CALCIUM TRANSIENTS IN ASYMMETRICALLY ACTIVATED SKELETAL MUSCLE FIBERS

G. TRUBE, J.R. LOPEZ, AND S.R. TAYLOR, *Department of Pharmacology, Mayo Graduate School of Medicine, Rochester, Minnesota 55901*

ABSTRACT Skeletal muscle fibers of the frog *Rana temporaria* were held just taut and stimulated transversely by unidirectional electrical fields. We observed the reversible effects of stimulus duration (0.1–100 ms) and strength on action potentials, intracellular Ca^{2+} transients (monitored by aequorin), and contractile force during fixed-end contractions. Long duration stimuli (e.g., 10 ms) induced a maintained depolarization on the cathodal side of a cell and a maintained hyperpolarization on its anodal side. The hyperpolarization of the side facing the anode prevented the action potential from reaching mechanical threshold during strong stimuli. Variation of the duration or strength of a stimulus changed the luminescent response from a fiber injected with aequorin. Thus, the intracellular Ca^{2+} released during excitation-contraction coupling could be changed by the stimulus parameters. Prolongation of a stimulus at field strengths above 1.1 × rheobase decreased the amplitude of aequorin signals and the force of contractions. The decreases in aequorin and force signals from a given fiber paralleled one another and depended on the stimulus strength, but not on the stimulus polarity. These changes were completely reversible for stimulus strengths up to at least 4.2 × rheobase. The graded decreases in membrane depolarization, aequorin signals, and contractile force were correlated with the previously described folding of myofibrils in fibers allowed to shorten in response to the application of a long duration stimulus. The changes in aequorin signals and force suggest an absence of myofilament activation by Ca^{2+} in the section of the fiber closest to the anode. The results imply that injected aequorin distributes circumferentially in frog muscle with a coefficient of at least 10^{-7} cm^2/s, which is not remarkably different from the previously measured coefficient of 5×10^{-8} cm^2/s for its diffusion lengthwise.

INTRODUCTION

When isolated single muscle fibers are stimulated by extracellularly applied unidirectional pulses of relatively long duration, the force of the resultant contraction is decreased. An explanation for this phenomenon was suggested by Rüdel and Taylor (1969), who recorded the microscopic features of skeletal muscle fibers shortening in response to transverse unidirectional stimuli. Undulations appear in the part of a fiber adjacent to the anode during shortening elicited by pulses of long duration (shown schematically in Fig. 1 *D*). Rüdel and Taylor inferred that this asymmetrical waviness indicates the folding of passively shortened inactive myofibrils that are bent upon themselves by the actively contracting parts of the fiber close to the cathode. Thus, these undulations were accounted for in the same way that Gonzalez-Serratos (1971) explained the uniform production of wavy myofibrils in a resting isolated fiber compressed longitudinally by extrinsic forces. Rüdel and Taylor (1969)

Dr. Trube's present address is II Physiologisches Institut der Universität des Saarlandes, Homburg/Saar, Federal Republic of Germany.

speculated that stimuli of long duration excite only those parts of a fiber that are facing the cathode. In the anodal regions, the membrane supposedly remains hyperpolarized during a stimulus of long duration, so that contraction is not activated in that part of the fiber (illustrated schematically in Fig. 1 *C*). We were able to record the membrane potentials during long duration transverse field stimulation and, for the first time, have obtained direct qualitative evidence for this supposition.

An important feature of excitation-contraction coupling is the intracellular Ca^{2+} transient (Ashley and Ridgway, 1970) that initiates and regulates contractile activation. Eisenberg et al. (1979) recently found that the distribution of horseradish peroxidase (HRP) in skeletal muscle cells was anisotropic. They pointed out that similar information concerning other probes of intracellular events, such as aequorin, was needed to allow the collection and careful analysis of data. Prolongation of the stimulus duration was used in the present work as a method to induce changes in Ca^{2+} transients and to associate the changes with the previously observed asymmetrical activation. The results permit us to deduce some features of the rate and extent of intracellular distribution of the Ca^{2+} indicator we used, namely the photoprotein aequorin (Blinks et al., 1978). The rate at which aequorin distributes longitudinally after microinjection has already been measured (Blinks et al., 1978) but its distribution in other directions is unknown. Lower limits for the coefficients of diffusion in a radial or circumferential direction can be estimated from our present experiments, and the results suggest that aequorin distribution occurs without marked anisotropy.

A preliminary report of some of these results has already been presented (Trube et al., 1979).

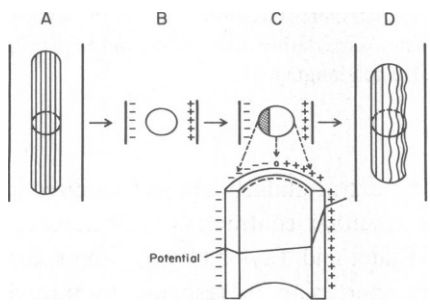

FIGURE 1

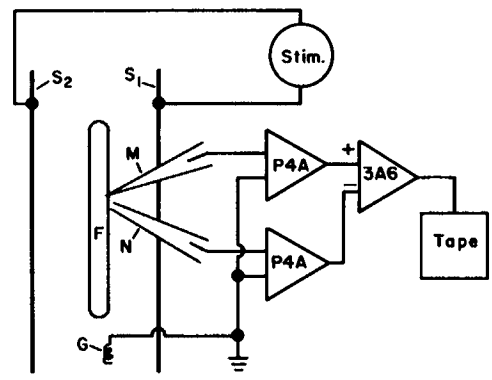

FIGURE 2

FIGURE 1 Schematic representation of muscle shortening and membrane polarization during a prolonged transverse DC stimulus. (*A*) Muscle fiber at rest seen in longitudinal section, between the stimulating plates. (*B*) Cross section at onset of the stimulus. − and + indicate the polarity of the electrodes. (*C*) Cross section at a later time during the stimulus. The hatching approximately indicates the segment that shortens without undulations and is activated (Rüdel and Taylor, 1969). The inset below illustrates the course of the electrical potential across the fiber. (*D*) Fiber shortening in response to a long stimulus. The myofibrils adjacent to the anode become wavy.

FIGURE 2 Schematic drawing of electrode arrangement and amplifier wiring for action potential recordings. M, intracellular microelectrode; N, extracellular microelectrode; F, muscle fiber; G, AgCl/Ag wire; S_1, S_2, platinum plates for stimulation; Stim, stimulator; P4A, high-input resistance amplifiers; 3A6, differential amplifier; Tape, tape recorder.

METHODS

Preparation and Mounting of Muscle Fibers

Single twitch muscle fibers were dissected from the tibialis anterior, semitendinosus, or iliofibularis muscles of the frog *Rana temporaria*. The fibers were mounted in a shallow bath placed on a temperature-controlled microscope stage. One tendon was attached to a force transducer (RCA Electro-Optics & Devices, RCA Solid State Div., Lancaster, Pa., model 5734). The resting striation spacing was measured from the diffraction pattern created by a He-Ne laser beam (Spectra-Physics Inc., Autolab Div., Santa Clara, Calif., model 155). All experiments were done at 15°C. Solutions in which aequorin signals and force were measured contained the following (in mM): NaCl, 115; KCl, 2.5; $CaCl_2$, 1.8; Na_2HPO_4, 2.15; NaH_2PO_4, 0.85. The pH was adjusted to 7.1 with NaOH or HCl. Curare (10^{-5} g/ml) was sometimes added to the solutions but produced no significant difference in the results to be described. Indirect stimulation of the fibers was evidently not a factor in the experiments.

