Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 1477–1481, March 1983
Physiological Sciences

Effects of tetracaine on charge movements and calcium signals in frog skeletal muscle fibers

(excitation-contraction coupling/displacement currents/absorbance signals)

JULIO VERGARA AND CARLO CAPUTO*

Department of Physiology, Jerry Lewis Neuromuscular Research Center and Ahmanson Laboratory of Neurobiology, University of California, Los Angeles, California 90024

Communicated by Susumu Hagiwara, November 29, 1982

ABSTRACT **Intramembrane charge movements in skeletal muscle fibers contain a tetracaine-sensitive component that can be isolated by the use of this drug. The time course and voltage dependence of this component, studied in relation to antipyrylazo III absorbance signals, suggest its direct involvement in the calcium release process in muscle.**

When frog skeletal muscle fibers are depolarized for tens of milliseconds or longer, contraction is activated if the membrane potential is made more positive than a threshold value of about −50 mV (1–3). The contractile activation is controlled by the potential across the membrane of the transverse tubular system (T system) (4). Supposedly, it is this voltage that plays a major role in regulating the release of Ca from the sarcoplasmic reticulum (5). Depolarization of the T-system membranes has been reported to be associated with charge movements which may represent a reorientation of charges, confined to the tubular membrane, that in turn may be related to the release of Ca from the sarcoplasmic reticulum (6, 7). The experimental evidence supporting the involvement of charge movement in the coupling between T-system depolarization and contractile activation in muscle has been substantially increased since their discovery by Schneider and Chandler (6–9).

In addition, there have been several indications that the electrical characteristics of the charge movements recorded in voltage-clamped skeletal muscle fibers are far from simple (9–14). One of the most remarkable complexities is that there are at least two distinct components in the charge movement records with apparently different voltage dependences (10, 11). Adrian and Peres (11) suggested that these components originated from the movement of two different intramembrane charges, Q_β and Q_γ. They suggested that Q_β would be responsible for the early approximately exponential charge movement and Q_γ, for the second component also called the "hump." This second component has been reported to be blocked by tetracaine at millimolar concentrations (8, 12, 13, 15) and by dantrolene sodium at concentrations of 10–15 μg/ml (14, 15). The blockage of the second component in charge movement records by tetracaine was first reported by Almers and Best (8, 13). These authors suggested, however, that the total amount of charge mobilized in charge movement records was the same in tetracaine-treated fibers as in control fibers. This finding restricted the importance of the second component as a physiologically relevant component of the charge movement and is in contradiction with the results reported by Huang (12) and in this paper.

We report here experiments in which charge movements and Ca signals, detected with the metallochromic Ca indicating dye antipyrylazo III (APIII), were recorded simultaneously (16–18). The experiments were designed to investigate the involvement of the hump (or second component), observed in charge movement records, in the Ca release process in skeletal muscle fibers. Tetracaine was used as a pharmacological tool to isolate the hump and study its voltage dependence and time course in comparison with absorbance records.

MATERIALS AND METHODS

Single muscle fibers were dissected from the semitendinous muscle of the frog (*Rana catesbiana*). All the experiments reported in this paper were performed in cut single-muscle fibers voltage-clamped in a three-vaseline-gap chamber (19) modified to allow for optical recordings (20). The general methods used in the dissection and mounting of the fibers and the electrophysiological and optical techniques were identical to those described elsewhere (20).

The potential of the segment of muscle fiber in a central pool of solution (pool A, see ref. 19) can be electronically controlled at any desirable level. In these experiments the membrane potential was held at −100 mV, and depolarizing steps of potential were used to depolarize the fiber briefly. The overall speed of the potential control circuit allowed changes in the membrane potential of the fiber segment in pool A in <15 μsec. The fiber in pool A was illuminated (by means of a fiber optic) with monochromatic light coming from a 100-W tungsten halogen lamp and filtered by interference filters of set wavelengths (Ditric Optics, three cavity filters). The image of the muscle fiber was formed on the active surface of a photovoltaic photodiode (EG & G Applied Research, Princeton, UV215) which transformed the light intensity into electrical current.

