A Chemical Method for Intracellular Loading of the Calcium Indicator Aequorin in Mammalian Skeletal Muscle (43344)

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Abstract. **The bioluminescent calcium indicator aequorin was loaded into bundles of skeletal muscle fibers from the rat extensor digitorum longus by macroinjection, a technique previously applied only to cardiac muscle. After loading, the amplitude and time course of the twitch returned to control values, indicating lack of damage to the fibers. Individual light signals (i.e., calcium transients) were recorded during each twitch or tetanus without the need for signal averaging. The calcium transients obtained were qualitatively and quantitatively similar to those reported previously with microinjection of aequorin. Our data suggest that macroinjection may be the method of choice for loading aequorin into mammalian skeletal muscle.** [P.S.E.B.M. 1992, Vol 199]

Aequorin, a bioluminescent protein that emits light when it combines with free, ionized calcium (Ca^{2+}), 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 amphibian skeletal muscle fibers, in which injection during a single pipette penetration can produce bright aequorin signals. In contrast, the dense connective tissue network surrounding mammalian skeletal muscle has prevented wide application of the microinjection technique, and only two groups have reported success with this approach (3, 4) since the introduction of aequorin into skeletal muscle research in the 1970s (5). Moreover, microinjection is a long and tedious process in mammalian skeletal muscle, since the diameters of the fibers are smaller than in amphibian muscle and fibers are surrounded by dense connective tissue sheaths that cause a high percentage of unsuccessful penetrations. Often the resultant preparation is too dim to record individual aequorin signals and averaging of the calcium transients from multiple twitches or tetanic contractions is necessary. This is clearly a disadvantage for studying the changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) that may occur during individual twitches or tetanic contractions under non-steady state conditions in which light signals may change from one contraction to the next, making signal averaging undesirable. We have recently developed an alternative method for loading aequorin into mammalian and amphibian skeletal muscle that avoids many of the problems associated with microinjection and reproducibly provides light signals that are detectable without the need for signal averaging. We will describe in this paper only the loading of mammalian muscle cells.

Materials and Methods

Male Sprague-Dawley rats, 12 weeks old and weighing between 300 g and 400 g, were anesthetized with ether. The extensor digitorum longus muscles were removed from both legs and placed in a modified Krebs solution of 118 m*M* NaCl, 4.7 m*M* KCl, 1.2 m*M* KH_2PO_4, 0.6 m*M* $MgCl_2$, 25 m*M* $NaHCO_3$, 11 m*M* glucose, and 2.5 m*M* $CaCl_2$, and equilibrated with 95% O_2 and 5% CO_2 to a pH of 7.4 at 22.5°C. Bundles of 100–200 cells were carefully dissected free under a microscope. In order to ensure that the fibers had an optimal exposure to stimulating currents and solutions and that, at the same time, the aequorin injections were

