Autoradiographic Localization of Voltage-Dependent Sodium Channels on the Mouse Neuromuscular Junction Using ^{125}I -Alpha Scorpion Toxin. I. Preferential Labeling of Glial Cells on the Presynaptic Side

Jean-Louis Boudier,¹ Emmanuel Jover,² and Pierre Cau¹

¹Laboratoire de Biologie Cellulaire-Histologie, and ²Laboratoire de Biochimie, U.A. CNRS 1179, INSERM U. 172, Faculté de Médecine Nord de Marseille, 13326 Marseille Cedex 15, France

Alpha-scorpion toxins bind specifically to the voltage-sensitive sodium channel in excitable membranes, and binding is potential-dependent (Catterall, 1984). The radioiodinated toxin II from the scorpion *Androctonus australis* Hector (αScTx) was used to localize voltage-sensitive sodium channels on the presynaptic side of mouse neuromuscular junctions (NMJ) by autoradiography using both light and electron microscopy. Silver grain localization was analyzed by the cross-fire method. At the light-microscopic level, grain density over NMJ appeared 6–8 $\times$ higher than over nonjunctional muscle membrane. The specificity of labeling was verified by competition/displacement with an excess of native αScTx . Labeling was also inhibited by incubation in depolarizing conditions, showing its potential-dependence. At the electron-microscopic level, analysis showed that voltage-sensitive sodium channels labeled with αScTx were almost exclusively localized on membranes, as expected. Due to washout after incubation, appreciable numbers of binding sites were not found on the postsynaptic membranes. However, on the presynaptic side, αScTx -labeled voltage-sensitive sodium channels were localized on the membrane of non-myelin-forming Schwann cells covering NMJ. The axonal presynaptic membrane was not labeled. These results show that voltage-sensitive sodium channels are present on glial cells *in vivo*, as already demonstrated *in vitro* (Chiu et al., 1984; Schrager et al., 1985). It is proposed that these glial channels could be indirectly involved in the ionic homeostasis of the axonal environment.

In vertebrates, motor neurons control skeletal muscle cells through a special cholinergic synapse, the neuromuscular junction (NMJ). Both cell types are excitable, owing to the presence of voltage-sensitive sodium channels in their membranes. These

channels are responsible for the fast inward sodium permeability that generates the first phase of the action potential (Hodgkin et al., 1952; Adrian et al., 1970). In the presynaptic nerve ending, the neuronal action potential induces acetylcholine release. From the classic studies of Katz and Miledi (1967, 1969), it is well known that calcium entry into the nerve terminal is a necessary step in the triggering of acetylcholine release. However, the issue of impulse propagation in the nerve terminal, leading to the calcium entry, is still controversial (reviewed by McArdle, 1984), and raises the question of sodium channel distribution on the nerve ending surface. In the frog NMJ, electrophysiological experiments have shown a distribution of sodium channels along most of the length of the nerve terminal (Katz and Miledi, 1965, 1968; Braun and Schmidt, 1966; Mallart, 1984). However, in mouse, the sodium current is restricted to the preterminal part of the axon (Brigant and Mallart, 1982). On the postsynaptic side, it has recently been shown, using the patch-clamp technique, that there is a higher density of sodium channels near the NMJ (Beam et al., 1985; Caldwell et al., 1986). On the basis of light-microscopic studies of sodium channel localization, using fluorescent derivatives of toxins (Angelides, 1986) or an anti-sodium channel antibody (Dreyfus et al., 1986), these authors have claimed a colocalization of sodium channels and AChR. However, the limited resolution of fluorescent markers did not permit a clear distinction between the different structural components of the NMJ. More precise sodium channel localization on the NMJ should improve our understanding of the mechanisms of both the pre- and postsynaptic aspects of transmission.

It is well accepted that scorpion toxins specifically label voltage-sensitive sodium channels (reviewed by Catterall, 1980a). Sodium channels have 4 separate neurotoxin receptor sites (Couraud et al., 1982; Catterall, 1984). Alpha-scorpion toxin (αScTx) binds to neurotoxin site 3, which is related to the inactivation of the channel (Catterall, 1980a, 1984). High-affinity binding is voltage-dependent and is dramatically decreased by membrane depolarization. When purified sodium channels are incorporated into lipid vesicles, voltage-dependent, high-affinity binding of αScTx can be restored (Feller et al., 1985). Thus, αScTx is an excellent molecular probe for the study of voltage-sensitive sodium channels. However, αScTx affinity is lower for skeletal muscle cells than for neurons (Catterall, 1979, 1980b; Baumgold et al., 1983).

Scorpion toxins can also be used as markers for localization of the voltage-sensitive sodium channels, owing to the following properties: (1) the nonspecific binding is relatively low; (2) they

Received May 28, 1986; revised Sept. 28, 1987; accepted Sept. 29, 1987.

The authors gratefully acknowledge the technical assistance of Jeannine Bottini and Anne-Marie Athouel.

This work was supported by the DGRST (Grant 81 1571). We wish to thank Professor R. Couteaux for comments on ultrastructural problems and his interest in this work, Professor M. A. Williams for helpful advice about autoradiographic methods, A. Mallart for invaluable help in dissecting the nerve-muscle preparations and in interpreting the electrophysiological results, C. Burgunder for help in grain counting, and F. Couraud and M. Seagar for reviewing the text.

Correspondence should be addressed to Dr. Jean-Louis Boudier, Lab. Biologie Cellulaire-Histologie, Faculté de Médecine Nord, Blvd. Pierre-Dramard, 13326 Marseille Cedex 15, France.

Copyright © 1988 Society for Neuroscience 0270-6474/88/051469-10\$02.00/0

can be labeled with ^{125}I to a high specific activity, allowing their use for autoradiographic experiments; (3) because of their polypeptide nature, they can be cross-linked to proteins on their binding site by the usual microscopic fixatives. The autoradiographic localization of voltage-sensitive sodium channels using scorpion toxin as a marker has already been carried out on cultured nerve cells (Catterall, 1981; Boudier et al., 1985; Cau et al., 1985) and on tissue incubated *in vitro* (Dellmann et al., 1983).

In the present study, we used the ^{125}I -iodinated toxin II of the scorpion *Androctonus australis* Hector (αScTx) to localize voltage-sensitive sodium channels on the presynaptic structures of the mouse NMJ by both light- and electron-microscopic autoradiography (ARG). The choice of αScTx as marker and binding conditions were expected to promote visualization of presynaptic binding sites over muscle membrane sites. Distribution of labeling was analyzed by the cross-fire method (Blackett and Parry, 1973).

