Cell Tissue Res (1983) 233:463–469

Cell and Tissue Research

Ultrastructural evidence for monoamine uptake by vesicles of pineal sympathetic nerves immediately after their stimulation

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Summary. Bilateral electrical stimulation of the preganglionic fibers to the superior cervical ganglia of the rat markedly reduces the number of osmiophilic dense cores present in the nerve vesicles of the sympathetic fibers in the pineal gland. These cores owe their density to the presence of noradrenaline and serotonin in the vesicles. When sympathetic nerves of the pineal organ are exposed immediately after stimulation for a brief period to the false neurotransmitter 5-hydroxydopamine, either in vitro or in vivo, dense precipitates reappear in the electron-lucent vesicles. On the basis of these observations, it is concluded that the vesicles remaining in the nerves after releasing their neurotransmitter content have the capacity to take up and store monoamines. This provides a morphological correlate for the recent biochemical evidence suggesting that the vesicles in sympathetic nerves are reused after neurotransmitter release.

Key words: Sympathetic nerves – Synaptic vesicles – Vesicle recycling – Nerve stimulation – Pineal gland (rat)

The chemical transmitters used by neurons for synaptic communication are stored in the vesicles present in their terminal portions (De Robertis and Bennet 1955). Since these vesicles play a crucial role in the transmission of nerve impulses, numerous attempts have been made to define the stages of their life cycle by studying neurons that contain acetylcholine or noradrenaline (NA) under different experimental conditions (Zimmermann 1979). Noradrenergic neurons are particularly suitable for establishing the structural correlates of the release process because the stored catecholamines form an electron-dense precipitate with several fixatives used for ultrastructural studies (Pellegrino de Iraldi and De Robertis 1961; Snipes et al. 1968; Tranzer et al. 1969; Hökfelt 1971; Jaim-Etcheverry and Zieher 1971;

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Tranzer and Richards 1976). Thus, in this case, the neurotransmitter itself serves as a morphological marker for the integrity of vesicular function.

Recent investigations have confirmed the usefulness of peripheral sympathetic endings for the morphological analysis of transmitter release, both in vivo and in vitro (Coté et al. 1970; Basbaum and Heuser 1979; Wakade et al. 1982; Pollard et al. 1982). For example, in the rat the electrical stimulation of the preganglionic fibers running to the superior cervical ganglia dramatically reduces the number of dense cores in vesicles of sympathetic nerves that originate in these ganglia and innervate the pineal gland. Both NA and serotonin are contained in the cores of the large (LGVs) and small granular vesicles (SGVs) characteristic of these nerves (Jaim-Etcheverry and Zieher 1968; Pellegrino de Iraldi and Gueudet 1969). The disappearance of the cores from small vesicles after stimulation reflects the depletion of both amines as confirmed by cytochemical studies at the ultrastructural level (Jaim-Etcheverry and Zieher 1980a; Pellegrino de Iraldi and Corazza 1981).

Biochemical data are now available suggesting that noradrenergic vesicles can be reused after transmitter release (Wakade 1979; Wakade and Wakade 1982; Wakade et al. 1982). To examine this mechanism further, we analyzed the ability of vesicles in pineal nerves of the rat to take up and retain exogenous amines after stimulation. For this purpose, 5-hydroxydopamine (5-OH-DA), a false neurotransmitter that behaves like NA but strongly reacts with oxidizing agents such as osmium tetroxide and potassium dichromate was used, thus providing a useful tool for the ultrastructural identification of sympathetic nerve vesicles (Tranzer and Thoenen 1967). A preliminary account of these experiments was previously presented (Jaim-Etcheverry and Zieher 1980b).

Materials and methods

Male rats of the Wistar strain weighing 200–300 g were used in this study and kept in a controlled environment with lights on from 7 a.m. to 7 p.m. for more than three weeks prior to the experiments. Between 9 a.m. and 11 a.m., an incision was made in the neck of animals 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 sympathetic trunks were electrically stimulated with square wave pulses generated by a constant current stimulator. The pulses of 1 ms duration were delivered at supramaximal voltage, established by the exophtalmic response that also served to determine the effectiveness of the stimulation. The frequency at which the pulses were delivered and the total duration of the stimulation varied as described below. Anesthetized rats undergoing a similar surgical procedure but in which the passage of current through the electrodes was omitted served as controls (sham-operated animals).

