CHARGE MOVEMENTS MEASURED DURING TRANSVERSE-TUBULAR UNCOUPLING IN FROG SKELETAL MUSCLE

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ABSTRACT Capacity transients and slow asymmetric charge-movements are measured in frog skeletal muscle using the Vaseline-gap voltage-clamp technique. Capacity transients show a rapid phase lasting 10–30 μs, due to the charging of the surface membrane capacitance, and a slower phase lasting several milliseconds, consistent with the charging of the transverse tubular system (T-system). Exposure to isotonic CsF caused the ratio of the slowly-charging capacitance (c_{slow}) to the fast-charging capacitance to decline by 88 ± 9% (n = 16). Electron micrographs of four fibers treated with CsF show disruption and disorganization of the T-system and sarcoplasmic reticulum membranes and a >90% decrease in the number of dyads and triads. The role of CsF was investigated: (*a*) Fibers exposed to CsF internally or externally, exhibit slower and less complete loss of c_{slow} than fibers exposed both internally and externally. (*b*) Little loss of c_{slow} occurs during the external exposure to CsF. The bulk of loss occurs only after the fiber is returned to Ca^{++}-containing solution. (*c*) Elevated external Ca^{++} causes more rapid and more complete loss of c_{slow}. The time-course of c_{slow} loss is gradual, occurring over a period of 10 min to 2 h. The progressive loss of c_{slow} is accompanied by a progressive decline in the peak of the slow asymmetric charge-movement and a progressive slowing of charge movement kinetics. These effects are qualitatively accounted for by including gradual tubular uncoupling in a distributed model of charge movement proposed by B. Simon and K. G. Beam (1985, *J. Gen. Physiol.*, 85:21–42).

INTRODUCTION

In electrically excitable cells, asymmetric charge-movements have been detected that are hypothesized to arise from voltage-induced molecular rearrangements of membrane proteins (Schneider and Chandler, 1973; Armstrong and Bezanilla, 1974; reviewed by Almers, 1978; and by Armstrong, 1981). These charge movements have been implicated in the voltage-dependent regulation of ionic conductances in nerve and muscle cells, where they are called "gating currents" and also in the process of excitation-contraction (E-C) coupling in skeletal muscle where they are called "charge movement." In E-C coupling, an action potential propagates into the transverse-tubular system (T-system) from the sarcolemma, and the ensuing depolarization of the T-system membrane leads to release of Ca^{++} from the sarcoplasmic reticulum (SR). The slow asymmetric-charge movement of skeletal muscle has been hypothesized to link this T-system depolarization to the subsequent release of Ca^{++}.

The hypothetical role of charge movement in E-C coupling requires that most of the charge be located in the T-system. However, evidence for such a tubular location of charge movement is scant and indirect. One piece of evidence comes from the effect of glycerol shock on charge movement. Treatment of skeletal muscle with hypertonic glycerol, after a sudden return to isotonic solution, causes electrical discontinuity and morphological disruption of the T-system (Howell and Jenden, 1967; Eisenberg and Eisenberg, 1968; Franzini-Armstrong et al., 1973; Valdiosera et al., 1974). Chandler et al. (1976) sought to use glycerol shock to determine if the charge-movement molecules are located in the T-system. They found that glycerol treatment removed only ~40% of the tubular capacitance, while it eliminated 77% of the charge movement. Although these results are not inconsistent with a tubular location of the charge, they indicate that the effect of glycerol shock on charge movement may not depend solely on the electrical uncoupling of the T-system. Thus, their results do not exclude the possibility that the charge resides in the surface membrane and is affected by glycerol treatment in some manner that is independent of effects on the T-system.

Additional evidence for a tubular location of the mobile charge comes from experiments on charge-movement kinetics. Simon and Beam (1985*a*, *b*) investigated the

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source of the slow rising phase of charge movement that gives the current a rounded peak. They found that a simple kinetic scheme for the movement of charge could entirely account for the time-course of ON-, OFF-, and STEPPED-OFF charge movements as long as the delays inherent in the distributed morphology of the T-system were also accounted for in the model. Without the incorporation of tubular delays, a complicated kinetic scheme for charge movement is required to fit the rising phase (Horowicz and Schneider, 1981).

Here I draw on both previous approaches and describe treatments with isotonic CsF that cause gradual electrical uncoupling of the T-system from the surface membrane of skeletal muscle fibers. Charge movements measured at various stages during this uncoupling show progressive distortion of their kinetics. When models for progressive uncoupling of the T-system are incorporated into the distributed model of Simon and Beam (1985*b*) the calculated charge movements mimic the distortion seen experimentally, supporting the idea that the mobile charge resides in the T-system.

METHODS

Single fibers were dissected from semitendinosus muscles of large (11–17 cm) northern bullfrogs (*Rana catesbiana*) and voltage clamped using the Vaseline gap technique (Hille and Campbell, 1976). This technique is based on the standard method for voltage clamping nodes of Ranvier of myelinated axons. Vaseline seals are used to isolate an annular patch of membrane in one pool of the recording chamber containing an external solution such as Ringer's. Since this annulus is analogous to the node of Ranvier, it will be called the artificial node and the pool bathing it will be called the nodal pool. Voltage inside the artificial node is measured and current is passed to control that voltage, through ends of the muscle fiber that have been cut in pools containing a Ca-free internal solution. In these experiments the fiber ends are cut 2–3 mm from the nodal pool, and then they are cut again about halfway across the fiber diameter ~650 and 900 μm from the center of the nodal pool.

A small flap of plastic film is applied over the seals to hold the solution level above the top surface of the fiber. As previously described (Campbell and Hahin, 1984), this eliminates the thin layer of solution across the top of the fiber that would otherwise contribute to a nonuniform resistance in series with the sarcolemma. The remaining resistance in series with the sarcolemma (0.5–1.5 ohm · cm^2) was estimated from the "hop" in voltage produced by a rapid step in membrane current and compensated electronically. A much larger and nonuniform resistance represented by the lumen of the T-system is in series with the tubular membrane. This resistance is not compensated and is reflected in the slow time-course of charging the tubular membrane capacitance that is the subject of this paper.

Data acquisition and analysis are as described previously (Campbell, 1983). Briefly, the output of the current-to-voltage converter is filtered with a four-pole Bessel filter, amplified, filtered again at the same frequency, digitized by a 12-bit A/D converter, and stored on floppy diskettes by a laboratory minicomputer. For charge-movement recordings, the preliminary subtraction of linear capacity and leakage currents is accomplished with an analog transient generator, permitting the remaining current to be recorded at high gain without exceeding the range of the A/D converter. The remaining linear current is eliminated digitally by subtracting an appropriately scaled control record elicited by voltage steps from −120 to −90 mV.

