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THE VOLTAGE-DEPENDENT SODIUM CHANNEL IS CO-LOCALIZED WITH THE ACETYLCHOLINE RECEPTOR AT THE VERTEBRATE NEUROMUSCULAR JUNCTION

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SUMMARY. Isolated motor endplates from mouse intercostal muscles can be obtained after subcellular fractionation. On these motor endplates, localization of the nicotinic receptor and of the voltage-dependent Na^+ channel coincides as demonstrated by double labeling with rhodamine α-bungarotoxin and a specific anti-Na^+ channel monoclonal antibody. High density of Na^+ channel at the motor endplate is confirmed by the enrichment in TTX binding sites as compared to the crude homogenate. In contrast isolated motor endplates are almost completely devoid of Ca^{2+} channel antagonist binding sites.

Co-localization of the nicotinic receptor and of the Na^+ channel is also observed on fixed isolated muscle fiber and on thick frozen sections from adult mouse muscle.

INTRODUCTION. The mechanism of action potential generation in skeletal muscle after post-synaptic excitation is crucially dependent upon the local distribution of specific synaptic proteins near or at the motor endplate. Voltage-dependent Na^+ channels are not uniformly or randomly distributed along the length of a normal skeletal muscle fiber. Patch-clamp experiments have recently shown that Na^+ current densities in muscle are higher near the motor endplate (1).

Na^+ channels can be visualized using radiolabeled toxins such as tetrodotoxin (TTX) (2), or scorpion toxins (3). They can also be identified using monoclonal or polyclonal antibodies (4-7).

The purpose of this work is to analyze the cytolocalisation of Na^+ channels at the neuromuscular junction by using a functional monoclonal antibody against the Na^+ channel (7). This monoclonal antibody, 72-38, has a high affinity for the Na^+ channel and acts at the receptor site for a Brazilian scorpion toxin Tityus γ toxin and has the

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same electrophysiological effects as the polypeptide toxin itself : it shifts the voltage-dependence of Na^+ channel activation and produces spontaneous activity in nerve and muscle (7).

METHODS. Motor endplates were isolated as previously described (8) after subcellular fractionation of 3-week-old mouse intercostal muscle. Isolated motor endplates and whole gastrocnemius muscle were fixed for 1 hr in 4% paraformaldehyde made in phosphate buffered saline (PBS) at pH 7.4. Thick frozen sections were fixed for 10 min. The preparations were washed twice in PBS 0.1 M glycine and once in PBS.

Gastrocnemius muscles were teased apart in small bundles of 2-10 muscle fibers.

Fixed motor endplates, teased fibers and sections were incubated for 1 hr in 0.2 µg/ml rhodamine α-bungarotoxin, in 4% goat serum in PBS, washed 6-fold, 10 minutes each, and further incubated in 50 µg/ml of anti Na^+ channel monoclonal antibody during 15 hours. After 2 washings in 4% goat serum PBS and PBS alone, the preparations were incubated for 7 hrs with a biotinylated goat anti-mouse IgG monoclonal antibody (50 µg/ml). After adequate washing, the preparation was incubated with fluorescein coupled streptavidin (Cappel, 1/50) during 15 hours.

Binding experiments using ^3H labeled TTX derivatives were carried out as previously described (9). Membrane fractions (0.5 mg/ml) were incubated 15 min at 4°C in 100 mM Tris-Cl at pH 7.4 with increasing concentrations of tritiated ligands. Aliquots were filtrated under vacuum on GF/C glass fiber filters (Whatman) and rapidly rinsed with 3 times 5 ml of ice-cold 100 mM Tris-Cl buffer at pH 7.4. ^3H radioactivity on filters was counted in a Packard Tricarb 4000 instrument using Biofluor (NEN). Non-specific binding was determined in the presence of 1 µM TTX. The presence of Ca^{2+} channels was investigated using the tritiated Ca^{2+} channel inhibitor (+)[^3H]PN 200-110 (10).

The mouse monoclonal anti-Na^+ channel antibody 72-38 was obtained as previously described (7).

RESULTS AND DISCUSSION. Rhodamine α-bungarotoxin specifically labels (Fig. 1a) the nicotinic receptor in isolated motor endplate obtained after subcellular fractionation (8) of 3-week-old mouse intercostal muscle. These isolated motor endplates have a well-kept structure and were obtained in a very good yield as judged by the recovery of the muscle nicotinic receptor which ranged between 40 and 50% of the total acetylcholine receptor content.

Double labeling experiments of the isolated motor endplates were carried out with rhodamine α-bungarotoxin for the nicotinic receptor and with the mouse monoclonal anti-Na^+ channel antibody indirectly labeled with fluorescein streptavidin using goat anti-mouse IgG for the Na^+ channel. Staining for the nicotinic receptor coincides very well with staining of the Na^+ channel (Fig. 1a and b). Non-specific monoclonal antibodies used in the same conditions and a monoclonal antibody against the voltage-dependent Ca^{2+} channel (a generous gift of S. Vandaele) did not stain the isolated motor endplates.

