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THE MODE OF ACTION OF TETRABENAZINE ON PERIPHERAL NORADRENERGIC NERVES

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1 Tetrabenazine (100 mg/kg i.p. in rats) greatly decreased catecholamine-induced histofluorescence in the iris, hepatic portal vein, inferior vena cava and mesenteric blood vessels 4 h after injection. Fluorescence returned to normal by 24 h after injection.

2 The extent of this depletion (4 h after tetrabenazine) was similar to that seen 18 h after reserpine (0.5 mg/kg i.p.)

3 Incubation of tissues taken from rats 4 h after this dose of tetrabenazine with noradrenaline 5×10^{-6}M restored the intraneuronal fluorescence as well as the electron density of noradrenergic vesicle cores viewed with the electron microscope. No such repletion was seen on incubation of tissues from reserpine-treated rats with noradrenaline under the same conditions.

4 Incubation of tetrabenazine-treated hepatic portal veins with noradrenaline also reinstated the normal response to electrical stimulation of the intramural nerves. This did not occur with reserpine-treated veins.

5 The interpretation that tetrabenazine exerts a reversible depleting effect on the noradrenergic vesicle is supported by the demonstration that it exerts no monoamine oxidase inhibition.

Introduction

Tetrabenazine has been found to decrease the noradrenaline content of brain and, to a lesser extent, of peripheral organs (Quinn, Shore & Brodie, 1959; Pletscher, Brossi & Gey, 1962). Häggendal (1968) found that tetrabenazine exerted a noradrenaline-depleting effect on rat heart, submandibular gland and skeletal muscle which was maximal 4 h after treatment. Levels of noradrenaline had returned to normal 36 h after treatment. Reserpine, on the other hand, is known to produce a noradrenaline depletion, recovery from which is not complete until several days after the injection. This has led to the belief that the effect of reserpine on the vesicle is irreversible and repletion is attendant upon the arrival, in the terminals, of unaffected vesicles from the cell body (Dahlström, Fuxe & Hillarp, 1965). Studies on constricted noradrenergic neurones from tetrabenazine-treated cats showed that recovery of noradrenaline levels could occur by noradrenaline biosynthesis within the axon (Tomlinson, Till & Mayor, 1975).

The present study was designed to examine the mode of action of tetrabenazine on the noradrenaline stores of peripheral noradrenergic terminals by comparison with the well-established properties of reserpine. A preliminary communication of some of the results has been given (Tomlinson, 1977).

Methods

This study was performed on 28 male Wistar rats of weight range 220–220 grams. Rats were killed by a blow on the head and bled from the throat, tissues were then removed for morphological examination or experiments *in vitro* (see below).

Incubation of tetrabenazine and reserpine-depleted tissues with noradrenaline in vitro

The iris, inferior vena cava and mesentery were removed from rats either 4 h after tetrabenazine (100 mg/kg, i.p.) or 18 h after reserpine (0.5 mg/kg, i.p.). The tissues were washed in Krebs solution gassed with 95% 0_2 and 5% CO_2 at 37°C. They were then incubated with either normal Krebs solution (controls) or Krebs solution containing noradrenaline 5×10^{-6}M. The incubates were gassed as above, kept at 37°C and incubation was continued for 15 minutes. At the end of incubation the tissues were given two

5 min washes in fresh Krebs solution to remove any unbound noradrenaline and then the veins, mesenteries and some of the irides were processed for fluorescence histochemistry. For each treatment (control and noradrenaline-incubated) for each rat one hemi-iris was fixed for electron microscopy.

Fluorescence histochemistry

This was performed on whole mounts of iris, inferior vena cava, hepatic portal vein and duodenal to ileal mesentery after treatment with glyoxylic acid (as the monohydrate: Fluka) in aqueous solution as described by Furness & Costa (1975). The preparations were examined in a Zeiss Photomicroscope.

Electron microscopy

Rat irides were fixed, with glutaraldehyde, sodium dichromate and osmium tetroxide, by the method described as optimal for the preservation of granular vesicles in noradrenergic nerves by Tranzer & Richards (1976). After dehydration in graded ethanols and embedding in epoxy resin (Araldite: Taab), ultra thin transverse sections were cut on a Reichert OMU 3 ultramicrotome and stained on the grid with aqueous lead citrate (Reynolds, 1963). Sections were examined in a Phillips EM300. Counts of varicosity and vesicle numbers were made at the microscope without prior knowledge of the treatment applied to each specimen.