Materials and Methods

Toxins. The toxin II from the scorpion *Androctonus australis* Hector was purified and radioiodinated as previously described (Miranda et al., 1970; Rochat et al., 1977). Specific activities of about 1000 Ci/mmol were obtained. The ^{125}I - α -neurotoxin (αNTx) from the snake *Naja mossambica* was a generous gift from P. Bougis (Laboratoire de Biochimie, Faculté de Médecine Nord, Marseille, France).

In vitro muscle-nerve preparation. The mouse (BL7 strain) triangularis sterni muscle was dissected out together with its innervation and maintained *in vitro*, as previously described (McArdle et al., 1981). The oxygenated superfusion solution contained (in mM) NaCl, 137; KCl, 5; CaCl_2 , 2; MgCl_2 , 0.8; glucose, 11; HEPES, 25; and Tris base, pH 7.2. Excitability was controlled by checking for the presence of contractions elicited by the stimulation of an intercostal nerve through a suction electrode (square pulses, 2 V, 0.1 msec, 2 Hz).

Toxin incubation conditions. Standard incubation medium was Na^+ - and Ca^{2+} -free; it consisted of (in mM) choline chloride, 140; KCl, 5.4; MgCl_2 , 0.8; glucose, 10; HEPES, 25; and Tris base, pH 7.2. For αScTx total binding experiments, 1 nM of ^{125}I - αScTx was added, and for non-specific binding, 1 nM of ^{125}I - αScTx plus 0.2 μM of native αScTx was added. Incubation was at 20°C for 30 min, followed either by 3 short rinses of about 30 sec each in the incubation buffer or, in some cases, by a 30 min rinse. For incubation in the presence of 1 mM veratridine, choline chloride was replaced by 140 mM NaCl. For experiments in high K^+ , KCl concentration was raised to 145 mM.

αNTx was incubated in the standard medium under the same conditions, except for toxin concentrations: 0.5 nM of ^{125}I - αNTx for total binding; 0.5 nM of ^{125}I - αNTx plus 0.25 μM of native toxin for nonspecific binding.

Light microscopy. Muscles were fixed for 1 hr at 20°C in a solution of 2.5% glutaraldehyde plus 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). They were then processed for AChE staining (Karnovsky and Roots, 1964). A strip of tissue containing nearly all the junction was then dissected out and cut into blocks. These were postfixed in OsO_4 2% solution, dehydrated in ethanol, and embedded in Epon. Sections, 2 μm thick, were collected on slides which were dipped into Kodak NTB2 emulsion. After 16–20 d exposure, development was carried out in Kodak D19b, and sections were stained with toluidine blue.

Quantitative analysis was performed by visual grain counting using a $100\times$ objective. Only sections with the plane perpendicular to the muscle fiber axis were used. The image of a 1.8- μm -radius circle was superimposed on the image of the section with a camera lucida. Sections were screened by systematic sampling. When a NMJ was encountered, the center of the circle was placed on the dense line of the AChE reaction at one end of the NMJ and then moved along the line for one diameter length as many times as necessary to cover the entire NMJ. The number of grains falling in the circles was recorded. Then the center of the circle was placed on a nonjunctional muscle membrane in the vicinity of the NMJ, and the number of grains falling in the same number of circles as for the NMJ was also recorded. A set of preestablished rules was used for positioning the circle on structures.

Electron microscopy. Before sectioning, the processing of tissue was the same as for light microscopy, except that AChE staining was omitted. The ARG was performed by the dipping method (Larra and Droz, 1970). The emulsion was Ilford L4, developed in Kodak D19b after 45–52 d of exposure.

For quantitative evaluation, only sections perpendicular to the muscle cell axis were used. The background and general grain density on tissue were first determined by direct grain counting at the electron microscope, using the quadrat method (Williams, 1977) at $6000\times$ magnification. For the cross-fire analysis, 3 grids/tissue block from 3 different blocks were scanned, and all the NMJ profiles were photographed. They were viewed at a final magnification of $25,115\times$ (checked with a calibration grating replica). About 160 NMJ profiles were recorded.

Cross-fire analysis. The purpose of this analysis was to overcome the problem of radiation spread by analyzing intermingled putative sources in a complex structure (Blackett and Parry, 1973, 1977; Salpeter et al., 1978; Williams and Downs, 1978). Briefly, a hypothetical set of source-grain couples was generated. Their distribution allowed construction of a matrix of the relationships between grains and sources in the tissue (cross-fire matrix). Mathematical adjustment of the generated grains to the observed grains gives the actual source distribution. Thus, the most important step in the method is the division of the tissue into putative source and grain compartments (Williams, 1977). In the present study, membranes were possible sources and should be considered as separate compartments. But their virtual absence of section surface prevented generated grains and sources to hit them. Thus, a small circle was used to attribute grains and sources to compartments. This gives the membranes an artificial surface section, and therefore improves their sampling (Williams, 1977). A 2.5-mm-diameter circle was chosen because it allowed sufficient sampling of membranes without generation of numerous compound compartments. In actual practice, this diameter even enabled pre- and postsynaptic membranes to be treated as separate compartments. The tissue was thus divided into 10 putative grain and source compartments (Table 1). Overlays of generated source-grain sets (1 source/6 grains) were superimposed on the screen of the viewing box, and positions of sources and grains recorded in the cross-fire matrix. Real observed grains were also attributed to grain compartments with a 2.5 mm circle. Distances between sources and grains in generated sets were calculated from the ^{125}I universal curve of Salpeter et al. (1977), with $\text{HD} = 180$ nm. [HD was defined by Salpeter et al. (1977) as the distance from a radioactive line source which contains 50% of the developed grains derived from the source.] This HD was empirically set as the value giving the best adjustment of the cross-fire matrix on the basis of several preliminary analyses. Calculations and drawing of overlays were performed by programs run on a microcomputer connected to a plotting device (Cau, 1988). Adjustment of the matrix to the observed grain vector was made using the method of Newton-Raphson (Noble, 1964), and standard errors were computed following Salpeter et al. (1978).

Results

Light microscopy

Inspection of the 2 μm sections allowed reliable identification of all the NMJ. The AChE reaction product was clearly visible as a dark blue line covering a sector of the muscle fiber membrane (Fig. 1). In addition to this main criterion, the limit of the muscle cell looked irregular at the NMJ, and the sole plate was often visible as an underlying light zone in the axoplasm, containing 1 or 2 nuclei. Cells and processes often covered the structure.

