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AN ELECTROPHYSIOLOGICAL ANALYSIS OF THE EFFECTS OF RESERPINE ON ADRENERGIC NEUROMUSCULAR TRANSMISSION

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1 An electrophysiological study has been made of the effects of depleting synaptic vesicles (i.e. small vesicles < 60 nm diameter) of their transmitter with reserpine on the quantity of transmitter released by nerve impulses, using the amplitude of the synaptic potential as a measure of transmitter release.

2 Pretreatment of adrenergic nerve terminals with reserpine sufficient to deplete the terminals of 70% of their noradrenaline (NA) did not change the total number of synaptic vesicles in the terminals, but did reduce the number with a large granular core as well as the quantity of NA released by a single nerve impulse by 80%.

3 Pretreatment of adrenergic nerve terminals with reserpine and iproniazid, to decrease vesicular NA but enhance cytoplasmic NA, had the same effect on synaptic vesicles and on the NA released by a single nerve impulse as did reserpine alone.

4 During a short train of impulses at high frequencies in reserpine pretreated terminals, the quantity of NA released by successive impulses increased until a steady-state release was reached comparable to that in untreated preparations. This facilitated release could be quantitatively predicted in terms of the addition of the individual potentiations introduced by each impulse in the train.

5 These results are consistent with the idea that each quantum of transmitter is stored in a synaptic vesicle, and that these may be released by nerve impulses directly from the terminal by a process of exocytosis.

Introduction

The vesicle hypothesis suggests that the structural basis for the storage and release of transmitter at synapses is the vesicle, which contains a single quantum of transmitter which is released on arrival of an impulse at a nerve terminal by the process of exocytosis (Katz, 1969; Smith, 1971, 1973). Extensive biochemical studies of adrenergic synaptic vesicles have shown that noradrenaline (NA) can be almost entirely removed from vesicles, and therefore from the nerve terminal, with reserpine (Stjarne, 1964; van Orden, Bensch & Giarman, 1967; Boadle-Biber & Roth, 1972; Enero & Langer, 1973). This procedure is known to elevate intraneuronal extravesicular NA when the enzyme monoamineoxidase is inhibited (van Orden *et al.*, 1967). However, if the synaptic vesicles in sympathetic nerve terminals are depleted of their NA content by reserpine, there is little difference in the contractile force developed by a smooth muscle during stimulation of its nerve supply with short trains of impulses (van Orden *et al.*, 1967; Ambache & Zar, 1971 Wakade & Krusz, 1972; Ambache, Dunk, Verney & Zar, 1972). In the present work an electrophysiological study has been made of the effects of altering the amount of NA in vesicles and in the intraneuronal cytoplasm on the quantity of NA released by nerve impulses.

Methods

Electrophysiology

The isolated vas deferens preparation of the mouse (Bennett, 1973) was used in all studies of adrenergic transmission. Mice were killed by cervical fracture and both vas deferens dissected free and pinned out in a perspex organ bath. Preparations were bathed in a modified Krebs-Ringer-bicarbonate solution of the following ionic composition (mM): Na 151, K 4.7, Ca 1.8, Mg 1.2, Cl 142, H_2PO_4 1.3, SO_4 1.2, HCO_3 16.3,

glucose 7.8. The solution was bubbled with a gas mixture containing 95% O_2 and 5% CO_2 and maintained at 35-37° C while flowing through the organ bath at 1-3 ml/minute.

The intramural sympathetic nerves of the vas deferens were stimulated through two platinum-ring electrodes separated 1 mm apart and placed round the vas deferens, with stimuli of 50 V amplitude and 0.01-1.0 ms duration. Intracellular potentials were recorded from smooth muscle cells with glass microelectrodes filled with 2M KCl and having resistances from 30-70 MΩ. The signals were led through a high impedance unity gain amplifier, displayed on an oscilloscope screen and photographed on moving film. The amplitude of the excitatory junction potentials (e.j.ps) in the smooth muscle were graded with the stimulus so that those potentials did not exceed 10 mV amplitude during trains of impulses, so as to avoid serious errors due to non-linear summation (Martin, 1955).

