Axonal transport of muscarinic receptors in vesicles containing noradrenaline and dopamine-β-hydroxylase

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Presynaptic muscarinic receptors labeled with [^{3}H]dexetimide and noradrenaline in dog splenic nerves accumulated proximally to a ligature at the same rate of axonal transport. After fractionation by differential centrifugation, specific [^{3}H]quinuclidinyl benzilate or [^{3}H]dexetimide binding revealed a distribution profile similar to that of dopamine-β-hydroxylase and noradrenaline. Subfractionation by density gradient centrifugation showed two peaks of muscarinic receptors; the peak of density 1.17 contained noradrenaline and dopamine-β-hydroxylase whereas that of density 1.14 was devoid of noradrenaline. Therefore the foregoing experiments provide evidence that presynaptic muscarinic receptors are transported in sympathetic nerves in synaptic vesicles which are similar to those containing noradrenaline and dopamine-β-hydroxylase. This suggests a possible coexistence of receptor and neurotransmitter in the same vesicle.

Muscarinic receptor *Axonal transport* *Dopamine-β-hydroxylase* *Synaptic vesicle*

1. INTRODUCTION

In sympathetic nerves, axoplasmic flow is responsible for the transport of noradrenaline and dopamine-β-hydroxylase from the cell body to the nerve terminals [1,2]. Noradrenaline release from nerve endings is controlled by various regulatory mechanisms which imply, namely, the occurrence of presynaptic muscarinic receptors: the stimulation by acetylcholine of these receptor sites elicits an inhibitory effect on noradrenaline release [3,4]. More direct evidence for this regulatory mechanism was provided when muscarinic receptors were found to accumulate on both sides of a ligature in dog splenic nerves, thus demonstrating a bidirectional axonal flow for these receptors [5,6]. Similar results were then obtained in cat hypogastric nerve [7] and in rat sciatic nerve [8]. We now report on the axoplasmic transport and the subcellular localization of muscarinic receptors from dog splenic nerves.

2. MATERIALS AND METHODS

Mongrel dogs were anesthesized with pentobarbital (30 mg/kg i.v.) and the splenic nerve fibers were dissected near the bifurcation of the splenic artery. A silk thread ligature was tied around the splenic nerve. At different times after the operation, 1-cm segments proximal to the ligature were removed; from control animals, the whole nerve was dissected. After removing connective tissue, the splenic nerves were cut in small pieces and then homogenized in 30 vols of 0.25 M sucrose with a Duall homogenizer. The total homogenate so obtained was used as such.

2.1. *Differential and density gradient centrifugation*

The total homogenate was submitted to differential centrifugation to give 5 pellets and a final supernatant as in [9]; P_1 fraction at $6596 \times g_{av}$ for 8 min; P_2 and P_3 at $20203 \times g_{av}$ for 15 and 30 min,

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respectively; P_4 and P_5 at 55364 × g_{av} for 22 and 35 min, respectively. The pellets were suspended in 2 vols of distilled water and used immediately for binding assays or stored at −20°C for enzyme and protein measurement.

For density gradient experiments, a particulate fraction was prepared as follows: after having centrifuged total homogenate at low speed (5000 rpm, 10 min), the obtained pellet was resuspended in 30 vols of 0.25 M sucrose and again homogenized and centrifuged. Both supernatants were pooled, filtered through one layer of surgical gauze and centrifuged at 30000 rpm for 30 min in a Ti65 Rotor of a Spinco centrifuge. The pellet was suspended in 1–2 vols of 0.25 M sucrose; 4 ml was layered on a 36-ml continuous gradient of sucrose (0.4–2 M) and allowed to equilibrate at 2°C in a vertical VTI rotor (Spinco centrifuge) for 1 h at 30000 rpm. Then 16 fractions of 2.5 ml were collected and 1.5 ml was diluted with distilled water for immediate use in binding assays, the remainder being stored at −20°C.

2.2. *Binding assays and marker enzymes*

Binding on muscarinic receptors was carried out using either [^{3}H]dexetimide (spec. act. 17 Ci/mmol; I.R.E., Fleurus, Belgium) or [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) (spec. act. 31 Ci/mmol; NEN, Boston). Binding was performed with 0.1 nM [^{3}H]dexetimide and 0.5 nM [^{3}H]QNB in 5 ml of 50 mM phosphate buffer (pH 7.4) and the rapid filtration technique was used as in [10]. Before use, the filters were washed with 1% BSA to reduce non-specific binding. Specific binding was the difference between total binding and binding in the presence of 2 × 10^{-7} M dexetimide (blank).

