Structure-linked latency of muscarinic receptors in axonal transport

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Muscarinic receptors that accumulated above a ligature in rat sciatic nerves were labelled in vitro under isotonic conditions with *N*-[^{3}H]methylscopolamine. The addition of 0.005% of digitonin doubled the binding in proximal segments above and close to the ligature but not in the intermediary segments between two ligatures. Osmotic shock and freeze-thawing treatments also enhanced the binding. Digitonin did not affect the affinity of muscarinic receptors but revealed a greater number of sites by increasing the membrane permeability to the hydrophilic ligand. We conclude that presynaptic muscarinic receptors that undergo fast axonal transport in rat sciatic nerves exist under a latent form because they are associated with vesicles. This is the first demonstration of a structure-linked latency for receptors.

Axonal transport *Muscarinic receptor* *Latency* *Synaptic vesicle* N-*Methylscopolamine*

1. INTRODUCTION

In the absence of protein synthesis in nerve terminals, axoplasmic transport mechanisms are responsible for delivering macromolecules to nerve endings. The first evidence for axonal transport of neuroreceptors was provided when presynaptic muscarinic receptors were found to accumulate on both sides of a ligature in dog splenic nerves [1]. This finding was confirmed in nerves of various species but namely in rat sciatic nerves [2,3]. Subcellular fractionation of dog splenic nerves revealed that muscarinic receptors coexist with a neurotransmitter, noradrenaline, in the same vesicles [4].

Two conditions must be fulfilled to demonstrate structure-linked latency of an enzyme: (i) the enzyme must be associated with a subcellular organelle and (ii) the membrane of the particle must be impermeable to the substrate. The concept of structure-linked latency of acid hydrolases was determinant for the identification of lysosomes [5]. Other enzymes like dopamine β-hydroxylase were also found under a latent form [6,7].

In applying this concept to binding studies, we found that the muscarinic receptors accumulated above a ligature in rat sciatic nerves are under a latent form when the binding is performed with *N*-[^{3}H]methylscopolamine ([^{3}H]NMS).

2. MATERIALS AND METHODS

Male Wistar rats (200 ± 20 g) were anaesthetized with ether and a silk thread ligature was placed around both sciatic nerves. In some experiments a double ligature was tied on the same nerve at a distance of 1 cm. 16 h later, 3 mm segments of sciatic nerves, two above (proximal to) and two below (distal to) the ligature were removed. The segments pooled from 4 different nerves were homogenized in 3.5 ml isotonic phosphate buffer (310 mosM, pH 7.4) with a type PT-10 Polytron homogenizer (Kinematica, Switzerland) setting at 11000 rpm for 10 s. In some experiments, homogenization was performed in distilled water.

2.1. *Binding and enzyme assays*

The muscarinic receptor binding assay was car-

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ried out in a total volume of 1.1 ml isotonic phosphate buffer using 0.5 nM [^{3}H]NMS (spec. act. 85 Ci/mmol, NEN) and 1 ml homogenate. After incubation at 37°C for 20 min, 5 ml cold buffer was added and the rapid filtration technique was carried out as described in [8]. Before use, the GF/B Whatman filters were washed with 0.5% BSA. Specific binding was defined as the difference between total binding and binding in the presence of 2×10^{-7} M dexetimide.

The free activity of dopamine-β-hydroxylase was measured with tyramine in isotonic acetate buffer (pH 5.0, 310 mosM) as in [6]; the octopamine formed was assayed as in [9].

3. RESULTS

To characterize muscarinic receptors moving in axons through fast transport mechanisms, two ligatures were placed in rat sciatic nerves; 16 h later the proximal P_1 and P_2 segments (above the most proximal ligature) and an intermediary I segment (between both ligatures) were removed and muscarinic receptors and dopamine-β-hydroxylase were measured. Fig.1 shows a marked accumulation of [^{3}H]NMS-binding sites and dopamine-β-hydroxylase activity in the P_1 segment close to the ligature. Digitonin markedly increased the binding and the enzyme activity in this segment when compared to values obtained in isotonic conditions and without detergent. By contrast, the intermediary I and the P_2 segment were minimally affected by the addition of detergent. Fig.2 shows that the effect of digitonin in the [^{3}H]NMS-binding assay was concentration-dependent in the P_1 segment but not in the intermediary I segment. 0.005% was the optimal concentration of digitonin since higher concentrations inhibited the binding. Fig.3 shows that osmotic shock, digitonin and freeze-thawing (3 cycles), alone or in combination, increased [^{3}H]NMS binding in the P_1 segment of ligated sciatic nerves.