The metallochromic Ca indicator APIII was introduced inside the muscle fiber by diffusion from the cut ends (16–18). The final concentration of APIII in the segment of the fiber in pool A was estimated, from capillary calibrations and measurements of resting absorbance, to be about 0.2 mM in these experiments. Tension development and the consequent movement artifact normally elicited by depolarizing pulses were blocked by the presence of EGTA diffused from the cut ends (18, 20). EGTA apparently does not interfere with the Ca release process from the sarcoplasmic reticulum as evidenced by the dyes arsenazo III and APIII (18). Although various wavelengths were used to record absorbance signals during these experiments, only three of them are represented in the optical traces shown below: 710, 790, and 550 nm. At 710 nm, APIII shows a maximum in the absorbance difference spectrum obtained by titration with Ca in *in vitro* calibration experiments

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: T system, transverse tubular system; APIII, antipyrylazo III.

* Permanent address: Centro de Biofisica y Bioquimica, IVIC, Caracas, Venezuela.

(17, 21). For this reason, we selected this wavelength to monitor myoplasmic Ca concentration changes. Also, contamination by absorption effects of possible Mg concentration changes is minimal (18, 21). APIII does not show significant absorbance at 790 nm and therefore this wavelength has been used to record small movement artifacts that may remain unblocked by the presence of EGTA inside the fiber. The movement artifacts have been scaled according to the resting light intensities measured at 710 and 790 nm in order to obtain the corrected absorbance records shown below. All the records have been normalized by the resting absorbance recorded at 550 nm to allow for comparison with previous reports in the literature (16–18).

The cut ends of the fiber were immersed in an internal solution containing 120 mM cesium aspartate, 3 mM $MgSO_4$, 3 mM Tris EGTA, 3 mM ATP, 5 mM phosphocreatine, and 1 mM APIII. The external solution (in pool A of the vaseline-gap-chamber) had the following composition: 60 mM tetraethylammonium sulfate; 3 mM tetraethylammonium 3-(*N*-morpholino)propanesulfonate; 3 mM Rb_2SO_4; 1 mM $CaSO_4$; 4 mM $MgSO_4$; 20 mM glucose. The internal and external solutions were at 240 mOsm.

RESULTS

Charge Movements and Ca Signals. Absorbance signals and charge movements were simultaneously recorded by using the P/4 pattern of stimulation (22–24) (also see legend of Fig. 1) to subtract the linear components in the current recordings. The absorbance signals were not recorded during the subtracting pulses. Fig. 1 shows the results of an experiment in which the charge movements and absorbance signals were recorded for depolarizations to −40, −35, −30, −25, and −20 mV. The records shown in Fig. 1 were obtained without signal averaging in order to minimize the length of the experiments. The fast transient components in the current traces correspond to the contribution of the Na gating currents. They have been altered by the electronic filtering (frequency devices, 6-pole low-pass Bessel filter) used in these experiments with a cut-off frequency of 5 kHz. These Na gating currents have been described in this preparation (23) and their contribution can be partially eliminated from the slow charge movement records by using short depolarizing prepulses (unpublished data). We have preferred to show the records containing the fast Na gating current contribution in order to avoid further complications introduced by the use of prepulses.

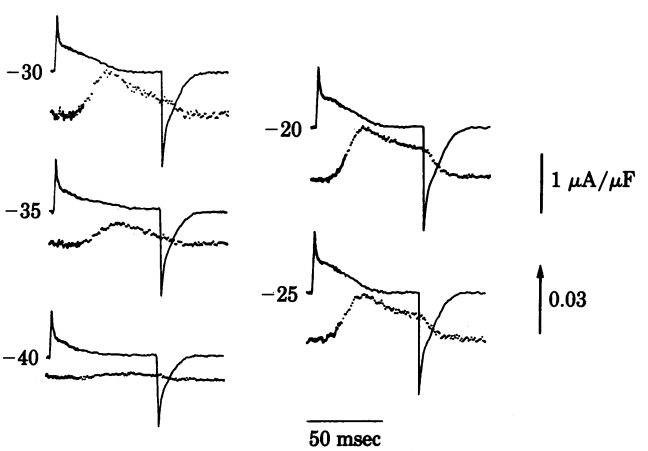

FIG. 1. Records of asymmetrical displacement currents (continuous lines) and APIII absorbance signals (dotted lines) associated with voltage pulses to −40, −35, −30, −25, and −20 mV from a holding potential of −100 mV. Each asymmetry current trace was obtained by subtracting four current records associated with voltage steps of P/4 amplitude (applied from a holding potential of −130 mV) from the current record generated by a voltage step of amplitude P (applied from the holding potential of −100 mV). The membrane capacity, used to normalize the asymmetry current records, was calculated by integration of current records of long duration (>80 msec) and small amplitude (<40 mV). These records were taken at the beginning and at the end of the experiment. The APIII absorbance signals were corrected from a small contribution of remaining movement artifact recorded at 790 nm. The arrow represents an absorbance increase of 0.03 and refers to $\Delta A = (\Delta A_{710} - \Delta A_{790})/A_{550}$. Temperature was 9°C.

