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Stimulation-Depletion of Serotonin and Noradrenaline from Vesicles of Sympathetic Nerves in the Pineal Gland of the Rat

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Summary. The pineal gland of the rat receives a rich nervous supply originating from the superior cervical ganglia. These fibers contain serotonin in addition to their neurotransmitter, noradrenaline. Cytochemical studies at the ultrastructural level have shown that both amines are present in the cores of the granular vesicles that are characteristic of these nerves. It is presently shown that the bilateral electrical stimulation of the preganglionic fibers innervating the ganglia markedly reduces the number of small sites reacting cytochemically for both noradrenaline and serotonin, these sites corresponding to the cores of small granular vesicles, while the larger reactive sites (cores of large vesicles) remain unaltered. The vesicles are retained in nerve terminals after stimulation, as observed in conventionally processed tissues, although with altered sizes and shapes. Apart from these cytochemical and structural changes, nerve stimulation also reduces the endogenous noradrenaline content of the pineal gland. Thus, both noradrenaline and serotonin are released from their storage sites in pineal sympathetic nerves after electrical stimulation in vivo. This suggests the possibility that several substances with presumed transmitter or modulatory functions might be simultaneously released by nerve impulses from a given nerve terminal.

Key words: Sympathetic nerves – Synaptic vesicles – Noradrenaline – Serotonin – Pineal gland – Rat.

Ultrastructural studies have shown that terminal varicosities of peripheral adrenergic axons where noradrenaline is highly concentrated contain vesicles displaying dense cores in tissues fixed in metallic oxidants (Pellegrino de Iraldi and De Robertis 1961; Grillo 1966; Hökfelt 1971). These cores result from the presence of the amine inside the vesicles; they disappear after depleting noradrenaline from

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sympathetic terminals with various drugs and can be restored by exogenous amines (Hökfelt 1968; Jaim-Etcheverry and Zieher 1971a). The majority of the amine-storing vesicles of adrenergic varicosities are of small diameter (400 Å), the small granular vesicles (SGVs), while approximately 5 % of the total vesicular population is represented by the large (1,000 Å) granular vesicles (LGVs). These vesicles not only have a storage function, they also participate in noradrenaline synthesis and play a key role in its release by nerve impulses (Smith 1971; Geffen and Livett 1971; Fillenz 1977). There is evidence suggesting that the neurotransmitter is liberated from these nerves by exocytosis, i.e., direct extrusion of the entire vesicular content to the extracellular space after fusion of the vesicular and axonal membranes (Smith 1971; Fillenz 1977). In relation to this mechanism, it is important to bear in mind that various types of molecules such as adenine nucleotides, enzymatic and non-enzymatic proteins, are present in vesicles of sympathetic terminals that may also contain other monoamines in addition to noradrenaline. For example, with specific cytochemical procedures it has been possible to localize serotonin in both types of vesicles in the adrenergic fibers innervating the pineal gland of the rat (Jaim-Etcheverry and Zieher 1968; Pellegrino de Iraldi and Gueudet 1969). Such fibers constitute the sole nervous supply of the gland, originate in cell bodies located in the superior cervical ganglia and, once inside the pineal, accumulate serotonin that is actively synthesized by parenchymal cells (Axelrod 1974). Noradrenaline and serotonin are not only simultaneously present in these nerves but, as suggested by indirect evidence, may even coexist within their vesicles (Jaim-Etcheverry and Zieher 1971b; Rubio et al. 1977). Thus vesicular serotonin might be released along with noradrenaline by impulses reaching sympathetic nerves in the pineal. To analyze this possibility, we studied these fibers with cytochemical procedures for the ultrastructural localization of serotonin and catecholamines after bilateral electrical stimulation of the preganglionic nerves to the superior cervical ganglia.

Materials and Methods

Male Wistar rats (200–300 g) used in this study were kept under diurnal lighting conditions (light period from 7 a.m. to 7 p.m.) for at least three weeks before the experiments were performed (between 9 a.m. and 11 a.m.). Rats were anesthetized with chloral hydrate (350 mg/kg i.p.), the preganglionic nerves to the superior cervical ganglia were exposed bilaterally and mounted on bipolar platinum electrodes. Both preganglionic cervical sympathetic trunks were stimulated with square wave pulses generated by a constant current stimulator (25 Hz, 1 ms pulse duration) for 20 min at supramaximal voltage. This was determined by the exopthalmic response produced by the stimulation, the effectiveness of which was also monitored by that response. Controls were obtained by treating rats in the same manner but omitting the passage of current through the electrodes. After stimulation (11 rats) or sham-stimulation (8 rats), the animals were decapitated and the pineal gland quickly removed.

