Simultaneous Measurement of Ca^{2+} in Muscle with Ca Electrodes and Aequorin

Diffusible Cytoplasmic Constituent Reduces Ca^{2+}-independent Luminescence of Aequorin

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ABSTRACT Estimates of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) were made essentially simultaneously in the same intact frog skeletal muscle fibers with aequorin and with Ca-selective microelectrodes. In healthy fibers under truly resting conditions $[Ca^{2+}]_i$ was too low to be measured reliably with either technique. The calibration curves for both indicators were essentially flat in this range of $[Ca^{2+}]$, and the aequorin light signal was uniformly below the level to be expected in the total absence of Ca^{2+}. When $[Ca^{2+}]_i$ had been raised to a stable level below the threshold for contracture by increasing $[K^+]_o$ to 12.5 mM, $[Ca^{2+}]_i$ was 38 nM according to aequorin and 59 nM according to the Ca-selective microelectrodes. These values are not significantly different. Our estimates of $[Ca^{2+}]_i$ are lower than most others obtained with microelectrodes, probably because the presence of aequorin in the cells allowed us to detect damaging microelectrode impalements that otherwise we would have had no reason to reject. The observation that the light emission from aequorin-injected fibers in normal Ringer solution was below the level expected from the Ca^{2+}-independent luminescence of aequorin in vitro was investigated further, with the conclusion that the myoplasm contains a diffusible macromolecule (between 10 and 30 kD) that interacts with aequorin to reduce light emission in the absence of Ca^{2+}.

INTRODUCTION

The studies described in this paper were undertaken with the related goals of explaining an intrinsic paradox in the light signals recorded from resting frog skeletal muscle fibers containing the Ca^{2+}-regulated bioluminescent protein aequorin, and resolving an apparent inconsistency between the results obtained in such

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cells with aequorin and with Ca^{2+}-selective microelectrodes. The paradox was the finding that the light emission from healthy aequorin-injected frog skeletal muscle fibers at rest appeared to be below the level measured in vitro in the absence of Ca^{2+}; the inconsistency was that in several published studies on resting frog skeletal muscle fibers, most of them carried out with Ca^{2+}-selective microelectrodes (Tsien and Rink, 1980; Lopez, Alamo, Caputo, DiPolo, and Vergara, 1983; Weingart and Hess, 1984), values for $[Ca^{2+}]_i$ (in the range of 50–150 nM) were obtained that would be expected to have increased the luminescence of aequorin well above the Ca^{2+}-independent level (Allen, Blinks, and Prendergast, 1977; Blinks, Wier, Hess, and Prendergast, 1982).

Although it was reported some years ago (Blinks, Wier, and Snowdowne, 1980) that estimates of $[Ca^{2+}]_i$ made with aequorin in frog skeletal muscle fibers at rest were in the vicinity of 80 nM, we now realize that those measurements were influenced by two systematic errors: a small error in the in vitro calibration curve for aequorin resulting from the use of reflective rather than purely absorptive filters (Moore, 1986), and an overestimate of the $[Mg^{2+}]$ in the cytoplasm of the muscle fibers. In more recent experiments by Moore (1986), the error resulting from the use of reflective filters was eliminated and an estimate of $[Ca^{2+}]_i$ of 39 nM was arrived at on the basis of the assumption that $[Mg^{2+}]_i$ was 3 mM (Hess, Metzger, and Weingart, 1982; Lopez, Alamo, Caputo, Vergara, and DiPolo, 1984). The relevance of this assumption is that Mg^{2+} antagonizes the effect of Ca^{2+} on aequorin, and therefore the calibration curve for aequorin must be determined in the same $[Mg^{2+}]$ as in the cytoplasm. Later studies by Alvarez-Leefmans, Gamiño, Giraldez, and Gonzáles-Serratos (1986) indicated that $[Mg^{2+}]_i$ was somewhat lower than previously believed, and that it was influenced by experimental conditions, particularly $[Mg^{2+}]_o$. Recent measurements made with a new and more reliable ion exchanger under the exact conditions of our experiments indicated that the $[Mg^{2+}]_i$ is ~ 1 mM (Blatter, 1990). When one applies Moore's measurements of aequorin luminescence from resting skeletal muscle fibers to an aequorin calibration curve determined in 1 mM Mg^{2+}, one obtains the seemingly impossible result that the light emission from most resting fibers is below the level that would be expected from the amount of aequorin in the cell even if the cytoplasm were totally free of Ca^{2+}.

There are three potential explanations for this paradox: (*a*) there is still an error in our aequorin calibration curve; (*b*) Moore's measurements of the light emission of resting muscle fibers were somehow in error; or (*c*) there is something in the cytoplasm of living muscle fibers that alters the properties of aequorin. As a first step in deciding among these possibilities, we reexamined our methods for determining Ca^{2+} concentration–effect curves for aequorin in vitro. We found no additional sources of error. We next set out to repeat Moore's determinations, measuring aequorin luminescence from intact aequorin-injected single skeletal muscle fibers, and obtained results virtually identical to his. This left us with the strong suspicion that the third explanation might be correct, and apparently with a substantial discrepancy between the estimates of $[Ca^{2+}]_i$ in frog muscle obtained with aequorin and Ca-selective electrodes. To resolve this discrepancy we undertook to measure $[Ca^{2+}]_i$ in the same cells and essentially simultaneously with the two techniques. Simultaneous measurements with two indicators are particularly helpful when, as in

this case, unexpected or anomalous results are obtained with one of them (Requena, Whittembury, Tiffert, Eisner, and Mullins, 1984). In this case the presence of aequorin allowed us to exclude results obtained from damaging microelectrode impalements. When this was done, we found that the two techniques gave similar results, and that $[Ca^{2+}]_i$ in healthy frog muscle cells at rest was considerably lower than most previous studies with Ca electrodes had indicated. We went on to test the possibility that the Ca^{2+}-independent luminescence of aequorin might be reduced by some constituent of the myoplasm, and the results of exploratory studies indicate that to be the case.

Some of these results have appeared in abstract form (Blatter and Lee, 1989).

METHODS

Fiber Preparation and Experimental Arrangements

Single twitch fibers were dissected from the tibialis anterior muscles of *Rana temporaria* stored at 4°C. The fibers were microinjected with aequorin prepared (Blinks, Mattingly, Jewell, van Leeuwen, Harrer, and Allen, 1978) and injected (Blinks, 1989) as described previously. The injected fibers were transferred to an apparatus constructed around an ellipsoidal reflector for efficient and omnidirectional light gathering (Blinks, 1982, 1989); it was modified for use with Ca^{2+}-selective microelectrodes by the addition of two independently mounted micromanipulators. The fiber was stimulated to twitch at 5-s intervals while its length was adjusted to give maximum twitch tension and its position in the reflector was adjusted to give the brightest light signals; stimulation was then discontinued. Microelectrode measurements were made with the fiber withdrawn from the reflector. The apparatus was arranged so that the fiber could be moved into or out of the reflector very quickly, and returned precisely to its original position. Thus, although aequorin signals could not be measured while the microelectrodes were in place, light measurements could be made within 1 min after the electrodes had been withdrawn. All measurements, including calibrations, were made at room temperature, which did not vary more than one degree from 19°C. The standard Ringer solution had the following composition (mM): 115 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 2.15 Na_2HPO_4, and 0.85 NaH_2PO_4, pH 7.1. The high-potassium solution contained (mM) 12 NaCl, 93 sodium propionate,[1] 12.5 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 2.15 Na_2HPO_4, and 0.85 NaH_2PO_4 (pH was adjusted with HCl).