For short trains of impulses (< 100) to the intramural sympathetic nerves, the amplitude of each e.j.p. was normalized with respect to the first e.j.p. in the train and the normalized data for all sympathetic nerve terminals studied were then pooled. The changes in e.j.p. amplitude during a short train were compared with predictions based on the relationship between the effect of a conditioning impulse on the amplitude of the e.j.p. evoked by a subsequent test impulse and the interval between the two stimuli derived previously (Bennett, 1973). This is given by the equation $f = f_1 \ exp - (b_1 t)$ where f is the facilitation (equal to $(V - Vo)/Vo$ where Vo is the amplitude of a conditioning e.j.p. and V is the amplitude of a test e.j.p.), and f_1 is the facilitation at zero time (equal to 0.6) and b_1 is a rate constant of decay (equal to 0.17); if the successive facilitations introduced by each impulse simply add, then the growth of facilitation during a train is described by $f = k_1 \ (1 - exp(-b_1 t))$ where $k_1 = f_1 (exp \ b_1 \Delta t - 1)^{-1}$ and Δt is the interval between successive impulses (Mallart & Martin, 1967).

Electronmicroscopy

When a vas deferens was to be examined with the electronmicroscope it was first cut into small pieces (< 1 mm_3) and these were fixed in 3% glutaraldehyde in cacodylate buffer pH 7.3 for 1 h at 4-5° C and then washed overnight in 0.2 M cacodylate buffer (4.5° C). The vas deferens was then post-fixed in 2% OsO_4 in Palade's buffer pH 7.3 at 4-5° C for 2-2.5 h, rinsed briefly in tap water and stained en block with 10% uranyl nitrate solution in Palade's buffer (pH 7.3) for 1 h at 4-5° C. The vas was then dehydrated through a graded series of water-ethanol mixtures for 5-10 min and cleared in xylene. Infiltration in an Araldite mixture at room temperature for two days was followed by embedding in fresh Araldite in flat moulds; polymerization at 60° C was carried out for 1-2 days. Blocks were trimmed and silver to gold sections were cut on an LKB ultramicrotome. Sections were spread with xylene and picked up onto 200 mesh copper grids coated with a nitrocellulose film. The sections were stained on the grids with lead citrate and examined on a Phillips 200, 300 or 201 electronmicroscope. Ten or twelve electronmicrographs of adrenergic nerve-varicosity profiles were taken from one grid of sections from one block for each vas deferens examined and there was a total of 12 to 20 adrenergic varicosities in each set of micrographs; numbers of synaptic vesicles (i.e. small vesicles with diameter < 60 nm) with and without a granular core (taken to be a granule with a diameter of more than 25% of the vesicle diameter) were counted for each varicosity profile, and the area of the profile determined by tracing its outline on paper, cutting out the enclosed area, weighing it and multiplying by an appropriate factor for magnification and paper weight; in this way the number of synaptic vesicles per μm^2 of varicosity profile was determined.

Drugs

Reserpine Either mice were given intraperitoneal injections of 2.5 mg/kg reserpine (Serpasil, CIBA), 48, 24 and 2 h before they were killed and the isolated vas deferens then exposed to 1.0 μM reserpine (as described by van Orden *et al.*, 1967) in the bathing medium or the vas deferens from untreated mice were exposed to reserpine 10 μg/ml in the bathing medium for 2 h before experiments started.

Iproniazid In some cases the vas deferens was exposed to this drug in the bathing medium to a final concentration of 25 μg/ml for a period of 2 h before experiments started.

Results

The effects of depleting adrenergic vesicles of noradrenaline on the quantity of noradrenaline released by a single impulse

The smooth muscle syncytium of the mouse vas deferens receives a multiple adrenergic innervation (Furness, Campbell, Gillard, Malmfors, Cobb & Burnstock, 1970; Bennett, 1972, 1973), in which