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successful, the bundles were dissected so as to have a rectangular cross-sectional shape (except in two cases in which they had a triangular shape). The fibers with rectangular shapes had the following dimensions: base, 1.22–1.66 mm (average, 1.37 ± 0.14 mm); and height, 0.22–0.44 mm (average, 0.33 ± 0.06 mm). The triangular ones had a base of 0.55 and 0.77 mm and a height of 1.66 and 0.999 mm, respectively. The cross-sectional areas varied from 0.37 to 0.59 mm^2 (average, 0.48 mm^2). The length of the bundles at equilibrium length varied between 11- and 12.5 mm (average, 11.7 ± 0.6 mm). The bundles were vertically mounted on a holder in a muscle bath containing the same physiologic salt solution at 22.5°C. They were then attached to a force transducer (Statham UC2); muscle lengths were adjusted to the apex of the length-tension curve and muscles were stimulated to contract with square wave pulses of 0.5 msec in duration delivered via field electrodes placed at the base of the muscle holder. The geometrical arrangement of the electrodes was such that current passed through all the fibers during electrical stimulations. The stimulating current used was 1.5 times the minimum voltage necessary for activation of all the cells and produced no artifact in the light signals recorded, as described below. The corresponding tension response was considered to be the before-loading reference value. After cessation of stimulation, muscles were placed in a reduced $[Ca^{2+}]_o$ solution (1.9 m*M*) for 5 min before and during the macroinjection process (see below). In the initial experiments performed with mammalian skeletal muscles, the bathing solution was replaced by one without added Ca^{2+}. We found that when the bundles were bathed again with the 2.5 m*M* $[Ca^{2+}]_o$ after the macroinjection procedure, the recovery of twitch tension was poor. Therefore, we abandoned the 0 $[Ca^{2+}]_o$ step in lieu of the 1.9 m*M* $[Ca^{2+}]_o$. Under these conditions, the recovery of twitch tension and the intracellular incorporation of aequorin, after the loading period, were satisfactory, as described in Results. Therefore, the 1.9 m*M* $[Ca^{2+}]_o$ Krebs solution was the lowest $[Ca^{2+}]_o$ Krebs solution used in this procedure. Aequorin was prepared as described previously (1, 2) and loaded by macroinjection (6). The loading solution was composed of 154 m*M* NaCl, 155.4 m*M* KCl, 1.0 m*M* $MgCl_2$, 17 m*M* HEPES, 11 m*M* glucose, and 0.1 m*M* EDTA with aequorin (1 mg) (pH 7.4). The aequorin solution was loaded into short-shank glass micropipettes fashioned on a Flaming-Brown pipette puller (resistance, 1–4 MΩ when filled with 3 *M* KCl). The muscle holder and bundle were briefly raised from the bath into the air, the connective tissue sheath was penetrated with the micropipette, and 1–2 μl of the aequorin solution was injected 2–3 times into the interstitial space. This entire process required 30 sec or less. Afterward, the bundles were lowered into the bath and returned to a physiologic salt solution containing 1.9 m*M* $[Ca^{2+}]_o$ for 15 min after which $[Ca^{2+}]_o$ was increased to 2.5 m*M*. The muscles were allowed to equilibrate for 20 min and then were stimulated to contract. Light and tension responses were recorded simultaneously. Light was recorded as anode current from the photomultiplier tube (EMI 9635qA) and filtered with a 10-msec time constant. This degree of filtration may affect the time course of the brief skeletal muscle aequorin signal (Table I), but this effect seems small when compared with unfiltered tracings under the conditions of our experiment and markedly enhances the clarity of the signals (by reducing the random noise). Recordings were performed only after light and tension responses had reached a steady state. Experiments were performed at 22.5°C unless otherwise stated.

Experimental Protocol

Twitches and intracellular Ca^{2+} transients were recorded after reaching steady state. The temperature was subsequently reduced to 15°C and 12°C to determine the effects of temperature changes on tension and $[Ca^{2+}]_i$. Temperature was then returned to 22.5°C and a series of tetani was induced by stimulating at 5 Hz and increasing the frequency until the maximum tetanic force was obtained. This usually was achieved at

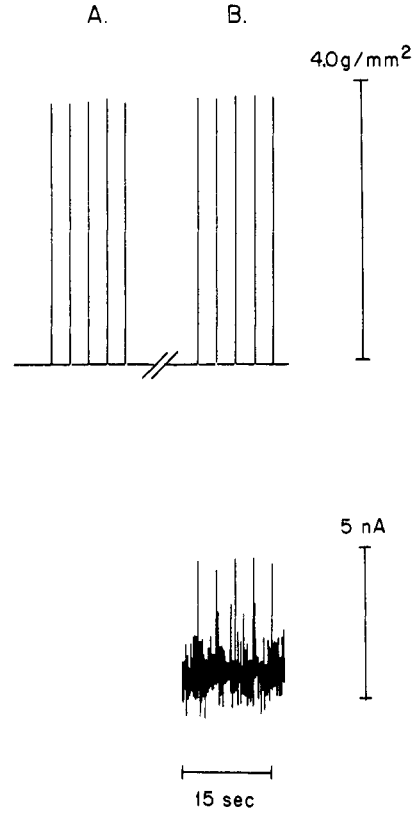

Figure 1. This figure shows the force at 22.5°C before the loading procedure (Panel A), and after the aequorin loading procedure (Panel B) to demonstrate peak twitch force recovery after the loading procedure in bundles of cells from the rat extensor digitorum longus. The lower right side demonstrates individual $[Ca^{2+}]_i$ transients present after the loading procedure. Note the one-to-one correspondence of the $[Ca^{2+}]_i$ transients and twitches. $[Ca^{2+}]_o$, 2.5 m*M* at 22.5°C. The muscle cross-sectional area was 0.55 mm^2.