For ultrastructural studies, each gland was disected in a drop of saline and its fragments were treated according to the procedure for the simultaneous cytochemical demonstration of noradrenaline and serotonin (glutaraldehyde-dichromate, GD reaction), or for the demonstration of serotonin (formaldehyde-glutaraldehyde-dichromate, FGD reaction), the specificities of which have been previously tested (Jaim-Etcheverry and Zieher, 1968b). For the GD reaction, tissue was fixed in 3 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 3 h, washed in 0.32 M sucrose in the same buffer, incubated in 2.5 % potassium dichromate in 0.1 M phosphate-citrate buffer, pH 5.0, overnight, dehydrated in ethanol and embedded in Epon. All operations were carried out at 4°C. The FGD procedure involved fixation of the tissue in 8 % paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, overnight before treatment as described for the GD reaction. Pineals were also fixed according to the

procedure described by Tranzer and Richards (1976): 1% glutaraldehyde and 0.4% paraformaldehyde in 0.1 M chromate-dichromate buffer, pH 7.2, for 10 min, incubated for 18 h in 0.1 M chromate-dichromate buffer, pH 6.0, refixed in 2% osmium tetroxide in the same buffer used for primary fixation for 1 h, dehydrated in ethanol and embedded in Epon. All operations were carried out at 4° C. Finally, tissue was also fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3, for 1 h, washed in 7% sucrose in the same buffer, and postfixed in 1% osmium tetroxide in cacodylate buffer for 1 h.

The content of endogenous noradrenaline in pineals of control and stimulated animals was determined with a radioenzymatic procedure (Coyle and Henry, 1973). Each pineal was homogeneized in 200 µl of 0.1 N perchloric acid and its noradrenaline content was assayed in triplicate. Noradrenaline was converted to its 3-0-methyl derivative by incubating 50 µl of the homogenate for 60 min at 37° C in the presence of catechol-0-methyl-transferase and 2.5 µCi of ^{3}H-S-adenosylmethionine (New England Nuclear, sp. act. 55–85 Ci/mmol). The ^{3}H-0-methyl derivatives formed were isolated by selective extraction, transferred to an aqueous phase and oxidized with sodium metaperiodate to separate derivatives of dopamine from those of noradrenaline. ^{3}H-methyl vanillin was extracted into toluene and the radioactivity determined by liquid scintillation spectrometry.

Results

The results obtained in pineals processed with the cytochemical method for the demonstration of serotonin (FGD reaction) were indistinguishable from those obtained in tissue processed with the technique showing the presence of both noradrenaline and serotonin (GD reaction), both in controls and in electrically stimulated rats. Pineals of rats, the preganglionic nerves of which were sham-stimulated, possessed structures containing clusters of mixed small and large reactive sites (Fig. 1a and c). Their size and frequency correspond to those of the dense cores observed in nerve vesicles in conventionally processed preparations. Stimulation markedly diminished the number of cores present in nerve processes (Fig. 1b and d). However, these could be positively identified due to the persistence of some scattered small reactive sites and of larger sites. The latter, corresponding to cores of LGVs, were little affected by stimulation, and they appeared to be present in approximately the same amount in control and stimulated nerves. A quantitative analysis of their occurrence in both situations could not be made, however, because the osmication (that this would require) interferes with the specificity of the cytochemical reaction. The cores of LGVs have, in addition to the amine, another component reacting with osmium, and therefore the presence or density of cores after osmium fixation is independent of their amine content (Jaim-Etcheverry and Zieher, 1969).