1. In vitro uptake of 5-hydroxydopamine: Preganglionic nerves were stimulated at 25 Hz for 20 min (total number of impulses 30000) in 11 rats. At the end of the stimulation period, the pineal was quickly removed, cut in two halves in a drop of saline and the fragments incubated for 10 min at 37° C in Krebs Ringer gassed with 95% O_2 – 5% CO_2 (composition of medium in g/l: NaCl 7.0; KCl 0.35; $CaCl_2$ 0.13; KH_2PO_4 0.16; $MgSO_4 \cdot 7H_2O$ 0.29; $Na_2PO_4 \cdot 2H_2O$ 2.92; glucose 1.0; pH adjusted to 7.4 with 1 M Na_2CO_3). One half of the gland was incubated in medium alone while the other (from the same gland) was incubated in Krebs Ringer containing 330 µg/ml 5-hydroxydopamine bromhydrate (5-OH-DA; F. Hoffmann La Roche, Basel Switzerland) (expressed as the free base). After a brief rinse in Krebs Ringer,

sham-stimulated (5) and stimulated glands (6) incubated in Krebs Ringer alone or with the addition of 5-OH-DA were fixed for electron microscopy.

2. In vivo uptake of 5-hydroxydopamine: In a group of 20 animals nerves were stimulated at 10 Hz for 60 min (total number of impulses 36000). At the end of the stimulation period, the rats received an i.v. injection of saline (controls) or 20 mg/kg 5-OH-DA, dissolved in saline, and were killed by decapitation 15 min after the injection. Pineals from sham-stimulated rats injected with saline (4) or with 5-OH-DA (3) and of rats stimulated and injected with saline (7) or with 5-OH-DA (6), were quickly removed and immersed in fixative for ultrastructural study.

For electron microscopy, pineal glands were processed according to the method described by Tranzer and Richards (1976). The primary fixation was carried out in a solution containing 1% glutaraldehyde and 0.4% paraformaldehyde in 0.1 M chromate-dichromate buffer, pH 7.2, for 10–15 min. The tissue was subsequently immersed for 18 h in 0.1 M chromate-dichromate buffer, pH 6.0, postfixed in 2% osmium tetroxide in the same buffer used for the primary fixation, dehydrated in ethanol and embedded in Epon. All operations were carried out at 4° C. Sections were observed under a Siemens Elmiskop I electron microscope after staining with lead citrate.

Results

The sympathetic fibers from the superior cervical ganglia that innervate the pineal gland of control rats (sham-stimulated) contained the characteristic population of small and large vesicles, the majority of them displaying an osmiophilic dense core (Fig. 1 A). Incubation of pineals of sham-stimulated rats in the presence of 5-OH-DA increased the number and the size of the dense cores of both types of granular vesicles (Fig. 1 B). In nerves of pineal glands removed from animals killed immediately after stimulation and incubated for 10 min in Krebs Ringer, the dense cores of small vesicles were markedly depleted and the majority of the remaining vesicles were electron-lucent (Fig. 1 C). Moreover, as previously described (Jaim-Etcheverry and Zieher 1980a; Pellegrino de Iraldi and Corazza 1981), while in controls the vesicles were round in shape, most of those emptied by electrical stimulation appeared flattened or elliptical and were larger in diameter. Neither the number nor the appearance of LGVs appeared to be modified by nerve stimulation. The addition of 5-OH-DA to the incubation medium caused the reappearance of the cores in most SGVs, irrespective of their shape although, in general, vesicles were less filled than in control nerves exposed to 5-OH-DA.

In rats killed 15 min after the injection of saline at the end of the electrical stimulation of both preganglionic trunks, small vesicles in sympathetic nerves were mostly electron-lucent because the dense cores were substantially reduced in number in comparison to controls (Fig. 2 A). When 5-OH-DA was injected after stimulation 15 min before killing the animals, dense cores reappeared in all the small vesicles, round, elliptical or tubular in shape (Fig. 2 B).

The morphological changes produced in pineal nerves by electrical stimulation were similar when preganglionic fibers were stimulated at 25 Hz for 20 min (Fig. 1 C) or at 10 Hz for 60 min (Fig. 2 B). Under both conditions, the total number of impulses was roughly the same.