Ca-selective Microelectrodes

Single-barreled microelectrodes were pulled and silanized as described previously (Blatter, 1990). The pipettes were first filled from the butt with an electrolyte solution that resembled the intracellular fluid in key respects and had a pCa of 6.9 (composition [mM]: 8.3 Na^+, 130 K^+, 0.5 Mg^{2+}, 2 Ca^{2+}, 5 HEPES, 4 EGTA, and 129.3 Cl^-). The tips were then filled with a mixture of 86% (by weight) of the calcium-sensing "cocktail" containing the neutral carrier ETH 1001 (#21048; Fluka Chemical Corp., Ronkonkoma, NY) and 14% polyvinyl chloride (PVC) (#81392; Fluka Chemical Corp.) (Oehme, Kessler, and Simon, 1976; Tsien and Rink,

[1] The substitution of propionate for chloride would be expected to cause some intracellular acidification, but judging from the records of De Hemptinne and Marrannes (1979), the initial change in pH is partly offset by cellular compensatory mechanisms and a new steady state is reached within ~20 min. Thus our comparative measurements will have been made under the same conditions, whether or not the acidification influenced $[Ca^{2+}]_i$. Both aequorin and Ca-selective microelectrodes are insensitive to pH changes of the magnitude likely to be encountered in living cells.

1981). The sensor-PVC mixture was diluted approximately fourfold in tetrahydrofuran, the tips of the pipettes were dipped into it, and suction was applied to the pipette to draw in a final column length of 50–200 μm.

A high-impedance electrometer (model FD 223; World Precision Instruments, Sarasota, FL) was used to monitor signals from the Ca-selective microelectrode and from a conventional microelectrode used to measure the transmembrane potential (E_m). The potential difference between ground and the Ca-selective microelectrode is designated as V_{Ca}. $V_{diff(Ca)}$ is the differential signal between the Ca-selective microelectrode and the reference microelectrode and corresponds to $[Ca^{2+}]_i$ ($V_{diff(Ca)} = V_{Ca} - E_m$).

Calibration Solutions

Both the Ca-selective microelectrodes and aequorin from the batch to be used intracellularly were calibrated in vitro in the same series of EGTA-buffered calibration solutions; these had an ionic composition similar to that of the cytoplasm of frog skeletal muscle (Na^+, K^+: MacDermott, 1987; Mg^{2+}: Blatter, 1990; see also reviews by Conway, 1957; Walker and Brown, 1977; Godt and Maughan, 1988.) Ca^{2+} concentrations were established where possible with known ratios of calcium and EGTA. Similar solutions without EGTA were used to extend the calibration curve for aequorin to Ca^{2+} concentrations above the range in which EGTA could be used as an effective calcium buffer. (In these solutions $[Ca^{2+}]$ was established by simple dilution of $CaCl_2$). The composition of the calibration solutions was (mM): 130 K^+, 8.2 Na^+, 1 Mg^{2+}, 1 EGTA, and 5 PIPES, pH 7.0; pCa varied between 5.4 and 7.4; Cl^- varied slightly with pCa, but always was within the range 130.05–130.1 mM. Concentrated solutions of KCl, KOH, NaCl, and PIPES were decalcified by passage through a column of Chelex 100 (Bio-Rad Laboratories, Richmond, CA). Calcium was added from a 1 M stock solution of $CaCl_2$ (#19046; BDH Laboratory Chemicals, Poole, UK), the exact concentration of which was checked both gravimetrically and by the titration method of Moisescu and Pusch (1975), for which dry EDTA was used as the primary standard. The EGTA (J. T. Baker, Inc., Phillipsburg, NJ) used to make the calibration solutions was recrystallized (Moore, 1986) and heat-dried (>24 h at 110°C). Its purity (determined by the Moisescu-Pusch titration method) was >99%. As a check on the correctness of the ratio of calcium to EGTA in our final calibration solutions, we estimated the apparent association constant for the CaEGTA complex (K_{CaEGTA}) with both indicators by matching calibration curves determined in the EGTA-buffered solutions (pCa 7.4–5.4) with curves determined with simple dilutions of $CaCl_2$ (pCa 5.9–2.0) (for a description of the method see Allen et al., 1977, or Blinks, 1982). Under the conditions of pH, temperature, $[Mg^{2+}]$, and ionic strength of our calibrations, log K_{CaEGTA} estimated with Ca-selective microelectrodes (12 measurements) was 6.43; that estimated with aequorin (9 measurements) was 6.39. The value calculated from "absolute" affinity constants (see, for example, Blinks et al., 1982) was 6.43.

Translation of Aequorin Signals into $[Ca^{2+}]_i$

The method was in principle that described by Allen and Blinks (1979), in which light signals recorded from the muscle cell are converted into units of fractional luminescence (L/L_{max}; for definition see legend to Fig. 1) and related to a calibration curve expressed in the same units. Fig. 1 shows a calibration curve for the batch of aequorin used to inject the muscle fibers, determined in vitro under conditions of temperature, $[Mg^{2+}]$, and ionic strength appropriate to our experiments. The apparatus and methods are described in detail elsewhere (Blinks, 1989). For these calibrations we used the same photomultiplier tube, dynode chain, high-voltage power supply, and electronic circuitry as for the intracellular measurements. The aequorin was preequilibrated with the same concentration of Mg^{2+} (1 mM) used in the calibration solutions.

L_{max} for the aequorin in the muscle cells was estimated by lysing the cell with Ringer solution containing 1% Triton X-100 while recording light emission under the same optical conditions used to measure light signals from the intact fiber. The total amount of light emitted was determined by integrating the photomultiplier anode current over the period (30–60 s) required for full discharge of the aequorin present in the muscle fiber. L_{max} was calculated as the product of this integral and the ratio of peak light intensity to total light emission (peak-to-integral ratio) determined in vitro at the peak of the calibration curve.

Calibration of Ca-selective Microelectrodes

Calibration was carried out in a chamber described by Weingart and Hess (1984), with the reference microelectrode and the Ca-selective microelectrode both immersed in the calibration solution. The signals from the two electrodes were recorded differentially, and the difference signal measured in high-potassium (12.5 mM K^+) Ringer solution ($[Ca^{2+}]$ = 1.8 mM) was defined as 0 mV. Only electrodes that gave stable measurements during the calibration process were used for measurements in muscle fibers, and all electrodes used for intracellular measurements were recalibrated after use. Experiments were discarded if there was a difference of 10 mV or more between points in the relevant range of the calibration curves determined before and after the impalement. 10 experiments survived this test (as well as all the other tests described under Results); the results of their calibrations are presented individually in Table I. (Note that 6 of the 10 experiments met a much more stringent test, and had differences between the two calibration curves of no more than 2.5 mV.) A calibration curve constructed from the average responses of the 10 electrodes of Table I (both before and after use) is shown as Fig. 2.