After incubation with ^{125}I - αScTx alone (total binding), silver grains were dispersed over the entire tissue, with spots of high density over the NMJ. However, these spots were not clearly delimited. The high density of grains very often extended outside the limits of the synapse itself (Fig. 1A). The labeling of the NMJ appeared to be very variable. AChE-positive NMJ labeled at the same low level as the surrounding structures were occasionally observed. However, no other structure in the tissue showed spots of high grain density. In preparations incubated with ^{125}I - αScTx plus an excess of native αScTx (nonspecific bind-

Figure 1. Autoradiographic localization of radioiodinated alpha-scorpion toxin (^{125}I - αScTx)-labeled voltage-sensitive sodium channels on transverse semithin sections. Staining for AChE appears as *dense lines* underlining sectors of myocyte membranes and reveals the neuromuscular junctions (*arrowheads*). *A*, Incubation with 1 nM ^{125}I - αScTx (total). Note the clusters of silver grains superimposed upon junctions. The diffuse labeling is due to binding to muscle cells. *B*, Incubation with 1 nM ^{125}I - αScTx plus 2 μM of native αScTx (nonspecific). *C*, *D*, Effect of depolarization. Incubation as for *A*. *C*, In the presence of 145 mM KCl. *D*, In the presence of 1 mM veratridine. Bar, 10 μm .

ing), the general density of grains was clearly lower, and high-density labeling of NMJ did not appear at all (Fig. 1*B*).

This observation was confirmed by a quantitative study of labeling (Fig. 2). The very low background allowed us to obtain a ratio of the general signal on muscle (NMJ excepted) to background greater than 50:1 in total binding experiments. The grain density was evaluated in the junctional and nonjunctional areas that could be considered as rough bands, including the membrane. This allowed us to take into account all the structures associated with the NMJ, such as glial cells covering the junction at the external side and membrane folds at the internal side. Junctional areas were centered on the AChE-positive part of the membrane, whereas nonjunctional areas were on the other, negative, part. When junctional regions were compared to nonjunctional regions in total binding experiments, NMJ showed a grain density about 6–8 $\times$ higher than a nonjunctional muscle membrane of the same length. Nonspecific labeling of junctional areas was very low: less than 10% of total labeling.

Voltage-dependent labeling of NMJ was tested by incubating ^{125}I - αScTx in depolarizing media, which should induce an inhibition of labeling. The result can be seen in Figure 1, *C*, *D*, as well as in Figure 2 for grain densities. Incubation in the presence of 145 mM KCl inhibited the labeling of NMJ, reducing

it to about one-third of the control value. Labeling was also clearly reduced by veratridine, though to a lesser extent.

Electron microscopy

Preparations subjected to this analysis were rinsed for 30 min after incubation. Direct observation first confirmed the preferential labeling of NMJ on preparations incubated with ^{125}I - αScTx alone (total binding). A more detailed study of grain distribution showed a preferential localization on cells that covered the NMJ (Fig. 3, *A*, *B*). Some grains were also seen on the nerve terminal and the muscle membrane. In preparations incubated with ^{125}I - αScTx plus an excess of native toxin (nonspecific binding), there was again no significant labeling. These observations correlate well with results of a global quantitative study shown in Figure 4. In total binding preparations, the grain density over junctional areas was found to be about 5 $\times$ higher than the density over the rest of the tissue. This ratio is in the same range that was found with light microscopy. Nonspecific labeling of NMJ was again less than 10% of the total. However, the apparent dispersion of grains over the NMJ due to the radiation spread (Fig. 3) prevented precise localization of the binding sites to the different structures of the NMJ. In particular, direct observation did not allow us to determine whether bind-

Figure 2. Light-microscopic autoradiography. Grain densities over areas of sections centered on neuromuscular junctions or nonjunctional myocyte membranes (see Materials and Methods for details). Units: grain number/10 μm^2 of section surface; mean $\pm$ 95% mean confidence limit (n). Nerve-muscle preparation was incubated either with 1 nM [^{125}I]- αScTx (Total) or with 1 nM [^{125}I]- αScTx plus 2 μM of native αScTx (Non-specific). *A*, Depolarization by incubation as for Total but in 145 mM KCl. *B*, Depolarization by incubation as for Total but in 1 mM veratridine. In both series, note for Total the difference in labeling between junctional and nonjunctional areas. Mean background density, 1.17×10^{-3} grains/ $\mu\text{m}^2 \pm$ SD 0.56×10^{-3} (35).

ing sites existed on the nerve terminal membrane. A cross-fire quantitative analysis was then performed.

As scorpion toxin receptor sites are on voltage-sensitive sodium channels, they are presumably located on plasma membranes. The negligible sampling of membranes was overcome by attributing both hypothetical and observed silver grains, as well as sources, to tissue compartments with a circle large enough

Table 1. Tissue division into grain and source compartments for the cross-fire analysis

Number	Name	Definition
1.	Muscle interior	Internal space delimited by the muscle cell surface membrane, including nucleus and cytoplasmic organelles
2.	Muscle membrane	Muscle cell surface membrane except the postsynaptic zone (folds)
3.	Axon interior	Internal space delimited by the axon terminal membrane, including cytoplasmic organelles
4.	Glial cell int.	Internal space delimited by the surface membrane of identified glial cells, including cytoplasmic organelles
5.	Junctional folds mmb.	Muscle postsynaptic membrane
6.	Glial cell mmb.	Surface membrane of identified glial cells
7.	Unident. processes	Membrane and interior of thin cell processes not formally identified
8.	Glial + axon mmbs.	Parts of glial and axonal membranes facing together and in close contact
9.	Axonal membrane	Presynaptic part of the axon terminal membrane facing the muscle postsynaptic membrane
10.	Intercell. space	All the external space, including connective tissue with collagen and sometimes capillary blood vessel

to give a certain section surface to membranes, and therefore to increase their sampling (see Materials and Methods). However, it should be noted that the circle also included cytoplasm and extracellular space. Thus, grains and sources attributed to a membrane compartment should formally belong to the near cytoplasm or extracellular space. The final division of the tissue into source and grain compartments is given in Table 1. Cells that cover the NMJ are generally accepted as being glial (Gauthier, 1976). They can be identified at the ultrastructural level by the presence of a basal lamina and/or very close contact with the axonal membrane. When both features were absent, these structures could not be formally identified as glial. In our samples, this applied only to long and thin cell processes, which were often heavily labeled (Fig. 3*A*). Thus, these structures were classified as a separate compartment, "unidentified processes."

