Vol. 162, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 14, 1989 Pages 402-407

A COMPARATIVE STUDY OF THREE METHODS FOR INTRACELLULAR LOADING OF THE CALCIUM INDICATOR AEQUORIN IN FERRET PAPILLARY MUSCLES

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Received May 22, 1989

We compared the results of loading the bioluminescent Ca^{++} indicator aequorin by standard microinjection techniques to those obtained with two new chemical approaches to loading that utilize low concentrations of Ca^{++} chelator; i.e., 1) Immersion and 2) Macroinjection. After loading with the immersion and macroinjection methods, twitch tension returned to pre-load values indicating lack of damage to the muscles. The aequorin signals obtained with all three methods were similar and converted to similar quantitative values for $[Ca^{++}]_i$. Our data suggest that chemical loading (in particular macroinjection) may be preferable to microinjection, particularly in muscles with increased connective tissue content.

Aequorin, a bioluminescent protein that emits light when it combines with free ionized calcium $[Ca^{++}]$, has been used to study excitation-contraction coupling processes in a wide variety of skeletal, cardiac and smooth muscle preparations (1). The most common method for loading aequorin into the cytoplasm of cells is by pressure-injection through fine-tipped glass micropipettes (2). This method works well for relatively large cells, such as skeletal muscle fibers, in which injection during a single pipette penetration can produce bright aequorin signals. By way of contrast, in cardiac muscle cells, microinjection is a long and tedious process, since usually 40 to 100 superficial cells must be penetrated and loaded in order to obtain a detectable light response. Due to the technical difficulty of microinjecting photoproteins, alternative approaches to loading have been explored in several laboratories, including liposome fusion, temporary disruption of cell membranes by ultrasound, exposure to hypoosmolar or "hyperpermeabilizing" solutions, centrifugation of cell suspensions, "scrape loading" of cell cultures and fusion of cells with photoprotein-loaded ghost cells (see 1 for review). Most of

0006-291X/89 $1.50

these techniques have not been successfully applied to cardiac or vascular smooth muscle, although hypoosmolar solutions have been used to load aequorin into dispersed rat ventricular cells (1). With regard to intact multicellular preparations of cardiac and vascular smooth muscle, the use of "hyperpermeabilizing" solutions has provided the only alternative to microinjection (3). As originally described by Sutherland, this method involves sequentially exposing muscles to three different solutions which are designed to make the cell membranes hyperpermeable, load photoprotein into the cells and reverse the hyperpermeable state. The rationale for this approach centered on the use of high concentrations of the Ca^{++}-chelator EGTA to render the sarcolemma permeable to aequorin. Incomplete contractile recovery of mammalian myocardium after loading led to modifications that resulted in the development of a useful alternative to microinjection which, however, still utilized high concentrations of EGTA (4,5). As described in the present report, we recently modified this approach so that the concentration of calcium chelator in the loading solutions can be reduced, thereby minimizing the chances of irreversible damage to the cell membranes. Moreover, we describe an approach for applying the aequorin technique to muscles with increased connective tissue content as occurs in many pathophysiologic states.

METHODS

Male ferrets, approximately 12 weeks of age and weighing 800-1000g were anesthetized with chloroform. Their chests were then quickly incised and the hearts removed and placed into a physiological salt solution that was constantly bubbled with a gas mixture of 95% O_2 and 5% CO_2. The composition of the salt solution was as follows (mM): NaCl 120, KCl 5.9, dextrose 11.5, $NaHCO_3$ 25, $NaH_2PO_4 \cdot H_2O$ 1.2, $MgCl_2 \cdot 6H_2O$ 1.2 and $CaCl_2$ 1.0. The pH of the solution was 7.4. The right ventricle was quickly opened and a right ventricular papillary muscle of 1 mm or less in diameter was selected and dissected free. The papillary muscle was then placed into a bath containing the oxygenated physiologic salt solution and the temperature was maintained at 30°C. The papillary muscle was attached to a tension transducer and stimulated to contract at 0.33 Hz using threshold voltage and square wave pulses of 5 msec duration delivered through a punctate platinum electrode located at the base. Muscles were loaded with aequorin by one of three methods described below. The preparation of aequorin for laboratory use and its reaction with Ca^{++} have been described in detail (1,2).