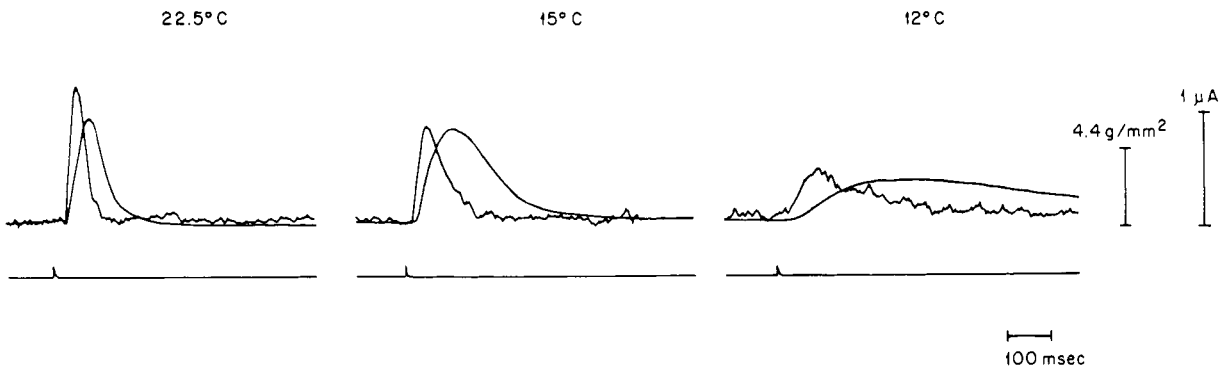

Figure 2. Representative experimental tracings of the effects of temperature on $[Ca^{2+}]_i$ transients and corresponding tension responses recorded from bundles of cells from the rat extensor digitorum longus that were macroinjected with aequorin. The aequorin light signals (i.e., $[Ca^{2+}]_i$ transients) are the noisy tracings; the tension tracings are the smooth tracings. A stimulus artifact is shown below each tracing. $[Ca^{2+}]_o$, 2.5 m*M*; pulse duration, 0.5 msec; frequency, 0.33 Hz at 22.5°C. The muscle cross-sectional area was 0.41 mm^2.

Table I. Peak Tension and Time Courses of Light and Tension Responses[a]

n	PT (g/mm^2)	TPT (msec)	TPL (msec)	$T_{50}T$ (msec)	$T_{50}L$ (msec)	$T_{80}T$ (msec)	$T_{80}L$ (msec)
8	2.8 ± 0.8	44 ± 2	27 ± 3	28 ± 1	20 ± 1	45 ± 3	35 ± 1

[a] Light and tension responses recorded in macroinjected bundles of cells from the rat extensor digitorum longus. $[Ca^{2+}]_o$, 2.5 m*M* at 22.5°C; *n* = number of muscles studied; PT, peak tension expressed as g/mm^2 to normalize for cross-sectional area; TPT, time to peak tension; TPL, time to peak light; $T_{50}T$, time to 50% relaxation from peak tension; $T_{50}L$, time to 50% decline from the peak light; $T_{80}T$, time to 80% relaxation from peak tension; $T_{80}L$, time to 80% decline from the peak light; values are mean ± SE.

frequencies between 75 Hz and 100 Hz. After the series of tetani, a full recovery of tension was allowed (30–60 min), after which a twitch was recorded and followed by lysis of muscle cell membranes. This was accomplished via exposure of the muscle to a solution containing 5% Triton X-100 in 50 m*M* $CaCl_2$ at 22.5°C in order to quantitate the total amount of aequorin in the cells, which can then be used to determine intracellular Ca^{2+} levels (Fig. 4). This fractional luminescence method allows the estimation of the light intensity (L_{max}) that would be recorded under the conditions of the experiment if all of the aequorin were instantly exposed to a saturating Ca^{2+} concentration. In order to calculate L_{max}, the area encompassed by the light signal during Triton X-100 exposure was measured and multiplied by the rate constant for aequorin consumption in the presence of saturating Ca^{2+}, 1.10 sec^{-1}. The rate constant determination was performed at 22.5°C after preincubation with 1 m*M* Mg (1). The L/L_{max} ratio was converted to a quantitative $[Ca^{2+}]_i$ concentration using an *in vitro* calibration curve (6, 7).