The cross-fire matrix of generated (hypothetical, systematically distributed) sources and related grains appears in Table 2. A total of 5047 sources and grains were applied to structures for 707 observed (real) grains. The total of generated grains gives the grain distribution corresponding to a random source distribution, as seen in the last 2 columns. As shown by the chi-square test, the observed and generated grain distributions are different, meaning that observed silver grains are not randomly distributed over the NMJ structures.

The optimized source density given in Table 3 is the result of adjusting the cross-fire matrix to the observed grain distribution. Calculated grains were obtained from the optimized source density values. The fit with the observed grain distribution

Figure 3. EM autoradiographic localization of alpha-scorpion toxin (α ScTx)-labeled voltage-sensitive sodium channels on neuromuscular junctions. Incubation with 1 nM 125 I- α ScTx (total binding). *A*, Overview of a heavily labeled neuromuscular junction, showing the general distribution of silver grains. *B*, Detail of a synaptic gutter. Note the predominant location of silver grains over the structures covering the nerve terminals. Bar, 1 μ m.

Table 2. "Cross-fire" table of generated (hypothetical) source-grain couple localization and of observed (real) grain localization

	1	2	3	4	5
Grain compartments	Muscle int.	Muscle mmb.	Axon int.	Glia int.	Folds mmb.
Source compartments					
1. Muscle interior	1631	25	6	0	49
2. Muscle membrane	37	34	7	0	1
3. Axon interior	1	0	92	2	6
4. Glial cell int.	2	1	0	145	0
5. Junction. folds mmb.	37	2	18	1	88
6. Glial cell mmb.	7	5	10	45	4
7. Unident. processes	1	0	0	2	0
8. Glial + axon. mmb.	1	0	8	3	0
9. Axonal membrane	2	2	17	1	11
10. Intercell. space	25	25	2	25	8
Total generated grains	1744	94	160	224	167
Observed grains	63	16	18	103	11
Chi-square test					
Regrouped compartments	1	2	3	4	5
Normalized generated grains	244.30	13.17	22.41	31.38	23.39
Observed grains	63	16	18	103	11
Chi ²	134.55	0.61	0.87	163.47	6.57

^a $df = 8$; $p < 0.001$.

bution has been considerably improved by the adjustment, as indicated by the new value of chi-square. Source density values are given in units of source generated on the compartment (Salpeter et al., 1978). The nonmembrane compartments were practically devoid of sources, as expected. An exception was the interior of glial cells, which showed a low, but appreciable, source density. Among membrane compartments, glial mem-

branes, glial plus axonal membranes, and also unidentified cell processes (see definition in Table 1 and earlier in this section) had source densities that were clearly higher than those in the other compartments, and their values are in the same range. This means that some of the α ScTx binding sites localized by light microscopy on the NMJ belonged to the membranes of the glial cells that covered the endplate. Density in α ScTx receptors appeared not to be appreciably different between these 3 membrane compartments. In contrast, presynaptic axonal membrane contained no sources. When they were in close contact, axonal and glial membranes could not be analyzed separately. Nevertheless, it should be stressed that this compound compartment had a source density close to the value of the glial membrane alone. Muscle membranes showed low source density, as expected. Despite this low level, the source density on the muscle nonsynaptic membrane (around the NMJ) was clearly higher than that on the postsynaptic membrane (folds), which showed no significant labeling.

Controls incubated with snake α NTx

Light microscopy of muscle incubated with 125 I- α NTx alone (total binding) showed that only NMJ were labeled by heavy spots of silver grains. Such labeling was absent on tissue incubated with 125 I- α NTx plus an excess of native toxin (nonspecific binding).

At the electron-microscopic level, silver grains were observed almost exclusively on the top of the folds of the postsynaptic membrane in total binding preparations.

Discussion

From the results presented above, it appears that α ScTx binding sites are more numerous at the NMJ than on the other parts of the muscle. An excess of native toxin inhibited 90% of labeling of NMJ at both light- and electron-microscopic levels, which attests to its specificity of labeling. Labeling of the NMJ was

Figure 4. EM autoradiography. Grain densities over areas of sections centered on neuromuscular junctions or nonjunctional myocyte membranes, or over Epon areas devoid of tissue for background (see Materials and Methods for details). Units: grain number $\times 10^{-3}/\mu\text{m}^2$ of section surface; mean $\pm$ 95% mean confidence limit (n). Nerve-muscle preparation was incubated either with 1 nM 125 I- α ScTx (Total) or with 1 nM 125 I- α ScTx plus 2 μM of native α ScTx (Nonspecific).

Table 2. Continued

6 Glia mmb.	7 Unident. process.	8 Glia + ax. mmb.	9 Axon. mmb.	10 Intercell. space	Total generated sources	
1	0	0	3	25	1740	0.345
6	1	0	2	25	113	0.022
2	0	13	5	3	124	0.025
40	0	0	0	25	213	0.042
1	0	0	6	15	168	0.033
97	0	7	3	66	244	0.048
1	73	0	0	70	147	0.029
7	0	12	0	4	35	0.007
1	0	3	7	3	47	0.009
45	85	1	1	1999	2216	0.439
201	159	36	27	2235	5047	1
140	93	22	3	238	Total	707
6	7	8 + 9		10	Total	
28.16	22.27	8.82		313.09	707	
140	93	25		238	707	
444.26	224.59	26.64		18.01	1022.57 ^a	

clearly reduced by incubation in depolarizing conditions. Inhibition of binding by membrane depolarization is a characteristic of α ScTx binding on voltage-sensitive sodium channels (Catterall, 1980a). Cross-fire analysis revealed that most of the junctional α ScTx binding sites were located on the glial cells that cover the endplate. However, presynaptic membrane was devoid of α ScTx binding sites. It should be stressed that densities obtained by the cross-fire analysis are expressed in units of generated sources deposited on each compartment (Salpeter et al., 1978). They are different from, though proportional to, binding site densities.