METHOD I: Microinjection. In preparation for microinjection, muscles were stretched to approximately 75% of the length at which maximal isometric tension developed and stimulation was discontinued. Aequorin was dissolved to a concentration of 1 mg/ml in a solution containing 150 mM KCl and 5 mM N-2-

hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes) buffer, pH 7.5. The aequorin solution was loaded into fine-tipped glass micropipettes through which potential was monitored to determine when cells had been penetrated, and was injected by the application of gas pressure; injection was terminated if membrane potential began to fall in order to minimize cell damage (for details, see 1,2). It was necessary to inject between 30 and 120 cells in order to obtain detectable light signals. After microinjection, the muscles were transferred to a specially designed light collecting apparatus (1). The muscles were allowed to equilibrate for 1.5 hours during which time they were stretched to the length at which maximal isometric tension developed. Light and tension responses were recorded simultaneously, as described previously (4). Light signals were recorded with a photomultiplier (EMI 9635 qA). Because the light levels were very low and photomultiplier shot noise was prominent, it was necessary to average successive signals (from 20 to several hundred, depending on the light intensity) to obtain a satisfactory signal-to-noise ratio. Signal averaging was performed only after responses had reached steady state. The light signals were amplified and recorded using a filter with a 10 msec time constant.

METHOD II: Immersion. Muscles were placed into the light collecting apparatus and allowed to equilibrate for 1.5 hours during which time they were stretched to the length at which maximal isometric tension developed. Muscles were loaded with aequorin by a modification of the chemical technique described previously (5). A recording of the isometric twitch at steady state was obtained (preload value); then the salt solution bathing the muscle was switched to one of similar composition, but without added $CaCl_2$. The distilled water used to prepare our solutions had a calcium content between 10^{-7}-10^{-6} M. Stimulation was continued until developed tension fell to 15-20% of its value in 1 mM [Ca^{++}]; then the stimulus was discontinued. The temperature of the bath was reduced to 22°C over a time course of 4-5 minutes; then the muscle holder was lifted out of the bath and quickly immersed into a small test tube containing solution (solution H) of the following composition (mM) at 22°C: NaCl 154, KCl 5.4, $MgCl_2$ 1.0, Hepes 12, glucose 11, EDTA 0.1; and bubbled with O_2; pH 7.4. After a 2 minute incubation period the muscle was switched to a loading solution containing solution H with 0.5 mg/ml of aequorin. Muscles were incubated for 20-40 minutes in this loading solution and then lowered back into the bath which had been washed two times with Ca^{++}-free salt solution at 22 C (note that contaminant Ca^{++} was in the 10^{-7}-10^{-6} M range). $CaCl_2$ was gradually added to the bath at 30 minute intervals to raise $[Ca^{++}]_o$ to the following levels (mM): 0.001, 0.01, 0.1, 1. After 30 minutes in 1 mM $[Ca^{++}]_o$, the muscles were rewarmed to 30 C over 15-30 minutes. Then stimulation was restarted and the muscles were allowed to equilibrate for 1.5 hours until steady state conditions were reached. Post-load light and tension were recorded as described above for microinjected fibers.

METHOD III: Macroinjection. In previous experiments, we have noted greater than usual difficulty in microinjecting or chemically loading aequorin into working myocardium from older animals and animal or human hearts with significant degrees of hypertrophy or fibrosis. Histological examination of muscle from these sources revealed an increased connective tissue content including an increased thickness of the epimysium that surrounds each papillary muscle or trabecular strip. The thickened epimysium not only prevents penetration of superficial cells by fine-tipped glass micropipettes but also impedes their contact with aequorin when immersed into loading solutions. We therefore investigated the

effects of injecting our loading solution beyond this connective tissue barrier among the outermost cells of the body of the papillary muscle. Short-shank glass micropipettes were fashioned on a Brown and Flaming puller; when filled with 3M KCl, tip resistances were 10-20 MΩ. To minimize damage to the muscles, pipettes were mounted on a micromanipulator calibrated for micromovements as well as for coarse adjustment. The muscles were handled as described above under Method II, up to the point of raising from the bath for immersion in solution H. Instead, the connective tissue coat of the muscle was penetrated with the micropipette and 1-2 μl of solution H plus aequorin was injected just beneath the epimysium. This process required approximately 30 seconds, after which the muscle was lowered into the bath containing no added Ca^{++}; 22°C. The addition of Ca^{++} and rewarming were accomplished exactly as described above for Method II.