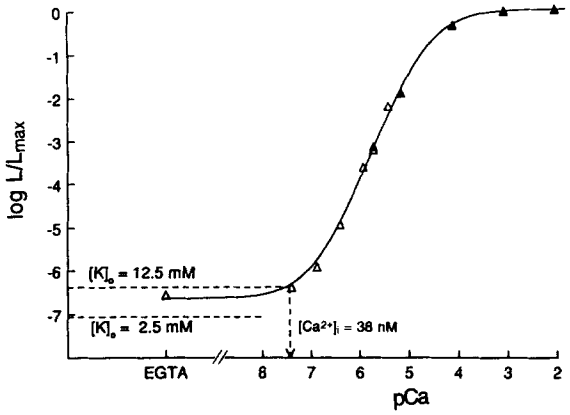

FIGURE 1. Ca^{2+} concentration–effect curve for aequorin determined in vitro. Peak light intensities (L) were measured when identical 10-μl aliquots of an aequorin solution were rapidly injected into 1-ml volumes of the various calibration solutions. Light intensities were normalized for the amount of aequorin present by dividing all values (L) by the peak light intensity (L_{max}) measured when the same amount of aequorin was injected into a saturating $[Ca^{2+}]$ (10 mM). Double logarithmic plot. Filled symbols indicate points for which $[Ca^{2+}]$ was established by simple dilution; open symbols indicate points for which calcium-EGTA buffers were used. EGTA indicates 1 mM EGTA and no added calcium (1 mM KCN was added subsequently to establish that the value was not elevated by contamination with Ag^+; see Allen et al., 1977). The dashed lines indicate estimates of fractional luminescence (L/L_{max}) determined from aequorin-injected fibers in $[K^+]_o$ = 2.5 and 12.5 mM. The curve was fitted by the two-state model (model B) of Allen et al. (1977), for which the constants used were K_R = 7,333,900 and K_{TR} = 162.

TABLE I

Summary of Comparative Measurements of $[Ca^{2+}]_i$ in Frog Skeletal Muscle Fibers with Aequorin and Ca-selective Microelectrodes

	Aequorin measurements				Measurements with Ca-selective microelectrodes						
	Before impalement		After impalement								
							Electrode calibration				
Experiment	Log(L/L_{max}) $[K^+]_o$ 2.5 mM	Log(L/L_{max}) 12.5 mM	Log(L/L_{max}) 12.5 mM*	Log(L/L_{max}) 12.5 mM‡	V_{diff} $[K^+]_o$ 12.5 mM	pCa	7.4	6.9	6.4	5.9	5.4
					mV				*mV*		
070888-1	−6.56	−6.43	−6.29	−6.29	−101.2	before	−98	−97	−93	−85	−72
						after	−93	−91.5	−88.5	−83.5	−72
071288-2	−7.07	−6.11	−6.07	−6.07	−105.5		−103	−98.5	−91	−81.5	−68.5
							−103.5	−98	−90.5	−80.5	−67
071488-2	−6.80	−6.24	−6.38	−6.38	−107.4		−100.5	−96	−90	−79.5	−66
							−103	−98	−90.5	−80	−65.5
072688-1	−7.49	−6.51	−5.13	−6.45	−105.6		−102	−99	−93	−82.5	−67
							−102.5	−97	−89.5	−77.5	−62.5
072688-2	−7.08	−6.51	−6.19	−6.25	−102.1		−102.5	−97	−89.5	−77.5	−62.5
							−101	−98.5	−93	−82.5	−67
072688-3	−7.08	−6.35	−4.86	−6.65	−113.5		−101	−98.5	−93	−82.5	−67
							−93.5	−91	−86	−77	−63.5
102888-2	−7.15	−6.35	−5.72	−6.31	− 88.1		−99.5	−98.5	−94	−85.5	−69.5
							−93.5	−92.5	−91.5	−84	−71.5
110188-1	−7.35	−6.30	−6.28	−6.38	− 92.8		−111.5	−109.5	−99.5	−89.5	−69.5
							−106	−103.5	−100	−87.5	−72.5
110188-2	−7.07	−6.33	−6.31	−6.31	−104.7		−109.5	−103	−95.5	−84	−67.5
							−100.5	−97	−90.5	−80	−66
110288-2	−6.98	−6.66	−6.54	−6.54	− 85.0		−109	−107	−100.5	−93.5	−77
							−102	−101.5	−100	−90	−74
Mean (±SD)	−7.06 (0.26)	−6.38 (0.16)	−5.98 (0.56)	−6.36 (0.16)	−100.6(9.1)	Mean	−101.8	−98.6	−93.0	−83.2	−68.4
Mean $[Ca^{2+}]_i$ (nM)	Below CIL	38	117	42	59	SD	4.97	4.61	4.18	4.41	3.85
n = 10 fibers											

*Measured immediately after removal of electrodes. ‡Measured after new steady state had been reached. CIL, Ca^{2+}-independent luminescence.

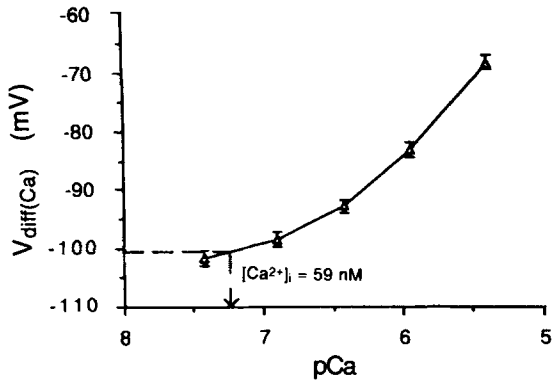

FIGURE 2. Calibration curve for Ca-selective microelectrodes. Average calibration curve (points indicate mean ± SEM) for the electrodes used to measure $[Ca^{2+}]_i$ in the 10 single muscle fibers listed in Table I. $[Ca^{2+}]$ was established with calcium-EGTA buffers. The dashed line indicates the average $V_{diff(Ca)}$ (−100.6 mV) measured intracellularly (in 12.5 mM K^+). This corresponds to a pCa_i of 7.23 (59 nM).

Muscle Extracts and Protein Solutions

The muscles of frog legs (*R. temporaria*) were soaked briefly in a solution containing (mM): 130 K^+, 8.2 Na^+, 1 Mg^{2+}, 5 PIPES, and 20 or 50 EGTA (pH 7.0, balance of anions = Cl^-); they were then chopped finely with a razor blade. The minced muscle was mixed with an approximately equal volume of the solution just described and stored overnight in a refrigerator, and the supernatant was recovered after centrifugation. The supernatant was subjected to ultrafiltration with membranes of various molecular weight cutoff levels (Diaflo YM2, YM10, YM 30, and PM 30 ultrafiltration membranes; Amicon, Beverly, MA).