Fig. 1 shows that the absorbance signals became larger and had steeper rising phases at large depolarizations compared to small depolarizations; these tendencies leveled off at potentials close to 0 mV (not shown in Fig. 1). The latency intervals between the initiation of the pulse (evidenced by the fast transients of the current records) and the rising phase of the absorbance signals also shortened dramatically with larger depolarizations. The most relevant characteristics of the charge movement records shown in Fig. 1 can be described as follows. At −40 mV they showed a fast transient component that corresponded to the previously described Na gating current, followed by a slow exponential relaxation to a base line during the pulse. This behavior is typical of records obtained with small depolarizations up to −40 mV. At −35 mV, a small deviation from the exponentiality in the slow relaxation phase was observed as a second component in the charge movement record that occurred almost concomitantly with the rising phase of the absorbance signal. At −30 mV, the second component became more conspicuous and occurred earlier, maintaining a correlation with the behavior of the absorbance signal. At −25 mV, the second component of the displacement current occurred even earlier and its presence in the total charge movement record could be inferred by comparison with the absorbance signal whose latency interval also was reduced. Finally, at −20 mV, the second component had become part of a continuous decaying phase in the asymmetry currents. At this potential, another method is necessary to reveal its presence in the charge movement record.

The second component of the displacement currents sometimes was more prominent than shown in Fig. 1 justifying the term "hump" used in the literature (8, 10, 14). This name is descriptive of what is observed in records like those at −35 and −30 mV in Fig. 1. At larger depolarizations the appearance of the second component was less conspicuous. The restricted potential range over which the hump appears has made this component difficult to quantify experimentally.

Isolation of the Hump. In an attempt to identify clearly the second component of the charge movement (hump), we compared the displacement currents before and during treatment with tetracaine. Fig. 2 shows the electrical and optical records

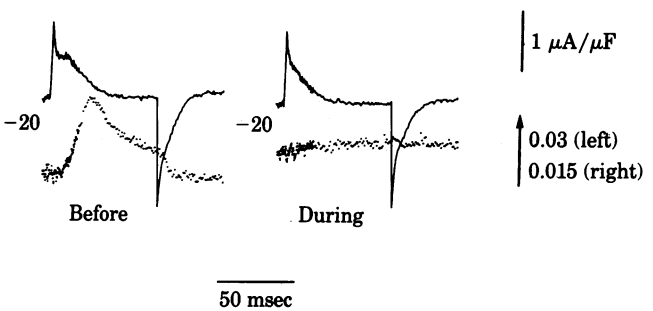

FIG. 2. Records of asymmetric displacement currents and APIII absorbance signals associated with voltage pulses to −20 mV. (*Left*) Control records. (*Right*) Records obtained after the addition of 0.5 mM tetracaine to the external solution in pool A. The conditions were the same as in Fig. 1.

obtained with a depolarizing pulse to −20 mV before and after the addition of 0.5 mM tetracaine to the external solution. This drug effectively blocked the absorbance signal (dotted traces), and it also significantly altered the time course of the displacement current. The current records display a more exponential relaxation during the pulse in the presence of tetracaine than before the drug was added. The effects of the tetracaine treatment were only partially reversible at concentrations >0.5 mM. At lower concentrations, better reversibility but less dramatic blockage was observed. In order to obtain a better pictorial view of the effects of tetracaine on charge movement records, we superimposed records before and during tetracaine (Fig. 3). The traces with a more exponential time course were obtained in the presence of tetracaine; for comparison, the control records are superimposed. At −40 mV there was virtually no difference between the control records and those in the presence of tetracaine (Fig. 3*A*). Also, the extra currents observed in the control traces had different time courses at −35 mV compared to −20 mV. One interesting characteristic of the records shown in Fig. 3*A* is that the currents recorded at the end of the depolarizing pulses ("off" currents) are different in records obtained in the presence of tetracaine than in the control records for the depolarizations to −35 and −20 mV. The difference was not appreciable at −40 mV.