Sodium channels on muscle membrane

Results on muscle membranes were not very significant with regard to the following factors: (1) *Leiurus V* α ScTx is known to have a relatively low affinity for muscle cell systems, unlike that for neuronal ones (Catterall, 1979, 1980b; Baumgold et al., 1983). The affinity of the *Androctonus* α ScTx for adult rat skeletal muscle cells *in vivo* is not known. These 2 α ScTx being very similar, it is highly likely that the affinity of *Androctonus* toxin is lower for muscle cells than for neurons, as with *Leiurus V* toxin. (2) Since toxin concentration in the incubation medium

Table 3. Result of the adjustment: optimized source density and table of grain localizations calculated from this density, plus χ^2 comparison with observed grain localizations

Compartments	1 Muscle int.	2 Muscle mmb.	3 Axon int.	4 Glia int.	5 Folds mmb.	6 Glia mmb.	7 Uni- dent. pro- cess.	8 Glia + ax. mmb.	9 Axon. mmb.	10 Intercell. space	Total sources
Opt. source density	0.024	0.257	0.000	0.294	0.081	1.189	1.235	1.226	0.000	0.027	4.333
Standard deviation	0.005	0.183	0.073	0.091	0.059	0.214	0.160	0.576	0.359	0.009	1.729
Calculated grains	63.51	16.42	25.16	103.06	13.44	139.75	92.71	23.07	4.67	238.80	Total calc. 720.59
Observed grains	63	16	18	103	11	140	93	22	3	238	Total obs. 707
Chi-square test											
Regrouped compartments	1	2	3	4	5	6	7	8 + 9		10	Total
Calculated grains	63.51	16.42	25.16	103.06	13.44	139.75	92.71	27.74		238.80	720.59
Observed grains	63	16	18	103	11	140	93	25		238	707
Chi ²	0.004	0.011	2.038	0.000	0.443	0.000	0.001	0.271		0.003	2.770 ^a

^a $df = 8$; $p > 0.90$.

was adjusted for neuronal binding, the binding site density on muscle membrane was probably underestimated. (3) In addition to this, it should be emphasized that cross-fire analysis was performed on preparations subjected to a 30 min rinse after toxin incubation. The effect of the toxin on muscle (i.e., fibrillations and irregular contractions) gradually disappeared during long rinses, although effects on nerve terminal currents did not (A. Mallart, personal communication). This could have been due to a progressive release of toxin from muscle, probably because of its relatively low affinity. Preparations that underwent short rinses were also examined with electron microscopy, and showed markedly higher labeling of muscle membranes. Thus, the very low labeling of muscle membranes appeared to be due mainly to a release of the toxin during long rinses. Although not relevant for muscle membrane, these binding conditions effectively highlighted the localization of toxin binding sites on the nonmuscular membranes of the NMJ. Further ARG experiments are in progress to study voltage-sensitive sodium channel distribution over the muscle membrane, using β ScTx and α ScTx in more favorable experimental conditions.

Sodium channels on the axon terminal

On available sections of NMJ, the part of the axon lying in the postsynaptic gutter has been sampled. The synaptic zone of the axon terminal membrane, which faces the membrane folds, is characterized by the presence of synaptic vesicles aggregated along ribbons of electron-dense material on its inner face—the active zones (Couteaux and Pecot-Dechavassine, 1970). Freeze-fracture preparations showed rows of intramembrane particles at these sites (Ellisman et al., 1976). The synaptic zone therefore appears as a highly organized membrane domain devoted to ACh release, and thus has been analyzed separately. Cross-fire analysis showed that the synaptic zone is clearly devoid of sodium channels. The other part of the membrane is in close contact with glial membrane. The source density on this compartment containing neuronal plus glial membrane is close to the value of the glial membrane alone. Thus, locating these sources on the glial membrane appears the most probable; this part of the axonal membrane, in such a case, would have either no sodium channels or a very low number of them.

This negative result casts doubt on the diffusion of the toxin in the synaptic gutter. To check this, nerve-muscle preparations were incubated with the α NTx of the snake *Naja mossambica mossambica* instead of the α ScTx. α NTx has the same M_r (about 7000 Da) as the α ScTx and is also highly positively charged (Rochat et al., 1974). It is known to bind to the AChRs (Tessier et al., 1978). As expected, specific labeling of AChRs at the top of the postsynaptic membrane folds was observed. Thus the snake toxin apparently diffuses into the synaptic gutter, and, by analogy, we concluded that it is very unlikely that the very similar α ScTx would not diffuse.

The absence or low density of sodium channels on all the axon segments included in the synaptic gutter is in good agreement with the conclusions of Brigant and Mallart (1982), who reported electrophysiological and pharmacological evidence that in the mouse, unlike the frog, the sodium current is restricted to the preterminal part of the axon, i.e., the segment between the end of the myelin sheath and the beginning of the synaptic gutter. These authors propose, therefore, that active depolarization of the preterminal part promotes a local circuit that induces a passive depolarization of the terminal part. Unfortunately, the

preterminal part is very short in our system, which makes it very difficult to observe on ultrathin sections.

Sodium channels on glial cells

The most surprising result of our study is that voltage-sensitive sodium channels are present on the glial cells that cover the NMJ. Although described in all reports on NMJ structure, these cells have generally received little interest. They have been placed in the same category as Schwann cells (Gauthier, 1976; Landon, 1982; Carry and Morita, 1984). Although they are peripheral glial cells, it should be emphasized that they do not form myelin. Some peripheral non-myelin-forming glial cells differ from myelin-forming Schwann cells in not expressing myelin-associated components (Brockes et al., 1980) and in containing glial fibrillary acidic protein (GFAP) (Jenssen et al., 1984). Surface antigens also appear to be different (Jenssen and Mirsky, 1984). Schwann cells at denervated frog NMJ are able to release ACh and thus induce EPSP (Dennis and Miledi, 1974). And the appearance of CAT in cultured rat Schwann cells is induced by coculture with myotubes (Brockes, 1984). Thus, non-myelin-forming Schwann cells covering NMJ seem to be different from other Schwann cells. They have, however, some properties that suggest that they may be functionally involved in neuromuscular transmission.

Some cell processes that could not be formally identified as glial were labeled at the same level as Schwann cells (see Table 1, Unidentified processes). An absence of close contact with the axon and especially of a basal lamina suggested at first that they were fibroblasts. However, by the following criteria, they were probably in fact glial cells: (1) the ultrastructural aspect was the same as for identified Schwann cells; (2) basal lamina was sometimes lacking on parts of the cells that were in close contact with the axon; and (3) labeling was in the same range as that of identified Schwann cells. Furthermore, it has been shown that Schwann cells in culture form a basal lamina only when they interact with axons (Bunge et al., 1982). It is possible, then, to assume that these cells are Schwann cells that, being separate from the axon, do not form a basal lamina.