Lyophilized preparations of frog muscle parvalbumin (P-6038; Sigma Chemical Co., St. Louis, MO) and bovine serum albumin (A-7638; Sigma Chemical Co.) were dissolved in an "intracellular" solution containing (mM): 130 K^+, 8.2 Na^+, and 10 EDTA (pH 7.0, balance of anions = Cl^-), and equilibrated with the same solution by gel filtration (G-25 Sephadex). Measurements of Ca^{2+}-independent luminescence were made in this solution; L_{max} in the presence of the proteins was determined in a solution of similar composition, except that the EDTA was replaced by 20 mM $CaCl_2$.

RESULTS

Determination of $[Ca^{2+}]_i$ in Frog Skeletal Muscle Fibers with Aequorin and Ca-selective Microelectrodes

In early experiments we confirmed that the resting $[Ca^{2+}]$ in healthy twitch fibers was too low to be measured in the usual way with aequorin, and were surprised to find that it was also too low to be measured with our Ca-selective microelectrodes. Even though neither method proved capable of providing reliable measurements of $[Ca^{2+}]_i$ under truly resting conditions, the main goals of the study might be accomplished almost as well if we could increase $[Ca^{2+}]_i$ to a steady level that would be measurable with both techniques, and we decided to try to accomplish that by increasing the $[K^+]$ of the bathing solution. To try to find a $[K^+]$ at which there would regularly be a stable elevation of $[Ca^{2+}]_i$ without a contracture, we tested the effects of various $[K^+]_o$ between 5 and 17.5 mM on aequorin-injected muscle fibers. We chose to make our comparative measurements in the presence of 12.5 mM K^+ because: (*a*) this concentration of K^+ always produced a substantial increase in the aequorin signal; (*b*) it never produced mechanical activation; and (*c*) the aequorin signal regularly leveled

off at a stable level in 12.5 mM K^+, whereas in higher $[K^+]$ it often did not. As is shown in Fig. 3 *A*, increasing $[K^+]_o$ from 2.5 to 12.5 mM usually had the effect of increasing the aequorin luminescence to a sharp peak, after which light emission came down to a steady state that was well above the level in 2.5 mM K^+.

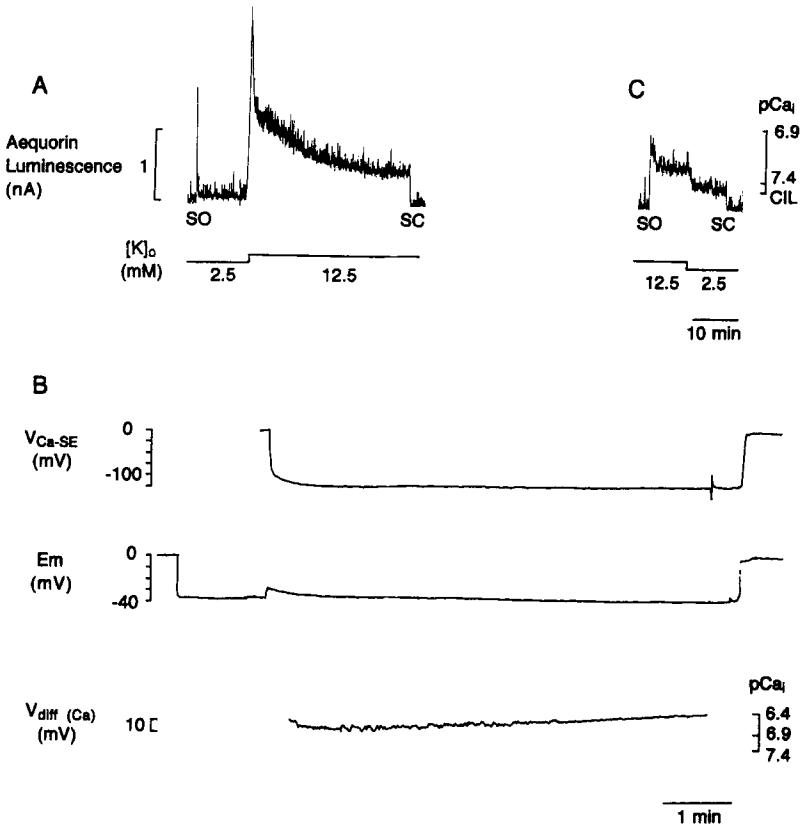

FIGURE 3. Intracellular Ca^{2+} signals recorded from a living frog skeletal muscle fiber with aequorin and a Ca-selective microelectrode. (*A*) Aequorin signal recorded from a muscle fiber in bathing solutions with $[K^+]_o$ = 2.5 and 12.5 mM before impalement by microelectrodes. The increase of $[K^+]_o$ caused a transient increase in the aequorin signal which subsided to a new (still elevated) steady state after several minutes. *SO* indicates shutter opened; *SC* indicates shutter closed. (*B*) Signals measured with the Ca-selective microelectrode during the time interval between *A* and *C*. From above downward: the signal recorded with the Ca-selective electrode ($V_{Ca\text{-}SE}$), the membrane potential (E_m), and the difference signal ($V_{diff(Ca)}$) corresponding to $[Ca^{2+}]_i$. (*C*) Aequorin signal recorded from the same fiber after withdrawal of electrodes, in bathing solutions with $[K^+]_o$ = 12.5 and 2.5 mM (experiment 102888-2).

Experimental Procedure

The resting glow from the aequorin-injected fiber was recorded in normal (2.5 mM K^+) Ringer solution for at least 10 min (often much longer) to verify that the fiber was in stable condition, and the $[K^+]_o$ of the perfusion solution was then increased to 12.5

mM. Time (usually 30 min or more) was allowed for the light emission to subside to a steady level. The fiber was retracted from the ellipsoidal reflector into the tissue bath and impaled with the two microelectrodes (usually 100–300 μm apart). The impalement of the two electrodes was maintained until the difference signal (which corresponds to $[Ca^{2+}]_i$) reached a steady level. The electrodes were then withdrawn, and the aequorin signal was measured again immediately thereafter. If there was a difference in the aequorin signals measured in 12.5 mM K^+ before and immediately after the electrode measurement (there was a difference in L/L_{max} of >0.2 log units in 4 of the 10 experiments listed in Table I), the fiber was kept in the high-potassium solution to determine whether the aequorin signal would recover; if it did, we assumed that the temporary elevation of $[Ca^{2+}]_i$ was the result of damage from the removal of the ion-selective electrode, and the results could be used. If there was no recovery the results of the experiment were not included in the comparison. The bathing solution was then switched back to normal (2.5 mM K^+) Ringer solution, and in most fibers the aequorin signal returned very nearly to the level observed at the beginning of the experiment. Results were discarded from those few experiments in which the resting glow remained elevated. At the end of the experiment the fiber was lysed with Triton X-100 to determine L_{max}, and the Ca-selective microelectrode was calibrated again. In the experiments of this series we made no further attempt to measure $[Ca^{2+}]_i$ with the Ca-selective electrodes under truly resting conditions (i.e., in 2.5 mM K^+) because prolonging the experiment would have weakened the comparison between the estimates made with the two methods in 12.5 mM K^+.