Capacitance Measurement

To measure the membrane capacitance of the artificial node, current is recorded without analog leak subtraction for a voltage step from −135 to −90 mV. With the Vaseline-gap method, the rapid phase of the capacity charging transient for such a voltage step is complete in 10–20 μs, although the remaining slow transient may last for 10–15 ms. To faithfully record the amount of charge moved during the rapid phase of charging, the current transient is slowed by filtering the signal at 10–20 kHz and the resulting signal is digitized at 100 kHz. This current record is then integrated by the computer to give the charge. The early, rapidly moving charge, Q_{fast}, is measured separately and used to estimate the rapidly charging portion of the membrane capacitance, c_{fast}, which presumably represents the capacitance of the surface membrane. Using this method, control measurements of capacitors of known value were accurate within ±4%. The total charge that flows over a 21.5-ms step is used to estimate the total capacitance, c_{total}. The slowly charging portion of the membrane capacitance, c_{slow}, is then calculated as $c_{total} - c_{fast}$. Measurements are given in the text as the mean ± the standard deviation.

Solutions

K-free Ringer's solution contained (in millimoles per liter) 110 NaCl, 2 $CaCl_2$, 5 CsCl, and 5 HEPES at pH 7.4. Charge movement solution contained 115 tetraethylammonium (TEA) bromide, 5 CsCl, 2 $CaCl_2$, 5 HEPES titrated to pH 7.4 with Tris and 10^{-6} M tetrodotoxin to block Na currents. The high-Ca^{++} Ringer's solution contained 20 $CaCl_2$, 90 NaCl, 5 CsCl, and 5 HEPES at pH 7.4. The CsF internal solution contained 120 CsF and 3 HEPES, adjusted to pH 7.3. Relaxing solution contained 112 Cs-aspartate, 4 Mg-aspartate, 7 Cs_2EGTA, 5 creatine phosphate, 3 ATP, and 7 HEPES adjusted to pH 7.3 with TEA-OH.

Electron Microscopy

The state of the internal membranes was studied in electron micrographs made from two control fibers (not exposed to CsF) and four "uncoupled" fibers that were exposed internally and externally to CsF. After voltage clamp measurements, while the fibers were still mounted in the recording chamber, they were fixed in a solution containing 150 mM Na cacodylate and 3% glutaraldehyde. After fixing for 30 min, the fibers were gently teased out from under the Vaseline seals, and trimmed with a scalpel so that the nodal region of the fiber occupied the central one-third of an ~250-μm fragment of fiber. This permitted identification of the nodal region during later sectioning. Fiber fragments were fixed for another 6–12 h, dried in ethanol and embedded in Epon.

Computations

The model used for the calculations of capacity transients and charge movements was developed by Simon and Beam (1985*b*) to describe charge movements in mammalian muscle, and details of the model can be found in their paper. The approach is that used by Adrian and Peachey (1973), in which the fiber cross-section is represented by a series of concentric annuli. The passive properties and the charge movement of individual annuli are lumped together, with neighboring annuli connected by lumped conductances, the values of which are computed from the luminal conductivity and geometric factors. The charge in each annulus is assumed to distribute between two states according to a first-order reaction with rate constants that depend on membrane voltage.

Values of model parameters were as given by Simon and Beam (1985*b*), with the following exceptions: R_a, the access resistance to the T-system, was increased from 60 to 100 ohm · cm^2; G_l, the luminal conductivity was 10 mS/cm; G_m, the conductance per unit area of the tubule membrane was 0.016 mS/cm^2; the radius was 80 μm (typical for the bullfrog fibers used); $\bar{V}$ and k, derived from a fit of the Boltzmann equation to an experimentally determined charge vs. voltage relationship,

were −15 and 18.4 mV, respectively; $\bar{\tau}$ derived from a fit of the model to the experimental charge movement measured for a step to 0 mV was 16 ms. For a given voltage step, current transients were calculated for the model with and without including charge movement. The charge movement was obtained by subtracting the current transient without charge movement from the transient including the charge movement, a procedure analogous to the digital subtraction of linear currents used to obtain the experimental charge movements.

All calculations presented here were made using 12 annuli. In tests, several calculations using 16 and 24 annuli were found to give virtually identical results. Computations were carried out in double precision using Fortran on a VAX 11/780 minicomputer (Digital Equipment Corp., Maynard, MA). To approximate the delay introduced by the electronic filter, the voltage step on the surface was assumed to rise with a time constant of 50 μs. Because this rate of charging is considerably faster than that used for the microelectrode voltage-clamp model, it was necessary to make the integration interval quite short (0.02 μs instead of the 0.5 μs used by Simon and Beam, 1985*b*) for accuracy during the initial charging period. Such a short integration interval is not required after about the first 0.1 ms. Therefore, to shorten the computation time the computer program doubled the integration interval whenever the second derivative of the voltage in the outermost annulus became smaller than a given tolerance (usually 10^{-5} times the remaining voltage in the step). The maximum integration interval was not permitted to exceed 2.56 μs because longer intervals sometimes caused instabilities in the computation.

RESULTS

Electrical Evidence of T-Tubular Uncoupling

A convenient way to normalize the current measured with the Vaseline-gap technique is to measure membrane capacitance. During the course of individual experiments it was frequently observed that the slowly charging portion of the muscle membrane capacitance (c_{slow}) decreases substantially with time, while the rapidly charging component (c_{fast}) decreases slightly or not at all. Because in the original description of the Vaseline-gap method (Hille and Campbell, 1976), the fiber was allowed to shorten considerably, and since severe shortening might be expected to put radial stress on the T-tubules, it was hypothesized that the loss of c_{slow} was caused by such shortening. Fig. 1 illustrates the loss of the slowly charging capacitance and demonstrates that this loss also occurs in rest-length fibers.

Fig. 1 shows current transients recorded from a fiber that was prevented from shortening during the mounting process. Both traces in Fig. 1 *A* exhibit pronounced early transients of current due to the charging of the surface membrane capacitance to the new voltage level. In addition to this rapid phase of charging, the capacity transient measured near the beginning of the experiment also exhibits a pronounced slow phase of charging, lasting several milliseconds, that is particularly evident at the higher gain of Fig. 1 *B*. The size and time-course of this slowly charging portion of the capacity transient is consistent with the idea that it represents the charging of the T-system