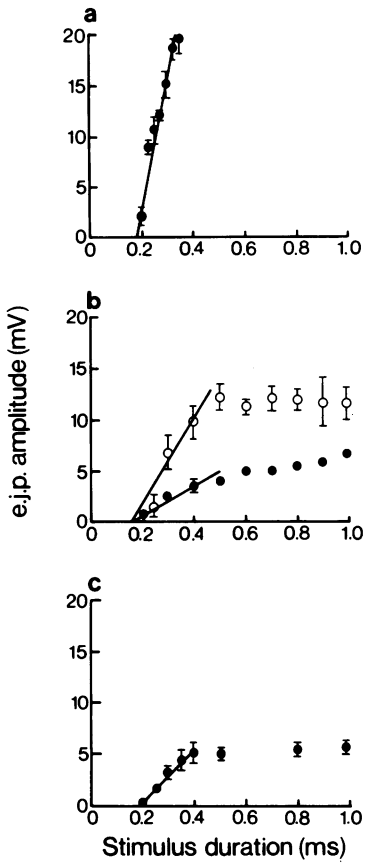

Figure 1 The relationship between the amplitude of the excitatory junction potential (e.j.p.) evoked by a single nerve stimulus and the duration of the stimulus before and after reserpine treatment. (a) Control: gradient of line 170 V/s; (b) reserpine 1 μg/ml (○) and 10 μg/ml (●): respective gradients 50 V/s and 20 V/s; (c) reserpine 10 μg/ml + iproniazid 10 μg/ml: gradient 25 V/s. Strength of stimulus 60 V in all cases. Vertical bars indicate s.e. mean for each point where it is larger than the size of the symbol; $n > 20$ from ten preparations.

the e.j.p. is reduced by over 90% by either phenoxybenzamine (50 μg/ml) or bretylium (50 μg/ml). An increase in the duration of the stimulus applied to these nerves gives an increase in the amplitude of the e.j.p. This increase in e.j.p. amplitude with stimulus duration is linear over the lowest stimulus durations (Figure 1a) up to e.j.p. amplitudes of about 15-20 mV, but over larger pulse durations (> 0.3 ms) the curve flattens off, presumably due to the effects of non-linear summation (Martin, 1955). The gradient of the linear part of the curve is 170 V/s which varies very little between cells in the one preparation or between preparations, probably because the e.j.p. in any smooth muscle cell is due to the effects of a large number of terminals releasing transmitter on other smooth muscle cells throughout the syncytium (Bennett, 1972). The gradient of this curve is proportional to the number of nerve terminals stimulated per given stimulus duration and the quantity of NA released by each stimulated terminal.

When the synaptic vesicles had been depleted of their NA by reserpine treatment, the gradient of the stimulus duration–e.j.p. amplitude curve decreased to 20 V/s (Figure 1b). At stimulus durations beyond 0.5 ms there was no further increase in e.j.p. amplitude, even though the e.j.p. was still only about 5 mV high, indicating that all the nerve terminals were now being stimulated at this duration. As it is unlikely that reserpine has any effect on the number of nerves releasing transmitter for a given set of stimulus conditions, as it has no effect on transmitter release by single impulses in the mouse phrenic nerve-diaphragm preparation (personal observations), then the reserpine effect is likely to be due to a decrease in the quantity of NA released from the nerve terminals. This decrease in the gradient of the e.j.p. amplitude-duration curve of 80% implies that reserpine has decreased the quantity of transmitter released from nerve terminals by a single impulse by this proportion.

This reserpine treatment caused a decrease of 80% in the percentage of small-diameter vesicles which had granular cores greater than one quarter the diameter of the vesicles (60.5 ± 9.9%) (30) before reserpine compared with 12.1 ± 0.5% (20) after reserpine (numbers of axon varicosity profiles studied given in brackets), without any significant change occurring in the total number of small-diameter synaptic vesicles in the nerve terminals. The similarity in the decrease in the quantity of NA released by a single impulse and in the number of vesicles with large granular cores after reserpine treatment suggests that the decrease in NA released is due to the depletion of the small vesicles of their NA content.

When NA is displaced from synaptic vesicles into the extravesicular but intraneuronal cytoplasm by reserpine, but protected from monoamineoxidase degradation by inhibition of this enzyme with iproniazid, there is an increase in the levels of cytoplasmic NA (van Orden *et al.*, 1967). However, this increase did not enhance the quantity of NA released by a single impulse over that following reserpine alone (Figure 1c). This observation implies that NA is released directly from the small synaptic vesicles following a nerve impulse, and not indirectly from the cytoplasm.