EXPERIMENTAL PROTOCOL

Comparison of the aequorin light signals from different muscles was performed by the method of fractional luminescence (1). To correct our resting and peak (at 3 second intervals of stimulation) light (L) values to fractional luminescence, we estimated the light intensity (Lmax) that would be recorded under the conditions of the experiment if all of the aequorin were instantly exposed to a saturating concentration of Ca^{++}.
Lmax was determined by lysing the cell membranes with a solution containing 4% Triton X-100 in 50 mM $CaCl_2$ at 30°C. Exposure to the solution of muscles loaded with aequorin resulted in contracture and a burst of light emission. After subtracting the contribution of background light, the integral of the area encompassed by the light signal during exposure to Triton X-100 was multiplied by the rate constant for aequorin consumption in the presence of saturating $[Ca^{++}]$, 2.11 sec^{-1}, in order to calculate Lmax. Determination of the rate constant was performed at 30 C after preincubation with 1 mM Mg^{++} (1). The ratio of L/Lmax was related to a quantitative Ca^{++} concentration by using a calibration curve determined in vitro (1,2).

RESULTS

Figure 1 shows the aequorin signals (i.e., intracellular Ca^{++} transients) and isometric twitches recorded from ferret papillary muscles loaded by each of the three methods. Note that the signals are qualitatively similar to those obtained by us and other investigators under similar experimental circumstances and with the high EGTA method of chemical loading we previously reported (4,5). As documented in Table 1, the force per unit cross-sectional area generated by the muscles was similar in each of the three groups. Note that neither immersion nor macroinjection affected the functional properties of the muscles as indicated by the comparison of pre-load vs. post-load force generating capacity. (Maximal isometric force generation was not determined prior to microinjection). The time courses of the calcium transients and isometric twitches were similar after all three methods of loading. Moreover, as shown in Table 1, quantitative calcium concentrations

