure of Goldberg & McCamen (1973). Ganglia were extracted with 1.0 ml of trichloroacetic acid (10%), the acid was removed by ether and 200 μl of the aqueous phase used for the determination of ACh (for details see Kwok & Collier, 1982). In this procedure, the blank value for each sample was the [^{32}P]-phosphorylcholine generated by an aliquot that was first treated with acetylcholinesterase to destroy its ACh.

In the experiments that measured surplus ACh in ganglia, the tissues were removed, desheathed, and incubated for 90 min (37°C) in Krebs solution with or without physostigmine (1.5×10^{-5} M); this medium always contained choline (3×10^{-5} M).

Measurement of choline uptake

Ganglia were desheathed and incubated (37°C or 4°C) for 5 min in Krebs solution containing [^{3}H]-choline. After incubation, tissues were rinsed, digested with Protosol and tissue radioactivity determined; uptake was calculated as the difference between accumulation at 37°C and 4°C. Some ganglia were denervated by removing a few mm of preganglionic trunk 2 weeks before the experimental test; surgery was with aseptic technique under halothane-N_2O-O_2 anaesthesia.

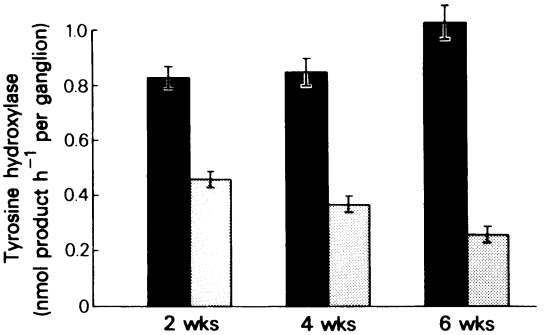

Figure 1 The effect of chronic administration of guanethidine (stippled columns) to rats on the tyrosine hydroxylase activity of an homogenate of the superior cervical ganglion; solid columns, saline-treatments. Each value is the mean for 5 rats; s.e.mean shown by vertical lines.

Expression of results

The measures are reported as the value assayed per ganglion. The protein content of the tissue homogenates or extracts was measured but was not used for the calculation of the results because guanethidine treatment appreciably increased protein content of ganglia; this phenomenon has been reported by others (e.g. Jensen-Holm & Juul, 1970) and probably results from lymphocytic infiltration associated with an immunologically mediated reaction (Manning *et al.*, 1982; 1983) All values are presented as mean ± s.e.mean and the significance of the difference between mean values was estimated by Student's *t* test.

Results

Postsynaptic markers

These experiments tested the effect of chronic administration of guanethidine on ganglionic tyrosine hydroxylase activity and α-bungarotoxin binding. Rats were given guanethidine for 2, 4 or 6 weeks after which their superior cervical ganglia were removed for the measurements which were compared to those made on ganglia from rats that had received injections of saline for the same period of time.

The results of the tyrosine hydroxylase measurement (Figure 1) were consistent with the production by guanethidine of an appreciable postsynaptic destruction. Thus, the drug clearly decreased activity by 40%, 56% and 75% after 2, 4 and 6 weeks of treatment. The tyrosine hydroxylase activity of ganglia from control rats tended to increase progressively during the time studied, possibly in response to repeated injections of saline.

The measurement of α-bungarotoxin binding (Figure 2) complemented the measurement of tyrosine hydroxylase. Toxin binding was reduced by 60% and 74% of the control values after 2 and 4 weeks treatment with guanethidine.

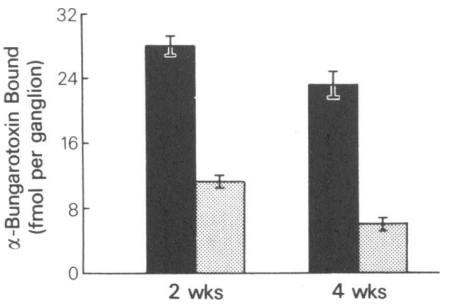

Figure 2 The effect of chronic guanethidine administration (stippled columns) to rats on the binding of α-bungarotoxin to desheathed superior cervical ganglia; solid columns, saline treatment. Each value is the mean from 4 rats; s.e.mean shown by vertical lines.