A low, but appreciable, level of binding was found inside Schwann cells. A possible explanation would be an overlapping of the radiation spread from 2 adjacent membrane sources, with most of the glial cells appearing on sections as fine processes. This explanation was tested by eliminating the glial interior as a source and leaving it as a grain compartment in the cross-fire table. After adjustment, the χ^2 reached only 15.3 instead of the 2.77 obtained previously. This supports the presence of sources in the glial cell interior. At first glance, scorpion toxin internalization would appear surprising. Internalization of sodium channels in neuroblastoma cells occurs at a slow rate: only 1.3% during 30 min at 37°C (Waechter et al., 1983). Thus it would not be expected to be appreciable in the time range used here (incubation plus rinse, 1 hr maximum). Using internalization as an explanation for labeling of the Schwann cell interior implies that the turnover of sodium channels in Schwann cells is much faster than that in neurons. New investigations are needed to probe this doubtful but important point.

The presence of sodium channels on Schwann cells *in vivo* was first shown in squid (Villegas et al., 1976), and then in the rabbit sciatic nerve after Wallerian degeneration (Ritchie and Rang, 1983). In culture, their presence has also been shown on human glial cells (Munson et al., 1979) and murine astrocytes

(Bowman et al., 1984; Bevan et al., 1985). Using patch-clamp techniques, sodium channels have been detected on cultured rat and rabbit Schwann cells (Chiu et al., 1984; Schrager et al., 1985), together with voltage-sensitive potassium channels. The results presented here are direct evidence for the presence of sodium channels on the non-myelin-forming Schwann cells of the mouse in normal *in vivo* conditions.

A discussion of the functional significance of voltage-sensitive sodium channels on Schwann cells can only be speculative. However, it seems difficult to defend the idea that these channels are only embryonic vestiges without a functional role, given evidence that they are functional and present in nonnegligible numbers (Schrager et al., 1985).

Schrager et al. (1985) and Gray and Ritchie (1985) proposed that Schwann cells synthesize sodium channels and then transfer them to the axon. This theory is based on the assumption that the axonal transport of proteins from the cell body cannot provide a sufficient amount of material to allow normal turnover of sodium channels in the membrane of the peripheral nerve. However, arguing against this interesting hypothesis is the evidence that the sodium channel appears as a quantitatively minor component of the axon membrane (Catterall, 1984). Also, results suggesting axonal transport of a tetrodotoxin-binding component have been obtained in rat peripheral nerve (Lombet et al., 1985).

Another group of functions of glia is related to the ionic homeostasis of neural extracellular spaces. Extracellular potassium concentration during stimulation of peripheral nerves may rise to levels high enough to alter conduction (Shanes, 1951). It is generally accepted that astrocytes in the CNS regulate the extracellular level of potassium by a mechanism of spatial buffering (Kuffler et al., 1984). As proposed by Bevan et al. (1985), voltage-dependent potassium and chloride channels could play a role in this mechanism. At the NMJ and the Ranvier node, another mechanism for extracellular K^+ buffering could be suggested, involving an active uptake through the sodium-potassium-adenosine triphosphatase ($Na^+-K^+-ATPase$) (reviewed by Walz and Hertz, 1983). This enzyme is also present on Schwann cells at the Ranvier node (Ariyasu et al., 1985). Schwann cells can therefore take up the extracellular K^+ released by the axon in the synaptic gutter during intense activity as at the Ranvier node. An increase in external K^+ concentration induces glial cell depolarization (Pentreath, 1982), as was recently observed in glia of the optic nerve (Konnerth and Orkand, 1986; Lev-Ram and Grinvald, 1986). Voltage-sensitive sodium channels would be activated during these glial depolarizations, and would trigger a signal for a metabolic regulation of the $Na^+-K^+-ATPase$, as already shown for the chick skeletal muscle (Wolitzky and Fambrough, 1986). This would regulate the glial buffering of external K^+ according to the neuromuscular activity. Other glial functions could be regulated in the same way. However, more direct functional involvement of glial sodium channels in conduction mechanisms cannot be considered without a knowledge of their density. New experiments are now in progress in order to assess the absolute channel density on the NMJ membranes.

Ultrastructural analysis of $\alpha ScTx$ binding on the presynaptic side of the mouse NMJ showed that voltage-sensitive sodium channels which bind $\alpha ScTx$ are mainly localized on glial cells covering the junction. These cells are probably more deeply involved in the transmission mechanism than expected. Further analysis of voltage-sensitive sodium channel localization at the

NMJ should provide new data on channel distribution at the muscular side.

