

## Immunocytochemical Demonstration of M<sub>1</sub> Muscarinic Acetylcholine Receptors at the Presynaptic and Postsynaptic Membranes of Rat Diaphragm Endplates

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### Summary

M<sub>1</sub>-muscarinic acetylcholine (ACh) receptors (M<sub>1</sub>R) were directly demonstrated immunocytochemically in electronmicroscopic images of rat diaphragm neuromuscular junctions (NMJ). Specific electron-dense granules were located at presynaptic nerve ending membranes and in the sarcolemma in the depths of postsynaptic folds. This first visualization of M<sub>1</sub>R on both sides of the NMJ is in agreement with previous pharmacological data on the regulatory role of M<sub>1</sub>R in quantal and non-quantal ACh release.

### Key words

Mammalian neuromuscular junction • Skeletal muscle • M<sub>1</sub> muscarinic receptor

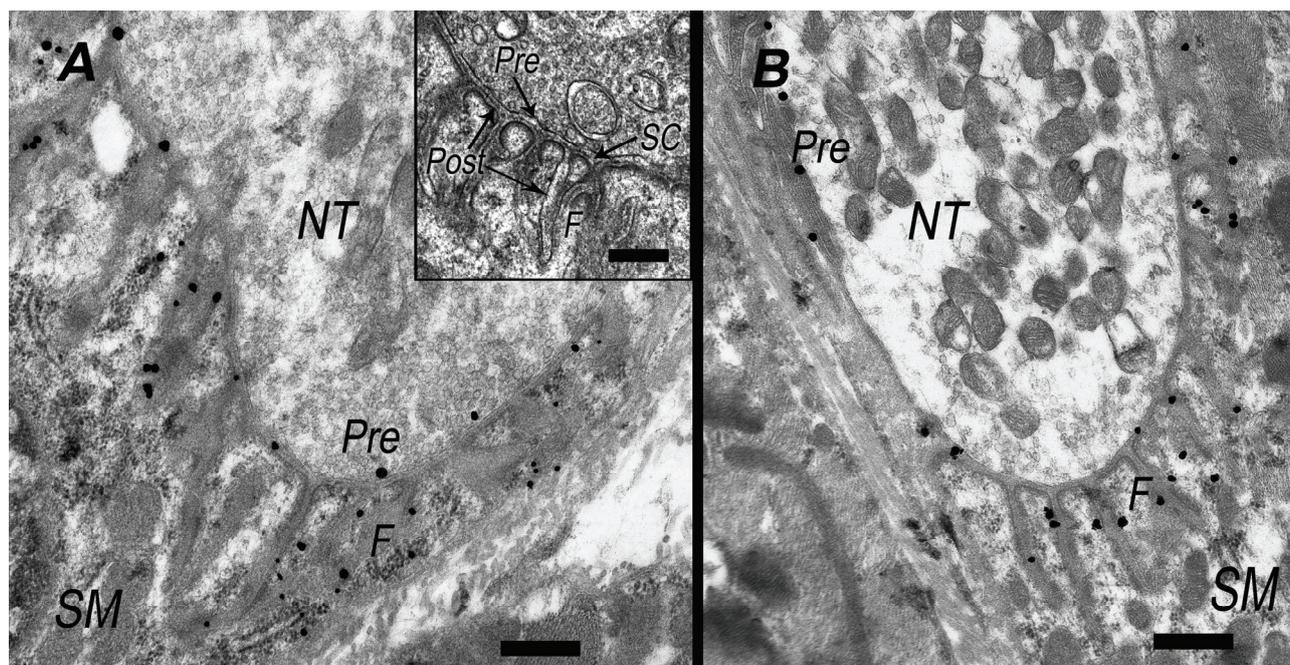
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Two groups of cholinergic receptors mediate the neurotransmitter function of acetylcholine (ACh): ionotropic nicotinic and metabotropic muscarinic receptors (MRs). MRs are monomeric proteins coupled to G-proteins. In vertebrates, there are M<sub>1</sub>R, M<sub>3</sub>R, and M<sub>5</sub>R that activate the G<sub>q/11</sub> family, and M<sub>2</sub>R and M<sub>4</sub>R