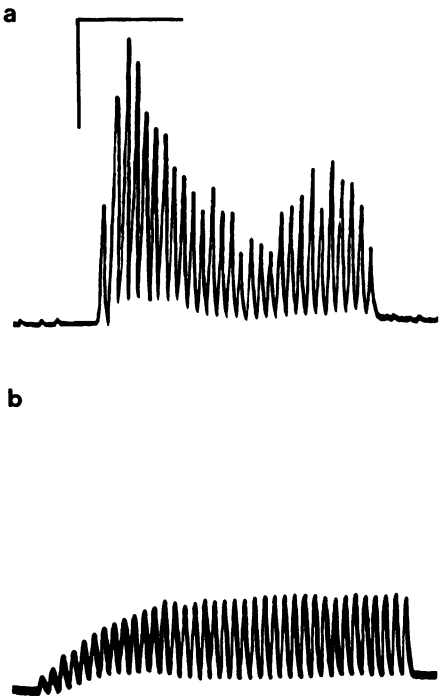

Figure 2 The effects of reserpine on the amplitude of the excitatory junction potential (e.j.p.) during short trains of high frequency impulses (10 Hz). (a) Control, (b) reserpine 10 μg/ml. Calibration: vertical, 5 mV; horizontal, 1 second.

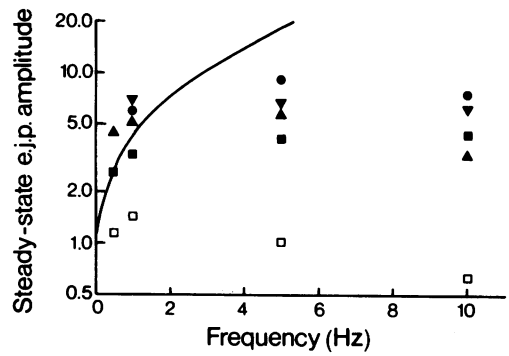

Figure 3 The effect of reserpine (10 μg/ml) and iproniazid (25 μg/ml) on the relationship between the amplitude of the steady-state excitatory junction potential (e.j.p.) reached during a short train of impulses and the frequency of stimulation. (□) Control values; (▲) reserpine *in vitro;* (▼) reserpine *in vivo* and reserpine *in vitro;* (■), reserpine + iproniazid *in vitro;* (●), reserpine *in vivo* and reserpine + iproniazid *in vitro*. The curve is the predicted relationship given in the methods. The s.e. mean was less than 15% of the mean for each point ($n > 6$).

The effect of depleting adrenergic vesicles of noradrenaline on the quantity of noradrenaline released by short trains of impulses

The amplitude of successive e.j.ps in a short-stimulus train in control preparations increases with the first few impulses and then either decreases (at high frequencies ⩾ 1 Hz; Figure 2a) or continues to increase (at low frequencies < 1 Hz) until a steady-state e.j.p. amplitude is reached after a few seconds of stimulation (Bennett, 1973). The relationship between the steady-state amplitude reached during a short train of impulses and the frequency of stimulation is shown in Figure 3: the steady-state amplitude at first increases with increasing frequency, then decreases until it is similar to or less than the first e.j.p. in the train. In the presence of reserpine or after pretreatment with this drug, the amplitude of successive e.j.ps increased progressively at both high and low frequencies (Figure 2b) until a steady-state e.j.p. amplitude substantially greater than the first e.j.p. was reached after a few seconds of stimulation (Figure 3). Thus, although depletion of NA from the synaptic vesicles greatly depressed the amplitude of the e.j.p. evoked by a single impulse, repetitive stimulation at high frequencies (⩾ 1 Hz) led to a sustained facilitation of e.j.p. amplitude, such that the absolute amplitude of the e.j.p. in the steady-state was similar to that of control preparations (Figure 2). The effect of a conditioning impulse on the amplitude of the e.j.p. due to a subsequent test impulse at different intervals is shown in Figure 4: the facilitatory effect of the conditioning impulse falls into two components, one which occurs at short intervals and lasts up to 200 ms, and the other which lasts for several seconds and has a time constant of about 6 s (Bennett, 1973). Reserpine has no effect on either the short or long time course facilitation (Figure 4).