Bundles of ~10 fibers were used for the membrane potential recordings. The fibers were freely suspended in the bath, just as they were in the other experiments, because a supporting structure would bend the equipotentials of the stimulating field. Therefore, the concentration of NaCl was increased to 230 mM to paralyze the fibers and prevent movement artifacts. Hypertonicity may have influenced the amplitude of the membrane potential recordings (Nastuk and Hodgkin, 1950). The effects of hypertonicity on the action potential as a whole, however, are evidently not substantial (Hodgkin and Horowicz, 1957).

Stimulation

Two bright platinum electrodes (45 mm long, 9 mm high) covered the walls of the chamber (21 mm apart) parallel to the longitudinal axis of the fiber. Square wave voltage pulses were delivered to the electrodes by a power amplifier (Hewlett Packard Co., Palo Alto, Calif., model 6824A), which received its input from a conventional stimulator (Grass Instrument Co., Quincy, Mass., models S48 and SIU5). The average field strength at rheobase for twitch responses was 3.1 V/cm (range 2.3–4.0 V/cm at 15°C). Twitches or tetani were elicited at intervals of 5 min. At more frequent intervals of stimulation the aequorin responses are rate dependent (e.g., see Blinks et al., 1978).

For the membrane potential recordings the plates were moved closer together (13 mm apart) to reduce the voltage needed to excite the fibers. Thus, the size of the stimulus artifact transmitted to the recording electrodes was reduced. The Hewlett Packard 6824A amplifier was replaced by a battery-powered amplifier (four 6-V batteries) mediated by a transistor (RCA 2N6254). This amplifier was controlled by an isolated stimulator (Devices, Medical Systems Corp., Great Neck, N.Y.; model 2533) to provide better isolation of the stimulation circuit from ground. The fibers were stimulated every 10–20 s during the membrane potential measurements.

Injection of Aequorin and Light Recording

Aequorin was supplied to us by J. R. Blinks (Blinks et al., 1976; Prendergast and Mann, 1978). Glass micropipettes with abruptly tapered tips (Taylor et al., 1979) were filled with a few microliters of aequorin, mounted on a micromanipulator, and, while under observation through a microscope, inserted into a muscle fiber at the edge closest to one of the two stimulating plates. Aequorin was injected by applying gaseous pressure to the butt of the pipette until slight swelling was observed. Subsequently, the micropipette was withdrawn from the cell and the bath (Blinks et al., 1978).

In most other kinds of experiments with aequorin it is desirable to inject several spots along the whole length of the muscle fiber in order to obtain a more uniform longitudinal distribution of aequorin (Taylor et al., 1979). When a fiber is injected at several points, however, the injected spots are not necessarily on the same line along the length of the cell, because segments may rotate during the impalement procedure or during a contraction. Because we wanted to see if it was possible to detect differences in calcium transients during asymmetrical activation that might be due to an asymmetrical circumferential or radial distribution of aequorin, only one or two impalements were made; each was <0.5 mm apart and not displaced arount the circumference of a cell. Fibers that were markedly noncircular when examined

by ordinary light microscopy were not used for the experiments described in this paper. The fibers were stretched to a striation spacing of 2.8 μm for the microinjections. In some experiments, the fibers were released to 2.2 μm afterwards, whereas in the other experiments we kept the fibers stretched because we wanted to minimize the possibility that the injected region might move to another position during the release. Fibers were also occasionally checked to see whether the injected spot rotated detectably during a tetanus.

After the injection, repositioning of the fibers, and visual checks mentioned above, the microscope was replaced by a photomultiplier tube (EMI Gencom Inc., Plainview, N.Y., model 9635B). These procedures usually took at least several minutes, which is why the first records were made with some delay after an injection (e.g., Fig. 6). The anode current of the photomultiplier was converted to a voltage signal and amplified; the time constant of this system was 10 ms. Light and force signals were displayed on a chart recorder (Gould, Inc., Cleveland, Ohio; Brush model 220) and recorded on FM tape (Ampex Corp., Redwood City, Calif., model SP-300). The signals on the tape were later replayed to an instrument computer (Nicolet Scientific Corp., Madison, Wis., model 1074) and finally displayed on an X-Y plotter (Hewlett Packard, model 7044A).

Recording of Action Potentials

Recordings of action potentials from preparations that are located between large stimulating plates are usually distorted by at least two types of stimulation artifacts.

Large capacitive spikes are caused when the stimulus is switched on and off, and the decay of such a spike can last longer than the action potential itself. This artifact depends on the degree of isolation of the stimulating circuit from the recording circuit, the size of the chamber, strength of stimulation, and the length of the microelectrode tip immersed in the solution. Another very important factor is the resistance of the recording microelectrode. By using microelectrodes of very low resistance (1.5–3 MΩ) we could reduce the duration of the capacitive spike artifacts to values that did not mask the action potential spikes (e.g., see Fig. 3).

The reference electrode, an Ag/AgCl wire, apparently became polarized during prolonged stimuli. The resulting instability of the reference potential could be eliminated from the measurement by recording the potential difference between two microelectrodes.

Our glass microelectrodes for the measurement of the intracellular potential (M in Fig. 2) were filled with 3 M KCl and had the dimensions of the micropipettes that we used for the injection of aequorin (Taylor et al., 1979). The tips of these electrodes are small enough to impale frog muscle fibers without causing detectable membrane damage (e.g., local contractures). But the shank of the electrodes taper very abruptly, which presumably helps to achieve the desired low electrical resistance. The glass microelectrodes for the measurement of extracellular potentials (N in Fig. 2) were filled with Ringer's solution and the tips were broken until we obtained a resistance of ~2 MΩ. Microelectrode M was inserted into a muscle fiber at the edge closest to one of the two stimulating plates. Microelectrode N was positioned on the outside of a cell as close as possible to the point of impalement. Both microelectrodes were connected to two high-input resistance amplifiers (Bioelectric Instruments Inc., Hastings-on-Hudson, N.Y.; model P4A). The bath was connected to ground by another electrode of low resistance (AgCl/Ag wire, G in Fig. 2) because these amplifiers have only single-ended inputs. This electrode was positioned approximately on an isopotential with the two microelectrodes. The output signals from the P4A amplifiers and their difference were observed directly on an oscilloscope (Tektronix Inc., Beaverton, Ore., model RM 565) and stored by the tape recorder. The stimulus current (I) was monitored by measuring the potential difference across a 1-Ω resistor (not shown in Fig. 2) in series with the stimulator.