After preliminary experiments in which it was learned that $[Ca^{2+}]_i$ in healthy frog muscle fibers under truly resting conditions was too low to be measured with either technique, in which it was established that raising $[K^+]_o$ to 12.5 mM would be optimal for our purposes, in which the $[Mg^{2+}]$ in our fibers was measured, and in which it was determined that it was necessary to stabilize the Ca-selective electrodes with PVC, we attempted comparative measurements on 26 fibers that had been successfully injected with aequorin and appeared healthy in all respects afterward. 10 of these fibers met the criteria for electrode response and calibration (see Methods) and for behavior of the aequorin signal before and after the electrode impalement as just discussed, and provided estimates of $[Ca^{2+}]_i$ with both techniques in 12.5 mM $[K^+]_o$; results from these experiments are presented in Table I.

$[Ca^{2+}]_i$ *Estimated with Aequorin*

In each of the 10 successful experiments the aequorin signal was recorded before and after the electrode measurements in Ringer solutions with normal (2.5 mM) and elevated (12.5 mM) $[K^+]$. In normal Ringer solution the fractional luminescence recorded from single skeletal muscle fibers at the start of the experiment (mean log $L/L_{max} = -7.06$, SD 0.26) was below the Ca^{2+}-independent luminescence (CIL) determined in vitro (log $L/L_{max} = -6.55$) in all of the 10 experiments included in Table I, and in 26 of 28 other fibers that were not included in the table for various reasons. (The mean log L/L_{max} for these other fibers was -7.04, SD 0.36.) In high-potassium Ringer, the average steady-state value of log L/L_{max} measured before microelectrode impalement was -6.38 (SD 0.16), corresponding to an average pCa_i of 7.42 (38 nM). Immediately (1–2 min) after the microelectrodes had been

withdrawn from the fibers the mean log L/L_{max} was −5.98 (SD 0.56), corresponding to a pCa_i of 6.93 (117 nM). There was no correlation between the amplitude of the rise of the aequorin signal on withdrawal and the $V_{diff(Ca)}$ detected by the electrode just before withdrawal, and when the aequorin signal had settled down to a steady state it was, on average, at almost exactly the same level as before the electrode measurement (log L/L_{max} = −6.36). These observations suggest that although the removal of the electrodes caused a transient increase in $[Ca^{2+}]_i$, no lasting damage was done to these 10 fibers by either the insertion or the removal of the electrodes. When the high-potassium Ringer was replaced with normal Ringer solution near the end of the experiment, the resting glow of the fibers declined below the estimated CIL again (mean L/L_{max} = −6.69, SD 0.27).

$[Ca^{2+}]_i$ *Estimated with Ca-selective Microelectrodes*

The mean value for $V_{diff(Ca)}$ measured in 10 fibers at $[K^+]_o$ = 12.5 mM was −100.6 mV (SD 9.1 mV), corresponding to an average pCa_i of 7.23 (59 nM). Although each electrode used in these 10 experiments was calibrated individually (twice), we were frequently (in 5 of 10 experiments) not able to convert individual measurements of $V_{diff(Ca)}$ into estimates of $[Ca^{2+}]$ because the value of $V_{diff(Ca)}$ recorded from the fiber was more negative than the lowest point on the corresponding calibration curve. Therefore, the average value for $V_{diff(Ca)}$ was used to estimate $[Ca^{2+}]_i$ from the average calibration curve for all 10 experiments (Fig. 3). (Our reasons for doing this will be considered in the Discussion.)

Cut Fiber Experiments

A small bundle (two to seven fibers) of intact muscle fibers was dissected free and placed in an aliquot of an effectively Ca^{2+}-free solution (of the same composition as that used for the preparation of the muscle extract), where both ends of the fibers were cut off. The bundle (cell volume 0.5–1.5 μl) was then transferred to a quartz cuvette containing a small (100 μl) volume of the same solution to which enough aequorin had been added to give a readily detectable Ca^{2+}-independent luminescence. Light emission from the solution was monitored in the assay apparatus used to establish the Ca^{2+} concentration–effect curve (see Methods). The solution was highly buffered with EGTA (20 or 50 mM) to avoid effects of Ca^{2+} that might be introduced with the fibers, and to permit maintenance of a physiological $[Mg^{2+}]$. It was established in similar aliquots of the aequorin solution without muscle fibers that the addition of 1 mM KCN did not reduce the light emission from this solution (a test for contamination with Ag^+, which increases the Ca^{2+}-independent luminescence, and which might be bound by the addition of tissues or proteins).

The addition of freshly cut fibers to the aequorin-containing solution led to a gradual decrease in aequorin luminescence over a period of 10–30 min (Fig. 4). There was no immediate reduction in light intensity, as would have been expected if the effect of adding the fibers had been a purely optical one. These observations suggested either that the cut muscle fibers released a diffusible factor that interacted with the aequorin in the surrounding solution, or that the aequorin was being bound and concentrated inside the fiber in such a way that its CIL was reduced. Evidence against the latter possibility was provided by the observation that no change in

aequorin luminescence occurred when the fibers with cut ends were first stored in a large volume of the Ca^{2+}-free solution overnight, and then transferred to the cuvette containing the aequorin.

The findings just described led us to the tentative conclusion that a diffusible substance (or substances) in the myoplasm was responsible for the change in the Ca^{2+}-independent luminescence of aequorin in the cuvette, and that the presence of this substance might very well account for the fact that the resting glow recorded from intact aequorin-injected muscle fibers was below the CIL expected for the amount of aequorin in the cell. Because in the absence of Ca^{2+} the substance appears to stabilize aequorin in a nonreactive state, we have referred to it as the myoplasmic aequorin-stabilizing substance (MASS).

Muscle Extracts

Addition of 20 μl of the muscle extract to 1 ml of an effectively Ca^{2+}-free solution containing aequorin reduced the Ca^{2+}-independent luminescence by ~10%. In

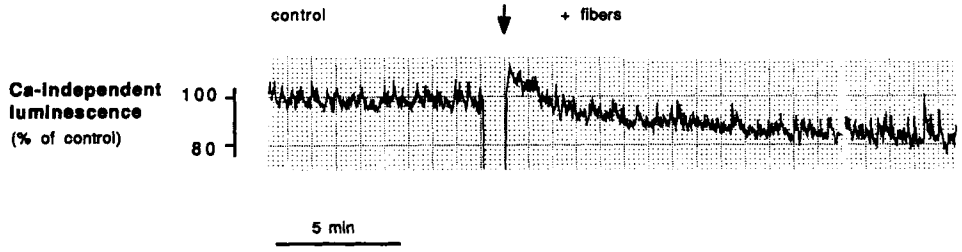

FIGURE 4. Effect of cut muscle fibers on the Ca^{2+}-independent luminescence of aequorin. Photomultiplier record of luminescence from 110 μl of an effectively Ca^{2+}-free solution containing aequorin. At the arrow a bundle of muscle fibers from which the ends had been freshly cut was introduced into the measuring cuvette, and the recording was resumed. The light intensity declined by ~20% over the next 20 min.

contrast to the cut fibers, the extract had an immediate effect. When identical aliquots of the muscle extract were added to EGTA-buffered solutions of pCa 7.4 and 6.9, the absolute change in light intensity was the same as in the Ca^{2+}-free solution. However, in relative terms the influence of the muscle extract became smaller as the Ca^{2+} concentration increased, and for practical purposes it was negligible at pCa 6.9.