Results

After aequorin loading, peak twitch tension recovered to 80–105% of the before-loading values, indicating a lack of significant damage to muscle fibers by the macroinjection technique, as shown in Figure 1. Figure 2 shows the aequorin signals (i.e., intracellular Ca^{2+} transients) and isometric twitches recorded from bundles of 100–200 rat extensor digitorum longus fibers at various temperatures. Note that decreasing the temperature caused direct and proportional reductions in both the amplitude of the $[Ca^{2+}]_i$ transient and peak tension and that the aequorin signal rose to a peak and then declined toward baseline before the corresponding mechanical events. In addition, marked prolongations of the $[Ca^{2+}]_i$ transient and tension responses were present at lower temperatures. These effects on both the amplitude and time course of light and tension were completely reversible when temperature was returned to the control value of 22.5°C. Table I shows the peak tension, normalized for cross-sectional area, and the time course parameters for the light and tension responses recorded at 22.5°C in the eight muscles studied. Figure 3 shows the light signal and isometric tension recorded during a fused tetanic contraction induced by 75 Hz of stimulation for a 1-sec duration. The shape and time course of the light signal is similar to those described previously by Blinks *et al.* (5). We used the method of fractional luminescence to calculate peak and resting $[Ca^{2+}]_i$ values (6) during twitches (0.83 ± 0.23 μM and 0.20 ± 0.04 μM, respectively) and during tetanic stimulation (1.16 ± 0.15 μM; $n = 7$). A representative tracing of the lysis by Triton X-100 is shown

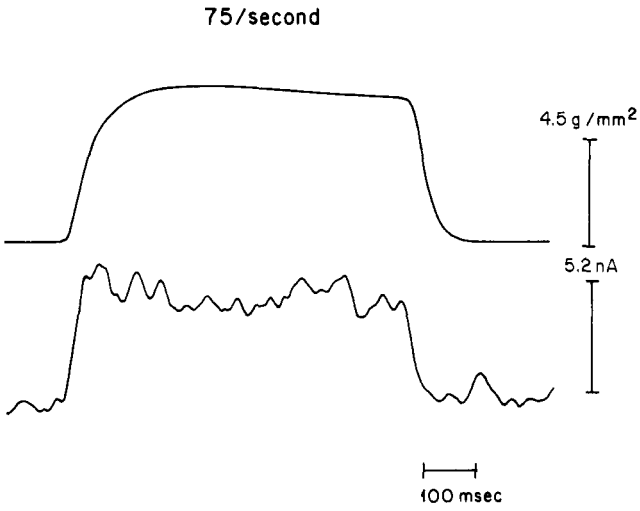

Figure 3. Representative experimental tracing of the tension and corresponding aequorin light response to a tetanus induced by 75 Hz of stimulation for 1 sec. $[Ca^{2+}]_o$, 2.5 m*M* at 22.5°C. The muscle cross-sectional area was 0.44 mm^2.

in Figure 4. Aequorin signals that were large enough to be interpreted on a twitch-to-twitch basis without the need for signal averaging were obtained in eight of eight preparations.

Discussion

The macroinjection approach described in the present study offers a significant practical advantage over microinjection techniques in terms of its simplicity; the skills and equipment required for microinjection are unnecessary. Moreover, the qualitative and quantitative features of the calcium signals recorded after loading by macroinjection are the same as those reported previously with microinjection techniques (2–6). The temporal relationship and correlation between the aequorin signal and tension development, plus the reversibility of temperature-induced changes in the amplitude and time course of the light and tension responses, indicate that the light signals were not artifacts unrelated to Ca^{2+} release during contractions (Figs. 2 and 3).