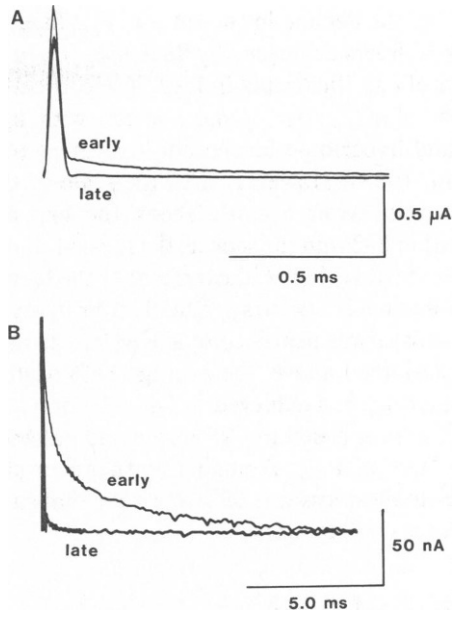

FIGURE 1 Loss of capacitance of a muscle fiber cut in CsF. To prevent shortening during the initial depolarization in CsF, the fiber was held at ~1.2 times rest length for the first 3 min after its transfer from Ringer's solution to isotonic CsF, and then allowed to return to rest length. In 12 control fibers visualized by phase contrast microscopy, this procedure resulted in a sarcomere spacing of 2.2 ± 0.1μm (mean ± SD). After mounting in the Vaseline-gap chamber, the ends of the fiber were cut in the CsF solution and Ringer's solution was flushed into the pool containing the artificial node. (*A*) Capacitative charging transients elicited by 45 mV depolarizations from −135 to −90 mV are displayed at low gain over a short time scale. (*B*) The same two capacity transients, displayed at high gain (the peak of the current records have been truncated) and at a slower sweep speed. The *upper trace* in each frame was recorded within a minute of flushing CsF from the nodal pool with K-free Ringer's solution. The *lower trace* in each frame was measured 22 min later. For the transient recorded after 1 min, the rapid spike of current carried 37.9 pC of charge and the total transient carried 210.2 pC. For the transient recorded 22 min later, the rapid spike of current carried 36 pC and the total transient carried 41.6 pC. c_{slow} calculated from the measured charge declined from 3.8 to 0.12 nF, while c_{fast} declined from 0.84 to 0.80 nF. Fiber 221, T = 18°C.

membrane through the distributed series resistance of the tubular lumen. Thus, in the transient recorded early in the experiment the ratio Q_{fast}/Q_{slow} is 1/4.5, close to the value expected for the ratio of surface to tubular capacitance in a frog muscle fiber 80 μm in radius (Almers et al., 1982). In the lower trace, recorded 22 min later, the slow phase of charging is almost entirely absent, with the ratio Q_{fast}/Q_{slow} equal to 1/0.15. In this experiment, c_{slow} declined by 97% while c_{fast} decreased by 5%, consistent with the idea that the T-system has become electrically uncoupled from the surface membrane. In 16 fibers that were both cut in CsF, and also exposed externally to CsF, c_{fast} decreased by 16 ± 12% (mean ± SD), while c_{slow} decreased by 90 ± 8%. Some of the loss of capacitance may be due to a slight flowing or shifting of the Vaseline seals during the course of the experiment. Thus, the loss of c_{slow} may be more accurately

described by the decline in the ratio of c_{slow}/c_{fast}, which in this group of fibers decreased by 88 ± 9%.

The loss of c_{slow} illustrated in Fig. 1 is electrically similar to the loss of c_{slow} after osmotic shock with hypertonic glycerol and hypertonic formamide treatment (Campbell and Hahin, 1983). However, the time-scale of the loss is quite different. After osmotic shock the loss of c_{slow} is evident within 2–3 min (as soon as the measurement can be made). By contrast, Fig. 2 illustrates that the loss of c_{slow} in fibers exposed to CsF occurs gradually over many minutes. In this fiber, loss was nearly complete within 22 min. In the 16 fibers described above, the average 88% decline in the ratio of c_{slow}/c_{fast} was achieved in 34 ± 19 min. All of the fibers that were exposed to CsF inside and outside showed significant loss of c_{slow}. Among the 16 fibers studied in detail, the smallest loss was 69% in a fiber that survived 35 min. before becoming leaky.

External Exposure to CsF Promotes Loss of c_{slow}

Previous work by others has demonstrated that under some conditions electrophysiological signals from the T-system, or events presumably triggered by T-system depolarization such as Ca^{++} release or contractile activation, can be studied using cut fibers (Vergara et al., 1978; Vergara and Bezanilla, 1979; Kovacs et al., 1979; Horowicz and Schneider, 1981). Because the various internal solutions used in those studies did not contain CsF, it seemed possible that exposure to CsF was involved in T-system uncoupling. Therefore, experiments were carried out to: (*a*) confirm the observations that suggested internal solutions without CsF preserve tubular connectivity; (*b*) determine the effect of exposing only the external membrane in the nodal pool to CsF; and (*c*) determine the effect of exposing only the fiber interior to CsF. Fig. 3 *A* demonstrates that c_{slow} is preserved when fibers are cut in Cs-aspartate relaxing solution instead of in CsF. The two capacity transients in Fig. 3 *A*, recorded 35 min apart, superimpose identically over their entire time-course, indicating no change in the slowly charging capacitance, or in the resistance connecting it with the surface membrane. Fig. 3 *B* shows that charge movements measured 35 min apart in this fiber are also virtually unchanged, demonstrating that cutting the fiber in relaxing solution also preserves the slow asymmetric charge movement. In three other fibers cut in relaxing solution and studied for up to 2.5 h, c_{slow} declined by <8% and total charge by <10%. These results confirm the observations of other investigators that cutting fibers in relaxing solution preserves T-system connectivity and charge movement function (Vergara et al., 1978; Vergara and Bezanilla, 1979; Kovacs et al., 1979; Horowicz and Schneider, 1981; Almers et al., 1981; Heiny and Vergara, 1982; Heiny et al., 1983). This preservation of c_{slow} and charge movement is in marked contrast to the capacity transients illustrated in Fig. 1 and to the average 88% decrease in c_{slow} seen over the same time-scale in the fibers treated with CsF.

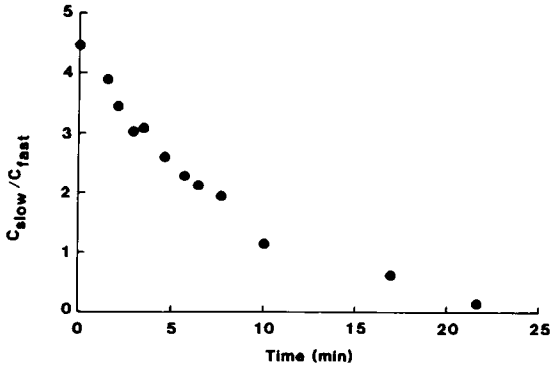

FIGURE 2 Time-course of the loss of capacitance. The ratio of the charge moved during the slowly charging portion of the capacity transient to the charge moved during the initial spike of current is plotted as a function of time for the fiber of Fig. 1.

In contrast to the preservation of capacitance and charge movement in Figs. 3 *A* and *B*, Fig. 3 *C* illustrates that c_{slow} in the same fiber is abruptly decreased after a brief exposure of the external membrane to CsF. Fig. 3 *D* demonstrates that along with this sudden loss of c_{slow} there is a simultaneous decrease in the peak of charge movement and a pronounced slowing of charge movement kinetics. This loss of charge movement will be considered later in more detail. In three fibers that were cut in relaxing solution and then transiently exposed externally to CsF, the average loss of c_{slow} was 43%.