In ultrafiltration experiments we found that the substance in the muscle extract responsible for reducing the CIL was largely retained by YM2 and YM10 membranes, and largely passed by YM30 and PM30 membranes, suggesting that it had a molecular weight between 10,000 and 30,000. This led us to suspect that the substance of interest might be a protein, and that in turn raised the possibility that the stabilizing effect which we had observed might not be a specific one, but an effect common to many proteins.

Effects of Some Soluble Proteins on Aequorin Luminescence

Two soluble proteins were tested for effects on Ca^{2+}-independent luminescence and on Ca^{2+}-activated luminescence in a saturating calcium concentration: frog parvalbu-

min, which is present in the cytosol of frog skeletal muscle at near millimolar concentrations (Gosselin-Rey and Gerday, 1977; Gerday, 1986; Godt and Maughan, 1988; Irving, Maylie, Sizto, and Chandler, 1989), and is therefore an obvious candidate to be the MASS, and bovine serum albumin, which served as a nonspecific protein control. Both proteins were studied in a final concentration of 1 mM. Their effects on aequorin luminescence are summarized in Table II.

Both frog parvalbumin and bovine serum albumin decreased the Ca^{2+}-independent luminescence of aequorin slightly, and both increased somewhat (by 6–8%) the total amount of light emitted by an aliquot of aequorin exposed to a saturating $[Ca^{2+}]$ (i.e., they increased the quantum yield of the luminescent reaction). There was also a small (1–5%) increase in L_{max} (the peak intensity of the flash of light recorded when aequorin was rapidly mixed with a saturating $[Ca^{2+}]$); the increases in L_{max} and total

TABLE II

Effects of Albumin and Parvalbumin on Aequorin Luminescence

	Control	Albumin (1 mM)	Parvalbumin (1 mM)
L_{max} (peak light)	100 ± 0.09 (39)	101 ± 0.11 (13)	105 ± 0.08* (21)
Total light (integrated signal)	100 ± 0.10 (39)	106 ± 0.14 (13)	108 ± 0.09** (21)
Ca^{2+}-independent luminescence (CIL)	100 (22)	94 ± 0.06* (7)	94 ± 0.04*** (10)
Average change in $\log(L/L_{max})$		−0.029	−0.044
Average peak-to-integral ratio (s^{-1})	1.12	1.06	1.10

All values normalized to % of corresponding control; mean, coefficient of variation, and number of measurements (n) are given.

No assumptions about the distribution of the data were made, and because of the small size of some samples we used a nonparametric procedure to compare the differences in the measured means for statistical significance. The test used was a two-tailed Wilcoxon rank sum test (Mann-Whitney U test; for test statistics and critical values see Ciba-Geigy, 1980). Asterisks indicate significance of difference from control values: *$P \leq 0.1$; **$P \leq 0.002$; ***$P \leq 0.001$.

light yield were not significantly different, so the increase in L_{max} is presumably entirely a reflection of the increase in the quantum yield. When the CIL is expressed in terms of fractional luminescence (i.e., as $\log L/L_{max}$), the effects of the two proteins on L_{max} accentuate their effects on the CIL somewhat by increasing the denominator in the expression L/L_{max} as well as by reducing the numerator. In 1-mM concentrations, parvalbumin and albumin reduced the CIL (expressed as $\log L/L_{max}$) by 0.044 and 0.029 log units, respectively (see Table II).

DISCUSSION

Interpretation of Measurements of Intracellular $[Ca^{2+}]$

In comparing the results obtained with the two methods used here, we need not be concerned about possible differences in the calcium concentrations of the calibration

solutions because the same solutions were used to calibrate both indicators. However, uncertainties of this sort are critically important in comparing our results with those of others. When Ca^{2+} buffers are used to establish the $[Ca^{2+}]$ of calibration solutions, failure to control pH adequately, or small errors in the concentration of calcium or of chelator (as may result from the presence of impurities in commercial preparations of EGTA) may produce substantial errors in $[Ca^{2+}]$. This is especially true when the buffer ratio (i.e., the ratio of Ca chelator to free chelator) is high (for discussion, see Thomas, 1982; Blinks, 1989). However, the composition of the calibration solutions can influence the results in other, less obvious ways.

Ca-selective Microelectrodes

All ion-selective electrodes suffer from interference by other ions. In the case of intracellular $[Ca^{2+}]$ measurements, the electrode must sense nanomolar concentrations of Ca^{2+} in the presence of concentrations of K^+, Na^+, and Mg^{2+} that are four to six orders of magnitude higher, and the sensor ETH 1001 suffers from significant interference by K^+ and Mg^{2+} (see, for example, Bührer, Gehrig, and Simon, 1988). Substantial variation has been reported in the concentrations of these ions in frog muscle fibers, both from study to study (e.g., Conway, 1957; Walker and Brown, 1977; Alvarez-Leefmans et al., 1986; MacDermott, 1987; Godt and Maughan, 1988), and within the same study. In particular, Alvarez-Leefmans et al. (1986) noted a very large fiber-to-fiber variation within a series of measurements of $[K^+]_i$ in frog skeletal muscle fibers (83 to 122 mM, with substantial variation even among fibers of the same muscle). There is also considerable variability in $[Mg^{2+}]_i$ (in 38 experiments on fibers of exactly the same kind used in this paper, Blatter [1990] found a range of $[Mg^{2+}]_i$ of 0.1–4.4 mM with mean $pMg_i = 3.03$, SD 0.42). However, within the ranges of $[K^+]$ and $[Mg^{2+}]$ found in living cells, the interference caused by variations of $[Mg^{2+}]_i$ is likely to be less than that from variations in $[K^+]_i$ (see Bührer et al., 1988). Although we initially planned to convert each estimate of $V_{diff(Ca)}$ into a Ca^{2+} concentration using the results of the calibration curve determined with the corresponding electrode, this proved to be impossible in practice because in about half of the experiments the $V_{diff(Ca)}$ measured inside the cell fell below the lowest point on the calibration curve for the same electrode. The reason for this was probably that the concentrations of other ions, particularly K^+, varied from cell to cell, while those in the calibration solutions were constant. We did not reject such experiments, because they were apparently technically satisfactory in all respects and to have done so probably would have biased our results in favor of fibers with a high $[K^+]_i$, whereas the $[K^+]$ of the calibration solutions reflected a presumably unbiased sample. However, to include such results in our averages we were forced to abandon our original plan of using paired estimates of $[Ca^{2+}]_i$ from individual fibers for purposes of statistical comparison. Unusually negative measurements of $V_{diff(Ca)}$ could not be converted to estimates of $[Ca^{2+}]$ for the individual experiments in question, so they were incorporated into a presumably unbiased estimate of mean $V_{diff(Ca)}$. This was then applied to an average calibration curve (that shown in Fig. 2) calculated from the results obtained with the same electrodes to obtain a mean value of $[Ca^{2+}]_i$. This is not the first time such an approach has been used: Blatter and McGuigan (1986) faced a similar problem in the use of Mg^{2+}-selective microelectrodes, and dealt with it in the same way.