Functional studies

Rat hepatic portal veins were suspended between parallel platinum wire electrodes in a 20 ml organ bath containing Krebs solution (composition mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 2.4, $NaHCO_3$ 30, NaH_2PO_4 1 and glucose 11.1) gassed with 95% O_2 and 5% CO_2 and maintained at 37°C. Contractions were recorded by means of an isotonic transducer (S.R.I.) driving a flat-bed recorder (Farnell). Field stimulation of intramural nerves was elicited every 4 min with 10 s trains of 200 μs pulses (1–20 Hz) at an amplitude of 140 V using a Farnell stimulator.

Drugs

Tetrabenazine (Nitoman: Roche) was given by intraperitoneal injection *in vivo* as a stable suspension prepared in 0.9% w/v NaCl solution (saline) by the addition of 1 drop of Tween 80 per 10 ml and agitation with an ultrasonic probe. Reserpine for injection was prepared by dissolving the pure substance (Serpasil: Ciba) in 10% ascorbic acid and subsequently adjusting the pH to 4.8 with 5% $NaHCO_3$. Monoamine oxidase was inhibited *in vitro* with tranylcypromine (Parnate: Ciba) at a concentration of 3×10^{-5} M. Vesicular re-uptake was tested *in vitro* with noradrenaline bitartrate (Sigma) at 5×10^{-6}M.

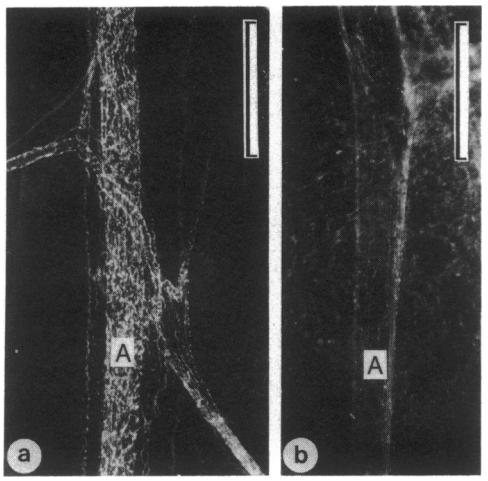

Figure 1 Fluorescence micrographs taken from fields of whole mounts of rat mesentery processed with glyoxylic acid (see Methods section). (a) Mesentery from a normal, untreated rat. A dense, fluorescent plexus of noradrenergic nerves with varicosities visible in the wall of the small artery (A). (b) Mesentery taken 4 h after tetrabenazine (100 mg/kg, i.p.). Most of the intramural nerve plexus is not fluorescent, indicating a marked depletion of noradrenaline. Marker lines denote 150 μm.

Results

Treatment of rats in vivo *with tetrabenazine and reserpine*

Depletion of peripheral noradrenaline by these drugs was assessed by fluorescence histochemistry of iris, inferior vena cava, hepatic portal vein and mesenteric blood vessels. The histofluorescence of noradrenergic nerves in these tissues after condensation with glyoxylic acid has been described and illustrated by Furness & Costa (1975). Figure 1a shows a field photographed from a portion of mesentery prepared from an untreated rat. In contrast, Figure 1b shows that fluorescence of arterial intramural nerves was not present in preparations taken from rats 4 h after a single intraperitoneal injection of tetrabenazine (100 mg/kg). Depletion was less marked 2 and 8 h after such an injection and neuronal fluorescence had returned to levels which were not detectably subnormal by 24 h after injection. Depletion and repletion of fluorescence followed a similar time course in all tissues examined, but the maximum depletion attained (4 h after injection) was not as great in the iris as in the vascular tissues.

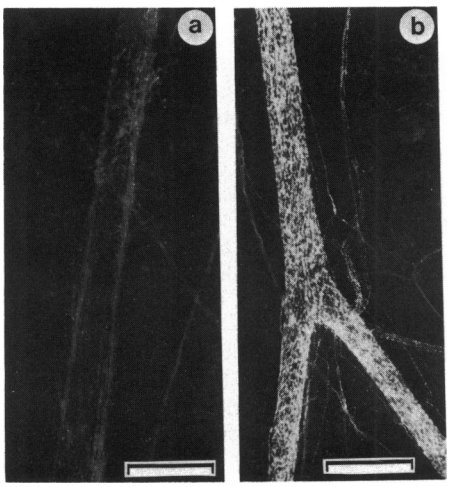

Figure 2 Fluorescence micrographs taken from two portions of the same rat mesentery, removed from the animal 4 h after tetrabenazine (100 mg/kg, i.p.). (a) Control mesentery which had been incubated in Krebs solution alone. The lack of fluorescence of intramural nerves in the artery is characteristic of a low noradrenaline content. (b) Mesentery which had been incubated with noradrenaline (5×10^{-6} M). The fluorescence of the nerve plexus has been restored by this treatment. Marker lines denote 150 μm.