RESULTS

Membrane Potential During Long Stimuli

The top traces in each of the four frames of Fig. 3 show records of the membrane potential (V_m) during transverse stimuli of 10 ms duration. The stimuli not only excited the membrane

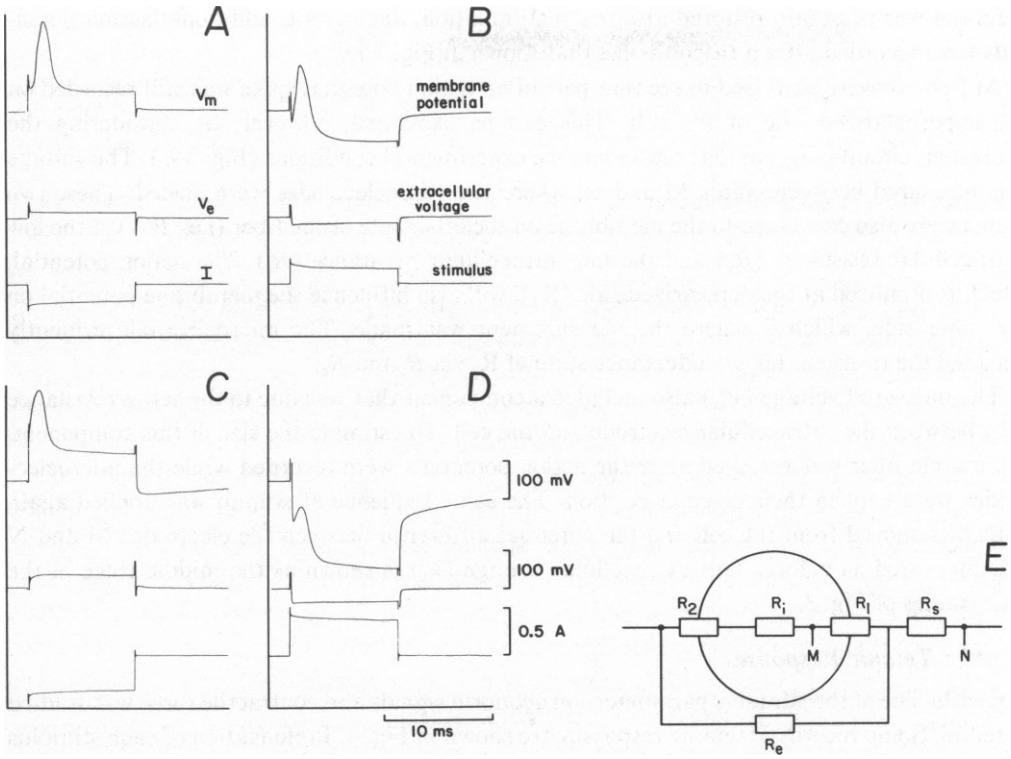

FIGURE 3 Membrane potentials (V_m) recorded during the application of transverse field stimuli of long duration (fiber 21.XII.79). The membrane potentials (top traces in panels *A*, *B*, *C*, and *D*) were recorded from one muscle fiber within a bundle of other fibers. In *A* and *C* the negative stimulating electrode (S_1 in Fig. 2) was on the side of the fiber where the membrane potential was recorded. In *B* and *D* the polarity of the stimulus was reversed. The records were taken in the sequence shown. The resting potential returned to the initial value of −85 mV after each response. The solution was hypertonic (the concentration of NaCl was 230 mM) and the fiber stretched to a striation spacing of 3.0 μm to suppress movement. V_m, Membrane potential recorded differentially between an intracellular and an extracellular microelectrode; V_e, Voltage between the two microelectrodes due to the potential gradient in the extracellular solution, recorded after removing the fiber; *I*, Total current between the stimulating electrodes; 0.5 A corresponds to a current density of 0.22 A/cm^2 and a field strength of 12 V/cm (calculated from the chamber dimensions and the specific resistivity of the solution). (Panel *E*) Simplified circuit diagram. The circle symbolizes a cross section of the muscle fiber. V_m in panels *A*, *B*, *C*, and *D* is the voltage measured by two microelectrodes between points M (intracellular) and N (extracellular). R_1 and R_2 include the membrane resistance and other electrical properties of the membrane on opposite sides of the fiber. R_e, extracellular resistance around the fiber; R_i, intracellular resistance across the fiber; R_s, series resistance between extracellular electrode N and the cell membrane.

to produce an action potential spike, but also caused a maintained deviation from the resting potential; the amplitude of this deviation depended on the polarity and the strength of the electric field. When the anode was on the side of the fiber where the membrane potential was recorded, the membrane was hyperpolarized (Fig. 3 *B* and *D*). The hyperpolarization became larger as the stimulus strength was increased (Fig. 3 *D* vs. *B*) until the superimposed action potential spike no longer reached mechanical threshold or even the resting potential (Fig. 3 *D*). No irreversible effects of the large hyperpolarization were detected. The resting

potential was promptly restored after each stimulation, and several additional action potentials were recorded after a response like that shown in Fig. 3 *D*.

At first we were surprised to see that part of an action potential spike was still recorded on the hyperpolarized side of the cell. This can be explained, however, by considering the equivalent circuit diagram that represents the experimental conditions (Fig. 3 *E*). The voltage was measured between points M and N, where the microelectrodes were placed. These two points were also connected to the membrane on the other side of the fiber (i.e., R_2) via the low extracellular resistance (R_e) and the low intracellular resistance (R_i). The action potential, which is produced at the depolarized side (R_2), will also influence the membrane potential on the other side, which is where the measurement was made. The microelectrode evidently detected the transient high conductance state of R_2 via R_i and R_e.

The measured voltage (V_m) also included a component that was due to the series resistance (R_s) between the extracellular electrode and the cell. To estimate the size of this component, the muscle fiber was removed after the action potentials were recorded while the microelectrodes were kept in their original position. The same sequence of stimuli was applied again with M removed from the cell and the potential difference between the electrodes M and N was measured as before. This extracellular voltage (V_e) is shown as the middle trace in the four frames of Fig. 3.

Tetanic Responses

The influence of the stimulus parameters on aequorin signals and contractile force was studied in tetani. Some records of tetanic responses are shown in Fig. 4. Prolongation of each stimulus in the train from 1–10 ms reduced the plateau of force by 45%, and reduced the integral of the light signal by almost the same fraction in this fiber (viz., 40%). From Rüdel and Taylor's (1969) results and from our membrane potential records (Fig. 3) we infer that opposite sides of the fiber were activated when the polarity of the stimuli was changed. But the light signal and the force of contraction were not markedly influenced by the polarity of the stimulus (Fig. 4 *B* and *C;* the difference between the integrals of the light signals is only 2.5%). The possibility that anode break excitation activated the cell on the hyperpolarized side was ruled out, because the rare occurrence of this phenomenon is readily detected in the force records (e.g., Rüdel and Taylor, 1969, Fig. 5). The lack of a difference between the records in panels *A* and *D* of Fig. 4 was expected since we presumed that the whole fiber was activated by stimuli of 1 ms duration.

Twitch Responses

Twitch responses were studied more extensively than tetani and some characteristic records are shown in Fig. 5. Pulses of 10 ms duration diminished the light transients and force by almost the same amount, regardless of the stimulus polarity. Light was decreased by 39% in Fig. 5 *A*, and by 46% in *B* when the polarity was reversed. Force was decreased by 47% in both *A* and *B*. The same responses are plotted again in Fig. 5 *C* and *D* after their peaks were normalized to the same heights. The normalized responses are alike in shape, position, and area, which shows that prolongation of the stimulus to 10 ms changed only the amplitude but not the time-course of the light transient and force of contraction.