References

- Adrian, R. H., W. K. Chandler, and A. L. Hodgkin (1970) Voltage clamp experiments in striated muscle fibres. *J. Physiol. (Lond.)* 208: 607-644.
- Angelides, K. J. (1986) Fluorescently labelled Na^+ channels are localized and immobilized to synapses of innervated muscle fibres. *Nature* 321: 63-66.
- Ariyasu, R. G., J. A. Nichol, and M. H. Ellisman (1985) Localization of sodium/potassium adenosine triphosphatase in multiple cell types of the murine nervous system with antibodies raised against the enzyme from kidney. *J. Neurosci.* 5: 2581-2596.
- Baumgold, J., J. B. Parent, and I. Spector (1983) Development of sodium channels during differentiation of chick skeletal muscle in culture. I. Binding studies. *J. Neurosci.* 3: 995-1003.
- Beam, K. G., J. H. Caldwell, and D. T. Campbell (1985) Na channels in skeletal muscle concentrated near the neuromuscular junction. *Nature* 313: 588-590.
- Bevan, S., S. Y. Chiu, P. T. A. Gray, and J. M. Ritchie (1985) The presence of voltage-gated sodium, potassium and chloride channels in rat cultured astrocytes. *Proc. R. Soc. Lond. [Biol.]* 225: 299-313.
- Blackett, N. M., and D. M. Parry (1973) A new method for analyzing electron microscope autoradiographs using hypothetical grain distributions. *J. Cell Biol.* 57: 9-15.
- Blackett, N. M., and D. M. Parry (1977) A simplified method of "hypothetical grain" analysis of electron microscope autoradiographs. *J. Histochem. Cytochem.* 25: 206-214.
- Boudier, J. A., Y. Berwald-Netter, H. D. Dellmann, J. L. Boudier, F. Couraud, A. Koulakoff, and P. Cau (1985) Ultrastructural visualization of Na^+ -channel associated 125-I alpha scorpion toxin binding sites on fetal mouse nerve cells in culture. *Dev. Brain Res.* 20: 137-142.
- Bowman, C. L., H. K. Kimelberg, M. V. Frangakis, Y. Berwald-Netter, and C. Edwards (1984) Astrocytes in primary culture have chemically activated sodium channels. *J. Neurosci.* 4: 1527-1534.
- Braun, M., and R. F. Schmidt (1966) Potential changes recorded from the frog motor nerve terminal during its activation. *Pfluegers Arch.* 287: 56-80.
- Brigant, J. L., and A. Mallart (1982) Presynaptic currents in mouse motor endings. *J. Physiol. (Lond.)* 333: 619-636.
- Brockes, J. P. (1984) Assays for cholinergic properties in cultured rat Schwann cells. *Proc. R. Soc. Lond. [Biol.]* 222: 121-134.
- Brockes, J. P., M. C. Raff, D. J. Nishigushi, and J. Winter (1980) Studies on cultured Schwann cells. III. Assays for peripheral myelin proteins. *J. Neurocytol.* 9: 67-77.
- Bunge, M. B., A. K. Williams, and P. M. Wood (1982) Neuron-Schwann cell interaction and basal lamina formation. *Dev. Biol.* 92: 449-460.
- Caldwell, J. H., D. T. Campbell, and K. G. Beam (1986) Na channel distribution in vertebrate skeletal muscle. *J. Gen. Physiol.* 87: 907-932.
- Carry, M. R., and M. Morita (1984) Structure and morphogenesis of the neuromuscular junction. In *The Neuromuscular Junction*, R. A. Brumback and J. Gerst, eds., pp. 25-64. Futura, New York.
- Catterall, W. A. (1979) Binding of scorpion toxin to receptor sites associated with sodium channels in frog muscle. Correlation of voltage dependent binding with activation. *J. Gen. Physiol.* 74: 375-391.
- Catterall, W. A. (1980a) Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. *Annu. Rev. Pharmacol. Toxicol.* 20: 15-43.
- Catterall, W. A. (1980b) Pharmacologic properties of voltage-sensitive sodium channels in chick muscle fibers developing *in vitro*. *Dev. Biol.* 78: 222-230.
- Catterall, W. A. (1981) Localization of sodium channels in cultured neural cells. *J. Neurosci.* 1: 777-783.
- Catterall, W. A. (1984) The molecular basis of neuronal excitability. *Science* 223: 653-660.
- Cau, P. (1988) BASIC programs to compute source densities from autoradiographic cross-fire matrices. *J. Microsc. (in press)*.
- Cau, P., A. Massacrier, J. L. Boudier, and F. Couraud (1985) Ultrastructural localization of voltage-sensitive sodium channels using 125-I alpha scorpion toxin. *Brain Res.* 334: 9-17.