Aequorin

Our estimate of the fractional luminescence (L/L_{max}) for aequorin-injected skeletal muscle fibers under truly resting conditions was not significantly different from those that had been obtained previously in this laboratory (Snowdowne, 1985; Moore, 1986). It was the recognition that this number had to be applied to an aequorin calibration curve appropriate to 1 mM Mg^{2+} that put us in the seemingly impossible position of having measured a "resting glow" that was on average ~0.5 log units below the CIL expected for the amount of aequorin in the cell. (Mg^{2+} reduces the CIL in a concentration-dependent manner in addition to antagonizing Ca^{2+} in the aequorin reaction [Blinks et al., 1982; Blinks and Moore, 1986].) Our confidence that a systematic error was not at the root of the discrepancy was greatly strengthened by the observation that the introduction of cut fibers into a solution of aequorin caused a gradual reduction of the CIL, and by the fact that our estimates of $[Ca^{2+}]_i$ in living muscle fibers bathed in 12.5 mM K^+ corresponded so closely with those made in the same fibers by a completely different technique. We are led to the conclusion that the cytoplasm of frog skeletal muscle fibers must contain a diffusible substance that decreases the Ca^{2+}-independent luminescence of aequorin.[2]

Our experiments with muscle extracts suggest that the myoplasmic aequorin-stabilizing substance reduces the Ca-independent luminescence of aequorin without appreciably influencing the response of the photoprotein to calcium:[3] when the Ca^{2+} rises to the point that the CIL represents a negligible fraction of the total light signal, the influence of the cytoplasmic factor effectively disappears. If this interpretation is correct, the effect of the unidentified cytoplasmic factor should be negligible at Ca^{2+} concentrations at which the total luminescence is more than, say, 10 times the CIL, and at lower $[Ca^{2+}]$ it should be possible to correct for its influence simply by adding the change in the CIL to the total light signal before referring the signal to the

[2] Of course, the possibility must be considered that the signals generated by our Ca^{2+}-selective microelectrodes were also influenced in some systematic way by an unknown component of the myoplasm. The fact that the Ca^{2+}-independent luminescence of aequorin is influenced by such a constituent is no reason to predict an effect of the same substance on the electrodes, because these two indicators sense $[Ca^{2+}]$ in such completely different ways. Nevertheless, it serves as a warning that the danger of interference cannot be ignored for any indicator. In contrast to the situation with aequorin, there has been no particular reason to suspect a problem of this sort with Ca^{2+}-selective microelectrodes, either in our results, or in those of other investigators who have used microelectrodes made with the sensor ETH 1001 in a wide variety of tissues (see Ammann, 1986, for references). However, V. Jacquemond, M. G. Klein, and M. F. Schneider (personal communication) have recently obtained direct evidence against the idea that the sensitivity of Ca^{2+}-selective microelectrodes is significantly influenced by contact with the myoplasm. In studies on three single frog muscle fibers in which the internal $[Ca^{2+}]$ was controlled with EGTA-buffered solutions by the cut-end technique (Kovacs, Rios, and Schneider, 1983), they found that the calcium potentials recorded by Ca^{2+}-selective microelectrodes were not significantly different when the controlled regions of the fibers were impaled with the microelectrodes, and when the electrodes were calibrated in solutions having the same ionic composition as those used to bathe the cut end of the fiber. Their Ca^{2+}-selective microelectrodes were fabricated in exactly the same manner as those used in our experiments, and the muscle fibers were from the same species.

[3] Note that a simple increase in the quantum yield of the luminescent reaction would not be expected to alter estimates of fractional luminescence, since all measurements of light intensity, including the estimate of L_{max}, would be increased in the same proportion.

aequorin calibration curve. We did this, making the assumption that the resting glow measured initially from the fibers in normal Ringer solution was at the Ca^{2+}-independent level, and this correction increased the aequorin-derived estimate of $[Ca^{2+}]_i$ in 12.5 mM K^+ Ringer (measured before microelectrode impalement) from 38 to 69 nM. Note (Table I) that although this value is closer than the uncorrected one to that obtained in the same fibers with the Ca-selective microelectrodes (59 nM), this should not be taken as proof of the validity of the correction procedure because neither of the values obtained with aequorin is significantly different from that obtained with the electrodes. Perhaps the most significant conclusion to be derived from this comparison is that the error introduced by the effect of the myoplasm on the Ca^{2+}-independent luminescence of aequorin is not important at Ca^{2+} concentrations above ~50 nM.

Comparison of Measurements of $[Ca^{2+}]_i$ Made with Aequorin and with Ca-selective Microelectrodes: Influence of Cell Damage

Although our measurements made with aequorin and Ca electrodes agreed well with each other, our estimates of $[Ca^{2+}]_i$ in partially depolarized frog muscle fibers (~50 nM) were significantly lower than most other estimates made with Ca-selective microelectrodes in frog skeletal muscle fibers at rest (50–150 nM) (Tsien and Rink, 1980; Lopez et al., 1983; Weingart and Hess, 1984). We believe there are four good reasons to think that our (lower) estimates are the more nearly correct: (*a*) The close correspondence between the estimates that we obtained in the same cells with aequorin and with Ca-selective microelectrodes gives us added confidence in the results given by both techniques. (*b*) While it is difficult to imagine why intracellular measurements with Ca electrodes might be spuriously low, there is a very obvious reason why they might be spuriously high: leakage at the site of impalement. (*c*) Because of the presence of the aequorin in the cells we studied we were able to detect those electrode impalements that resulted in long-lasting elevations of $[Ca^{2+}]_i$, and to exclude the corresponding microelectrode measurements from our averages. (*d*) While still very tentative, recent estimates of $[Ca^{2+}]_i$ in resting frog skeletal muscle fibers made with fura-2 (Baylor and Hollingworth, 1988; Klein, Simon, Szucs, and Schneider, 1988) have been lower than most of those made with Ca-selective microelectrodes.

A concern identified by Requena et al. (1984) in the comparison of measurements of $[Ca^{2+}]_i$ by different Ca^{2+} indicators was the potential existence of Ca^{2+} concentration gradients that might influence the measurements of the separate indicators in different ways. Although these investigators presented evidence for the existence of radial gradients of $[Ca^{2+}]$ in the squid giant axon, the best available evidence indicates that no such gradients exist in normal amphibian muscle fibers at rest. Ratio images obtained with fura-2 in *Xenopus* muscle fibers (Westerblad, Lee, Lamb, Bolsover, and Allen, 1990) revealed $[Ca^{2+}]$ gradients during low-sodium contractures, and during activity in fatigued muscles, but no gradients were detected by this method in normal or fatigued muscles during rest. In our experiments the most likely source of complicating $[Ca^{2+}]$ gradients would be leakage of Ca^{2+} at the ion-selective electrode impalement site. As Tsien (1983) has pointed out, one would expect Ca-selective microelectrodes to be particularly subject to interference from