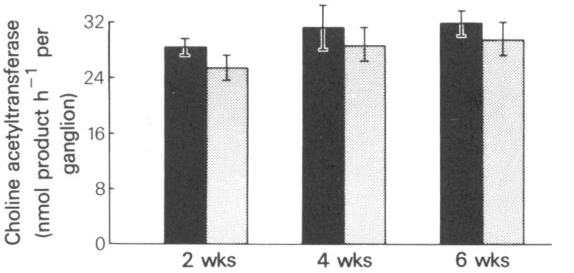

Figure 3 The effect of chronic administration of guanethidine (stippled columnes) to rats on the choline acetyltransferase activity measured on an homogenate of the superior cerival ganglion; solid columns, saline treatment. Each value is the mean from 5 rats; s.e.mean indicated by vertical lines.

Presynaptic markers

To test whether guanethidine administration altered presynaptic functions, the effect of 2–6 weeks of drug treatment on ganglionic choline acetyltransferase acitivity was measured. The activity of ganglia from drug-treated animals did not differ significantly ($P > 0.2$) from that of ganglia from the saline-treated controls (Figure 3).

Other experiments measured the ACh content of ganglia from animals treated 2 or 4 weeks with guanethidine. At both times (Figure 4) the ganglia

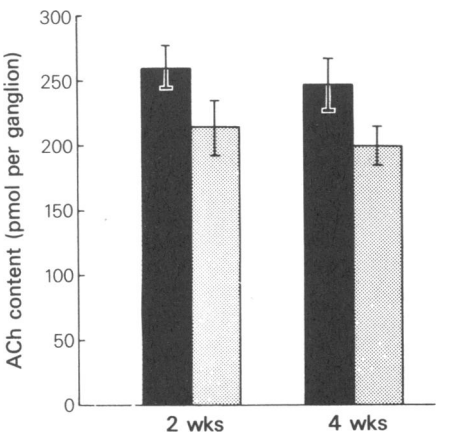

Figure 4 The effect of chronic administration of guanethidine (stippled columns) to rats on the acetylcholine content of the superior cervical ganglion; solid columns, saline treatment. Each value is the mean from 5 rats; s.e.mean shown by vertical lines.

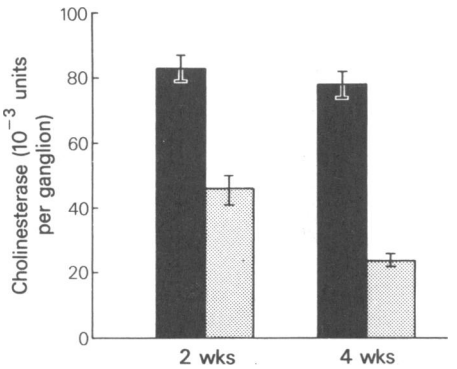

Figure 5 The effect of the chronic administration of guanethidine (stippled columns) to rats on the cholinesterase activity measured on an homogenate of the superior cervical ganglion. Enzyme activity was measured as the physostigmine-sensitive hydrolysis of acetylthiocholine; one unit of activity indicates 1 mmol of substrate hydrolysed per min. Each value is the mean for 5 rats; s.e.mean shown by vertical lines.