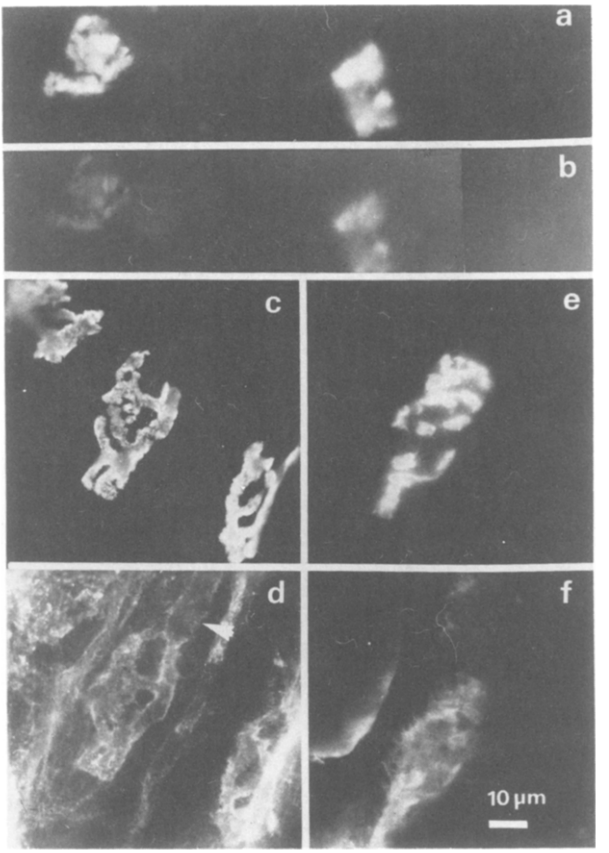

Figure 1 : Photomicrographs of adult mouse motor endplates double stained for acetylcholine receptors (AChR) (a, c, e) and voltage-dependent Na^+ channels (b, d, f). Fixed isolated motor endplates from 3-week-old intercostal muscles, teased muscle fibers and frozen sections from adult mouse sternocleidomastoid muscle.
All motor endplates are double labeled with rhodamine α-bungarotoxin and the anti-Na^+ channel monoclonal antibody. In the isolated motor endplate preparation the stainings are perfectly coincident at the resolution level of the light microscope (Fig. 1a, b). In the isolated muscle fibers the α-bungarotoxin staining (Fig. 1c) is also coïncident with the anti-Na^+ channel monoclonal antibody staining (Fig. 1d). The Na^+ channel antibody labeled in addition presynaptic structures, such as the myelin sheath of the preterminal myelinating Schwann cell (Fig. 1d; arrow head). In the frozen sections from adult sternocleidomastoid muscle, both postsynaptic structures, labeled with rhodamine α-bungarotoxin (Fig. 1e), and presynaptic elements, i.e. axon terminal branches are stained for the voltage-dependent Na^+ channel (Fig. 1f).

One of the available TTX-derivatives (11), ethylenediamine-TTX_I, specifically labels surface Na^+ channels in skeletal muscle (12). It has a lower affinity for T-tubule Na^+ channels. 1,4-Dihydropyridines such as PN 200-110 specifically label a protein which is presumably associated with the Ca^{2+} channel and which is primarily localized in the T-tubule structure (13).

Table 1

	en-TTX_I		(+)PN 200-110	
	K_D (nM)	B_{max} (pmol/mg)	K_D (nM)	B_{max} (pmol/mg)
H_T	1.5	0.92	0.75 ± 0.06	0.90 ± 0.20
C_I	1.9	3.3	0.64 ± 0.07	0.15 ± 0.03

The total muscle homogenate and the C_I subcellular fraction have been defined previously (8). C_I is the fraction the most enriched in motor endplates.

Table I shows that fraction C, which contains the isolated motor endplates is enriched in ethylene-TTX_I receptors as compared to the total muscle homogenate whereas the 1,4-dihydropyridine ((+)PN 200-110) receptor level in this fraction is decreased by a factor of about 6. This observation confirms that this fraction is definitely enriched in Na^+ channels.

Double labeling experiments on 4% paraformaldehyde fixed, isolated muscle fibers or fiber bundles from adult mouse gastrocnemius muscle were also carried out. Analyses at the light microscope level show, in this case too, that the nicotinic receptor and the Na^+ channel are co-localized at the motor endplate (Fig. 1c, d). The same type of staining was obtained on 10 µm thick frozen sections from adult mouse sternocleidomastoid muscle further documenting the remarkable coincidence between α-bungarotoxin staining and the staining with the anti-Na^+ channel monoclonal antibody (Fig. 1e, f). Staining also seemed to involve presynaptic elements such as Schwann cell membranes and axon terminals (Fig. 1d, f).

Two sets of observations have recently suggested the presence of relatively high densities of voltage-dependent Na^+ channel at the motor endplate : **(i)** the loose patch-clamp technique (1) has shown that Na^+ currents are greater (by a factor of 5 to 6) near the motor endplate than in extrasynaptic regions. The current density falls off with distance and reaches background levels 100-200 µm away from the motor endplate; **(ii)** Na^+ channels fluorescently labeled with Leiurus quinquestriatus toxin were found to be localized (and immobilized) to synapses formed in nerve-muscle cultures (14).

Our results using a different approach with monoclonal antibodies and TTX binding experiments on isolated motor endplates extend the very recently published observations of Angelides (14) to the mature neuromuscular junction. The morphological appearance of the postsynaptic fluorescent staining for Na^+ channel is very similar to the nicotinic receptor staining and seems to be located in the gutter-like structure described by Couteaux and Taxi (15) which corresponds to the primary unfolding of the motor endplate showing a restricted distribution to narrow lines of intense staining (see Fig. 1d). The distribution of nicotinic receptors has been studied in detail in numerous morphological (16-18) or electrophysiological (19, 20) studies of the vertebrate motor endplates which show that the density of this nicotinic receptor sharply decreases by 10-50 fold on the edge the subsynaptic membrane 10 µm or less from the motor endplate. At the ultrastructural level the nicotinic receptor has been shown to be highly concentrated at the crests of the secondary post synaptic folds (21, 22). We do not know how close could be the respective accumulations of acetylcholine receptor and Na^+ channel. Immunocytochemical studies at the electron microscope level should settle this point.

Since Na^+ channels and nicotinic receptors are closely spatially related, they may be concentrated at junctional sites by the same mechanism(s) and by the same factor(s) which are responsible for synapse stabilization.

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