Figure 5 shows the observed growth in e.j.p. amplitude during short trains of impulses at different frequencies compared with the growth predicted on the basis that the facilitation introduced by each impulse in the train simply adds (Bennett, 1973), for control muscles and in the presence of reserpine or reserpine plus iproniazid. There is good agreement between the predicted and observed growth at 0.5 Hz (Figure 5c) in both control and drug-treated preparations, but at higher frequencies (Figure 5a, b) the control values are much smaller than those predicted whereas the treated preparations show good agreement up to 5 Hz. The normalized

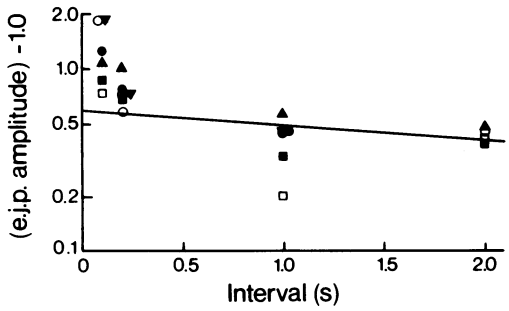

Figure 4 The action of reserpine (10 μg/ml) and iproniazid (25 μg/ml) on the effect of a conditioning impulse on the amplitude of an excitatory junction potential (e.j.p.) evoked by a subsequent test impulse. (□) and (○) Controls from two series of experiments; (■) reserpine and iproniazid *in vitro;* (●) reserpine *in vivo* and iproniazid *in vitro*; (▲) reserpine *in vitro*; (▼) reserpine *in vivo.* The s.e. mean was less than 15% of the mean for each point ($n > 6$). The line indicates a time constant of 6 seconds.

short-term steady-state e.j.p. amplitude reached at 5 Hz or 10 Hz in the presence of reserpine was about five times greater than the corresponding value in untreated preparations (Figure 3). As reserpine treatment caused about an 80% decrease in the NA released by the first impulse in a train, the NA released per impulse during the short-term steady-state in the presence of reserpine is of the same order as that in untreated preparations.

Discussion

The release of noradrenaline in the smooth muscle of the mouse vas deferens by impulses

The question arises as to whether the e.j.p. in response to a single nerve impulse in the mouse vas deferens is solely due to the release of NA. Although the mechanical response of the vas deferens to trains of nerve impulses is abolished by 6-hydroxydopamine (Furness *et al.*, 1970; Farnebo & Malmfors, 1971) and bretylium (Henderson, Hughes & Kosterlitz, 1972), it is possible that some transmitter is still released from nerves when preparations are exposed to these drugs, but that the e.j.p. is now reduced below threshold for initiation of the events leading to a mechanical response: thus although bretylium abolishes the mechanical response, the e.j.p. is not abolished, but is reduced to less than 10% of its control size; similarly, relatively low concentrations of phenoxybenzamine block the spontaneous contractions of the vas deferens which occur after treatment with 6-hydroxydopamine (Furness *et al.*, 1970), but they do not abolish the e.j.p., although it is reduced to less than 20% of its control size. However, when the degenerative effects of 6-hydroxydopamine on the adrenergic nerve terminals is complete, the e.j.p. is abolished (Furness *et al.*, 1970); this observation, together with the fact that the e.j.p. is unaffected by atropine (Bennett, 1973) or hyoscine (Furness *et al.*, 1970), suggests that the e.j.p. is solely due to the release of NA.

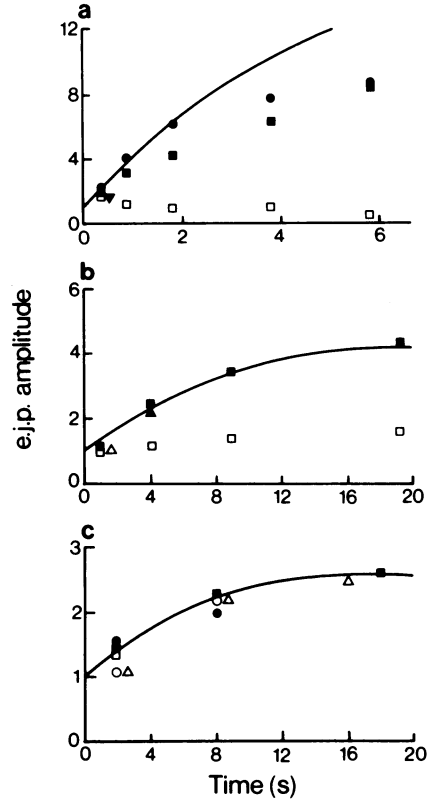

Figure 5 The effect of reserpine (10 μg/ml) and iproniazid (25 μg/ml) on the growth of the excitatory junction potential (e.j.p.) during short trains of impulses at different frequencies. (a) 5 Hz; (b) 1 Hz; (c) 0.5 Hz. (□) Control values; (●), reserpine *in vitro*; (■), (○) and (△), reserpine + iproniazid *in vitro*. Curves are the predicted relationship for the growth of the e.j.p. given in the methods section. The s.e. mean was less than 15% of the mean for each point ($n > 9$).