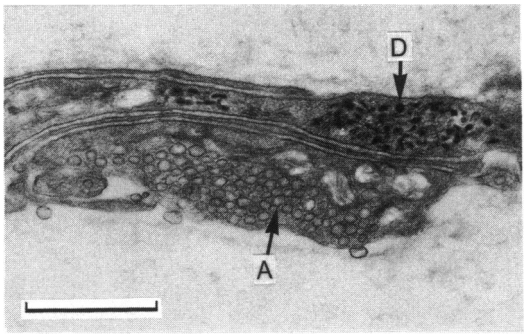

Figure 3 An electron micrograph taken from a section of iris of an untreated rat. Noradrenergic varicosities are readily characterized by the inclusion of dense-cored vesicles (D) in their axoplasm. The adjacent, presumably cholinergic, varicosity contains agranular vesicles (A). The bar denotes 0.5 μm.

Exploratory experiments were performed with reserpine to determine the dose which gave a maximum depletion similar to that obtained with tetrabenazine 100 mg/kg. This was attained 18 h after an intraperitoneal injection of reserpine at 0.5 mg/kg. Four hours after this dose of reserpine, neuronal fluorescence in the iris was normal and only slight depletion was observed in the vascular tissues. The neuronal fluorescence of these tissues was still detectably sub-normal 4 days after this dose of reserpine.

Experiments have shown that fluorescence of noradrenergic nerves, depleted *in vivo* with reserpine, cannot be restored by treatment *in vitro* with noradrenaline (Hamberger, 1967). The effects of such treatment on tetrabenazine-depleted tissues were therefore examined.

Uptake of noradrenaline in tetrabenazine- and in reserpine-depleted tissues

Tissues from tetrabenazine-treated rats incubated with noradrenaline showed a full restitution of neuronal fluorescence (Figure 2). No restitution of fluorescence was observed in tissues from reserpine-treated rats which had been incubated with noradrenaline. The functional integrity of $Uptake_1$ (see Iversen, 1965) in the latter was demonstrated by the inclusion of tranylcypromine (3×10^{-5} M) with noradrenaline in some of the incubates. Under these conditions inhibition of monoamine oxidase permitted the terminals to concentrate noradrenaline leading to a restitution of fluorescence.

The finding that fluorescence can be restored to tetrabenazine-depleted neurones by incubation with noradrenaline is open to two possible interpretations; either tetrabenazine could deplete the vesicles reversibly or it could inhibit monoamine oxidase in addition to an irreversible depleting effect on the vesicle. These hypotheses were tested in two ways. Various concentrations of tetrabenazine (10, 20 and 50 μg/ml) were included with noradrenaline in incubates for reserpine-depleted mesenteries. Under these conditions the amine-depleting effect of tetrabenazine on the vesicles would not counteract any restitution of fluorescence made possible by the hypothetical monoamine oxidase inhibition. However, no restitution of fluorescence occurred indicating that, at the concentrations employed, tetrabenazine did not protect the noradrenaline taken up by the terminals from monoamine oxidase.

The possibility that tetrabenazine promoted a reversible depletion of the noradrenergic vesicle was examined by electron microscopy. Irides from untreated rats, from reserpine-treated and from tetrabenazine-treated rats after incubation in Krebs solution with or without added noradrenaline (see above) were fixed and processed for electron microscopy. Figure 3 shows that this treatment permits the identification of noradrenergic varicosities by the characteristic inclusion of dense-cored vesicles in their axoplasm. Pharmacological depletion of vesicular noradrenaline reduces the formation of electron-dense material in the vesicle core upon reaction with the fixative (Tomlinson, 1975). Figure 4 shows that the number of dense-cored vesicles present