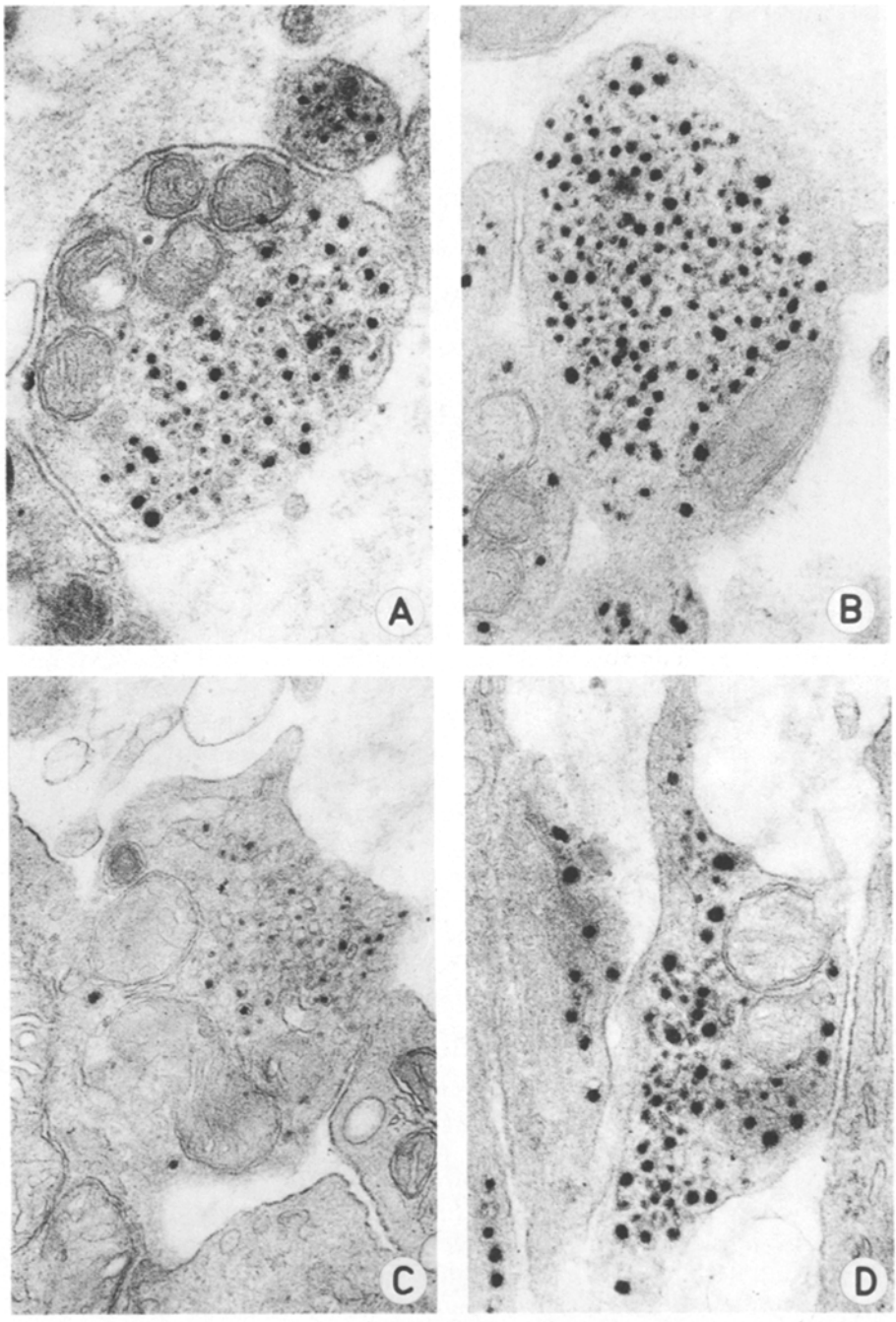

Fig. 1 A–D. Sympathetic nerve endings in the pineal gland of the rat processed according to the technique of Tranzer and Richards (1976). **A** Nerves of sham-stimulated rats contain dense osmiophilic cores in both small and large synaptic vesicles. **B** Exposure of nerves from sham-stimulated rats to 5-OH-DA in vitro (330 μg/ml) increases the size and density of the dense cores in nerve vesicles. **C** Most of the small vesicles are electron-lucent in sympathetic terminals of pineals incubated in Krebs Ringer for 10 min after the bilateral stimulation of preganglionic fibers. **D** In the other half of the pineal from the same stimulated animal incubated in the presence of 5-OH-DA, dense reactive cores reappear in the electron-lucent vesicles. × 56000