Light signals were never perfectly reproducible, and variations in peak amplitude made the

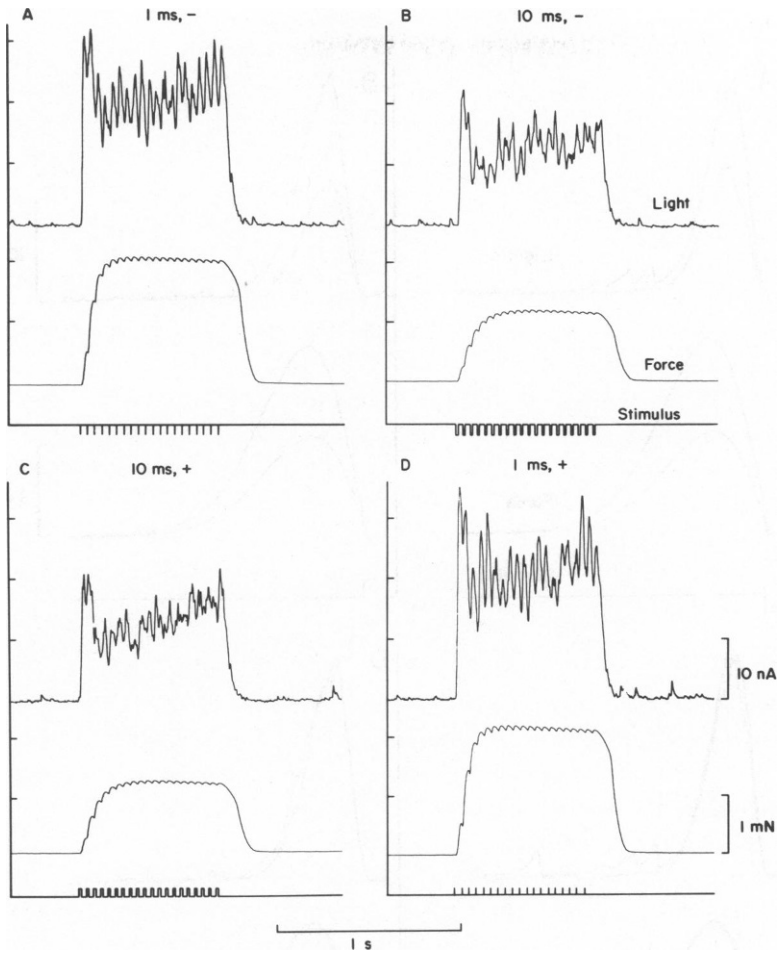

FIGURE 4 Records of tetanic responses to stimuli of short and long durations and two polarities (fiber 1.XI.79). Top traces, aequorin luminescence (The calibration refers to the photomultiplier current.); middle traces, force of contractions; bottom traces, stimulus marks. (*A*) The stimulating electrode adjacent to the side of the fiber injected with aequorin was negative. The individual stimuli were 1 ms long. (*B*) Same polarity of electrodes as in *A*, but the stimuli were 10 ms long. (*C*) Electrode adjacent to the injected side was positive and each stimulus was 10 ms in duration. (*D*) Same polarity of electrodes as in *C*, but the stimuli were 1 ms long. The records were taken at intervals of 5 min and in the sequence shown. Pulse strength, 2.6 × rheobase; striation spacing, 2.2 μm; frequency of stimulation, 25 Hz; temperature, 15°C.

detection of very small differences difficult. This was troublesome, particularly when the initial signal-to-noise ratio was small. Signal averaging, which is often used to obtain a measurable average of small events, is a suitable procedure when variations in the noise are essentially random. The fact that the signal in these experiments also showed variations prompted us to investigate the degree to which signals were in fact identical to each other.

As previously reported, aequorin responses to brief stimuli were essentially constant within a few minutes after an injection (Blinks et al., 1978). But when long duration stimuli were

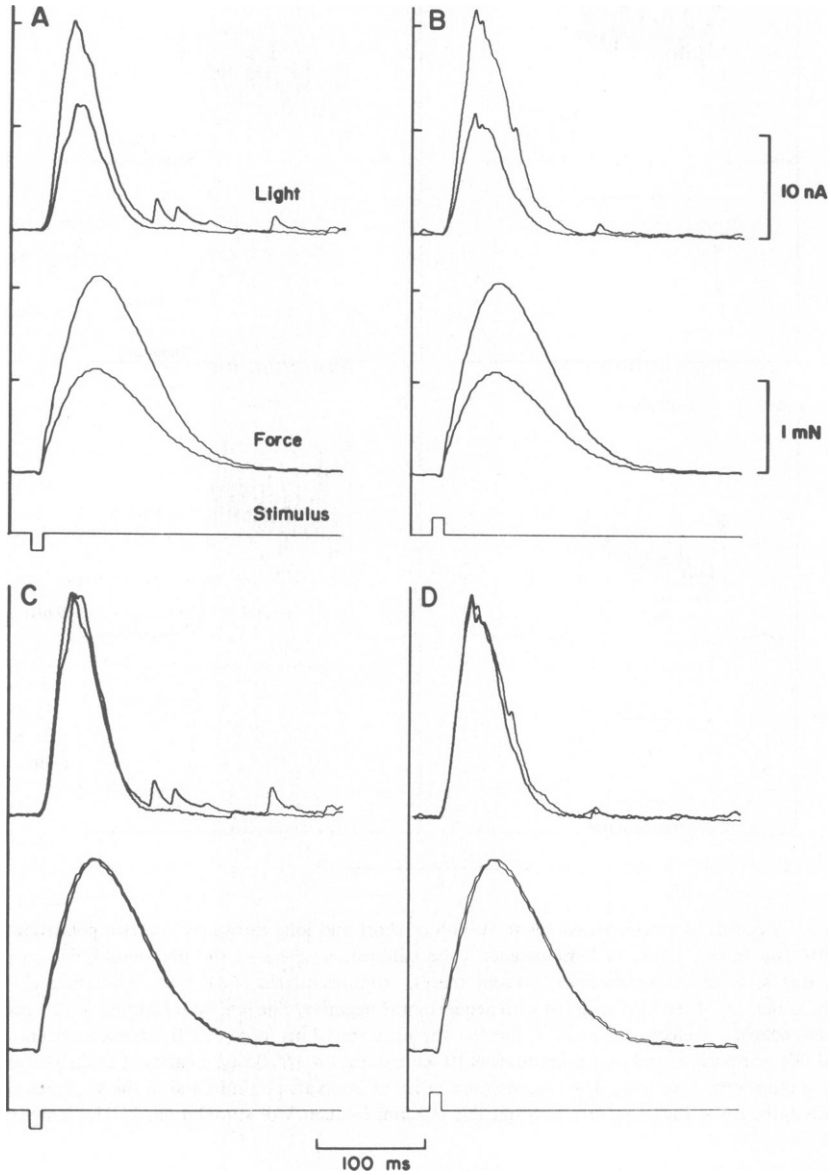

FIGURE 5 Superimposed records of twitch responses to stimuli of 0.5 ms and 10 ms duration (fiber 12.VI.79). (*A*) Responses to pulses that made the electrode adjacent to the injected side of the fiber negative. The response with the higher peaks of light and force was elecited by a stimulus of 0.5 ms duration; the weaker response was elicited by a 10-ms stimulus. The onset of both types of stimuli was at the same time. The pulse strength was 4.2 × rheobase. Striation spacing: 2.8 μm. The records shown are averages of 4 responses taken in a period between 15 and 95 min after the injection of aequorin. (*B*) Responses to stimuli of opposite polarity of those in *A*. All other specifications as in *A*. (*C*) Same responses as in *A* after normalizing their peak heights to the same value. (*D*) Normalized responses from panel *B*.