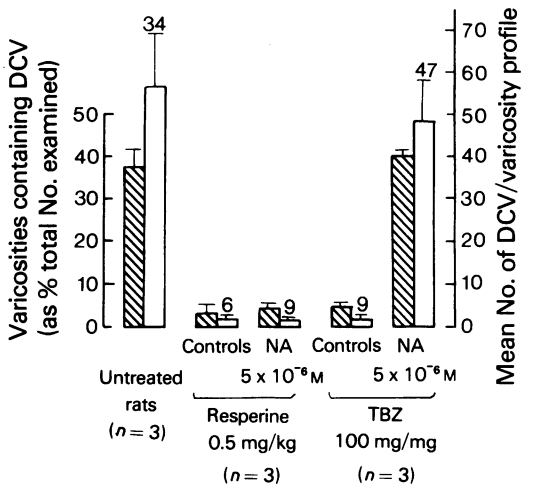

Figure 4 Histogram showing the effect of reserpine (18 h) and tetrabenazine (TBZ) (4 h) *in vivo* with and without subsequent incubation *in vitro* with noradrenaline on the number of recognizable noradrenergic varicosities (hatched columns) and the frequency of dense-cored vesicles (DCV) therein (open columns) in transverse sections of rat iris examined with the electron microscope. The varicosity percentages were calculated after examination of about 100 varicosities per animal (i.e. about 300 for each treatment). The figures above the open columns denote the number of varicosities used for vesicle counts. Vertical lines show s.e. mean (n = number of animals).

in varicosities was greatly reduced by either tetrabenazine or reserpine treatment. The numbers of recognizable noradrenergic varicosities and their dense-cored vesicles in reserpine-treated irides was not increased by incubation with noradrenaline. In contrast, *in vitro* treatment of tetrabenazine-depleted irides with noradrenaline increased the numbers of dense-cored vesicles and recognizable noradrenergic varicosities to levels similar to those estimated in normal, untreated irides.

Functional studies

Frequency/response curves to field stimulation of intramural nerves were obtained from hepatic portal veins from 5 untreated rats, 5 rats 4 h after tetrabenazine (100 mg/kg i.p.) and 5 rats 18 h after reserpine (0.5 mg/kg i.p.). The responses to each frequency of stimulation obtained from the veins, of the reserpine- and tetrabenazine-treated rats, were much smaller than those obtained from untreated veins (see Figure 5a and b). Subsequent addition of noradrenaline (5×10^{-6} M) to the organ bath for 15 min followed by wash-out and repetition of each frequency of stimulation did not alter the frequency/response relationship for the reserpine-treated veins (Figure 5b). However, the slope of the frequency/response curve obtained, after noradrenaline treatment, from the tetrabenazine-treated veins was elevated to the norm (see Figure 5a).

Discussion

The present findings show clearly that, after reserpine-treatment, noradrenaline-depleted neurones will not take up the amine from an incubate in detectable amounts. This confirms the classical findings of Lindmar & Muscholl (1964) and Iversen, Glowinski & Axelrod (1965). This inhibition of the uptake and storage capacity is restricted to the vesicle since inhibition of monoamine oxidase permits the $Uptake_1$ process (see Iversen, 1965) to concentrate noradrenaline within the neurone. This is also in agreement with earlier work (Fuxe & Hillarp, 1964). Examination of fluorescent nerve plexi in tissues from untreated rats shows that the varicosities exhibit a much brighter fluorescence that do the inter-varicosities and pre-terminal axons. However, in plexi which have been reserpine-treated and subsequently repleted with noradrenaline in the presence of monoamine oxidase inhibition all parts of the neurone exhibit similar fluorescence. This has been interpreted by the suggestion that fluophore formation in such tissues occurs with noradrenaline which is in the 'free' state in the axoplasm (Malmfors, 1965). Electron microscopy confirms this supposition by showing that such fluorescent nerves contain very few dense-cored vesicles (Richardson, 1964; Hökfelt, 1967).

The noradrenergic terminal exhibits quite different properties after depletion of its amine with tetrabenazine. Restitution of fluorescence occurs upon incubation of the tissue with noradrenaline in the absence of monoamine oxidase inhibition. Electron microscopy shows clearly that this noradrenaline uptake occurs, at least in part, in the vesicle. Indeed, the demonstration that tetrabenazine cannot act as a monoamine oxidase inhibitor, to 'protect' noradrenaline taken up into reserpinized terminals, indicates that most of the amine concentrated by nerves previously depleted with tetrabenazine must be stored in the vesicles.

The reversibility of the vesicular depletion by tetrabenazine has also been demonstrated by the functional studies. In tetrabenazine-depleted hepatic portal veins, which had been incubated subsequently with noradrenaline, the response of the smooth muscle to noradrenergic nerve stimulation was identical, at all stimulation frequencies, to that obtained from the veins of untreated rats.