Dopamine-β-hydroxylase was assayed as in [11]. Marker enzymes and protein were measured as in [12]. Noradrenaline was measured according to [13].

3. RESULTS

At different times after ligation, [^{3}H]dexetimide binding and noradrenaline were determined in 1-cm segments of dog splenic nerves above the ligature. Previous studies have shown that the [^{3}H]dexetimide binding sites in these sympathetic nerves are of muscarinic nature [6]. Fig.1 shows

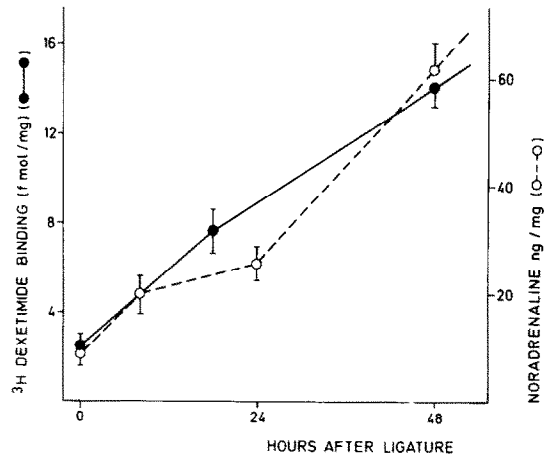

Fig.1. Time course of the accumulation of stereospecific [^{3}H]dexetimide binding and noradrenaline in 1-cm segments of dog splenic nerves above (proximal to) a ligature. Each point represents the mean value obtained from 5 or 6 animals (± SE).

that both noradrenaline and muscarinic receptors increased linearly as a function of time. Interestingly, the accumulation in the proximal part of the nerves was the same for both constituents. In contrast, the accumulation was not detectable when [^{3}H]levetimide, the pharmacologically inactive enantiomer of dexetimide, was used as ligand [6].

To determine the intracellular localization of muscarinic receptors in sympathetic nerves, various fractions of dog splenic nerves were prepared by differential centrifugation. Muscarinic receptors were measured in vitro in all the fractions using either [^{3}H]dexetimide or [^{3}H]QNB as ligand and their distribution profiles were compared to those of marker enzymes and of noradrenaline. Fig.2 shows the distribution pattern of muscarinic receptors after differential centrifugation; specific [^{3}H]dexetimide and [^{3}H]QNB binding were found mainly enriched in the P_3–P_5 fractions. The recovery, a little higher than 100%, was probably due to the presence in the total homogenate of substances inhibiting the binding. Interestingly, the distribution profiles of dopamine-β-hydroxylase, noradrenaline and 5′-nucleotidase were very similar to that of [^{3}H]QNB and [^{3}H]dexetimide binding. In contrast, cytochrome oxidase and *N*-acetylglucosaminidase revealed a quite different profile; they were mainly