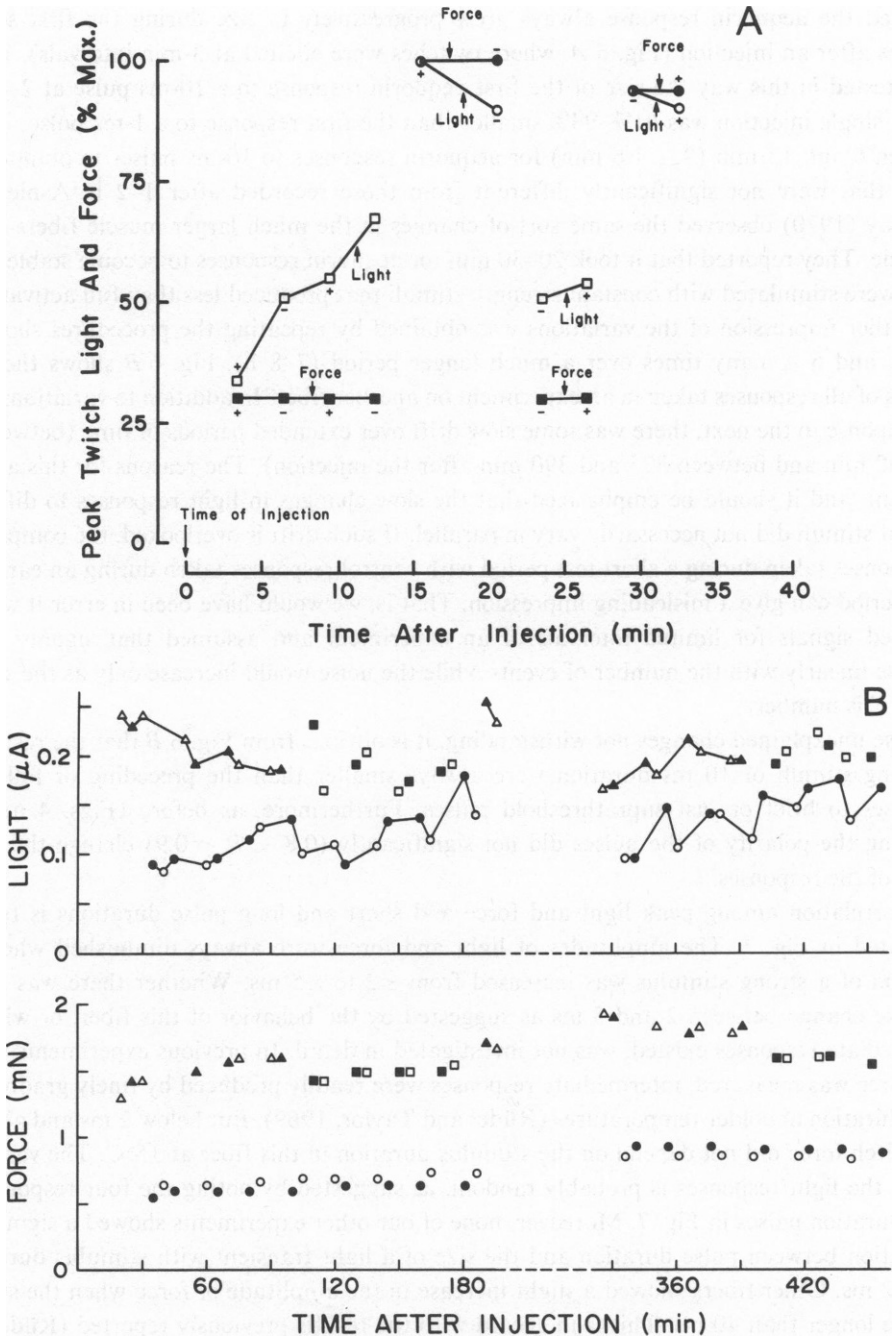

FIGURE 6 Reproducibility of light transients and twitch force. The peak amplitudes of the aequorin signals and the force of contractions are indicated for the first 10 responses taken early during the experiment of Fig. 6*A* (fiber 16.IV.81); all the responses taken during the experiment of Fig. 6*B* (fiber 31.V.79) are plotted. The lines connect points determined from responses to either 1- or 10-ms pulses and several times rheobasic strength. *A*, striation spacing, 2.2 μm; temperature, 15°C; field strength, 11.4 V/cm; □, ■, pulse duration 10 ms; ○, ●, pulse duration 1 ms. The strength of all pulses was 3 × rheobase. +, cathode opposite to the side of injection, −, cathode nearest to the side of injection. *B*, striation spacing, 2.8 μm; ○, ●, pulse duration 10 ms, strength 3.8 × rheobase; □, ■, pulse duration 10 ms, strength 1.1 × rheobase. △, ▲, pulse duration 1 ms, strength 3.8 × rheobase. Open symbols: cathode on the side where the aequorin had been injected. Filled symbols: anode on the side of the injection.

delivered the aequorin response always grew progressively in size during the first several minutes after an injection (Fig. 6 *A*, where twitches were elicited at 3-min intervals). In five fibers tested in this way the size of the first aequorin response to a 10-ms pulse at 2–4 min after a single injection was 61%–93% smaller than the first response to a 1-ms pulse; it took between 6 and 13 min (9 ± 1.6 min) for aequorin responses to 10-ms pulses to obtain peak values that were not significantly different from those recorded after 1–2 h. Ashley and Ridgway (1970) observed the same sort of changes in the much larger muscle fibers of the barnacle. They reported that it took 20–30 min for aequorin responses to become stable when fibers were stimulated with constant strength stimuli that produced less than full activation.

Another impression of the variations was obtained by repeating the procedures shown in Figs. 5 and 6 *A* many times over a much longer period (7–8 h). Fig. 6 *B* shows the peak heights of all responses taken in an experiment on another fiber. In addition to variations from one response to the next, there was some slow drift over extended periods of time (between 20 and 100 min and between 325 and 390 min after the injection). The reasons for this are not apparent, and it should be emphasized that the slow changes in light responses to different types of stimuli did not necessarily vary in parallel. If such drift is overlooked, the comparison of responses taken during a short test period with control responses taken during an earlier or later period can give a misleading impression. That is, we would have been in error if we had averaged signals for limited intervals of an experiment and assumed that signals would increase linearly with the number of events while the noise would increase only as the square root of this number.

These unexplained changes not withstanding, it is obvious from Fig. 6 *B* that the responses to strong stimuli of 10 ms duration were always smaller than the preceding or following responses to brief or just suprathreshold pulses. Furthermore, as before (Figs. 4 and 5), changing the polarity of the pulses did not significantly ($0.8 < P < 0.9$) change the mean values of the responses.

A correlation among peak light and force and short and long pulse durations is further illustrated in Fig. 7. The amplitudes of light and force were always diminished when the duration of a strong stimulus was increased from ≤ 2 to ≥ 5 ms. Whether there was a real stepwise change between 2 and 5 ms as suggested by the behavior of this fiber, or whether intermediate responses existed, was not investigated in detail. In previous experiments where only force was measured, intermediate responses were readily produced by finely grading the pulse duration at colder temperatures (Rüdel and Taylor, 1969). But below 2 ms and above 5 ms, twitch force did not depend on the stimulus duration in this fiber at 15°C. The variation among the light responses is probably random, as suggested by noting the four responses to 1-ms-duration pulses in Fig. 7. Moreover, none of our other experiments showed a significant correlation between pulse duration and the size of a light transient with stimulus durations below 2 ms. Other fibers showed a slight increase in the amplitude of force when the stimuli became longer than 40 ms. This is in accord with the results previously reported (Rüdel and Taylor, 1969), where it was noted that responses to extreme prolongation of a stimulus (i.e., beyond 20 ms) could be markedly nonreproducible for a given fiber.