Fig. 3*B* plots the difference between the displacement current records obtained before and during tetracaine in order to isolate the secondary component. The subtraction has been performed only for the −35 and −20 mV records because the −40 mV records shown in Fig. 3*A* did not reveal any appreciable differences. The rationale of this manipulation of the data is that it would allow us to describe the functional relevance of this component of the charge movements, eliminating the contribution of the tetracaine-insensitive component. The difference records shown in Fig. 3*B* are compared with the corresponding absorbance signals (dotted traces) recorded before the addition of tetracaine. At −35 mV the difference trace displayed a slow time course, peaking about 20 msec after the onset of the pulse (not explicitly shown in Fig. 3*B*) and slowly decaying toward a baseline that was not reached because the pulse was interrupted. At −20 mV, the difference current record peaked about 8 msec after the onset of the pulse and decayed to a baseline within 35 msec.

It should be observed in Fig. 3*B* that the absorbance traces show a voltage-dependent kinetic behavior similar to that of the current traces—namely, the absorbance trace obtained at −20 mV peaked and decayed faster than that recorded at −35 mV. Nevertheless, at both depolarizations the current traces preceded the corresponding absorbance traces. This observation has been reproduced at various suprathreshold depolarizations (giving detectable absorbance signals) in three other fibers in which this problem was studied.

Another observation related to Fig. 3*B* is that the total amount of charge (measured as the time integral of the current) that was mobilized at the on and off currents was 5.4 and 4.0 nC/μF, respectively, at −20 mV and 3.3 nC/μF for both at −35 mV. This rough conservation of the on and off total charge has been verified at several potentials (from −40 mV to 0 mV), indicating that the tetracaine-sensitive component of charge movement maintains this characteristic feature of displacement currents.

Voltage Dependence of Charge Mobilized in Different Components. An interesting consequence of the ability of tetracaine to abolish the hump of the charge movement signals is that it allows study of the relationship between charge movement and potential in the presence and absence of this tetracaine-sensitive component. In the presence of the drug (elimination of the hump component), a linear relationship was obtained in the range −50 to 0 mV (continuous line, Fig. 4). In contrast, when tetracaine was absent, the relationship deviated from linearity, starting at a potential of about −40 mV (broken line, Fig. 4). The arrow in Fig. 4 indicates the voltage at which the absorbance changes (associated with Ca release from the sarcoplasmic reticulum) start to be visualized.