The effect of cutting the ends of a fiber in CsF without exposing the exterior of the artificial node was examined in three fibers (not illustrated). In one, c_{slow} decreased by only 33% after 4.4 h. In another, c_{slow} decreased by 33% in 2.2 h. The artificial node of this second fiber was then transiently exposed to CsF, and 10 min after this treatment c_{slow} had declined to 15% of its original value. In the third fiber the external solution contained 10 mM Ca^{++} rather than the normal 2 mM Ca^{++}. In this fiber, c_{slow} decreased by 63% in 25 min. Potentiation of the loss of c_{slow} by elevated Ca^{++} is discussed next.

High External Ca^{++} Accelerates Uncoupling

Loss of c_{slow} is slight during the time that the artificial node is bathed in CsF. Indeed, fibers cut in CsF and bathed externally in CsF for periods of up to 2 h show little or no loss of c_{slow} (not shown). However, loss of c_{slow} occurs quite rapidly when a fiber soaked for a prolonged period in CsF is returned to Ca^{++}-containing solution, reinforcing the idea that external Ca^{++} is required for the uncoupling process. To explore this possibility further, the effect of elevated Ca^{++} on the uncoupling process was tested. Three fibers were cut in CsF solution and then the CsF in the nodal pool was replaced by Ringer's solution containing 20 mM Ca^{++}. In two of the fibers 98–99% of c_{slow} was lost in

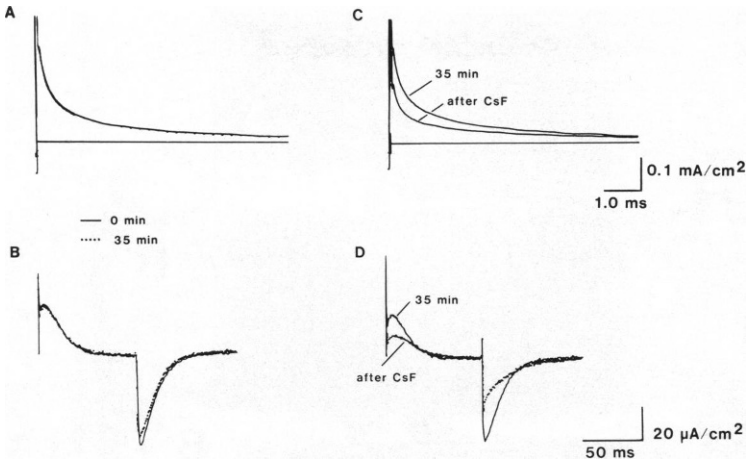

FIGURE 3 Transient external exposure to CsF promotes capacitance loss. The muscle fiber was dissected and mounted in the standard way except that the ends were cut in Cs-relaxing solution. (*A*) Capacity currents recorded at the beginning of the experiment (connected trace) and 35 min later (dots) are shown superimposed at the same gain. Because the gain is relatively high, the early spike of the capacity transient is off-scale. The two traces are identical, demonstrating that cutting in relaxing solution preserves the slowly charging portion of membrane capacitance. Current elicited by a step from −135 to −90 mV. Fiber bathed in charge movement solution. (*B*) Asymmetric charge movements recorded at the beginning of the experiment and 35 min later are shown superimposed. The voltage-clamp pulse was a step to +30 mV and a return to the holding potential of −90 mV. (*C*) The 35-min capacity transient shown in *A* is displayed superimposed on the transient measured 12 min later. In the interval between the two recordings the charge movement solution bathing the artificial node was changed to CsF and then, after 4 min in CsF, changed back to the Ca^{++}-containing charge-movement solution. This transient exposure to CsF caused the slowly charging portion of the membrane capacitance to decrease from 4.2 to 2.9 nF, and the rapidly charging capacitance to decrease from 0.66 to 0.58 nF. (*D*) Charge movements measured before and after the transient exposure to CsF. The 35-min charge movement from *B* is superimposed on the charge movement measured 12 min later, 8 min after the CsF treatment described in (*C*). Fiber 254, T = 5°C.

an average of 13 min; in the third, c_{slow} decreased 76% in 29 min. For comparison, in the five out of 16 fibers that showed >94% loss, the average time required for that degree of uncoupling was 44 min.

Ultrastructural Changes After Tubular Uncoupling

Four test muscle fibers were studied first electrically, and then examined electron micrographically to determine if ultrastructural changes were correlated to the loss of c_{slow} that is observed electrically. Test fibers were cut in CsF, bathed in either 2 (one fiber) or 20 mM Ca^{++} solution (three fibers) and studied electrically using the voltage clamp. Over a period of 30–60 min, these test fibers exhibited an average 92% decrease in c_{slow}. Fig. 4 illustrates the internal membrane disruption seen in the test fibers. In this fiber, exposed externally to 2 mM Ca^{++}, occasional triads may be seen (*A*). More typical in this and in the other test fibers was the appearance of large, disorganized vacuoles of SR membrane and the absence of triad and dyad structures. The disruption was sufficiently severe that it was impossible to be certain whether or not tubules remained. For this reason it was also impractical to attempt a quantification of tubular volume. Instead, the simpler procedure of counting clearly recognizable triad and dyad structures at the z-lines was used. Forty-two micrographs from random sites at different fiber depths were analyzed, and the four test fibers were found to contain 96% fewer dyads and triads than control fibers.

Asymmetric Charge Movements During Tubular Uncoupling

If the asymmetric charge-movement is located in the T-system, then it seems likely that progressive uncoupling of the T-system will cause progressive loss of the charge movement. Also, since the uncoupling of some portions of the T-system may diminish the access to remaining portions, it is reasonable to expect progressive changes in the kinetics of charge movement during progressive uncoupling. Such changes in charge-movement kinetics that were seen in Fig. 3 *D*, are shown in more detail in Figs. 5 and 6. The fiber of Fig. 5 was cut in CsF and also transiently exposed to CsF externally. The left-hand set of records were made 17 min after cutting the ends of the fiber in CsF, and 5 min after replacing the CsF in the nodal pool with Ringer's solution. During the course of the experiment, as c_{slow} decreases, the peak of the charge movement also decreases. In addition, the kinetics of the charge movement are progressively slowed, so that the time to peak has increased from 6 ms at 17 min to ~25 ms after 75 min. The slowed time-course of the altered charge-movement makes it difficult to estimate accurately the quantity of charge moved. In part this is because small differences in the choice of a baseline make large differences in the integral of the current record. Also, at the later stages of uncoupling, the falling phase of charge movement is not complete by the end of the 80 ms voltage pulse. Nevertheless, by 83 min, when only about 3% of the slowly