Changes produced by nerve stimulation in the overall morphology of the nerves were studied by using a fixation method that, unlike the cytochemical techniques so far described, involves osmication and reveals cell membranes, and at the same time effectively preserves the dense cores of the vesicles (Tranzer and Richards, 1976). In unstimulated nerves, most of the vesicles had a dense reactive core (Fig. 2a), and after stimulation, the cores remained unaltered in the LGVs but disappeared almost completely from the SGVs (Fig. 2b). Moreover, a change in the shape of the vesicles was apparent: they were mostly round in shape in control endings (Fig. 2c) but appeared flattened after stimulation (Fig. 2d). Numerous tubular profiles, empty vesicles of varying diameter and swollen mitochondria were also characteristic of stimulated terminals. The change in the shape of the vesicles produced by stimulation made it difficult to make comparative measurements between

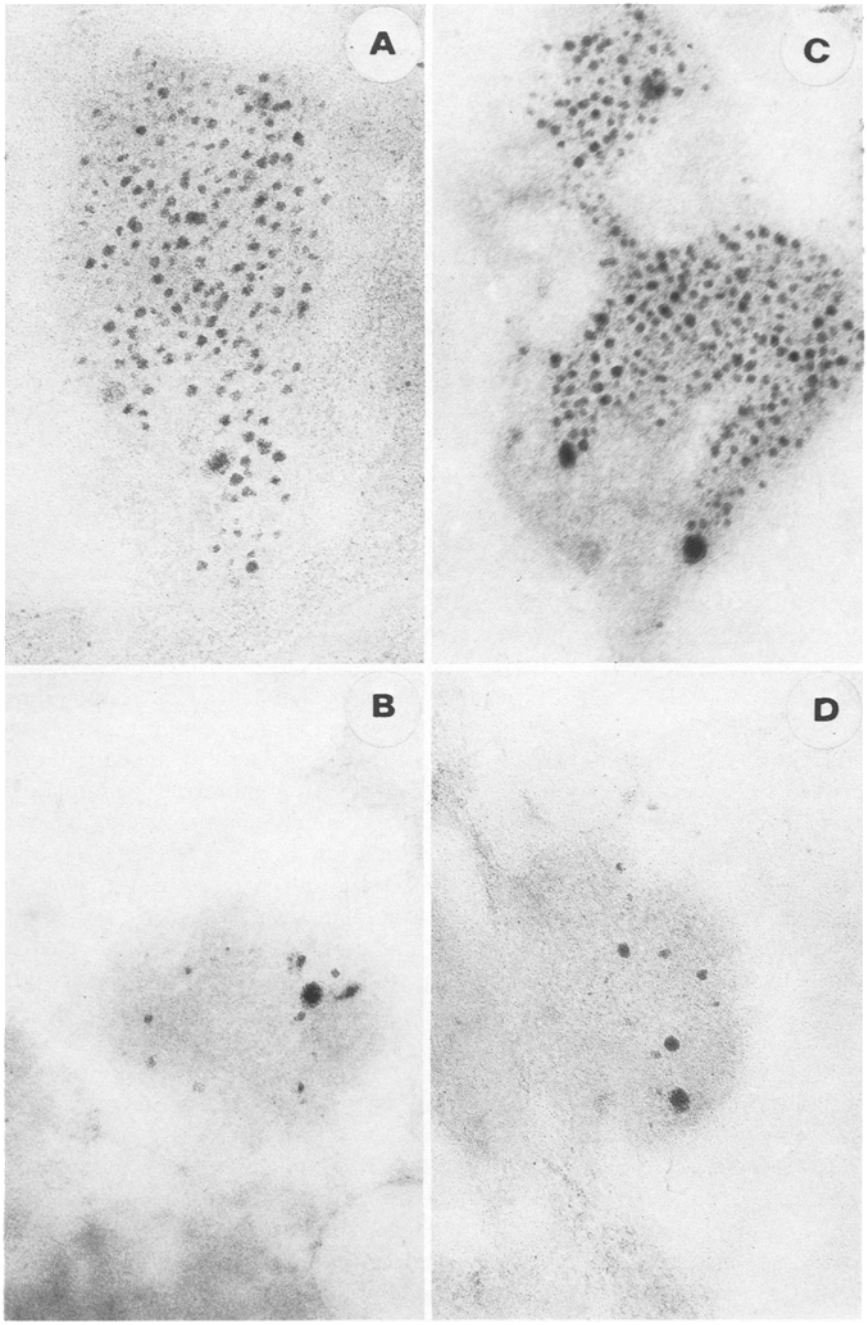

Fig. 1 A–D. Sympathetic nerve endings in the pineal gland of the rat. **A** Small and large reactive sites are observed in tissue from a sham-stimulated rat, processed for the demonstration of serotonin (FGD reaction). These correspond to the cores of small and large granular vesicles found in conventionally fixed tissue, respectively. × 50,000. **B** Tissue processed as in **A**. Preganglionic trunks of a rat bilaterally stimulated (25 Hz, 1 ms duration, 20 min). A few small and large reactive sites can be observed. × 55,000. **C** Similar to **A**, but tissue was processed with the GD reaction for the demonstration of catecholamines and serotonin. × 50,000. **D** Tissue processed as in **C**. Stimulation depletes the reactive sites corresponding to the cores of small granular vesicles. × 60,000