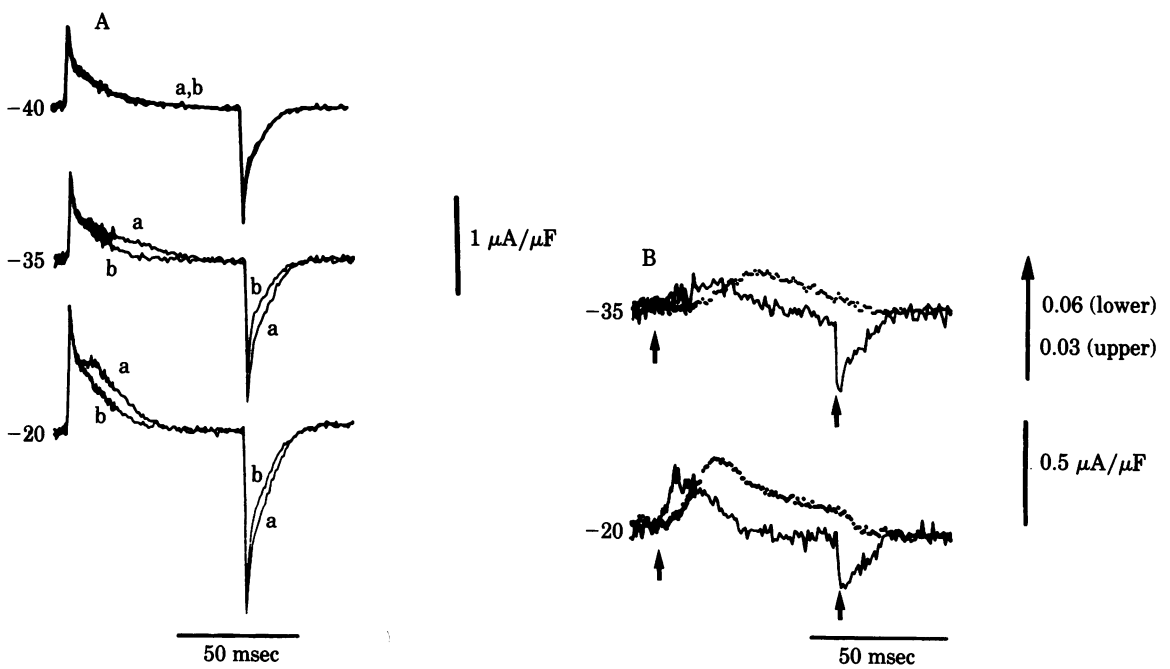

FIG. 3. (*A*) Superimposition of displacement current records obtained before (traces a) and after (traces b) the addition of 0.5 mM tetracaine. Current records elicited at −40, −35, and −20 mV are shown to illustrate the voltage dependence of the difference between the records (see text). (*B*) Superimposition of absorbance signals (dotted traces) and current difference traces (continuous traces) obtained at −35 and −20 mV. The current difference traces were computed by subtraction of trace b from trace a shown in *A*. The calibration arrow indicates direction and magnitude of absorbance signals as described in Fig. 1. The short arrows indicate the beginning and end of the pulses.

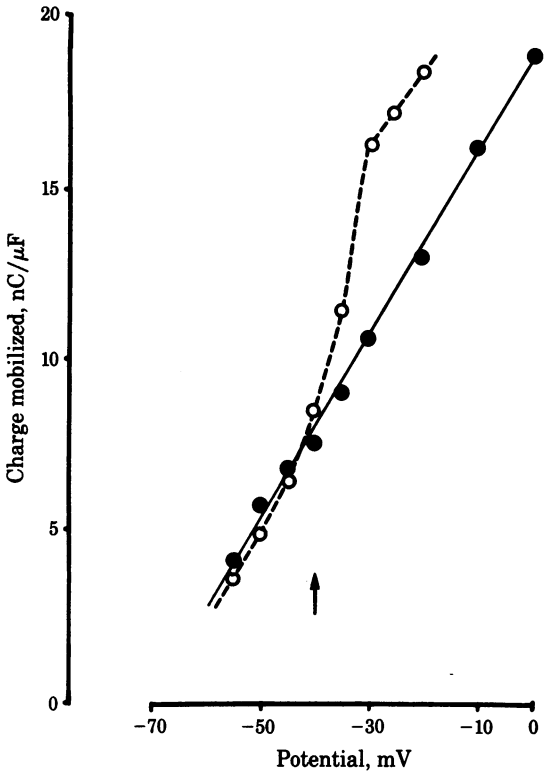

FIG. 4. Charge mobilized during depolarizing pulses to the potentials indicated. o---o, Control; •, in the presence of 0.5 mM tetracaine; ——, linear regression fitted to data represented by •. The parameters of this regression were: slope, 0.258 nC/μC; intercept, 18.1 nC/μF; linear correlation coefficient, 0.998. The data were obtained from the same fiber as in Fig. 3.

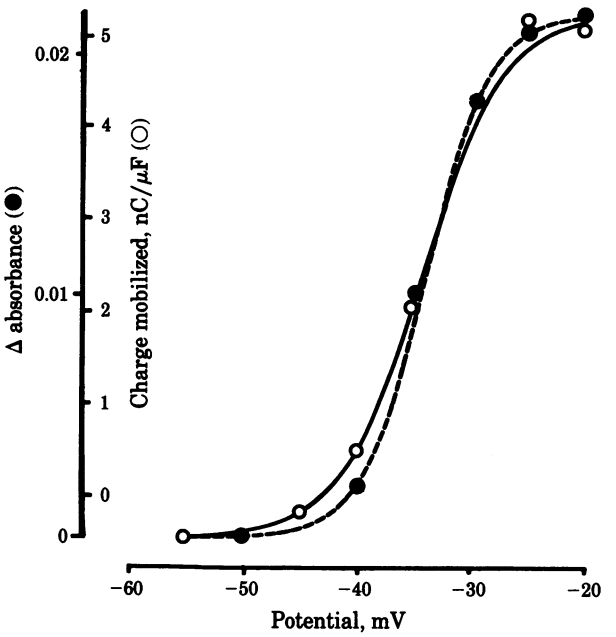

FIG. 5. The charge mobilized by the tetracaine-sensitive component of the charge movement (obtained by subtraction of the data in Fig. 4) (•) and the absorbance signals recorded by the APIII (previous to the treatment with tetracaine) (○) plotted against the membrane potential. o——o, Obtained by ΔQ (in nC/μF) = 5.7{1/(1 + exp −[(V + 34)/3.6])}; •---•, obtained by $\Delta A/A$ = 0.0215{1/(1 + exp −[(V + 34)/2.77])}.