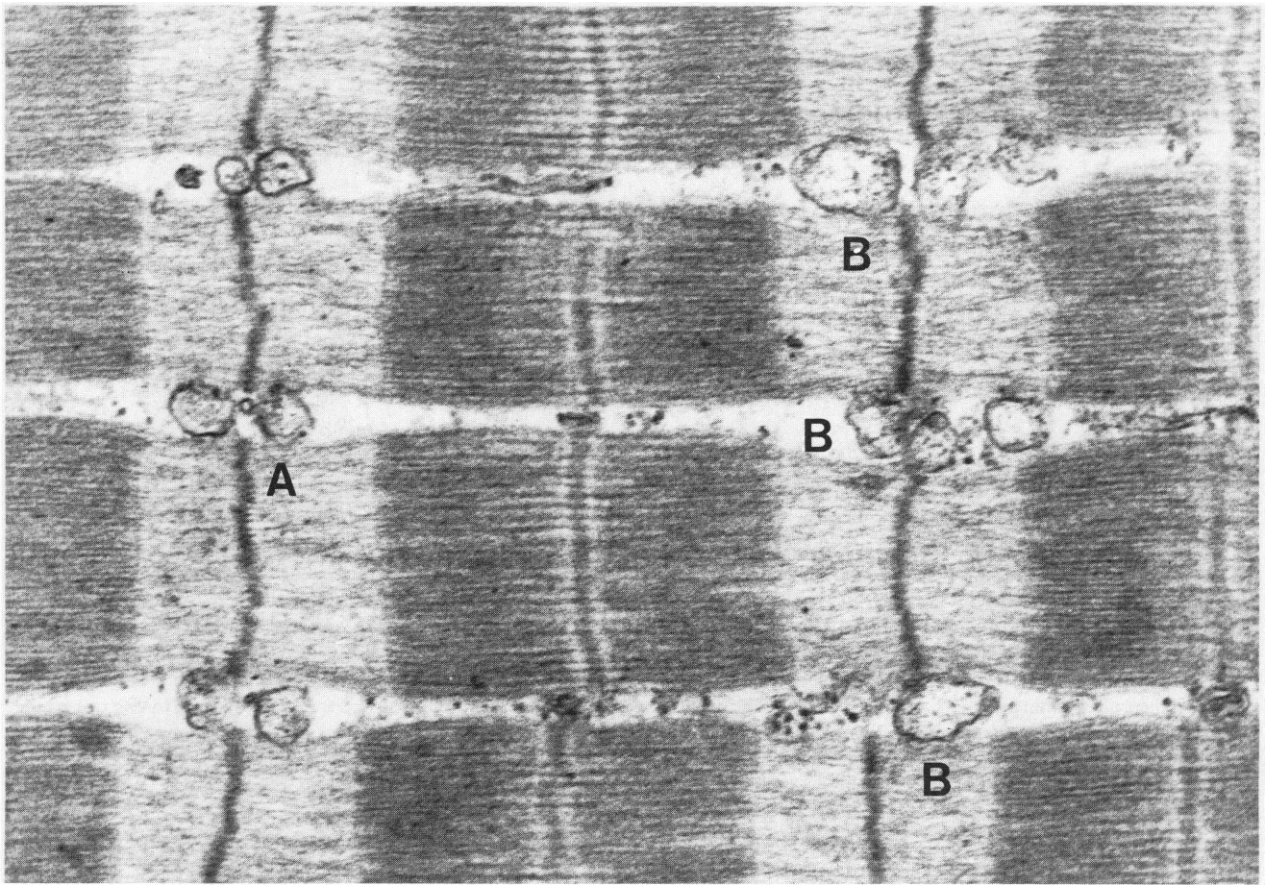

FIGURE 4 Electron micrograph of a test fiber exposed to CsF. The fiber was held at ~1.2 times rest length during the initial depolarization after the transfer to CsF solution, and then allowed to shorten to its slack length. Fiber ends were cut in CsF and then the solution in the nodal pool was changed from CsF to Ringer's solution. Before fixation and preparation for microscopy, the fiber was studied electrophysiologically. 25 min after flushing Ringer's solution into the nodal pool, the ratio of c_{slow} to c_{fast} had declined from 3.8 to 0.41 pC. This section, obtained from the region of the fiber axis, illustrates the variety of the disruption of membranes after CsF-treatment. Occasional triads appear to have relatively normal morphology (*A*). However, much more common in the intrafibrillar spaces at the z-line level are disorganized and swollen membrane vacuoles (*B*). Clear identification of transverse tubules at such sites is not possible. Control fibers that were similarly fixed while mounted in the recording chamber showed no such swollen vacuoles and contained numerous intact triads. x45,000.

charging capacitance remains, there is also very little charge movement remaining.

The sharp peak of current early in the charge-movement records is Na-channel gating current. In this fiber, in the 66 min between the time the records on the left and those on the right were obtained, the measured Na-channel gating charge increased slightly (from 5.9 to 6.2 pC). This increase probably reflects the difficulties in choosing the baseline for integration of the gating current in records exhibiting significant slow charge-movement (Campbell, 1983) rather than an actual increase in gating charge.

In an experiment like that illustrated in Fig. 5, in which the fiber is cut in CsF and in which the exterior of the fiber is also initially exposed to CsF, it is likely that there is some loss of c_{slow} and distortion of the charge movement before the first measurement can be made. In the experiment illustrated in Fig. 6, the fiber was mounted and cut while bathed in relaxing solution so that a control charge-movement with normal kinetics could be recorded before uncoupling of c_{slow} was initiated. After the control currents shown in the *left* column were recorded, the membrane in the nodal pool was briefly exposed to CsF. After this brief exposure, c_{slow} declined over the succeeding 27 min. As in Fig. 5, the progressive uncoupling of c_{slow} is accompanied by a progressive decrease in the peak charge-movement and a slowing of the time to peak. Similar results were obtained in five fibers in which charge movement was measured during the gradual loss of c_{slow}.

The Distributed Two-State Model Describes the Kinetics of Frog Muscle Charge Movement

Calculations were carried out to determine if a tubular location of charge movement could qualitatively account for the distortion of charge-movement kinetics seen during tubular uncoupling. It was necessary to start with a model that takes into account the effect of tubular geometry on