- Chiu, S. Y., P. Schrager, and J. M. Ritchie (1984) Neuronal-type Na⁺ and K⁺ channels in rabbit cultured Schwann cells. *Nature* 311: 156–157.
- Couraud, F., E. Jover, J. M. Dubois, and H. Rochat (1982) Two types of scorpion receptor sites, one related to the activation, the other to the inactivation of the action potential sodium channel. *Toxicon* 20: 9–16.
- Couteaux, R., and M. Pecot-Dechavassine (1970) Vesicules synaptiques et poches au niveau des "zones actives" de la jonction neuromusculaire. *C. R. Acad. Sci. [D] (Paris)* 271: 2346–2348.
- Dellmann, H. D., J. A. Boudier, F. Couraud, P. Cau, and J. L. Boudier (1983) Voltage-sensitive Na⁺ channels in the neurohypophysis of the rat as demonstrated by 125-I-labeled scorpion toxin. *Neurosci. Lett.* 35: 71–77.
- Dennis, M. J., and R. Miledi (1974) Electrically induced release of acetylcholine from denervated Schwann cells. *J. Physiol. (Lond.)* 237: 431–452.
- Dreyfus, P., F. Rieger, M. Murawsky, L. Garcia, A. Lombet, M. Fosset, D. Pauron, J. Barhanin, and M. Lazdunski (1986) The voltage-dependent sodium channel is co-localized with the acetylcholine receptor at the vertebrate neuromuscular junction. *Biochem. Biophys. Res. Commun.* 139: 126–201.
- Ellisman, M. H., J. E. Rash, L. A. Staehelin, and K. R. Porter (1976) Studies of excitable membranes. II. A comparison of specializations at neuromuscular junctions and nonjunctional sarcolemmas of mammalian fast and slow twitch muscle fibers. *J. Cell Biol.* 68: 752–774.
- Feller, D. J., J. A. Talvenheimo, and W. A. Catterall (1985) The sodium channel from the rat brain. Reconstitution of voltage-dependent scorpion toxin binding in vesicles of defined lipid composition. *J. Biol. Chem.* 260: 11542–11547.
- Gauthier, G. F. (1976) The motor end-plate structure. In *The Peripheral Nerve*, D. N. Landon, ed., pp. 464–494, Chapman and Hall, London.
- Gray, P. T. A., and J. M. Ritchie (1985) Ion channels in Schwann and glial cells. *Trends Neurosci.* 8: 411–415.
- Hodgkin, A., A. Huxley, and B. Katz (1952) Measurement of current-voltage relations in the membrane of the giant axon of *Loligo*. *J. Physiol. (Lond.)* 116: 424–448.
- Jenssen, K. R., and R. Mirsky (1984) Nonmyelin-forming Schwann cells coexpress surface proteins and intermediate filaments not found in myelin-forming cells: A study of Ran-2, A5E3 antigen and glial fibrillary acidic protein. *J. Neurocytol.* 13: 923–934.
- Jenssen, K. R., R. Thorpe, and R. Mirsky (1984) Molecular identity, distribution and heterogeneity of glial fibrillary acidic protein: An immunoblotting and immunohistochemical study of Schwann cells, satellite cells, enteric glia and astrocytes. *J. Neurocytol.* 13: 187–200.
- Karnovsky, J., and L. J. Roots (1964) A "direct-coloring" thiocholine method for cholinesterase. *J. Histochem. Cytochem.* 12: 219.
- Katz, B., and R. Miledi (1965) Propagation of electric activity in motor nerve terminals. *Proc. R. Soc. [Biol.]* 161: 453–482.
- Katz, B., and R. Miledi (1967) The timing of calcium action during neuromuscular transmission. *J. Physiol. (Lond.)* 189: 535–544.
- Katz, B., and R. Miledi (1968) The effect of local blockage of motor nerve terminals. *J. Physiol. (Lond.)* 199: 729–741.
- Katz, B., and R. Miledi (1969) Tetrodotoxin-resistant electric activity in presynaptic terminals. *J. Physiol. (Lond.)* 203: 459–487.
- Konnerth, A., and R. K. Orkand (1986) Voltage-sensitive dyes measure potential changes in axons and glia of the frog optic nerve. *Neurosci. Lett.* 66: 49–54.
- Kuffler, S. W., J. G. Nicholls, and R. A. Martin (1984) *From Neuron to Brain*, 2nd Ed., Sinauer, Sunderland, MA.
- Landon, D. N. (1982) The excitable apparatus of skeletal muscle. In *Abnormal Nerves and Muscles as Impulse Generators*, W. J. Culp and J. Ochoa, eds., pp. 607–631, Oxford U. P., New York, Oxford.
- Larra, F., and B. Droz (1970) Techniques radioautographiques et leur application au renouvellement des constituants cellulaires. *J. Microsc. (Paris)* 9: 845–880.
- Lev-Ram, V., and A. Grinvald (1986) Ca²⁺- and K⁺-dependent communication between central nervous system myelinated axons and oligodendrocytes revealed by voltage-sensitive dyes. *Proc. Natl. Acad. Sci. USA* 83: 6651–6655.
- Lombet, A., P. Laduron, C. Mourre, Y. Jacomet, and M. Lazdunski (1985) Axonal transport of the voltage-dependent Na⁺ channel protein identified by its tetrodotoxin binding site in rat sciatic nerves. *Brain Res.* 345: 153–158.
- Mallart, A. (1984) Presynaptic currents in frog motor endings. *Pflügers Arch.* 400: 8–13.
- McArdle, J. J. (1984) Overview of the physiology of the neuromuscular junction. In *The Neuromuscular Junction*, R. A. Brumback and J. W. Gerst, eds., pp. 65–119, Futura, New York.
- McArdle, J. J., D. Angaut-Petit, A. Mallart, R. Bournaud, L. Faille, and J. L. Brigant (1981) Advantages of the triangularis sterni muscle of the mouse for investigations of synaptic phenomena. *J. Neurosci. Methods* 4: 109–115.
- Miranda, F., C. Kopeyan, H. Rochat, C. Rochat, and S. Lissitzky (1970) Purification of animal neurotoxins. Isolation and characterization of eleven neurotoxins from the venoms of the scorpions *Androctonus australis* Hector, *Buthus occitanus tunetanus* and *Leiurus quinquestratus quinquestratus*. *Eur. J. Biochem.* 16: 514–523.
- Munson, R. J., B. Westermarck, and L. Glaser (1979) Tetrodotoxin-sensitive sodium channels in normal human fibroblasts and normal human glia-like cells. *Proc. Natl. Acad. Sci. USA* 76: 6425–6429.
- Noble, B. (1964) *Numerical Methods*, vol. I, Oliver and Boyd, Edinburgh.
- Pentreath, V. W. (1982) Potassium signalling of metabolic interactions between neurons and glial cells. *Trends Neurosci.* 5: 339–345.
- Ritchie, J. M., and H. P. Rang (1983) Extraneuronal saxitoxin binding sites in rabbit myelinated nerve. *Proc. Natl. Acad. Sci. USA* 80: 2803–2807.
- Rochat, H., J. Gregoire, N. Martin-Moutot, M. Menashe, C. Kopeyan, and F. Miranda (1974) Purification of animal neurotoxins: Isolation and characterization of three neurotoxins from the venom of *Naja nigricollis mossambica* Peters. *FEBS Lett.* 42: 335–339.
- Rochat, H., M. Tessier, F. Miranda, and S. Lissitzky (1977) Radioiodination of scorpion and snake toxins. *Anal. Biochem.* 82: 532–548.
- Salpeter, M. M., H. C. Fertuck, and E. E. Salpeter (1977) Resolution in electron microscope autoradiography. III. Iodine-125, the effect of heavy metal staining, and a reassessment of critical parameters. *J. Cell Biol.* 72: 161–173.
- Salpeter, M. M., F. A. McHenry, and E. E. Salpeter (1978) Resolution in electron microscope autoradiography. IV. Application to analysis of autoradiographs. *J. Cell Biol.* 76: 127–145.
- Schrager, P., S. Y. Chiu, and J. M. Ritchie (1985) Voltage-dependent sodium and potassium channels in mammalian cultured Schwann cells. *Proc. Natl. Acad. Sci. USA* 82: 948–952.
- Shanes, A. M. (1951) Potassium movement in relation to nerve activity. *J. Gen. Physiol.* 34: 795–807.
- Tessier, M., J. M. Grillo, and J. Mambrini (1978) Binding of 125I-toxin, from the spitting cobra *Naja mossambica mossambica*, at frog neuromuscular junctions. *Toxicon* 16: 682–686.
- Villegas, J., C. Sevcik, F. V. Barnola, and R. Villegas (1976) Grayanotoxin, veratrine, and tetrodotoxin-sensitive sodium pathways in the Schwann cell membrane of squid nerve fibers. *J. Gen. Physiol.* 67: 369–380.
- Waechter, C. J., J. W. Schmidt, and W. A. Catterall (1983) Glycosylation is required for maintenance of functional sodium channels in neuroblastoma cells. *J. Biol. Chem.* 258: 5117–5123.
- Waltz, W., and L. Hertz (1983) Functional interactions between neurons and astrocytes. II. Potassium homeostasis at the cellular level. *Prog. Neurobiol.* 20: 133–183.
- Williams, M. A. (1977) Quantitative methods in biology. In *Practical Methods in Electron Microscopy*, vol. 6, A. M. Glauert, ed., pp. 85–201, North-Holland, Amsterdam.
- Williams, M. A., and A. Downs (1978) An iterative approach to the analysis of EM autoradiographs. *J. Microsc.* 114: 157–178.
- Wolitzky, B. A., and D. M. Fambrough (1986) Regulation of the (Na⁺ + K⁺)-ATPase in cultured chick skeletal muscle. Modulation of expression by the demand for ion transport. *J. Biol. Chem.* 261: 9990–9999.