The dependence of the peak of the absorbance signals on membrane potential obtained in the absence of tetracaine and that of the hump component of charge movement, obtained by subtraction of the curves of Fig. 4, is shown in Fig. 5. Both processes have remarkably similar voltage dependences. Both curves were obtained by fitting Boltzmann relations of the form $Y = Y_{max}\{1/(1 + \exp - [(V - \bar{V})/K])\}$, in which Y represents either absorbance change or charge movement, to the data points. The charge movement curve (continuous line, Fig. 5) was generated by using Y_{max} = 5.7 nC/μF, K = 3.6 mV, and $\bar{V}$ = −34.2 mV. In Fig. 5 the charge movement curve starts at negative values for small depolarizations, giving positive values at potentials above −45 mV. These negative values are due to the fact that, at small depolarizations, the total charge mobilized in the presence of tetracaine is larger than that in the controls. This can be observed well in Fig. 4. We are not able to give a satisfactory explanation for this curious finding but it might be due to tetracaine blockage of residual conductances in the T system resulting in a more efficient depolarization of the deep regions of the T system. Alternatively, it might be due to tetracaine-induced nonlinear charge movements similar to those recently described for dipicrylamine in the squid axon preparation (25). The absorbance curve in Fig. 5 was generated by using the Y_{max} = 0.0215, K = 2.77 mV, and $\bar{V}$ = −34 mV. Even though there is a slight difference in the K values obtained for each data set (absorbance and charge movement), they are within the experimental error. In contrast, the correlation between the voltage dependence of the tetracaine-insensitive component (continuous line, Fig. 4) and the absorbance signals (broken line, Fig. 5) would be very poor. These results suggest that the release of Ca^{2+} from the sarcoplasmic reticulum is more closely related to the tetracaine-sensitive than to the tetracaine-insensitive component of the charge movement. Also, analysis of Fig. 5 shows that most of the nonlinearity of the total charge movement in Fig. 4 (broken line) comes from the steep nonlinearity of the 5.7 nC/μF contributed by the tetracaine-sensitive component.

DISCUSSION

It is clear from the results shown here that tetracaine can abolish the nonexponentially decaying component of the charge movement signal, usually referred to as the "hump." Simultaneously, tetracaine abolishes the absorbance signals recorded with the APIII, suggesting blockage of the Ca release from the sarcoplasmic reticulum. A possible alternative explanation for the blockage of the APIII absorbance signals could have been that tetracaine alters the spectral or Ca binding characteristics of the dye. However, we have explored this possibility in *in vitro* calibrations and found no significant effect of tetracaine (up to 2 mM) on the difference spectra recorded at several Ca concentrations and 0.5 mM dye dissolved in a 100 mM KCl/20 mM potassium morpholinepropanesulfonate, pH 6.9 (for a methodological description, see ref. 17). In view of the absence of these dye–tetracaine interactions, we favor the suggestion that tetracaine blocks release of Ca from the sarcoplasmic reticulum. This suggestion is in agreement with the results of Almers and Best (13) and with our results demonstrating that tetracaine abolished tension development and the Nile blue fluorescence signal in muscle fibers (26).

Our results suggest the possibility that it is the hump component of the charge movement signals that provides the voltage sensitivity for excitation-contraction coupling. This suggestion is reinforced by the close parallelism between the tetracaine-sensitive component and the Ca signals. The time relationship between these two signals clearly indicates that the former always precedes the latter, in agreement with the idea, suggested by Huang (27), that the hump may be a cause of the Ca signal.