It is likely that the amplitude of the e.j.p. is a measure of the amount of NA released by a single impulse as there is good correlation between the time course of changes in noradrenaline output from sympathetic nerves during long trains of impulses (Dearnaley & Geffen, 1966; Kopin, Breese, Kraus & Weise, 1968; Stjärne & Wennmalm, 1970) and changes in the e.j.p. amplitude (Bennett, 1973).

The effect of depleting vesicles of their noradrenaline on the amount of noradrenaline released by single impulses

The vesicle hypothesis has not been directly tested at cholinergic synapses because there has not been any method available to date to differentially deplete or load vesicular acetylcholine with respect to extravesicular acetylcholine. At the adrenergic synapse, evidence to suggest that NA is released directly from synaptic vesicles into the synaptic cleft has been obtained by biochemical examination of the constituents of adrenergic vesicles and comparison of these with the constituents appearing in the perfusate from organs on stimulation of their adrenergic nerve supply. In particular dopamine-β-hydroxylase has been shown to be present in adrenergic synaptic vesicles but not free in the nerve terminal cytoplasm, and to be released on stimulation of adrenergic nerves (de Potter, de Schaepdryver, Moerman & Smith, 1969; Geffen, Livett & Rush, 1969; Chubb, de Potter & de Schaepdryver, 1970, 1972; Bisby, Fillenz & Smith, 1973). Furthermore, the soluble cytoplasmic proteins of comparable size, such as dopa-decarboxylase, are not released (de Potter *et al.*, 1969; Geffen *et al.*, 1969).

The reserpine-treatment used in the present experiments depletes the nerve terminals of over 70% of their NA content (van Orden *et al.*, 1967) which is mostly due to the removal of NA from vesicles as extravesicular NA levels are elevated by this procedure (van Orden *et al.*, 1967; Boadle-Biber & Roth, 1972) especially in the presence of monoamineoxidase inhibitors (Potter, 1967; Smith, 1972). The 80% decrease in the amplitude of the e.j.p. after reserpine is likely to be due to a similar percentage drop in the quantity of NA released by a single impulse, as reserpine has little α-adrenoceptor blocking capacity (Flemming & Trendelenburg, 1962; Trendelenburg & Weiner, 1962). Taken in conjunction with the biochemical studies this result suggests that NA is released by exocytosis from the small granular synaptic vesicles.

The effect of reserpine on the quantity of noradrenaline released by short trains of nerve impulses

The reason that the contractile response of smooth muscle to short trains of impulses (< 100 impulses at > 5 Hz) in excitatory adrenergic nerves is not depressed when the transmitter is depleted from most of the synaptic vesicles by reserpine (van Orden *et al.*, 1967; Ambache & Zar, 1971; Ambache *et al.*, 1972; Wakade & Krusz, 1972), is probably due to the growth in facilitation of the e.j.p. which occurs in the presence of reserpine compared with the usual depression which occurs in its absence (Bennett, 1973). Thus, the amplitude of the first e.j.p., which is greatly depressed by reserpine treatment, soon facilitates to control values after a few impulses. The action of reserpine may be similar to that of the α-adrenoceptor blocking drugs in promoting transmitter release, which is discussed in the following paper (Bennett & Middleton, 1975).

Because the effector response to adrenergic nerve stimulation during short trains of impulses is little affected by reserpine treatment, it has been suggested that the store of noradrenaline required for normal transmission must be extremely small (Antonaccio & Smith, 1974; van Orden, Schaefer, Antonaccio & Smith, 1974), and may be related to the small number of granular vesicles which remain in the nerve terminals after reserpine treatment (van Orden *et al.*, 1974). The present work suggests a different explanation, related to the fact that reserpine has two actions on the nerve terminal: on the one hand it depletes synaptic vesicles of their NA, leading to a decrease in the amount of transmitter released by a single nerve impulse; on the other hand reserpine alters the neurosecretory mechanisms so that increasing numbers of quanta are released by successive impulses during a short train, until the synaptic potential reaches a size comparable to that in controls.

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