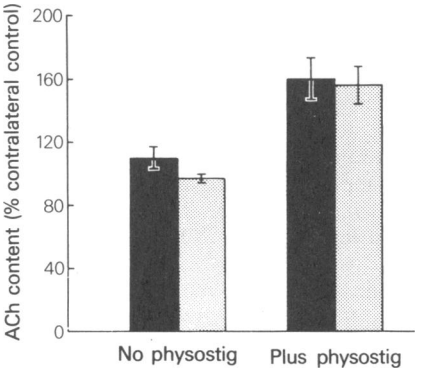

Figure 6 The change of the acetylcholine (ACh) content caused by incubation with or without physostigmine (physostig) in superior cervical ganglia from rats treated for 4 weeks with guanethidine (stippled columns) or saline (solid columns). Each animal provided two ganglia: one was extracted without incubation to provide the control ACh content and the other was incubated before extraction and its ACh content expressed relative to its control. The value for 100% in these experiments was 218 ± 10 pmol. Each value is the mean for 6 rats; s.e.mean shown by vertical lines.

from drug-treated animals had some 18% less ACh than had their saline-treated controls, but this difference did not reach statistical significance ($P > 0.1$ for both groups).

Cholinesterase activity and surplus acetylcholine formation

The consequence of the administration of guanethidine for 2 or 4 weeks on ganglionic cholinesterase activity is illustrated by Figure 5. Drug treatment resulted in 47% and 67% loss of enzyme activity after 2 and 4 weeks, respectively.

Surplus ACh accumulation was measured using ganglia from rats treated for 4 weeks with either guanethidine or saline. The left and the right superior cervical ganglion was removed from each rat: one ganglion was extracted immediately for determination of its ACh content and the other was desheathed for incubation. Twelve rats provided ganglia that were incubated in the presence of physostigmine, and ganglia from 12 other rats were incubated in its absence. Half of each group had received guanethidine and the others had been given saline. The results of these experiments (Figure 6) show that ganglia incubated in the absence of physostigmine contained no more ACh than their non-incubated controls, but the ganglia incubated with physostigmine significantly ($P > 0.01$) increased their ACh content. This increase in ACh store, due to the accumulation of surplus ACh, was apparent in both groups: ganglia from guanethidine-treated animals did not differ ($P > 0.8$) from the control ganglia in their accumulation of surplus ACh.

Choline uptake

Initial experiments on ganglia from untreated animals characterized the kinetics of choline uptake. Ganglia were incubated for 5 min with 1–100 μM [^{3}H]-choline and temperature-dependent accumulation was measured. The result indicated two transport processes, one of higher apparent affinity ($K_m = 2.5$ μM) than the other ($K_m = 67$ μM). To test the effect of guanethidine-treatment, rats were drug-treated or saline-treated for 4 weeks, ganglia were removed, and choline uptake measured (2.5 μM substrate concentration). These results showed (Figure 7) that there was little difference between guanethidine-treated ganglia and control ganglia,

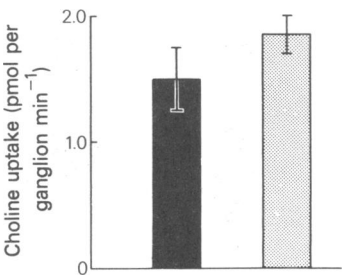

Figure 7 The effect of the chronic administration of guanethidine (stippled columns) to rats for 4 weeks on choline uptake by desheathed superior cervical ganglia; solid column, saline-treated. Each value is the mean from 5 rats; s.e.mean shown by vertical lines.

suggesting that choline uptake was not into adrenergic cell bodies. The result might suggest that choline uptake by ganglia was, indeed, into cholinergic nerve terminals, despite evidence to the contrary (Collier & Katz, 1974; Higgins & Neal, 1982a). Thus, we compared choline uptake by ganglia that had been denervated for 14 days to that of their contralateral control ganglia. As previously reported, denervation also had no significant effect on measured choline uptake: it was $84 \pm 10\%$ that of control.