Fig.2. Effect of increasing concentrations of digitonin on muscarinic receptors measured with [^{3}H]NMS under isotonic conditions in the proximal P_1 and intermediary I segments of ligated sciatic nerves (cf. fig.1). The value of I, which represents non-moving sites, was subtracted from that of the P_1 segment ($P_1 - I$). Results are means of 4 experiments (± SE).

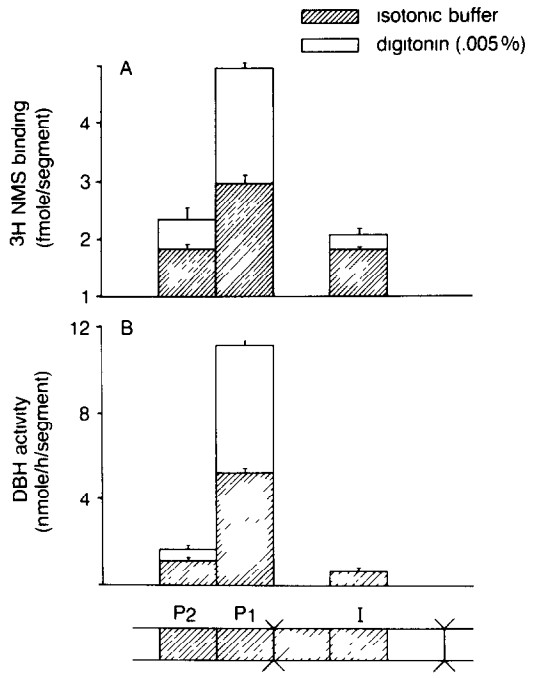
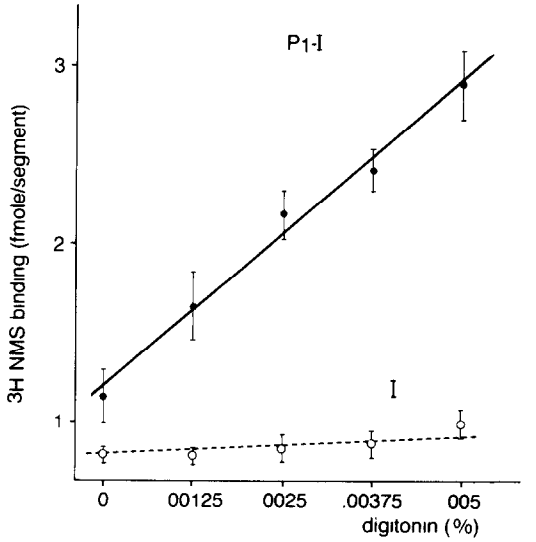


Fig.1. Effect of digitonin on muscarinic receptors measured in vitro with [^{3}H]NMS and on dopamine-β-hydroxylase in different segments of rat ligated sciatic nerves. Two proximal P_1 and P_2 and an intermediary I 3-mm segment were removed 16 h after ligation and then homogenized in isotonic phosphate buffer. Binding and enzyme activity were measured in the presence and absence of 0.005% digitonin. Results are means of 4 experiments (± SE).



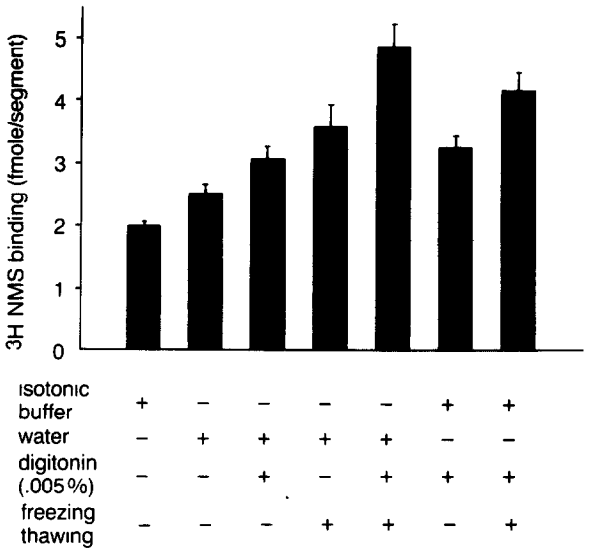


Fig.3. Effect of osmotic shock digitonin and freezing (3 cycles), alone or in combination, on muscarinic receptors measured in vitro with [^{3}H]NMS in P_1 segments of ligated sciatic nerves. Results are means of 4 experiments (± SE).