A graded relation between stimulus strength and the amplitudes of twitch force and light transients was always found for 10-ms pulses, whereas responses to short pulses (1 ms) were not influenced by the pulse strength (e.g., Fig. 8). In most fibers, twitch force was reduced in

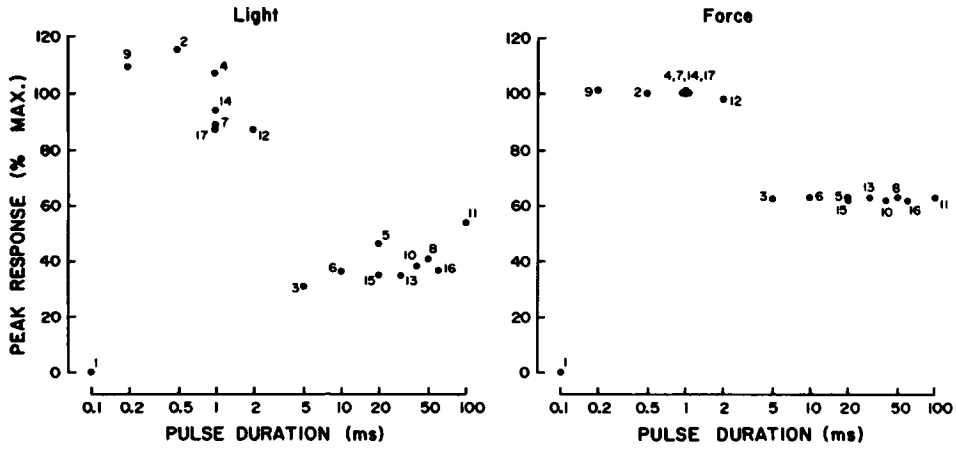

FIGURE 7 Influence of the pulse duration (fiber 9.V.79A). The points indicate the amplitudes of aequorin transients and twitch force of single responses. The mean amplitudes of the responses to pulses between 0.2 and 2 ms duration was defined as 100%, and the amplitudes of all single responses were expressed as a percentage of this mean (% MAX). The ascribed numbers indicate the sequence in which the responses were elicited, all from the same fiber. The first response was taken 50 min after the injection of aequorin. The pulse strength was 4 × rheobase; the negative electrode was always on the side where the injection had been made. Striation spacing, 2.2 μm; temperature, 15°C.

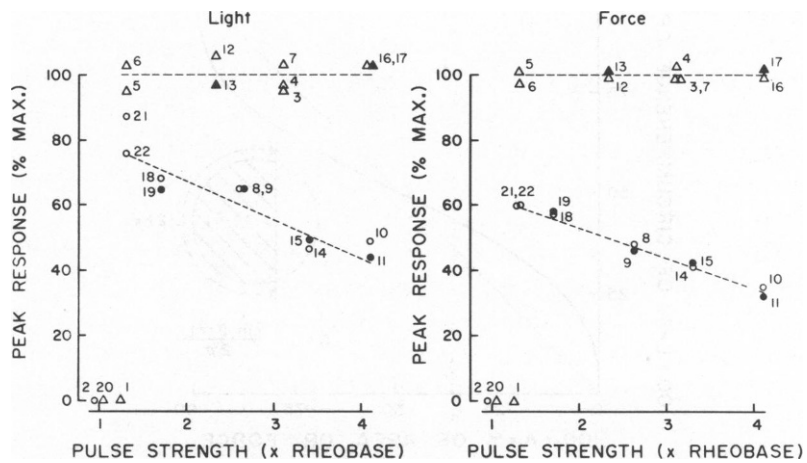

FIGURE 8 Influence of the pulse strength for pulses of 1-ms duration (△, ▲) and 10-ms duration (○, ●) (fiber 1.XI.79). The mean of the amplitudes of the responses to pulses of 1-ms duration was defined as 100%. All symbols indicate single responses, the ascribed numbers give their sequence. The first suprathreshold stimulus (response No. 3) was given 7 min after the injection of aequorin. △, ○, cathode on the side where the injection had been made; ▲, ●, stimulus of the opposite polarity; striation spacing, 2.2 μm; temperature, 15°C.

response to 10-ms stimuli even when the stimulus strength was low ($\leq 1.3 \times$ rheobase). A comparable depression at low strength was never seen as clearly in the light transients. One would expect a greater depression in light than in force if aequorin did not diffuse radially into the fiber and if it stayed close to its surface, and if simple assumptions and geometry were applicable to the correlations among stimulus strength, peak light, and peak force. This is discussed in more detail in the next section and illustrated by Fig. 9. As shown in Figs. 5, 6 *B*, and 8, pulses of either polarity caused similar decreases in the amplitudes of the light signals and essentially equal decreases in the amplitudes of the contractions.

DISCUSSION

Membrane Potential Changes During Asymmetrical Activation

As far as we are aware, records of the changes in membrane polarization during the application of long field stimuli to muscle have been shown before only for crayfish fibers, which normally develop graded electrical and contractile responses (Sugi and Kosaka, 1964). Our membrane potential recordings show that a strong and long field stimulus hyperpolarizes the membrane at the anodal side of an excitable muscle fiber sufficiently to prevent an action potential from reaching mechanical threshold (Fig. 3 *D*). These results support earlier suggestions about why activation is blocked in the vicinity of the anode during a current pulse (e.g., Katz, 1939; Kuffler, 1946; Taylor, 1953; Rüdel and Taylor, 1969).

After the cessation of the action potential spike, pulses of the same absolute magnitude but opposite polarity cause much stronger hyperpolarizations than depolarizations (Fig. 3 *C* and

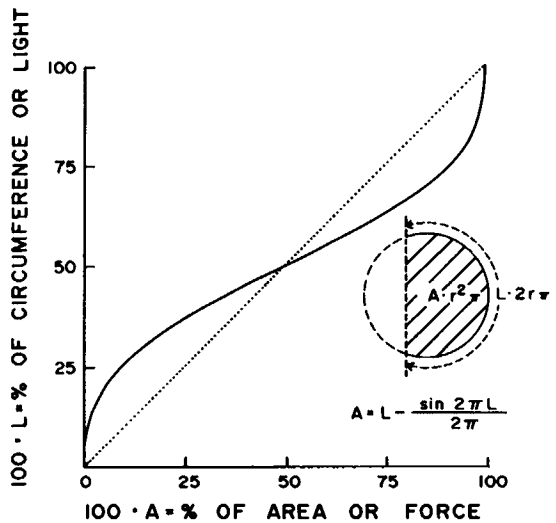

FIGURE 9 Arc length of a circle segment as a function of the area of the segment. The arc length of the segment is expressed as a percentage (L) of the circumference of the full circle and the area of the segment as a percentage (A) of the area of the circle. The relation between L and A is given by the continuous line. The dotted line was added to illustrate how much this relation deviates from a 1:1 proportionality. *Inset:* A is proportional to the hatched area in the circle and L to the continuously drawn part of the circumference. The graph was calculated from the expression given in the right lower corner.

D). Narahashi et al. (1960) showed previously that intracellularly applied depolarizing currents caused smaller deflections of the membrane potential than hyperpolarizing currents in TTX-poisoned frog muscle fibers. They explained the asymmetry by suggesting it was related to delayed rectification, i.e., an increase of the potassium conductance during prolonged depolarization. In addition, strong prolonged hyperpolarizations have been shown to decrease potassium permeability in frog skeletal muscle (Almers, 1972). Therefore, the membrane resistance during extracellular stimulation can be expected to decrease on the cathodal side and increase on the anodal side. Then the voltage across the membrane adjacent to the anode will be largest, as seen in Fig. 3.