The inverse possibility—that the hump might be an effect of the massive release of Ca in a restricted space—still has to be considered as plausible. However, our results strongly suggest that, if the latter possibility were correct, care has to be taken to eliminate this secondary contribution to the total charge movement because we demonstrated that it is not negligible. Finally, it is important to mention that the effects of tetracaine on charge movement records reported here are similar, if not identical, to those of dantrolene sodium reported by Hui (14). The parameters found by Hui to describe the voltage dependence of the dantrolene sodium-sensitive component of the charge movement (Q_γ in his nomenclature) are almost the same as those reported here. Hui (15) has also reported results on the effects of tetracaine on charge movement that are in general agreement with those reported in this paper. It is interesting that two unrelated drugs, tetracaine and dantrolene sodium, have the same effect on a component of the charge movement because it suggests that they share a general mechanism of action in the excitation-contraction coupling process.

We thank Dr. P. Palade for helpful suggestions on this manuscript, Mr. Victor Cordova for help with the electronic equipment, and Miss Gretchen Wooden for her secretarial help. This work was supported by grants from the Muscular Dystrophy Association of America (Project 6, Jerry Lewis Neuromuscular Research Center Grant to University of California, Los Angeles) and by Public Health Service Grant AM-25201.

1. Kuffler, S. W. (1946) *J. Neurophysiol.* **9**, 367–377.
2. Hodgkin, A. L. & Horowicz, P. (1960) *J. Physiol. (London)* **153**, 370–385.
3. Adrian, R. H., Chandler, W. K. & Hodgkin, A. L. (1969) *J. Physiol. (London)* **204**, 207–230.
4. Huxley, A. F. & Taylor, R. E. (1958) *J. Physiol. (London)* **144**, 426–551.
5. Ebashi, S., Endo, M. & Ohtsuki, I. (1969) *Q. Rev. Biophys.* **2**, 351–384.
6. Schneider, M. F. & Chandler, W. K. (1973) *Nature (London)* **242**, 244–246.
7. Chandler, W. K., Rakowski, R. F. & Schneider, M. F. (1976) *J. Physiol. (London)* **254**, 245–286.
8. Almers, W. (1978) *Rev. Physiol. Biochem. Pharmacol.* **82**, 97–190.
9. Horowicz, P. & Schneider, M. F. (1981) *J. Physiol. (London)* **314**, 565–593.
10. Adrian, R. H. & Peres, A. R. (1977) *Nature (London)* **267**, 800–804.
11. Adrian, R. H. & Peres, A. (1979) *J. Physiol. (London)* **289**, 83–97.
12. Huang, C. L.-H. (1981) *J. Physiol. (London)* **313**, 187–205.
13. Almers, W. & Best, P. M. (1976) *J. Physiol. (London)* **262**, 583–611.
14. Hui, C. S. (1981) *Biophys. J.* **33**, 152a (abstr.).
15. Hui, C. S. (1982) *Biophys. J.* **39**, 119–122.
16. Kovacs, L., Rios, E. & Schneider, M. F. (1976) *Nature (London)* **279**, 391–396.
17. Palade, P. & Vergara, J. (1981) in *The Regulation of Muscle Contraction: Excitation-Contraction Coupling*, eds. Grinnell, A. D. & Brazier, M. A. B. (Academic, New York), pp. 143–160.
18. Palade, P. & Vergara, J. (1982) *J. Gen. Physiol.* **79**, 679–707.
19. Hille, B. & Campbell, D. T. (1967) *J. Gen. Physiol.* **67**, 265–293.
20. Vergara, J., Bezanilla, F. & Salzberg, B. M. (1978) *J. Gen. Physiol.* **72**, 775–800.
21. Scarpa, A., Brinley, F. J. & Dubyak, G. (1978) *Biochemistry* **17**, 1378–1386.
22. Bezanilla, F. & Armstrong, C. M. (1977) *J. Gen. Physiol.* **70**, 549–566.
23. Vergara, J. & Cahalan, M. (1978) *Biophys. J.* **21**, 167a (abstr.).
24. Vergara, J. & Bezanilla, F. (1981) in *The Regulation of Muscle Contraction: Excitation-Contraction Coupling*, eds. Grinnell, A. D. & Brazier, M. A. B. (Academic, New York), pp. 67–77.
25. Fernandez, J., Taylor, R. E. & Bezanilla, F. (1982) *Nature (London)* **297**, 58–62.
26. Caputo, C., Vergara, J. & Bezanilla, F. (1979) *Nature (London)* **227**, 401–402.
27. Huang, C. L.-H. (1981) *J. Physiol. (London)* **320**, 381–391.