leakage at the site of impalement because of the very low concentration of Ca^{2+} inside the cell, and the very large electrochemical gradient tending to drive Ca^{2+} across the cell membrane. The fact that the tip of the electrode is very close to the leak (no more than a few microns away) makes interference all the more likely, though this problem is not unique to Ca electrodes. Leakage can be demonstrated with a second indicator, and aequorin is particularly well suited for this because its nonlinear concentration–effect curve makes it highly sensitive to local "hot spots" of $[Ca^{2+}]$. (Our experience has been that when aequorin-injected cells are impaled with ordinary microelectrodes, leakage around the electrode is much easier to detect through changes in aequorin luminescence than through changes in the apparent membrane potential.) In these experiments our experimental arrangement allowed us to detect persistent leaks in the membrane, and to discard the results of experiments in which they occurred. Among the 26 fibers in which we made a serious attempt to make measurements with both methods, there were five in which apparently successful microelectrode impalements produced a rise in the aequorin signal that did not subside or even increased with time. In these fibers there was no visible injury to the membrane, and the elevation of $[Ca^{2+}]$ was not great enough to produce a local contracture, so without the presence of a second Ca^{2+} indicator we would have had no reason to suspect that membrane damage had occurred. It seems likely that our ability to reject the results of experiments like these accounts largely for the fact that our estimates of $[Ca^{2+}]$ in partially depolarized fibers were lower than most previous ones made with Ca electrodes in truly resting frog muscle. On the other hand, the close correspondence between our (carefully screened) estimates of $[Ca^{2+}]_i$ obtained with aequorin and with Ca electrodes suggests that significant leakage around the electrode need not always occur.

The last few years have seen the publication of several studies of intracellular $[Ca^{2+}]$ in skeletal muscle made with the fluorescent indicator fura-2. Of these, two are of particular relevance here, because (*a*) they were carried out on frog muscle, and (*b*) the authors devised strategies to calibrate the fura-2 signals in situ, rather than depending on reference to in vitro calibration curves. Baylor and Hollingworth (1988) arrived at a (tentative) estimate of 20 nM for $[Ca^{2+}]_i$ in intact frog muscle fibers at rest, which fits rather well with our estimate of $\sim$50 nM in partially depolarized fibers. Klein et al. (1988) obtained a somewhat higher estimate (49 nM) with fura-2 in cut-end muscle fibers, but pointed out that this estimate was probably high because it was made about an hour after the fibers were set up, and $[Ca^{2+}]_i$ tends to rise progressively during the lifetime of such preparations.

Considerably higher levels have been reported from experiments with fura-2 in mammalian muscle fibers. Iaizzo, Klein, and Lehmann-Horn (1988) estimated that $[Ca^{2+}]_i$ was 80 nM in normal pig muscle at rest, and Iaizzo, Seewald, Oakes, and Lehmann-Horn (1989) obtained a value of 110 nM for normal human muscle. However, there are major uncertainties about the interpretation of these measurements, stemming primarily from the fact that fluorescence ratios measured in vivo were applied to calibration curves determined in vitro. It has been shown in other studies that a large fraction of the indicator is bound to constituents of the myoplasm and undergoes significant changes in its chemical and optical properties as a result (Konishi, Olson, Hollingworth, and Baylor, 1988; Blatter and Wier, 1990). Because of

this, Westerblad et al. (1990) and Lee, Westerblad, and Allen (1991) expressed their results obtained in *Xenopus* muscle fibers only in terms of fluorescence ratios, refraining altogether from making estimates of absolute Ca^{2+} concentrations with fura-2. Unfortunately, there is no way of comparing results expressed only as fluorescence ratios from one study to another because the ratios depend critically on the optical properties of the apparatus.

Although the use of two different Ca^{2+} indicators simultaneously in the same cell can provide important information that would not be evident from the use of either one alone, there have been only a few reports of studies of this sort. Those carried out in muscle fibers have been prompted mostly by concerns about the ability of one of the indicators to track rapid Ca^{2+} transients (Baylor, Hollingworth, Hui, and Quinta-Ferreira, 1985; Baylor and Hollingworth, 1988; Konishi, Hollingworth, Harkins, and Baylor, 1991), or a desire to extend the range of $[Ca^{2+}]$ covered by one indicator alone (Klein et al., 1988). The only previous use of aequorin and Ca electrodes simultaneously in the same cells was in the study by Requena et al. (1984), carried out on the giant axon of the squid.

The Myoplasmic Component Influencing Aequorin Luminescence

The fact that the CIL of aequorin is decreased by a soluble constituent of the myoplasm of frog skeletal muscle fibers has both theoretical and practical implications. From the theoretical standpoint we found it particularly interesting that the myoplasmic factor decreased the CIL of aequorin without apparently influencing the response to Ca^{2+}, at least at the low and intermediate levels of $[Ca^{2+}]_i$ we investigated. In this respect the effect of the MASS is distinctly different from that of Mg^{2+}, the only other substance presently known to decrease the CIL. From the practical point of view, the selective effect of the MASS on CIL is fortunate, in that the effect seems to be significant only at the very bottom of the aequorin calibration curve, and there it seems to be possible to correct rather simply for it in the manner described in the previous section. However, we have not yet studied the effects of the MASS on the whole of the Ca^{2+} concentration–effect curve of aequorin, or on the kinetics of aequorin's reaction with Ca^{2+}.

The MASS either has a molecular weight in the range 10,000–30,000, or is tightly bound to something of that size. It seems probable, though by no means certain, that a soluble cytoplasmic constituent of that size is a protein. Parvalbumin is the most abundant soluble protein in frog skeletal muscle, and its molecular weight ($\sim$11,500) falls within the range established for the unknown MASS. Although parvalbumin did exhibit some stabilizing effect on aequorin, the effect of 1 mM frog muscle parvalbumin (approximately the concentration in frog skeletal muscle) was far too small to account for the observed depression of the CIL in intact muscle fibers or for the effect of cut muscle fibers on aequorin luminescence in vitro. Bovine serum albumin had an effect even smaller than that of parvalbumin. It seems, therefore, that while there may be a small nonspecific aequorin-stabilizing effect of proteins generally, the aequorin-stabilizing effect of myoplasm is not attributable to that or to a specific effect of parvalbumin, but to a specific effect of some other substance, probably a protein, that we have not yet identified.

As far as we are aware, there has been only one other report suggesting that the

properties of aequorin might be modified by interaction with a constituent of the cytoplasm. Baker, Hodgkin, and Ridgway (1971) reported that in extruded axoplasm from the squid giant axon, aequorin appeared to have a considerably lower sensitivity to Ca^{2+} than in a Ca- and pH-buffered solution containing 550 mM KCl and 10 mM Mg^{2+}. Since the total Mg concentration of the axoplasm was ~10 mM (and the free $[Mg^{2+}]$ must have been considerably lower), they suggested that the axoplasm might contain substances that inhibited the aequorin reaction. Their results might also have been obtained if the axoplasm contained a substance that increased the affinity of EGTA for Ca^{2+}, or (more likely) a substance or organelle that was capable of sequestering Ca^{2+} within the range of $[Ca^{2+}]$ under study. If indeed the result of Baker et al. (1971) reflects an interaction between aequorin and a constituent of squid axoplasm, the properties of that constituent appear to be basically different from those of the myoplasmic aequorin-stabilizing substance in that the Ca^{2+} concentration–effect curve for aequorin in squid axoplasm (log-log plot) was shifted to the right in a roughly parallel fashion, whereas the MASS appears to have an influence on the Ca^{2+}-independent luminescence only.

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