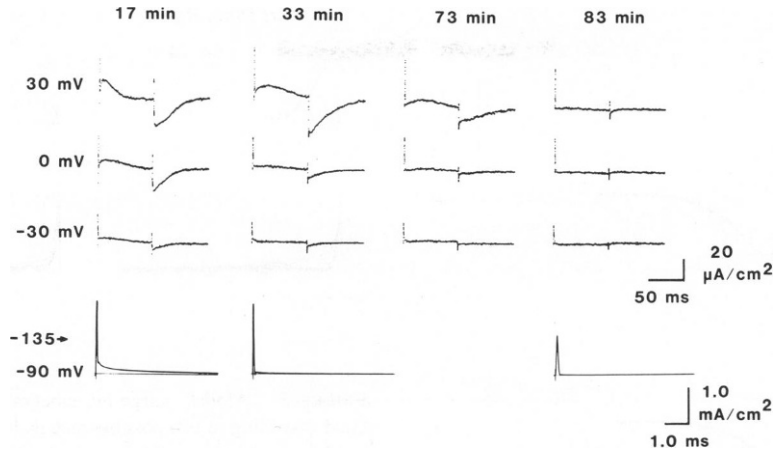

FIGURE 5 Charge movements recorded during the progressive loss of slow capacitance. The muscle fiber was bathed in CsF during the mounting process, the fiber ends were cut in CsF, and then after mounting, Ca^{++}-containing charge-movement solution was flushed into the nodal pool. In each column, charge movements measured at three different voltages are shown. At the bottom of the column are shown the capacity transients recorded at the same time as the charge movements. (There was no capacity transient measured at 73 min. Note that the peak of the capacity transient measured at 83 min is attenuated due to filtration at 10 kHz, although the charge carried during the early spike of current is similar to that of the 33-min trace.) 17 min after cutting the ends of the fiber in CsF, c_{fast} = 0.67 and c_{slow} = 5.33 nF. After 33 min, c_{fast} = 0.61 , c_{slow} = 1.12 nF. After 83 min, c_{fast} = 0.58 and c_{slow} = 0.14 nF. Fiber 255, T = 6°C.

charge-movement kinetics. Such a model has been proposed by Simon and Beam (1985*b*). They found that the delayed rising phase of charge movement (seen as the rounded peak in the charge movement records) could be accounted for entirely by delays in tubular membrane voltage inherent in the distributed geometry of the T-system. In their model, the charge movement itself has simple two-state kinetics, with voltage-dependent rate constants governing the distribution and the rate of movement of the charge between the two states. Fig. 7 demonstrates that this distributed two-state model, which was developed to describe charge movement in mammalian muscle, also provides a good fit to frog muscle charge movements. The major disagreement between the model and the measured charge-movements occurs at early times and is largely due to the rapidly moving Na gating charge.

Calculated Charge Movements and Capacity Transients During Simulated Tubular Uncoupling

Calculations of the distributed two-state model were carried out for three different models of tubular uncoupling: (*a*) The access resistance between the exterior and the

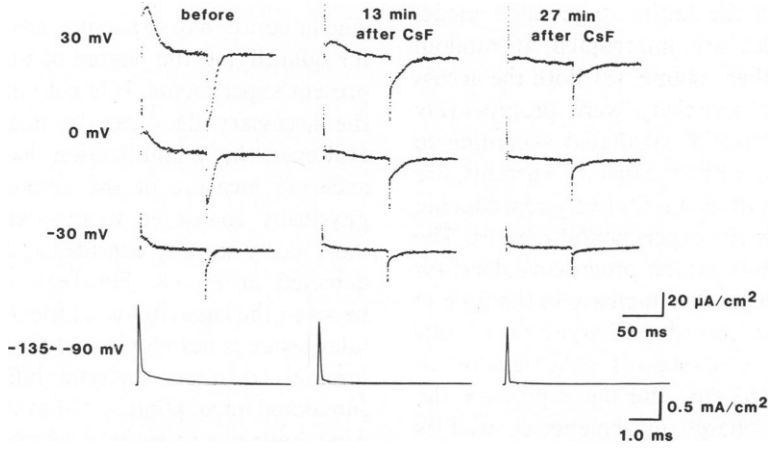

FIGURE 6 Charge movements recorded before and after external exposure to CsF. The fiber was bathed in Cs-relaxing solution during the mounting procedure to avoid exposing the membrane of the artificial node to CsF. After mounting, the ends of the fiber were cut in CsF and the Cs-relaxing solution in the nodal pool was replaced by charge-movement solution. The records in the *first column* were obtained 17 min after cutting the ends of the fiber in CsF. At this point, c_{fast} was 0.73 and c_{slow} was 3.18 nF. Following this set of recordings, the solution in the nodal pool was changed to CsF for 3 min and then changed back to charge movement solution. Four min later c_{slow} had declined to 2.11 nF (not shown). The traces in the *middle column* were recorded 13 min after the CsF treatment, at which time c_{fast} was 0.72 nF, and c_{slow} was 1.66 nF. The right-hand set of traces was recorded 29 min after the CsF treatment when c_{fast} = 0.66 and c_{slow} = 0.91 nF. Fiber 259, T = 6°C.

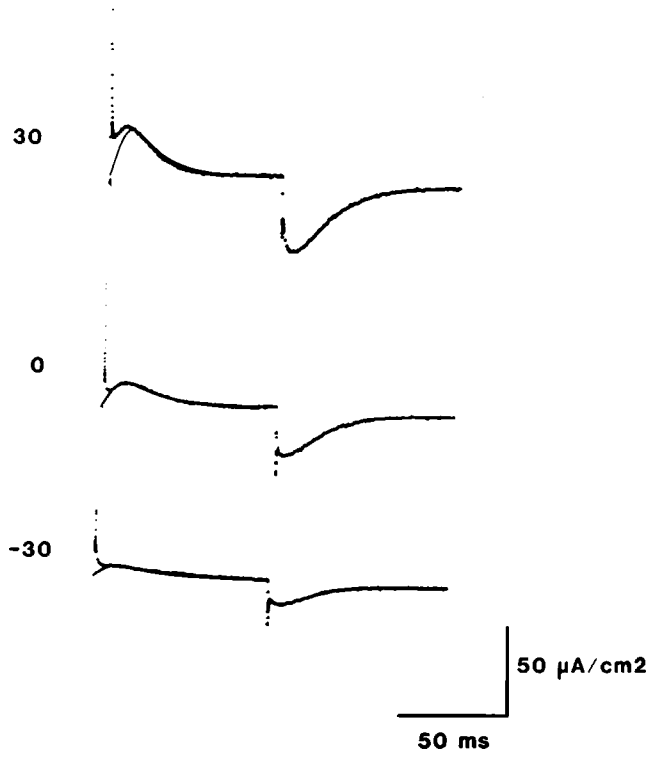

FIGURE 7 The distributed model fits frog muscle charge-movements. Charge movements elicited by steps to three different potentials are shown superimposed on ON-charge-movements calculated from the Simon and Beam model (1985*b*). Fiber 258, ends cut in Cs-relaxing solution, T = 5°C.