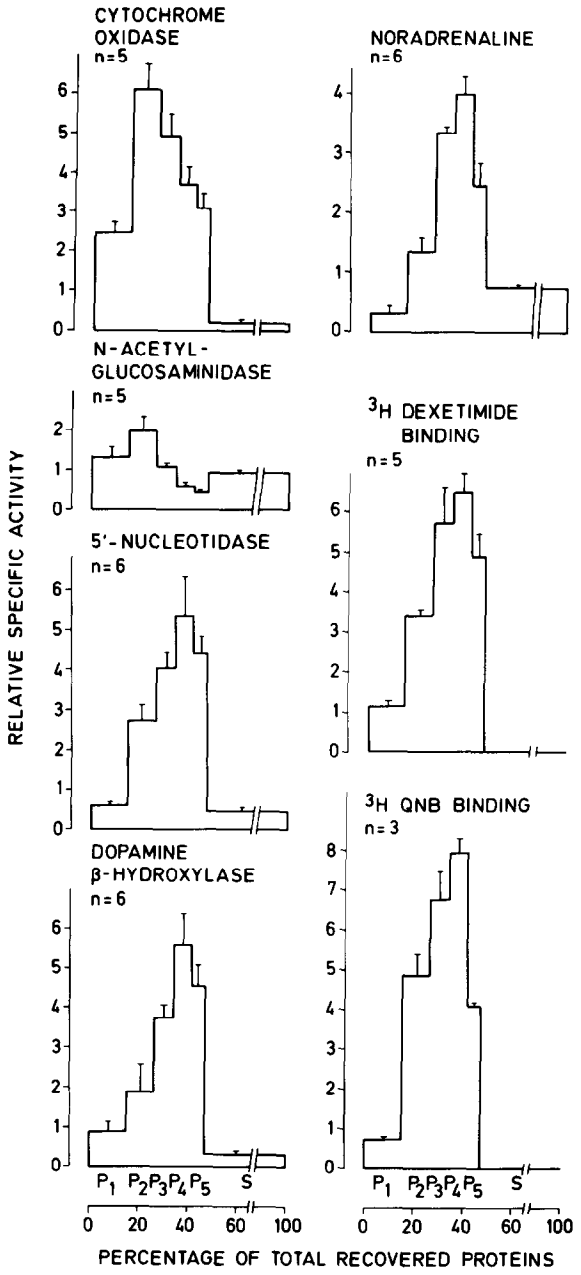

Fig.2. Distribution patterns of [^{3}H]QNB and [^{3}H]dexetimide binding, noradrenaline and marker enzymes in subcellular fractions of dog splenic nerves. The results are presented graphically in histograms following [14]. The ordinate represents the relative specific activity (RSA), i.e., the percentage of activity in a given fraction against the percentage of protein recovered in this fraction. The results are means of different determinations (n) (± SE). Recovery of [^{3}H]dexetimide and [^{3}H]QNB binding, noradrenaline and 5′-nucleotidase was 122, 108, 102 and 92%, respectively.

recovered in the first fractions, the highest relative specific activity being in the P_2 fraction. Note that a large amount (73%) of the lysosomal enzyme appeared in the supernatant, thus in a non-sedimentable form, which suggests that lysosomes had undergone much damage during the homogenization. In contrast, noradrenaline-containing vesicles appeared to be much less affected by this treatment.

Subfractionation of muscarinic receptors from dog splenic nerves was carried out by submitting a particulate fraction, which involves the P_2–P_5 fractions, to equilibration in sucrose density gradients. Because of the high non-specific binding of [^{3}H]dexetimide in sucrose solutions, [^{3}H]QNB was preferred for labelling muscarinic receptors. Fig.3 shows that in isopycnic centrifugation, [^{3}H]QNB binding revealed two peaks; the first one equilibrated in zones of high density (peak 1.17); this peak corresponds to the distribution profile of noradrenaline and partly of the dopamine-β-hydroxylase. The second peak of [^{3}H]QNB binding

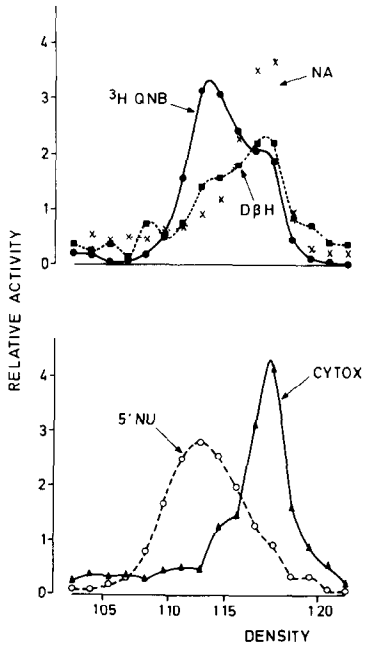

Fig.3. Distribution of [^{3}H]QNB binding, dopamine-β-hydroxylase, noradrenaline, cytochrome oxidase and 5′-nucleotidase after isopycnic centrifugation of a particulate fraction from dog splenic nerves in a sucrose gradient. All recoveries ranged between 92 and 100%.