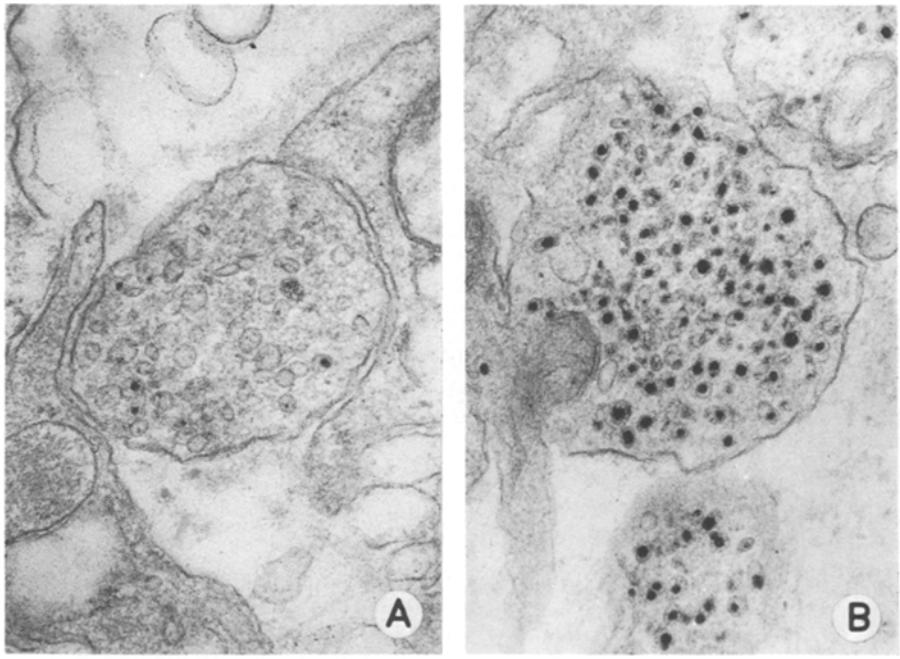

Fig. 2A–B. Sympathetic nerve endings in the pineal gland of the rat processed according to the technique of Tranzer and Richards (1976). **A** The vesicles of nerves of stimulated animals injected with saline immediately after stimulation and killed 15 min later are markedly depleted of their dense cores. **B** When 5-OH-DA (20 mg/kg i.v.) is injected after stimulation, the dense reactive precipitates reappear. × 56000

Discussion

Stimulation of the preganglionic fibers that run to the superior cervical ganglia markedly depletes the dense cores characteristic of the vesicles found in the postganglionic sympathetic terminals of the pineal gland of the rat (Jaim-Etcheverry and Zieher 1980a; Pellegrino de Iraldi and Corazza 1981). This observation confirms previous reports showing that different forms of nerve stimulation, either in vivo or in vitro, release endogenous NA from its vesicular storage sites (Fillenz 1977; Pollard et al. 1982).

Recent evidence also suggests that when nerve stimulation depletes these vesicles of their transmitter content, they retain the ability to take up and release NA or related amines (Wakade 1979; Wakade and Wakade 1982; Wakade et al. 1982; Pellegrino de Iraldi and Corazza 1983). The present results provide morphological evidence supporting such a mechanism since vesicles in pineal terminals depleted of their dense cores by nerve stimulation are capable of actively accumulating the false neurotransmitter 5-OH-DA. The exposure of sympathetic nerves after stimulation to 5-OH-DA either in vivo or in vitro causes the reappearance of dense cores in the small electron-lucent vesicles.

Several observations suggest that this uptake process takes place in the

same vesicles present in the nerves after stimulation: 1) The majority of small vesicles are electron-lucent after stimulation and almost all of them have a dense core after exposure to 5-OH-DA. 2) This exposure is brief, making it difficult to assume that the vesicular population is totally replaced in such a short interval, particularly in the in vitro situation. 3) The morphological characteristics of the vesicular profiles with dense precipitates after exposure of stimulated nerves to 5-OH-DA are similar to those observed in the electron-lucent vesicles that remain after stimulation and differ from the aspect of the vesicles in resting nerves. 4) In stimulated nerves exposed to 5-OH-DA, vesicles seem to be less filled by the dense precipitate that in control nerves exposed to the false neurotransmitter.

These observations support the growing body of evidence indicating that the vesicles remaining in sympathetic nerves after stimulation are capable of synthesizing, taking up, retaining and releasing exogenous neurotransmitters (Wakade and Wakade 1982). From this type of analysis, it is not possible to determine whether the vesicles found in the nerves after stimulation are exactly the same ones that were depleted of their cores or whether they have been reformed by some process, such as the proposed recycling after fusion of their membranes with that of the terminal (Heuser and Reese 1973; Basbaum and Heuser 1979; Zimmermann 1979). In any case, the vesicles either maintain or regenerate very rapidly the mechanisms required for the Mg^{2+}-ATP-dependent uptake of the neurotransmitter and for the formation of the complex in which it is stored. The careful analysis of the morphological correlates of the processes of release and re-uptake of transmitters in sympathetic nerve vesicles after stimulation will undoubtedly contribute to clarify the crucial problem posed by the fate of these complex organelles after release of neurotransmitters.

Acknowledgements. This work was supported by grants from CONICET, SUBCYT and the Cargill Foundation, Argentina. The technical assistance of Mrs. Alba M. de Novara and Ms. Silvia Trejo is gratefully acknowledged.

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Accepted May 13, 1983