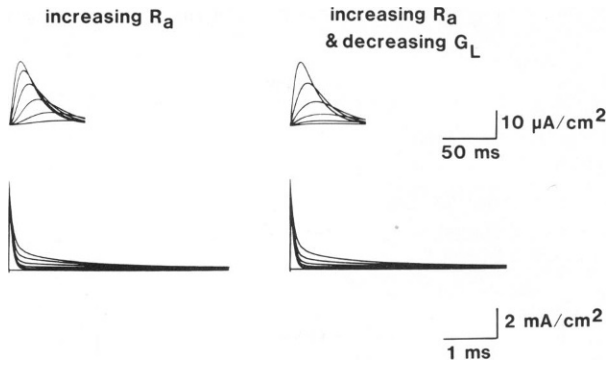

FIGURE 8 Model charge-movements and capacity transients calculated according to two possible models for the progressive uncoupling of the T-system. *Above:* charge movements calculated for voltage steps to +30 mV. The *upper trace* in each set is identical and corresponds to the +30 mV trace of Fig. 7. *Left:* the progressively smaller currents were calculated by assuming that R_a increased by a factor of 2 for each successive trace. No other parameters of the model were changed. *Right:* R_a was increased by a factor of 2 and G_l was decreased by a factor of 2 for each successive calculation. *Below:* the corresponding sets of capacity currents displayed at a lower gain and over a shorter time scale.

tubular lumen was progressively increased. This is roughly equivalent to disconnecting an increasing number of individual openings from the T-system to the outside. (*b*) The resistivity of the tubular lumen was progressively increased. This is equivalent to deposition of nonconducting material throughout the lumen of the T-system. It is also roughly equivalent to the initial stages of a model whereby individual tubules are interrupted at random locations throughout the fiber volume. (*c*) Both the access resistance and the lumen resistivity were progressively increased. Fig. 8 shows currents calculated according to models 1 and 3. The two sets of capacity currents are similar to each other, as well as qualitatively reproducing the decrease in c_{slow} seen in the experimental records. The calculated charge movements exhibit progressive decrease in peak current and the progressive increase in the time to peak in the experimental records. Charge movements calculated from model 2 (not shown) gave less of an increase in the time to peak and did not reproduce the kinetics of the measured charge movements as well as models 1 and 3.

DISCUSSION

CsF Promotes Uncoupling of the T-system

The slowly charging portion of the membrane capacitance has a magnitude and a charging time-course that is consistent with the idea that c_{slow} represents the capacitance of the T-system membrane. The loss of c_{slow} observed after CsF treatment is mimicked by osmotic shock using hypertonic glycerol or hypertonic formamide (Campbell and Hahin, 1983). Since such osmotic shock is known to cause T-tubular disruption (Howell and Jenden, 1967; Eisenberg and Eisenberg, 1968; Franzini-Armstrong et al., 1973) the similar effects of the CsF and glycerol treatments on the capacity transients support the conclusion that c_{slow} represents the tubular capacitance, and that c_{slow} loss reflects tubular uncoupling.

Quantification of Uncoupling

The integration of capacity transients gives a useful means for quantifying the degree of uncoupling observed in the present experiments. It is not only easily applied, but also the necessary data can be acquired in a few seconds. However, this quantification does not necessarily give an accurate measure of the amount of tubular membrane physically connected to the extracellular space. This is most easily seen by considering the models of uncoupling depicted in Fig. 8. For both models electrical contact between the capacitance of the T-system and the extracellular space is not eliminated until the access resistance or luminal resistance become infinite. Instead, they give functional uncoupling by (*a*) so slowing the charging of the capacitance as to make it effectively uncoupled over reasonable time intervals, and (*b*) by decreasing the effective length constant of the tubular system, resulting in incomplete charging of membrane deep within the fiber. A more detailed characterization of the electrical equivalent circuit of the T-system during the uncoupling process would be carried out best using impedance measurements.

Mode of Action of CsF in Uncoupling the T-system

The principal difference between the relaxing solution that preserves c_{slow} and the CsF internal solution is the presence or absence of F^-. Originally, CsF was chosen as the internal solution for the Vaseline-gap method in part because the solubility product of CaF_2 (3.4×10^{-11} M^3 at 18°C) is sufficiently low that sarcoplasmic Ca^{++} is prevented from reaching the contractile activation threshold. CsF inside the fiber potentiates the uncoupling process but by itself promotes very slow and possibly incomplete uncoupling. It is the combination of internal CsF and transient external exposure to CsF, followed by external Ca^{++}-containing solution, that produces the most complete and the most rapid uncoupling. In addition, increased extracellular Ca^{++} accelerates the uncoupling. For these reasons, it is suggested that tubular uncoupling is caused by precipitation of CaF_2 inside the tubules. One possibility considered early in the course of this work was that CaF_2 physically plugs the tubular lumen, increasing the access resistance and possibly the luminal resistivity. However, this hypothesis is not consistent with the pronounced disruption of the internal membranes in the electron micrographs. Instead, the loss of tubules and triads and the severe distortion of SR morphology suggest that CaF_2 acts by generally disrupting internal membranes. Interestingly, this disruption occurs without noticeable effect on the surface membrane.

The electron micrographs show a disruption of internal membranes after CsF treatment that is far more severe than that caused by glycerol-shock treatment. Thus, after glycerol-shock, up to a third of the triads are normal in appearance even though extracellular markers are excluded from >90% of the tubules (Eisenberg and Eisenberg, 1968; Franzini-Armstrong et al., 1973). Following glycerol shock, large vacuoles formed by the swelling of T-tubules are frequently seen, but in contrast to the CsF-treated muscle, the appearance of the SR is not greatly affected by glycerol treatment.

A New Method for Tubular Uncoupling

The ability to uncouple the T-system of muscle by a transient external exposure to CsF may prove generally useful in studies of muscle physiology. For those attempting to adapt this method to intact single fibers or to bundles of fibers, several considerations are worth comment. When CsF is not present inside the fiber, the uncoupling resulting from an external exposure to CsF is less complete than when CsF is also inside the fiber. Therefore, it may be useful to alternate between CsF and Ca^{++}-containing solution several times to obtain the desired degree of uncoupling. It seems likely that elevating the Ca^{++} concentration beyond the 2–20 mM used in the present experiments may help to promote maximum uncoupling. All of the experiments described here involved singly dissected fibers in the Vaseline-gap voltage-clamp chamber, a system in which extremely rapid exchange of bathing solutions is possible. Since such rapid changes of solution may serve to promote the precipitation of CaF_2 in the T-system, it may be necessary to use single fibers or very small bundles and a rapid-flow chamber to achieve maximum uncoupling with intact fibers.

State of the T-system in Cut Muscle Fibers Studied with the Vaseline-Gap Method

The present work resolves some seemingly contradictory results obtained using gap voltage-clamp methods on skeletal muscle. On one hand, many studies using the Vaseline-gap method have reported surprisingly little evidence of tubular Na current (Hille and Campbell, 1976; Pappone, 1980; Collins et al., 1982; Almers et al., 1982; Campbell, 1983). In addition, it has been reported that the slow charge-movement thought to reside in the tubular membrane is quite labile in cut muscle fibers (Campbell, 1983). On the other hand, other investigators using sucrose-gap and Vaseline-gap methods, have measured a prominent delayed component of Na current thought to arise from tubular Na channels (Mandrino, 1977; Vergara, 1981), and many groups have used the Vaseline-gap method to study charge movement and other currents (or physiological responses) that are presumed to depend on T-system connectivity (Vergara et al., 1978; Vergara and Bezanilla, 1979; Kovacs et al., 1979; Horowicz and Schneider, 1981; Almers et al., 1981; Heiny and Vergara, 1982; Heiny et al., 1983). In an impedance study the T-system was found to be quite labile in cut muscle fibers, with little evidence of connectivity in contracted or shortened fibers. However, in the same study, the T-system was found to be normal in fibers with normal striation patterns (Moore and Tsai, 1983). These apparent contradictions appear to be resolved by the present observations that (*a*) the T-system is disrupted in cut fibers exposed to CsF, (*b*) that this disruption may be variable, particularly in the first 10–30 min of an experiment due to the gradual time-course of disruption, and (*c*) in agreement with earlier results from others, tubular connectivity is maintained in fibers cut in F^--free relaxing solutions.