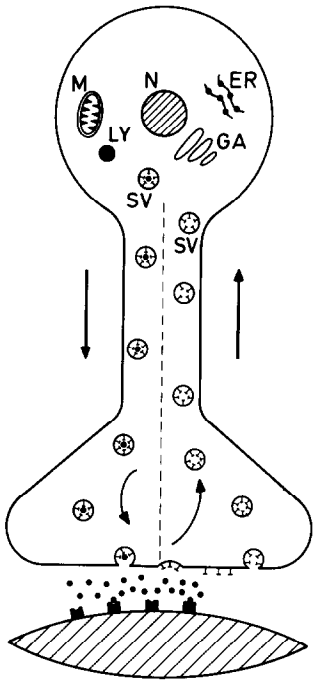

Fig.4. Model illustrating the bidirectional axonal transport in sympathetic nerves of muscarinic receptors which are or are not associated with noradrenaline in synaptic vesicles (SV). N, nucleus; M, mitochondrion; ER, endoplasmic reticulum; GA, Golgi apparatus; LY, lysosome.

was found in areas of lower density (peak 1.14); here only dopamine-β-hydroxylase was present but the amount of enzyme was less than in the peak 1.17. 5′-Nucleotidase was recovered in lower sucrose concentrations (1.13).

4. DISCUSSION

The foregoing results indicate that presynaptic muscarinic receptors in dog splenic nerves are transported in synaptic vesicles like noradrenaline and dopamine-β-hydroxylase. Sympathetic nerves are known to contain muscarinic receptors [5–8]; the recent observation that muscarinic receptors from rat spleens markedly decreased following chemical sympathectomy with 6-hydroxydopamine supports this view [15]. Similar results were recently obtained in our laboratory (in preparation).

In sympathetic nerves, fast axonal flow was reported for noradrenaline and dopamine-β-hydroxylase [1,2,16] and more recently for muscarinic receptors [5,6]. All the other enzymes so far measured from mitochondria, lysosomes, plasma membrane or cytosol were found to move at a much slower rate than the synaptic vesicles [17,18]. Both muscarinic receptors and noradrenaline accumulated above a ligature on the splenic nerves at the same rate which was estimated to be 1.2 mm/h, a value which is in good agreement with previous values reported for dopamine-β-hydroxylase and noradrenaline [1,2,16]. This suggests that both could be located within the same organelle.

Fractionation experiments provided more direct evidence for close association of muscarinic receptors with synaptic vesicles. First after differential centrifugation, the muscarinic receptors labelled either with [^{3}H]QNB or [^{3}H]dexetimide were recovered in particles sedimenting at relatively high speed together with dopamine-β-hydroxylase, noradrenaline and 5′-nucleotidase. In contrast, mitochondrial and lysosomal enzymes revealed a completely different pattern, which rules out a mitochondrial or lysosomal localization for the receptors.

Sucrose density gradients revealed two peaks (density 1.17 and 1.14) which exactly corresponded to those of dopamine-β-hydroxylase and to that of noradrenaline. 5′-Nucleotidase was found to equilibrate in a region of lower density and did not accumulate rapidly on both sides of a ligature (not shown) thus confirming that marker enzymes of plasma membranes are not associated with vesicles containing noradrenaline and dopamine-β-hydroxylase [9,18–20]. Consequently, muscarinic receptors in dog splenic nerves seem to be associated with dense vesicles (1.17) containing noradrenaline and dopamine-β-hydroxylase and with less dense (1.14) vesicles devoid of noradrenaline but containing dopamine-β-hydroxylase. Two peaks of dopamine-β-hydroxylase have been found previously [9,20,21], peak 1.17 being always more pronounced than peak 1.14. The fact that less muscarinic receptors were found in peak 1.17 could be due to the presence of noradrenaline which might inhibit the binding; the IC_{50} of noradrenaline in [^{3}H]dexetimide binding was 20 mM, a concentration which is relatively high but not incompatible with the high content of noradrenaline in the synaptic vesicles [22].

From our results a model can be proposed (fig.4). Synaptic vesicles containing noradrenaline, dopamine-β-hydroxylase and muscarinic receptors (presumably the 'dense-core vesicles') are synthesized and assembled in the cell body of sympathetic nerves and flow down towards the nerve terminals. There, noradrenaline and a part of dopamine-β-hydroxylase are released by exocytosis [19,21,22] and the receptors are externalized, thus becoming functional. After internalization or endocytosis, the receptors together with membrane-bound dopamine-β-hydroxylase could return to the cell body where they are either recycled or degraded. A recycling phenomenon directly in the nerve terminals cannot be excluded. Such a model implies that acetylcholine could play a role as a signal raising the internalization of muscarinic receptors in the nerve terminals [23].

Our experiments suggest the possible coexistence of a receptor and a neurotransmitter in the same organelle. It is too early to decide whether such an association is the rule in neurotransmission.

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