Aequorin Signals and Contractile Force During Asymmetrical Activation

In our experiments the force of contraction during prolonged stimuli was decreased in a way similar to that seen previously (Rüdel and Taylor, 1969). The phenomenon now seems to be adequately explained by the previous observations of myofibrillar folding during fiber shortening and our present records of membrane potentials. On the basis of this evidence that a long stimulus completely inhibits calcium release in a known segment of a muscle fiber, one would expect the aequorin signal to decrease if aequorin was not excluded from the myofilament lattice. Long-chain polymers can evidently be excluded from the myofilament lattice (Godt and Maughan, 1977). But aequorin, which has a Stokes radius of ~19 Å (Prendergast and Mann, 1978), should not be excluded simply because of its size from the spaces among filaments thought to be a few hundred Angstroms apart and separated by an aqueous medium (Huxley, 1971).

Eisenberg et al. (1979) found recently that the distribution of HRP introduced into frog muscle fibers through nicks in the sarcolemma, was only *C*-shaped after 30 min of diffusion. HRP evidently travels up to 2 mm longitudinally, only 1–20 μm in a radial direction, and only partially around the fiber periphery within 30 min. Therefore, Eisenberg et al. (1979) proposed that the radial, circumferential, and longitudinal diffusion coefficients may have a ratio of ~1:10:100. The markers they studied diffused in a markedly anisotropic manner in frog muscle cells. As they suggest, the distribution of intracellular probes such as aequorin must be considered when one is attempting to interpret signals from such a probe (Blinks et al., 1978). In contrast, our experiments imply that at the time they observed only *C*-shaped distribution of HRP (e.g., ~30 min after an injection for the experiment shown in Fig. 6 *B*), the distribution of aequorin must have been at least O-shaped. The observation that an aequorin signal was decreased when the field was of either polarity indicates that aequorin spread from the injected spot to the opposite side of the fiber. The full details of aequorin distribution are not explicitly shown by these experiments. For example, we have not sought to estimate nondiffusional factors such as the force provided by an injection itself or the possibility that aequorin may bind to other substances in a cell. But the aequorin concentration evidently changed from zero, at the moment and site of injection, to equal levels on both sides of the fiber in at most several minutes, that is, a time at which Eisenberg et al. (1979) found only limited distribution of some marker substances. A lower limit for the circumferential diffusion coefficient of aequorin can be estimated from this information if a simple model for the diffusion is assumed (see Appendix).

Uncertainty of Radial Diffusion

Force and light signals were decreased by prolonged stimuli of increasing strength in an approximately parallel fashion (Fig. 8). Most simply, long stimuli may induce calcium release in one part of the fiber as usual, and not release it at all in the other part (we assume that a region where calcium release may be only partially reduced is relatively small). Then, one might infer from the parallelism in the decreases that force and light are produced in the same subunits of the cell, and that part of the aequorin has to be in the core of the muscle fiber if the entire cross section of a fiber is at least partially activated (Gonzalez-Serratos, 1971; Costantin and Taylor, 1973). Model calculations, however, show that such a conclusion cannot be proven from the present experimental data (see Fig. 9). Force probably is proportional to the cross-sectional area of the activated part of the fiber, i.e., to the area ($A \cdot r^2 \pi$ in Fig. 9) of a circular segment. (We shall assume for simplicity that the fiber cross section is a perfect circle, although it rarely is). If aequorin did not diffuse into the core of the fiber, but stayed close to its surface, light would be proportional to the arc length ($L \cdot 2r\pi$) of the same segment. The relation between the parameters L and A is given by the curved, solid line in Fig. 9. In the case of the other extreme, if light were produced from the whole activated volume of the cell, one would expect a simple 1:1 proportion between the relative decreases of light and force (indicated by the dotted line in Fig. 9). The difference between the predictions of the two models is largest when the area, or force, is decreased to ~90 or 10%. Even at these points light would deviate from a 1:1 proportion by 17% at most. In our experiments, however, using stimulus strengths from 1.2–4 × rheobase, force was decreased to values between 70 and 25% (e.g., Fig. 8; see also Rüdel and Taylor, 1969, Fig. 3). Moreover, the variation in the amplitudes of the light transients is approximately as large as the difference between the two theoretical relations shown in Fig. 9. Thus, experimental values with an adequately large signal-to-noise ratio in the regions where both models differ most were not observed in practice. From experiments of the type shown in this paper, a firm conclusion about the radial distribution of aequorin cannot be made unless the variations observed during an experiment can be greatly reduced. The signal-to-noise ratio was, in general, low in these experiments because (*a*) fibers were injected at only one spot and contained less aequorin than usual, and (*b*) long duration stimuli could decrease the light signal by as much as half without affecting the noise. Fortunately, however, signal averaging was not necessary to detect light responses in these experiments. Signal averaging would have misled us because of long-term variations such as those shown in Fig. 6 *B*. Furthermore, aequorin is probably not a single homogeneous molecular species, and different species might distribute at different rates. Various isoaequorins have different properties; they are, for example, electrophoretically heterogeneous although they seem to have the same molecular weight and emission spectra (Blinks et al., 1976; Prendergast and Mann, 1978). In addition, various relations between a surface-related parameter and a volume-related parameter other than the ones shown in Fig. 9 can be constructed if noncircular shapes of the fiber cross section (Blinks, 1965) are taken into account. A triangular or an elliptically shaped fiber, for example, yields model results that differ less than the maximum 17% difference obtained with a hypothetical circularly shaped fiber. A very accurate knowledge of the fiber shape in the injected region of each fiber would be necessary to construct a reasonable theoretical fit of these experimental data.

APPENDIX

An Estimate for the Diffusion Coefficient of Aequorin

To obtain a simple model for the diffusion of a substance around the periphery of a muscle fiber we made the following assumptions: (*a*) The source of the substance is an instantaneously deposited amount of aequorin at a point, i.e., all the microinjected substance is concentrated at one point on the fiber periphery at time $t = 0$. (*b*) Radial diffusion to the core of the fiber is much slower than circumferential diffusion and can be neglected (Eisenberg et al., 1979). (*c*) The spread of the substance follows the diffusion equation (Crank, 1975, Eq. 1.11, p. 6):

$$\frac{\partial Q}{\partial t} = D_z \frac{\partial^2 Q}{\partial_z^2} + D_s \frac{\partial^2 O}{\partial_s^2} . \tag{1}$$

The meanings of the symbols are as follows: Q, concentration of diffusing substance; t, time; z, length in longitudinal direction; s, length going around the periphery of the fiber; D_z, coefficient of longitudinal diffusion; and D_s, coefficient of circumferential diffusion. For our boundary conditions the solution of Eq. 1 can be expressed as a product of the solutions of two one-dimensional problems, i.e., purely longitudinal and purely circumferential diffusion (Crank, 1975, pages 24 and 25). Since we are only interested in the time-course of the circumferential diffusion, we shall omit the factor for longitudinal diffusion in the following considerations. The solution $Q'(x, t)$ for diffusion from a point source into an infinite one-dimensional space is known (e.g., Crank, 1975, Eq. 2.6, page 12):

$$Q' = \frac{M}{2\sqrt{\pi Dt}} \exp(-x^2/4Dt), \tag{2}$$

where x is distance and M is the total amount of aequorin. The circumference of a fiber, however, is a finite length that rejoins itself. If S is one-half the circumference of the fiber (i.e., $2S$), we see that the points $s + 2S$, $s + 4S$, etc., and $s - 2S$, $s - 4S$, etc. all fall on one another. The solution $Q_s(s, t)$ of the diffusion equation for this space can be constructed from Eq. 2 by superposition (see, e.g., Crank, 1975, sections 2.2.2. and 2.2.4.):

$$Q_s = \sum_{i=-\infty}^{\infty} Q'(s + 2i \cdot S, t) . \tag{3}$$

For the source point ($s = 0$) and the most opposite point ($s = S$) Eq. 3 can be simplified because of the symmetry of Eq. 2:

$$Q_s(0, t) = Q'(0, t) + 2 \cdot \sum_{i=1}^{\infty} Q'(2i \cdot S, t) \tag{4}$$

$$Q_s(S, t) = 2 \cdot \sum_{i=0}^{\infty} Q'[(2i + 1) \cdot S, t)]. \tag{5}$$

We are mainly interested in the ratio of the concentrations (R) on both sides of the fiber and obtain from Eqs. 2, 4, and 5

$$R = \frac{Q_s(S, t)}{Q_s(0, t)} = \frac{2 \cdot \sum_{i=0}^{\infty} \exp[-(2i + 1)^2 \cdot S^2/4D_s t]}{1 + 2 \cdot \sum_{i=1}^{\infty} \exp[-4i^2 S^2/4D_s t]} . \tag{7}$$