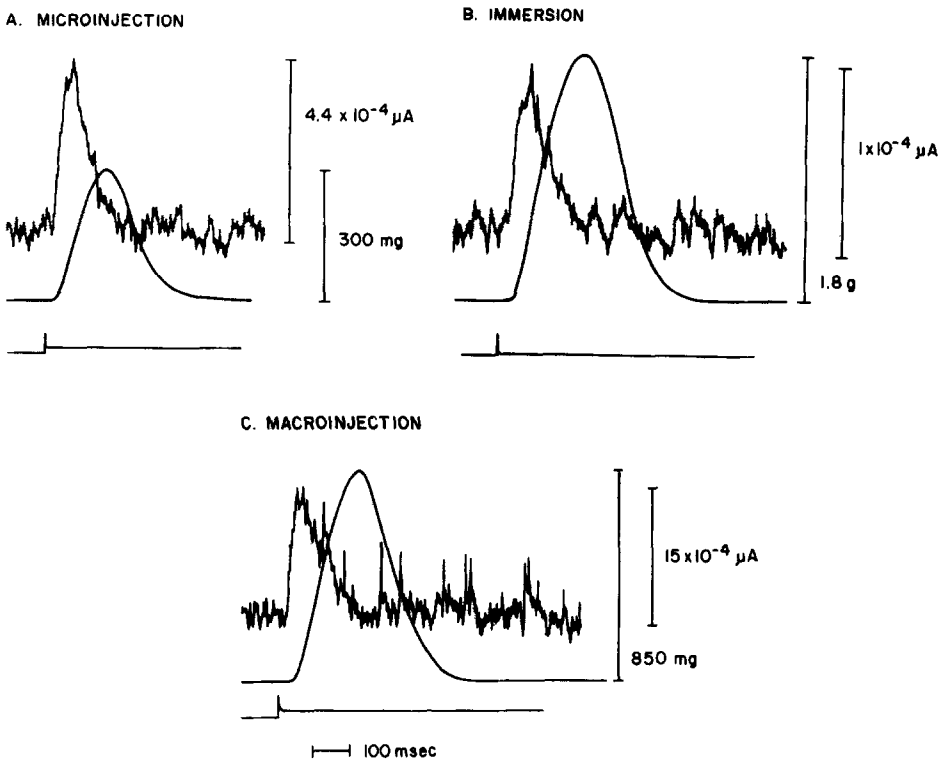

Figure 1: Aequorin signals (upper tracings), isometric twitches (middle tracings) and stimulus artifact recorded from ferret papillary muscles loaded with aequorin by : A Panel, microinjection; B panel, immersion; C Panel, macroinjection. See text for details. Preparations were stimulated to contract at 0.33 Hz, 30°C, $[Ca^{++}]_o$ = 1 mM. A represents the average of 1000 responses, B, 700 responses and C, 64 responses.

during the peak of the transient were also similar. Note that resting Ca^{++} was not detectable above background in any muscles from the immersion group, in 2 of the microinjected fibers or in 3 of the macroinjected group. However, peak Ca^{++} was easily determined in all of the fibers.

DISCUSSION

These results have several important ramifications. First, they provide direct documentation that the results obtained with chemical loading are equivalent to those obtained with direct microinjection of aequorin into the sarcoplasm (1), which is currently the standard technique for photoprotein loading. Second, the similarly of the present results to those obtained using our high EGTA method (4,5) indicate that high concentrations of calcium chelators are not necessary to produce reversible membrane hyperpermeability and successful intracellular loading of

Table 1: Comparison of structural and functional values

	MICROINJECTION	IMMERSION	MACROINJECTION
N	7	6	9
$\frac{\text{Pre-load T}}{\text{Post-load T}}$ x 100 (%)	---	103 ± 6.5	97 ± 10.4
PT (mN/mm^2)	7.2 ± 1.6	8.6 ± 3.3	5.2 ± 2.4
TPT (msec)	179 ± 13.2	192 ± 11	192 ± 5.9
$T_{50}T$ (msec)	95 ± 10.5	114 ± 10	116 ± 15.6
TPL (msec)	68 ± 6.4	59 ± 6.5	54 ± 4.1
$T_{50}L$ (msec)	50 ± 6.8	45 ± 7.0	49 ± 3.0
RL Detectable in N	5/7	0/6	6/9
log RL/L_{max}	-5.5 ± 0.2	---	-5.4 ± 0.2
Resting $[Ca^{++}]_i$ (M)	$2.3(\pm 0.6)\times10^{-7}$	---	$2.4(\pm 0.6)\times10^{-7}$
PL Detectable in N	7/7	6/6	9/9
log PL/L_{max}	-4.5 ± 0.2	-4.3 ± 0.25	-4.2 ± 0.2
Peak $[Ca^{++}]_i$ (M)	$5.9(\pm 1.0)\times10^{-7}$	$7.6(\pm 2.2)\times10^{-7}$	$8.4(\pm 1.6)\times10^{-7}$

L = Aequorin light signal; T = Isometric Tension; N=number of values in each group; PT=peak isometric tension; TPT=time to PT; $T_{50}T$= T to 50% decline from PT; TPL=TP light; $T_{50}L$=T to 50% decline from PL; RL= resting L; i.e., L from unstimulated muscle; PL = peak L; i.e., L at peak of Ca^{++} transient. Quantitative values determined by method of fractional luminescence (1). There were no statistical differences among values in the three groups by ANOVA analysis. Value = mean ± S.E.

photoproteins. Third, the chemical approaches described in the present study offer a significant practical advantage over alternative techniques in terms of their simplicity; the skills and equipment required for microinjection are unnecessary. Finally, the macroinjection approach we have developed may offer significant advantages over other techniques for loading aequorin into preparations with a high connective tissue content, without impairing the functional integrity of the preparation.

ACKNOWLEDGMENTS

Support: HL31117, H01611 to JPM and an AHA Fellowship to YK.

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