Evidence for the Tubular Location of Charge Movement

The progressive decrease in the peak of charge movement and the progressive slowing of charge-movement kinetics follow the time course of tubular uncoupling. Charge movements calculated from the distributed model of Simon and Beam (1985*b*) mimic the progressive changes in charge movement kinetics when progressive tubular uncoupling is included in the model. Taken together, these results lend considerable support to the hypothesis that the bulk of the charge resides in the tubular membrane. Conversely, if we assume that the effect of CsF treatment

on the slow charge-movement is mediated by the uncoupling of the T-system, then the observation that charge movement becomes vanishingly small when c_{slow} becomes negligible suggests that very little of the slow charge resides in the surface membrane.

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REFERENCES

Adrian, R. H., and L. D. Peachey. 1973. Reconstruction of the action potential of frog sartorius muscle. *J. Physiol. (Lond.).* 235:103–131.

Almers, W. 1978. Gating currents and charge movements in excitable membranes. *Rev. Physiol. Biochem. Pharmacol.* 82:96–190.

Almers, W., R. Fink, and P. T. Palade. 1981. Calcium depletion in frog muscle tubules: the decline of calcium current under maintained depolarization. *J. Physiol. (Lond.).* 312:177–207.

Almers, W., R. Fink, and N. Shepherd. 1982. Lateral distribution of ionic channels in the cell membrane of skeletal muscle. *In* Disorders of the Motor Unit. D. L. Schotland, editor. John Wiley & Sons, N.Y. 349–365.

Armstrong, C. M. 1981. Sodium channels and gating currents. *Physiol. Rev.* 61:644–683.

Armstrong, C. M., and F. Bezanilla. 1974. Charge movement associated with the opening and closing of the activation gates of the Na channels. *J. Gen. Physiol.* 63:533–552.

Bastian, J., and S. Nakajima. 1974. Action potential in the transverse tubules and its role in the activation of skeletal muscle. *J. Physiol. (Lond.).* 63:257–278.

Campbell, D. T. 1983. Sodium channel gating currents in frog skeletal muscle. *J. Gen. Physiol.* 82:679–701.

Campbell, D. T., and R. Hahin. 1983. Functional disruption of the T-system of cut muscle fibers bathed in solutions of normal tonicity. *Biophys. J.* 41(2, Pt. 2):177*a*.(Abstr.)

Campbell, D.T., and R. Hahin. 1984. Altered sodium and gating current kinetics in frog skeletal muscle caused by low external pH. *J. Gen. Physiol.* 84:771–788.

Chandler, W. K., R. F. Rakowski, and M. F. Schneider. 1976. Effects of glycerol treatment and maintained depolarization on charge movement in skeletal muscle. *J. Physiol. (Lond.).* 254:285–316.

Collins, C. A., E. Rojas, and B. Suarez-Isla. 1982. Activation and inactivation characteristics of the sodium permeability in muscle fibers from *Rana temporaria*. *J. Physiol. (Lond.).* 324: 297–318.

Eisenberg, B., and R. S. Eisenberg. 1968. Selective disruption of the sarcotubular system in frog sartorius muscle. *J. Cell Biol.* 39:451–467.

Franzini-Armstrong, C., R. A. Venosa, and P. Horowicz. 1973. Morphology and accessibility of the 'transverse' tubular system in frog sartorius muscle after glycerol treatment. *J. Membr. Biol.* 14:197–212.

Heiny, J. A., and J. Vergara. 1982. Optical signals from surface and T-system membranes in skeletal muscle fibers: Experiments with the potentiometric dye NK2367. *J. Gen. Physiol.* 80:203–230.

Heiny, J. A., F. M. Ashcroft, and J. Vergara. 1983. T-system optical signals associated with inward rectification in skeletal muscle. *Nature (Lond.).* 301:164–166.

Hille, B., and D. T. Campbell. 1976. An improved Vaseline gap voltage clamp for skeletal muscle fibers. *J. Gen. Physiol.* 67:265–293.

Horowicz, P., and M. F. Schneider. 1981. Membrane charge moved at contraction thresholds in skeletal muscle fibers. *J. Physiol. (Lond.).* 314:595–633.

Howell, J. N., and D. J. Jenden. 1967. T-tubules of skeletal muscle: morphological alterations which interrupt excitation-contraction coupling. *Fed. Proc.* 26:553.

Kovacs, L., E. Rios, and M. F. Schneider. 1979. Calcium transients and intramembrane charge movement in skeletal muscle fibres. *Nature (Lond.).* 279:391–396.

Mandrino, M. 1977. Voltage-clamp experiments on frog single skeletal muscle fibres: evidence for a tubular sodium current. *J. Physiol. (Lond.).* 269: 605–625.

Moore, L. E., and T. D. Tsai. 1983. Ion conductances of the surface and transverse tubular membranes of skeletal muscle. *J. Membr. Biol.* 73:217–226.

Pappone, P. A. 1980. Voltage clamp experiments in normal and denervated mammalian skeletal muscle fibers. *J. Physiol. (Lond.).* 306:377–410.

Schneider, M. F., and W. K. Chandler. 1973. Voltage dependent charge movement in skeletal muscle: a possible step in excitation contraction coupling. *Nature (Lond.).* 242:244–246.

Simon, B., and K. G. Beam. 1985*a*. Slow charge movement in mammalian skeletal muscle. *J. Gen. Physiol.* 85:1–19.

Simon, B., and K. G. Beam. 1985*b*. The influence of transverse tubular delays on the kinetics of charge movement in mammalian skeletal muscle. *J. Gen. Physiol.* 85:21–42.

Valdiosera, R., C. Clausen, and R. Eisenberg. 1974. Impedance of frog skeletal muscle fibers in various solutions. *J. Gen. Physiol.* 63:460–491.

Vergara, J. 1981. Sodium currents in muscle fibers. *Biophys. J.* 33(2, Pt. 2):151*a*.(Abstr.)

Vergara, J., and F. Bezanilla. 1979. Tubular membrane potentials monitored by a fluorescent dye in cut single muscle fibers. *Biophys. J.* 25(2, Pt. 2):201*a*.(Abstr.)

Vergara, J., F. Bezanilla, and B.M. Salzberg. 1978. Nile Blue fluorescence signals from cut single muscle fibers under voltage or current clamp conditions. *J. Gen. Physiol.* 72:775–800.