connected with the pertussis-toxin-sensitive G<sub>i</sub>/G<sub>o</sub> family. For a long time, the existence of MRs at the typically nicotinic NMJ was doubted (Magazanik and Vyskocil 1969). From the seventies onwards, data has accumulated on the modulation of nicotinic transmission at the NMJ by various MRs (Das *et al.* 1978, Somogyi *et al.* 1987, Alves-do-Prado *et al.* 1992, Urazaev *et al.* 2000, Furlan and Godinho 2005, Garcia *et al.* 2005, Malomouzh *et al.* 2007, Dudel 2007, Oliveira *et al.* 2009, Wright *et al.* 2009). M<sub>1</sub>R agonists increase but M<sub>2</sub>R agonists decrease the number of released ACh quanta during nerve stimulation (Santafe *et al.* 2003), particularly when cholinesterases were inhibited or absent (Minic *et al.* 2002). Also, non-quantal ACh release at the rat NMJ was lowered when M<sub>1</sub>R were activated. In this case, ACh modulates its own release by negative feedback via the activation of postsynaptic M<sub>1</sub> receptors, Ca<sup>2+</sup>-calmodulin-dependent postsynaptic stimulation of NO synthesis and its release from the muscle, whereupon it acts to inhibit the secretion of non-quantal ACh from nerve terminals (Malomouzh *et al.* 2007). Postsynaptic M<sub>1</sub>R were also suggested to form part of the ACh/NO/Cl<sup>-</sup> transporter cascade that stabilizes the muscle resting membrane potential and protects it *via* non-quantal ACh from early postdenervation depolarization (Urazaev *et al.* 2000, Vyskocil 2003, Vyskocil *et al.* 2009). However, to date there has been no direct ultrastructural evidence of MRs at the presynaptic or postsynaptic membranes. In this paper, we demonstrate the ultrastructural distribution of



**Fig. 1.** Distribution of  $M_1R$  at neuromuscular junction of rat diaphragm. **A, B**, electron microscopic pictures from two NMJ of two rats. Inset, main ultrastructure features of the NMJ. Pre, presynaptic membrane; SC, synaptic cleft; Post, postsynaptic membrane with folds (F). Immunogold particles are located close to the presynaptic membrane of the nerve terminal (NT) and to the postsynaptic membrane of skeletal muscle (SM), where they are mostly present in the depths of junction folds. Scale bars: 0.5  $\mu\text{m}$ .

$M_1R$  on both sides of the rat diaphragm NMJ.

Immunocytochemistry was performed on the diaphragms of 6 Wistar rats of both sexes in accordance with the European Communities Council Directive (86/609/EEC). The diaphragm was dissected from ether-anesthetized rats and fixed with 4 % paraformaldehyde in phosphate buffer solution (PBS, 0.1 M  $\text{NaH}_2\text{PO}_4$ , 0.1 M  $\text{Na}_2\text{HPO}_4$ , pH 7.2) followed by 0.2 % glutaraldehyde in PBS (2 h). Small rectangular pieces from the diaphragm were post-fixed with 0.5 % osmium tetroxide for 1 h. The samples were then embedded in LR White resin (Ted Pella, Redding, CA, USA). For immunolocalization, ultrathin sections (90 nm) were: (a) blocked (15 min) in Tris-buffered saline (TBS, 0.25 M Tris-HCl, pH 7.5) with 0.05 % Triton X-100 plus 5 % normal goat serum (Sigma, St. Louis, MO, USA); (b) incubated (2 h) with primary anti-muscarinic ( $M_1$ ) acetylcholine receptor rabbit antibodies (Sigma, Cat. No. M9808) diluted 1:200 with TBS plus 5 % normal goat serum; (c) washed three times in TBS; (d) incubated (2 h) with secondary goat anti-rabbit antibodies coupled to 5 nm colloidal gold (Sigma), diluted 1:100 with TBS plus 0.2 % bovine serum albumin; and (e) washed in TBS (pH 8.2) and purified water. Silver enhancement of gold particles conjugated to the secondary antibodies was carried out using the BBInternational Silver Enhancing Kit (Ted Pella). Control experiments were performed by omitting

primary antibodies. After washing in purified water, sections were contrasted in 5 % uranyl acetate and 30 % lead citrate. The samples were then observed under a JEOL 1200 SX electron microscope (Tokyo, Japan).