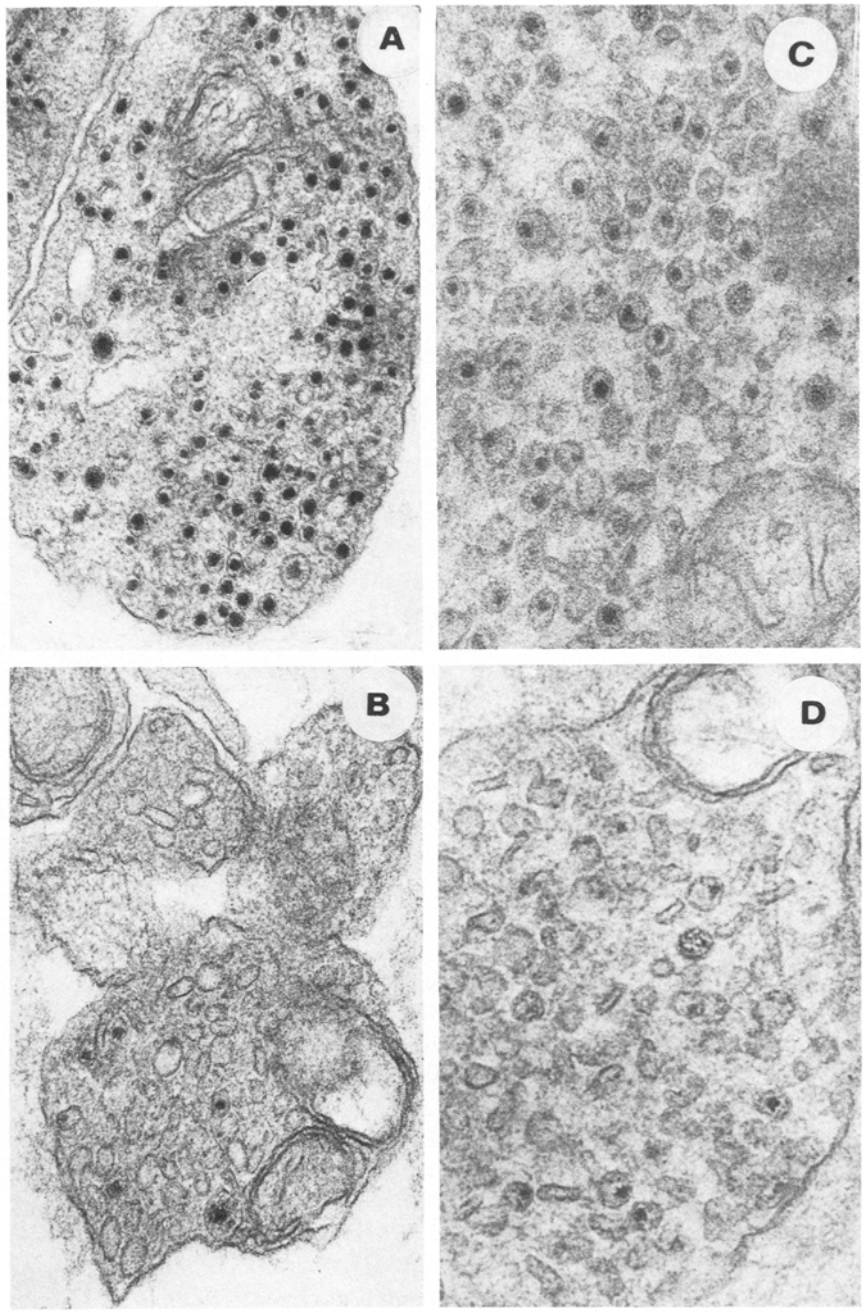

Fig. 2A–D. Sympathetic nerve endings in the pineal gland of the rat. **A** Terminal of a control rat processed according to the technique of Tranzer and Richards (1976). Most of the vesicles contain a dense core and are round in shape. A few large granular vesicles can be observed. × 52,000. **B** Tissue processed as in **A**. Preganglionic trunks of a rat electrically stimulated. Most of the small vesicles are empty except for a few that contain a very small dense core. A large granular vesicle is also present. × 60,000. **C** Nerve terminal in the pineal of an unstimulated rat. Tissue fixed in glutaraldehyde and osmium. Numerous small, round granular vesicles are present. × 87,000. **D** Nerve stimulation depletes the dense cores from most of the small vesicles and changes their shape. Tubular profiles, flattened vesicles and large empty vesicles, as well as a swollen mitochondrion can be observed. Tissue processed as in **C**. × 89,000