The macroinjection technique is a modification of the chemical approaches to aequorin loading we originally developed for use in cardiac and vascular smooth muscle (6). There are two main differences between the method described by Kihara and Morgan (6) and the one we described here for skeletal muscle: (i) we used a 1.9 m*M* $[Ca^{2+}]_o$ solution as the lowest Ca^{2+} Krebs solution instead of the 1×10^{-7}–1×10^{-6} *M* and we returned directly from the 1.9 m*M* Krebs to the 2.5 m*M* $[Ca^{2+}]_o$ Krebs; and (ii) the myocytes exposed transiently to the small bolus of loading solution experienced high K. KCl was raised from 5.4 to 155.4 m*M* in the loading solution and could have produced some degree of local depolarization before being diluted or washed away from the interstitium. Additionally, in contrast to our previous methods, ATP was omitted and EDTA was substituted for EGTA (6). The precise mechanism by which aequorin loads from the interstitium into the sarcoplasm of cardiac and skeletal muscle cells by this technique is not known and these alterations in the procedure were largely determined by trial and error.

It has been shown that large molecules can cross through a small fraction of the membrane in the giant nerve of the squid (8). Since the amount of aequorin needed inside the muscle cells to provide detectable light signals is in the picomolar range, this might be a mechanism by which aequorin is incorporated into the cells. The incorporation of aequorin does not appear to be related to active uptake or pinocytosis, since the process can occur at 2°C in cardiac and smooth muscle, but may be related to a reversible increase in sarcolemmal permeability produced by the loading conditions (9). Although we did not reduce the external divalent cation concentration enough to hyperpermeabilize the cell membrane, the aequorin solution injected may produce local permeabilization of membranes in the injected area because of its high EDTA content (0.1 m*M*). In 1978, Fabiato (10) showed that a cardiac muscle made hyperpermeable by skinning is able to incorporate aequorin simply by exposure to an aequorin-containing solution and he proposed that skinned cardiac cells may have the ability to concentrate aequorin. The same may hold true for muscle cells in general. All of the methods that to date have been shown effective for loading large molecules into cells—sonic shock, osmotic shock, and exposure of cells to low $[Ca^{2+}]_o$ or high EDTA—may share the common effect of reversibly disrupting the continuity of the sarcolemma either physically or by removing the Ca^{2+} ions that appear necessary for maintenance of the structural integrity of the cell membrane (9, 11, 12). The mechanical effects of the injection process may also play some role; however, aequorin can be loaded into papillary muscles (6) and suspensions of isolated cardiac myocytes simply by incubating them in solutions similar to those employed in the present experiments. In our experience, incubation does not appear to work well with skeletal muscle, probably because of the dense connective tissue sheaths (i.e., epimysium and perimysium) that surround the fibers and that may slow diffusion of aequorin.

It is important to note that we were able to obtain individual calcium transients without the need for signal averaging in 100% of the muscles studied. In our experience, the chances of obtaining a successful experiment with microinjection of aequorin are much lower. The relative success rate becomes particularly important in studies of scarce types of skeletal muscles, such as those available from human donors or from animals with pathophysiologic conditions. Particularly in these conditions, we believe that our macroinjection approach will not only provide data that are supplemen-