To show that digitonin did not affect the binding affinity or the pharmacological properties of [^{3}H]NMS binding, Scatchard analysis and displacement curves were performed in homogenates of P_1 and P_2 segments in the presence and absence of detergent. Table 1 shows that neither the K_d nor the IC_{50} values for dexetimide, atropine or levetimide (inactive enantiomer) were altered by the presence of 0.005% digitonin. The values in table 1 are similar to those reported in [3,5].

Table 1

Affinity and pharmacological characterization of [^{3}H]NMS binding in the P_1 and P_2 segment of rat ligated sciatic nerves in the presence and absence of 0.005% digitonin

	[^{3}H]NMS binding			
	Segment P_1		Segment P_2	
Digitonin	−	+	−	+
K_d (nM)	0.43	0.49	0.47	0.5
IC_{50} (nM)				
Dexetimide	6.3	5.7	7.1	5.6
Atropine	16	10	9	10
Levetimide	15000	10000	16000	19000

4. DISCUSSION

The foregoing experiments demonstrate a structure-linked latency for muscarinic receptors during axonal transport in rat sciatic nerves. The number of sites accumulating above a ligature and labelled in vitro with the hydrophilic ligand [^{3}H]NMS markedly increased when 0.005% digitonin was added to the homogenate prepared in isotonic conditions. Similarly, osmotic shock and repeated freeze-thawing cycles also enhanced the binding. This means that under iso-osmotic conditions, the labelled ligand cannot reach the receptors that are located within organelles presumably because the membrane of vesicles prevents its access which, however, is facilitated by digitonin (fig.4). In contrast, the detergent did not significantly increase the binding in an intermediary segment between two ligatures; in this case, the muscarinic receptors were not moving. Consequently, the marked difference between the latency in the proximal P_1 segment and the intermediary I segment clearly distinguishes two populations of muscarinic receptors: the latent sites are associated with vesicles and are mobile,

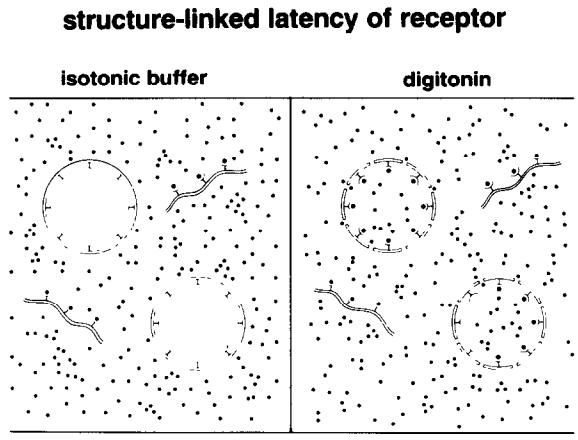


Fig.4. Model illustrating the concept of structure-linked latency for receptors. In isotonic conditions, low binding indicates that the ligand cannot reach the receptors located within the vesicles. In the presence of detergent, the vesicle membranes become more permeable to the ligand.

moving along the axon, whereas the non-latent sites are immobile, not located in axons but presumably associated with the membranes of blood vessels.

The concept of structure-linked latency when applied to receptor sites may provide new insights into the topology of a receptor within the membrane; indeed, muscarinic receptors which are transmembrane proteins appear to be oriented and only accessible to ligands in the interior of the vesicles (fig.4); binding sites of vesicle receptors are, thus, located only on one side. The latent receptor concept also implies that the receptors in vesicles are not functional because the neurotransmitter, here, acetylcholine is not present in vesicles of sympathetic neurones. Latent receptors are, therefore, 'stored' receptors. This work confirms that presynaptic muscarinic receptors are transported in rat sciatic nerves within vesicles as had been demonstrated in dog splenic nerves [4]. The concept of structure-linked latency may represent a new tool to study the ligand-induced internalization of receptors.

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