(At this step the factors for longitudinal diffusion drop out.) The terms in the series of Eq. 7 decrease very rapidly with increasing i as long as $\exp(-S^2/4D_s t)$ has a value not too close to 1. R increases from 0 to 1 as the concentration of the diffusing substance approaches an equal value around the whole periphery of the fiber. The speed at which R approaches the value 1 depends on the circumference $2S$ and the diffusion coefficient D_s. From our experiments we know that R must have been close to 1 after ~9 min (Fig. 6*A*). Assuming 300 μm for the fiber circumference and $D_s = 10^{-7}$ cm^2 s^{-1}, one obtains $R = 0.7$ for $t = 9$ min from Eq. 7. On the presumption that the radial diffusion of aequorin is much slower, we conclude, therefore, that the diffusion coefficient for circumferential diffusion of aequorin must be $\leq 10^{-7}$ cm^2 s^{-1}.

If radial diffusion can not be neglected, the solution of the diffusion equation for our boundary conditions (source at a point on the periphery of the fiber) becomes difficult, but with simplifying assumptions for the shape of the fiber cross section the procedure of reflection and superposition (Crank 1975, section 2.2.2.) can again be used. The result is an expression similar to Eq. 7. From those considerations we conclude that a coefficient larger than $3 \cdot 10^{-8}$ cm^2 s^{-1} for radial diffusion would also be sufficient to explain the lack of a difference between responses to stimuli of opposite polarity. Ashley and Moisescu (1975) found a value of 10^{-7} cm^2 s^{-1} for the diffusion of aequorin into a bundle of barnacle myofibrils, and Blinks et al. (1978) estimated the coefficient for longitudinal diffusion in frog muscle as $5 \cdot 10^{-8}$ cm^2 s^{-1}. Both values are close to the ones given above and, therefore, consistent with our limiting values for the rates.

We thank L. Wussow for the illustrations and the preparation of the manuscript, L. A. Wanek for skilled assistance with the experiments, and P. J. Griffiths for comments and criticisms. We are indebted to L. W. Engel for expanding the product solution in the Appendix in a Fourier series of eigenfunctions to prove that the solution is unique.

This work was supported by grants from the National Science Foundation (77-22442), Consejo Nacionalde Investigaciones Cientificas y Tecnologicas of Venezuela, the U.S. Public Health Service, (NS 14268) and 77-983 from the American Heart Association.

Received for publication 25 November 1980 and in revised form 21 July 1981.

REFERENCES

Almers, W. 1972. The decline of potassium permeability during extreme hyperpolarization in frog skeletal muscle. *J. Physiol. (Lond.).* 225:57–83.

Ashley, C. C., and D. G. Moisescu. 1975. The part played by Ca^{2+} in the contraction of isolated bundles of myofibrils. *In* Calcium Transport in Contraction and Secretion. E. Carafoli, F. Clement, and A. Margreth, editors. North-Holland Publishing Co., Amsterdam. 517–525.

Ashley, C. C., and E. B. Ridgway. 1970. On the relationships between membrane potential, calcium transient, and tension in single barnacle muscle fibres. *J. Physiol. (Lond.).* 209:105–130.

Blinks, J. R. 1965. Influence of osmotic strength on cross-section and volume of isolated single muscle fibres. *J. Physiol. (Lond.).* 177:42–57.

Blinks, J. R., F. G. Prendergast, and D. G. Allen. 1976. Photoproteins as biological calcium indicators. *Pharmacol. Rev.* 28:1–93.

Blinks, J. R., R. Rüdel, and S. R. Taylor. 1978. Calcium transients in isolated amphibian skeletal muscle fibres: detection with aequorin. *J. Physiol. (Lond.).* 277:291–323.

Costantin, L. L., and S. R. Taylor. 1973. Graded activation in frog muscle fibers. *J. Gen. Physiol.* 61:424–443.

Crank, J. 1975. The Mathematics of Diffusion. Clarendon Press, Oxford. 2nd edition.

Eisenberg, B. R., R. T. Mathias, and A. Gilai. 1979. Intracellular localization of markers within injected or cut frog muscle fibers. *Am. J. Physiol.* 237:C50–C55.

Godt, R. E., and D. W. Maughan. 1977. Swelling of skinned muscle fibers of the frog. Experimental observations. *Biophys. J.* 19:103–116.

Gonzalez-Serratos, H. 1971. Inward spread of activation in vertebrate muscle fibres. *J. Physiol. (Lond.).* 212:777–799.

Hodgkin, A. L., and P. Horowicz. 1957. The differential action of hypertonic solutions on the twitch and action potential of a muscle fibre. *J. Physiol. (Lond.).* 136:17–18P.

Huxley, H. E. 1971. The structural basis of muscular contraction. *Proc. Roy. Soc. Lond. B Biol. Sci.* 178:131–149.
Katz, B. 1939. Electric Excitation of Nerve. Oxford University Press, London 1–151.
Kuffler, S. W. 1946. The relation of electrical potential changes to contracture in skeletal muscle. *J. Neurophysiol. (Bethesda).* 9:367–377.
Narahashi, T., T. Deguchi, N. Urakawa, and Y. Ohkubo. 1960. Stabilization and rectification of muscle fiber membrane by tetrodotoxin. *Am. J. Physiol.* 198:934–938.
Nastuk, W. L., and A. L. Hodgkin. 1950. The electrical activity of single muscle fibers. *J. Cell. Comp. Physiol.* 35:39–73.
Prendergast, F. G., and K. G. Mann. 1978. Physical and chemical properties of aequorin and the green fluorescent protein from *Aequorea forskalea. Biochemistry.* 17:3448–3453.
Rüdel, R., and S. R. Taylor. 1969. The influence of stimulus parameters on contractions of isolated frog muscle fibres. *J. Physiol.* 205:499–513.
Sugi, H., and K. Kosaka. 1964. Summation of contraction in single crayfish muscle fibres. *Jpn. J. Physiol.* 14:450–467.
Taylor, R. E. 1953. The contractile process is not associated with potential changes. *J. Cell. Comp. Physiol.* 42:103–123.
Taylor, S. R., H. H. Shlevin, and J. R. Lopez. 1979. Calcium in excitation-contraction coupling of skeletal muscle. *Biochem. Soc. Trans.* 7(part 4):759–764.
Trube, G., J. R. Lopez, L. A. Wanek, and S. R. Taylor. 1979. Reversible decrease of aequorin responses in muscle fibers by prolongation of a transverse d.c. stimulus. *Physiologist.* 22:125.