This reversibility of depletion explains the rapid (relative to reserpine) recovery of noradrenaline levels *in vivo* after tetrabenazine-induced depletion. Other

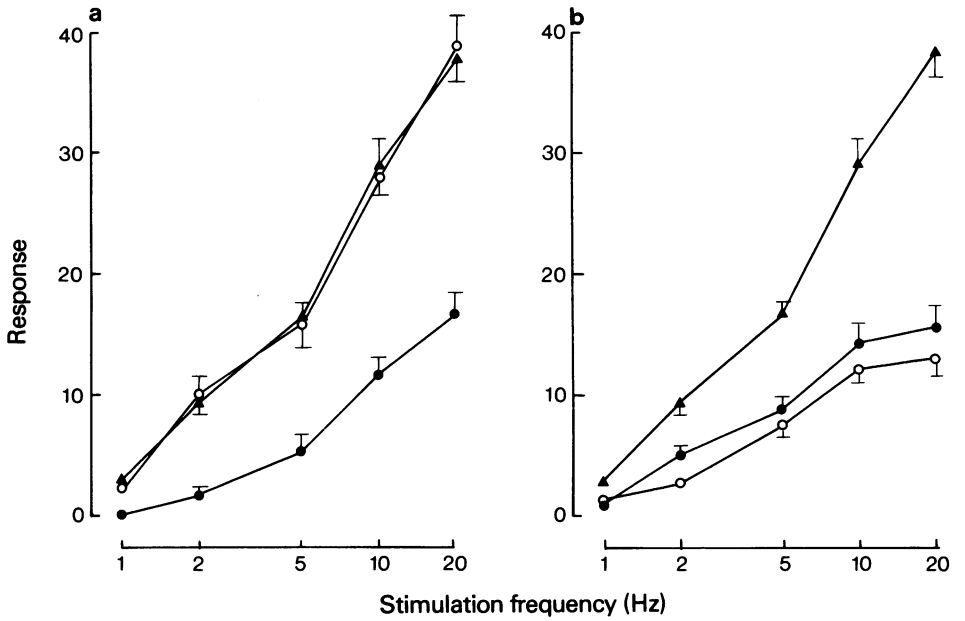

Figure 5 (a) Frequency/response curves obtained by field stimulation of hepatic portal veins from untreated rats (▲) and from rats 4 h after tetrabenazine (100 mg/kg) before (●) and after (○) incubation with noradrenaline 5×10^{-6} M for 15 minutes. (b) Frequency/response curves obtained by field stimulation of hepatic portal veins from untreated rats (▲) and from rats 18 h after reserpine (0.5 mg/kg) before (●) and after (○) incubation with noradrenaline 5×10^{-6} M for 15 minutes. In both (a) and (b) response is expressed in arbitrary units of longitudinal contraction. $n = 5$ rats for each treatment. Vertical lines show s.e. means.

studies (Tomlinson *et al.*, 1975) have shown that repletion occurs with equal rapidity in a population of vesicles isolated in noradrenergic nerve trunks between two ligatures. Thus, repletion is not dependent on the arrival of unaffected vesicles synthesized in the cell body. This repletion is prevented by inhibition of tyrosine hydroxylase (Tomlinson *et al.*, 1975).

The reversible nature of the tetrabenazine-induced depletion does not account for the apparently large difference between the time taken for maximum depletion by tetrabenazine (4 h) and by a dose of reserpine producing a quantitatively similar effect (18 h). However, there is, evidence to indicate that this difference may be associated with a low bioavailability of reserpine. Hamberger (1967) has shown that the use of reserpine in the form of its phosphate (as opposed to the conventionally used free base) produces a much more rapid depletion of noradrenaline.

Tetrabenazine is currently achieving a wide clinical use in the treatment of chorea (McLellan, Chalmers & Johnson, 1974). It is unlikely that the present study provides any useful information in this context. The dosage employed shows that it is of low potency as a depletor of peripheral noradrenaline and its effect on the central nervous system may be mediated via other transmitter systems. Side-effects which may be related to depletion of peripheral noradrenergic nerves have not been reported. Indeed, Kidd & McLellan (1972) found that a patient who took an overdose of tetrabenazine (15–20 mg/kg, orally) was normotensive, albeit in the supine posture.

However, the present study does demonstrate, that tetrabenazine is an extremely useful pharmacological tool, exhibiting a unique mode of action and thereby enabling the preparation of noradrenergic vesicles, depleted of their amine, but with their storage and release capacities intact.

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