On all 38 slices from 6 stained endplates, immunocytochemical labeling gave a positive reaction with antibodies to  $M_1R$  (Fig. 1). Electron-dense granules of colloidal gold were located close to the plasma membrane, being almost completely absent from subcellular particles. They were found presynaptically close to the membrane of nerve endings and at postsynaptic muscle sarcolemma, along the synaptic clefts. Granules were absent perisynaptically and outside the synaptic contact. None were seen at the nerve ending membranes 30–50  $\mu\text{m}$  from the synaptic contacts or in extrasynaptic regions of the sarcoplasm (checked on 14 slices from 3 endplates, not shown). Postsynaptically, the granules were mostly located in the depths of postsynaptic folds, where voltage-sensitive  $\text{Na}^+$  channels and ankyrin G molecules have also been found (Bailey *et al.* 2003). Very few were at the tops of the fold crests. The appearance of granules at the postsynaptic membrane was more frequent than at the presynaptic terminal and we estimated approx. 8 granules per 5  $\mu\text{m}$  length on the presynaptic membrane and approx. 25 granules on the postsynaptic membrane and folds adjacent to 5  $\mu\text{m}$  of presynaptic membrane (Fig. 1). It has been shown using

radioisotopic ligands that MRs are present in the membrane fraction from cultured rat muscle, in denervated diaphragms and in myoblasts from newborn rats (Reyes and Jaimovich 1996, Furlan and Godinho 2005). Using Western blot analysis, chemiluminescent immunological assay and immunocytochemistry, some authors demonstrated several MR subtypes at the adult and newborn rat diaphragm in the vicinity of the NMJ, without determining their precise ultrastructural distribution. Here we demonstrated the presence of M<sub>1</sub>R at both pre- and postsynaptic membranes and this can help to resolve some of the controversies concerning the effects of muscarinic drugs at the NMJ, which can be considered either to be acting non-specifically, on nicotinic receptors (Hong and Chang 1990) or on muscarinic autoreceptors (Das *et al.* 1978, Hong and Chang 1990, Alves-do-Prado *et al.* 1992). This data also supports our conclusions about the role of presynaptic and postsynaptic M<sub>1</sub>R in the regulation of non-quantal ACh release and the “trophic” action of motor nerves on skeletal muscle (Urazaev *et al.* 2000, Vyskocil 2003, Malomouzh *et al.* 2007). Postsynaptic evidence of M<sub>1</sub>R also strengthens other hypotheses concerning how these receptors are involved in the action of reactive oxygen species (McArdle *et al.* 2004, Shakirzyanova *et al.* 2009) and retrograde mediators such as NO (Stamler and

Meissner 2001, Malomouzh *et al.* 2007) and CO (Sitdikova *et al.* 2007).

The density of M<sub>1</sub>R appears low when compared with the density of nicotinic receptors at the fold crests and voltage-dependent Na<sup>+</sup> channels inside the endplate folds (Madhavan and Peng 2005). However, even low levels of MRs can be highly effective physiologically, because they are coupled to an amplifying cascade of G-proteins and could be localized to caveolae, omega-shaped invaginations of the plasma membrane. Caveolae provide a scaffold for multiple G-protein receptors and membrane-bound enzymes, thereby orchestrating signaling into the cell's interior. It would be interesting to study whether M<sub>1</sub>R are associated with structural proteins, e.g. caveolin 1 or 3, as was demonstrated for M<sub>2</sub>R and M<sub>3</sub>R in the heart, urinary bladder and airway smooth muscles (Schlenz *et al.* 2009).

### Conflict of Interest

There is no conflict of interest.

### Acknowledgements

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