diameters of unstimulated and stimulated nerves; however, vesicles appeared to be larger in stimulated nerves. Preliminary observations show that the morphological and cytochemical changes produced in pineal nerves by stimulation for 60 min at 10 Hz are similar to those described after 20 min at 25 Hz.

By using a sensitive radioenzymatic method for the determination of noradrenaline, it was possible to show that the stimulation of pineal nerves significantly reduces the levels of the amine in the gland. In control sham-stimulated rats, the content of noradrenaline in the pineal (mean value of 10 determinations $\pm$ s.e.m.) was 5.22 ± 0.75 ng/pineal. After bilateral stimulation of preganglionic nerves, noradrenaline was reduced by 42% ($p < 0.01$ when compared with controls, t test) (3.02 ± 0.33 ng/pineal).

Discussion

On the basis of evidence obtained from biochemical studies, Fillenz (1977) and others (Nelson and Molinoff, 1976) have proposed that SGVs constitute the storage sites of the neurotransmitter pool readily available for release by stimulation. This view has received morphologic support from the description of changes produced in the nerves of the vas deferens by in vitro stimulation (Coté et al., 1970; Basbaum and Heuser, 1979) as well as from the present results obtained after stimulation of pineal nerves in vivo. This mode of stimulation seems closer to the physiological situation since the stimulus does not act directly on the nerve terminal but must traverse the ganglionic synapse to reach the sympathetic neurons. The reduction produced by electrical stimulation in the number of small reactive sites in nerves processed with the FGD reaction for serotonin as well as after the GD reaction showing noradrenaline and serotonin, indicates that both amines are depleted from their vesicular stores. While SGVs are greatly modified by nerve stimulation, LGVs do not seem to undergo important changes; their number and appearance are not altered by nerve stimulation in vitro as shown by the use of conventional procedures (Basbaum and Heuser 1979), and they retain cytochemically demonstrable monoamines in pineal nerves stimulated in vivo. These findings are compatible with the concept that LGVs represent the storage site of a less readily releasable pool of the amine (Fillenz 1977).

The modifications produced by nerve stimulation in the shape and size of the vesicles are similar to those described in adrenergic nerves after in vitro electrical stimulation (Basbaum and Heuser 1979) and also after the administration of high doses of reserpine (Pellegrino de Iraldi and De Robertis 1961; Hökfelt 1973). These morphological changes are most probably related to the process of vesicle recycling that seems to take place after release (Basbaum and Heuser 1979).

Nerve stimulation triggers several mechanisms involved in the maintenance of neurotransmitter content at a level compatible with synaptic function, e.g., the acceleration of its synthesis (Fillenz 1977). Thus, it is not surprising to find controversial results concerning the effects of nerve stimulation on endogenous transmitter stores (Van Orden et al. 1972; Fillenz and Howe 1975). They most probably reflect differences in the balance between the intensity and duration of stimulation and the mechanisms maintaining transmitter supplies for release.

Changes in the content of noradrenaline were studied because those of neuronal serotonin are difficult to identify due to the presence of a large pool of extraneuronal amine contained in the pinealocytes and regulated by the activity of adrenergic nerves. The depletion of noradrenaline (42%) was lower than expected from the intensity of the cytochemical changes described. This discrepancy could be justified by the fact that (i) some cores remained in the SGVs, (ii) by the amine contained in the LGVs, or (iii) by its storage in some compartment either inside or outside the vesicles not demonstrable by the cytochemical procedure used. Almost certainly the sensitivity of the cytochemical method for the ultrastructural localization of the amines is much lower than that of biochemical assays.

The liberation of noradrenaline and serotonin from the vesicles of pineal sympathetic nerves raises the question of the relevance of this mechanism for the control of pineal activity and, more generally, of the physiological significance of the simultaneous release of several substances with presumed transmitter or modulatory functions stored within a single nerve terminal.

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