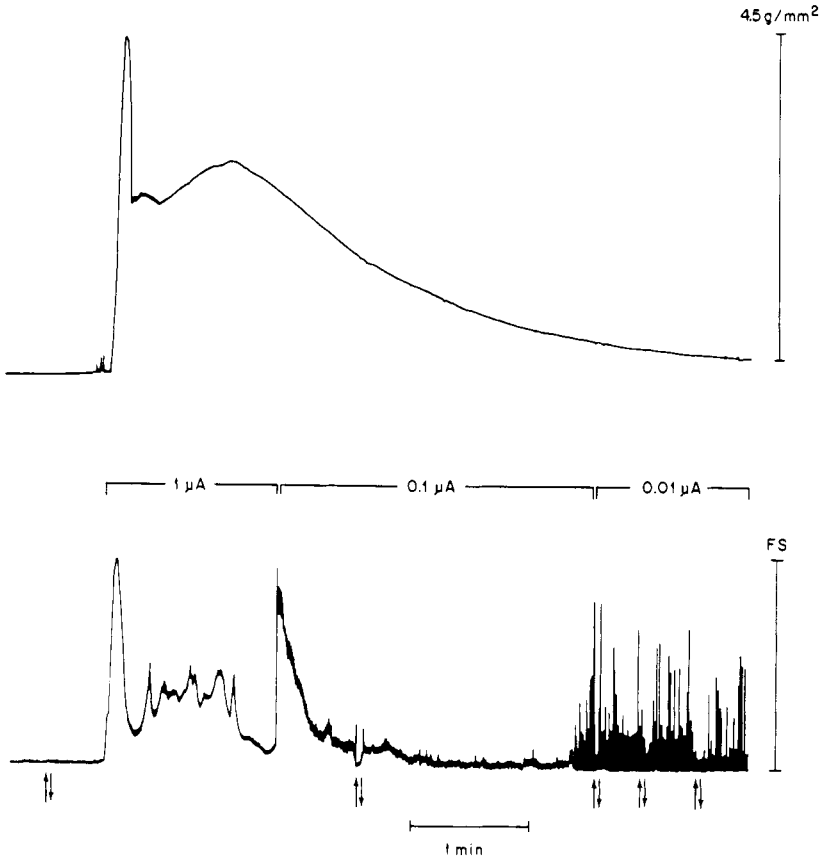

Figure 4. An experimental record of the force (top panel) and light signals (lower panel) obtained in response to detergent (i.e., Triton X-100) lysis. Details of the fractional luminescence technique are provided in Materials and Methods. FS indicates full scale on 1 μA, 0.1 μA, and 0.01 μA, respectively. Shutter open and shutter closed are indicated by ↑↓, respectively. This procedure was repeatedly performed in order to check for baseline drift of the recording, which did not occur during the few minutes required for complete cell lysis and aequorin consumption. The muscle cross-sectional area was 0.44 mm².

tary and complementary to microinjection, but may also offer distinct advantages in terms of ease of performance, percentage of successful experiments, and chances of obtaining individual aequorin signals that are bright enough for analysis on a twitch-to-twitch basis.

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1. Blinks JR. Intracellular [Ca^{2+}] measurements. In: Fozzard HA, Haber E, RB Jennings, Eds. The Heart and Cardiovascular System. New York: Scientific Foundations, pp671–701, 1986.
2. Blinks JR, Mattingly PH, Jewell BR, van Leeuwen M, Harrer GC, Allen DG. Practical aspects of the use of aequorin as a calcium indicator: Assay, preparation, microinjection and interpretation of signals. Methods Enzymol **57**:292–328, 1978.
3. Eusebi F, Miledi R, Takahashi T. Calcium transients in mammalian muscles. Nature **284**:560–561, 1980.
4. Fryer MW, Neering IR, Stephenson DG. Effects of 2,3-butanedione monoxime on the contractile activation properties of fast- and slow-twitch rat muscle fibers. J Physiol **407**:53–75, 1988.
5. Blinks JR, Rudel R, Taylor SR. Calcium transients in isolated amphibian skeletal muscle fibers: Detection with aequorin. J Physiol **277**:291–323, 1978.
6. Kihara Y, Morgan JP. A comparative study of three methods for intracellular loading of the calcium indicator aequorin in ferret papillary muscles. Biochem Biophys Res Commun **162**:402–407, 1989.
7. Allen DG, Blinks JR. Calcium transients in aequorin-injected frog cardiac muscle. Nature **273**:509–513, 1978.
8. Mullins LJ. Ion and molecule fluxes in squid axons. Ann NY Acad Sci **137**:830–836, 1966.
9. Morgan JP, DeFeo TT, Morgan KG. A chemical procedure for loading the calcium indicator aequorin into mammalian working myocardium. Pflugers Arch **400**:338–340, 1984.
10. Fabiato A. Myoplasmic free calcium concentration reached during the twitch of an intact isolated cardiac cell and during calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned cardiac cell from the adult rat or rabbit ventricle. J Gen Physiol **78**:457–497, 1981.
11. McClellan GB, Winegrad S. The regulation of the calcium sensitivity of contractile system in mammalian cardiac muscle. J Gen Physiol **72**:737–764, 1978.
12. Winegrad S. Studies of cardiac muscle with a high permeability to calcium produced by treatment with ethylenediaminetetraacetic acid. J Gen Physiol **58**:71–93, 1971.