Discussion

The present experiments used guanethidine treatment to induce a significant destructive lesion to adrenergic cell bodies in rat superior cervical ganglia, an effect for which there is considerable morphological evidence (see Introduction for references). The loss of tyrosine hydroxylase activity measured in our experiments indicates that guanethidine produced this effect in our rats, and this measure is consistent with other results (Johnson & O'Brien, 1976). We also measured α-bungarotoxin binding to ganglia: this parameter was decreased somewhat more than was the tyrosine hydroxylase measure. Although the functional significance of α-bungarotoxin binding sites is unclear (Brown & Fumagalli, 1977) toxin binding sites appear to be on adrenergic cell bodies (Fumagalli *et al.*, 1976; Fumagalli & DeRenzis, 1980), and the present result is consistent with this.

The present experiments show no important changes of presynaptic markers at times when guanethidine had caused clear postsynaptic destruction. Thus, choline acetyltransferase activity was well maintained following guanethidine treatment, and ACh levels were not changed by much, showing the selectivity of the drug-induced postsynaptic lesion. These results suggest that there are no important adaptive changes of these two presynaptic markers in response to loss of the target cells; one might expect an increased rate of impulse activity in the presynaptic neurone in response to loss of postsynaptic activity, and there is some evidence for this in guanethidine-treated rats (Rodionov *et al.*, 1981). In acute experiments, increased presynaptic activity can result in a significant increase of the amount of ACh stored in sympathetic ganglia (Birks, 1978), but this seems not manifest in the present situation for the ganglionic ACh content of the drug-treated animals was slightly less, not more, than that of the controls.

The maintained choline acetyltransferase activity in ganglia of the guanethidine-treated rats clearly indicates a presynaptic location for this enzyme. Previous evidence that this is so was provided mainly from the demonstration that presynaptic degeneration results in loss of activity (Banister & Scrase, 1950). It remained possible (see Collier & Kwok, 1982) that denervation removed an influence of the preganglionic neurone necessary to maintain postganglionic enzyme, as is now known to be so for acetylcholinesterase (Davis & Koelle, 1981). The present result removes some of this uncertainty.

As already mentioned, cholinesterase in ganglia is located mainly postsynaptically and the present demonstration that guanethidine treatment produces appreciable loss of esterase is consistent with that idea. We used this observation that guanethidine-treated animals lost cholinesterase to augment our knowledge about the likely site of accumulation of surplus ACh, an extra store of ACh that accumulates in ganglia when tissue cholinesterase is inhibited (Birks & MacIntosh, 1961). If surplus ACh accumulates presynaptically, as suggested by Collier & Katz (1971), ganglia from drug-treated animals should behave normally, but if surplus ACh accumulates postsynaptically, as suggested by Tuček (1978), they would not. Thus, if the postsynaptic structure that accumulates surplus ACh retained its integrity, but not its esterase, ganglia from guanethidine-treated animals would accumulate ACh in the absence of physostigmine; if the postsynaptic structure that accumulates surplus ACh was destroyed by guanethidine, much less surplus ACh would accumulate in these ganglia. The experiments showed neither of these results: ganglia from guanethidine-treated animals, like normal ganglia, did not form surplus ACh in the absence of physostigmine, but did so in its presence to the the same extent as did control ganglia. Thus, this experiment is consistent with the idea that surplus ACh accumulates presynaptically due to the inhibition of some nerve terminal associated esterase.

The present experiments that measured choline uptake by ganglia add more to confusion than to clarity: they confirm previous demonstrations (Collier & Katz, 1974; Higgins & Neal, 1982a) that preganglionic denervation does not affect measured choline uptake, and they show, in addition, that guanethidine treatment had singularly little effect. Thus, it appears that choline uptake measured on resting ganglia is into neither preganglionic structures nor into adrenergic cell bodies; it presumably occurs into supporting cells or into intraganglionic cells. If this is so, choline uptake as measured in resting tissue has little relationship to ACh synthesis; clearly the activated choline uptake during or just following transmitter release (Collier & Katz, 1974; Collier & Ilson, 1977; Higgins & Neal, 1982b) is the physiologically important mechanism.

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