

[48] Studies on Intact Sarcoplasmic Reticulum: Patch Clamp Recording and Tension Measurement in Lobster Split Muscle Fibers

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Introduction

The sarcoplasmic reticulum (SR) of muscle fibers is of critical importance to contraction: it regulates the intracellular movement of calcium ions that links the signals sent by the nervous system to the chemical reactions that fuel contraction. The SR membranes thus need to be understood in detail even though they are inside a muscle fiber, inaccessible to typical experimentation.

Sarcoplasmic reticulum membranes are accessible in skinned muscle fibers. Skinned muscle fibers are broadly defined as preparations in which removal or disruption of the sarcolemma exposes the highly ordered intracellular space to extracellular solutions. Skinned fibers bridge the gap between intact and isolated systems. Biochemical or physical properties characterized in isolated systems under well-controlled conditions have additional constraints *in situ*, imposed by geometry, diffusion, and interaction with other pathways, whereas many parameters in the intact fiber are unknown or uncontrolled. Skinned fibers can bridge the gap, maintaining a topologically realistic matrix with many of the normal constraints on specific reactions and the relations between them.

One way to examine the influence of Ca^{2+} ions on the properties of the contractile machinery is to employ tension measurements. This method bypasses the T-membrane depolarization step of excitation-contraction coupling; it activates isolated bundles of myofibrils directly in Ca^{2+} -buffered solutions. Activation by this procedure has the advantage that one can readily control the ionic environment around the contractile elements. In tension experiments, lobster skinned fibers respond to caffeine in a dose-dependent manner. They sustain many cycles of contractures and reloading.

The patch clamp is a powerful tool for studying ion channels with molecular, even atomic resolution. The technique has not often been used to study channels from internal membranes (such as SR and endoplasmic reticulum) because they are inaccessible in intact fibers, hidden behind the plasma membrane. If the plasma membrane of vertebrate muscle fibers is removed, giving the patch pipette access to the SR, gigaseals are hard to form, presumably because of mechanical interference from the myofibrils

(however, see Stein and Palade¹) which fill over 90% of most muscle fibers.²

Gigaseals might form more easily in muscles with fewer myofibrils and more SR, so we investigated muscles evolved to produce sound. They have few myofibrils and profuse SR,³⁻⁵ probably because they are synchronous and fast, contracting at more than 100 Hz.^{6,7} The remotor muscle of the lobster second antenna was chosen because (1) it has the highest reported content of SR (~70%, v/v),^{3,5} compared to about 34% in synchronous insect muscle⁸ and probably a similar figure in the brain heater muscle of billfish.^{9,10} (2) Excitation-contraction coupling in crustacean muscle is quite similar to that in vertebrate skeletal muscle.¹¹⁻¹⁶ We have split such fibers, exposing the SR, and used the patch clamp technique to examine channels in their native state. We use the words split and skinned in this chapter to imply the mechanical removal of the sarcolemma by dissection. The remotor muscle is a practical preparation: it is large enough to handle, and it is easy to obtain because lobsters are widely distributed commercially. Fibers were prepared by microdissection and split in relaxing saline.^{5,17-19} Pipettes readily formed gigaseals to this preparation, allowing the study of the behavior of single channels from the SR membrane.

In both tension measurement and patch clamp recording, the skinned lobster remotor muscle fibers seem as viable as most skinned preparations. Some of these results have already been previously presented,²⁰⁻²³ and

¹ P. Stine and P. Palade, *Biophys. J.* **54**, 357 (1988).

² B. R. Eisenberg, in "Handbook of Physiology, Section 10: Skeletal Muscle" (L. D. Peachey and R. H. Adrian, eds.), p. 73. Williams & Wilkins, Baltimore, Maryland, 1983.

³ J. Rosenbluth, *J. Cell Biol.* **42**, 534 (1969).

⁴ D. J. Scales, P. Kidd, T. Yasumura, and G. Inesi, *Tissue Cell* **14**, 163 (1982).

⁵ M. Villaz, M. Ronjat, M. Garrigos, and Y. Dupont, *Tissue Cell* **19**, 135 (1987).

⁶ M. Mendelson, *J. Cell Biol.* **42**, 548 (1969).

⁷ D. Young and R. K. Josephson, *Nature (London)* **309**, 286 (1984).

⁸ R. K. Josephson and D. Young, *J. Exp. Biol.* **118**, 185 (1985).

⁹ B. Block, *News Physiol. Sci.* **2**, 208 (1987).

¹⁰ B. Block, G. Meissner, and C. Franzini-Armstrong, *Fed. Proc.* **46**, 812 (1987).

¹¹ C. C. Ashley and E. B. Ridgway, *J. Physiol. (London)* **209**, 105 (1970).

¹² J. P. Reuben, P. W. Brandt, M. Berman, and H. Grundfest, *J. Gen. Physiol.* **57**, 385 (1971).

¹³ P. W. Brandt, J. P. Reuben, and H. Grundfest, *J. Gen. Physiol.* **59**, 305 (1972).

¹⁴ T. J. Lea, *Pflugers Arch.* **406**, 315 (1986).

¹⁵ T. J. Lea and C. C. Ashley, *Membr. Biol.* **61**, 115 (1981).

¹⁶ M. P. Timmerman and C. C. Ashley, *FEBS Lett.* **209**, 1 (1986).

¹⁷ M. Endo, M. Tanaka, and Y. Ogawa, *Nature (London)* **228**, 34 (1970).

¹⁸ M. Endo and Y. Nakajima, *Nature (London) New Biol.* **246**, 216 (1973).

¹⁹ M. Endo, *Physiol. Rev.* **57**, 71 (1977).

²⁰ J. M. Tang, J. Wang, and R. S. Eisenberg, *Biophys. J.* **51**, 48a (1987).

²¹ J. M. Tang, J. Wang, and R. S. Eisenberg, *J. Gen. Physiol.* **94**, 261 (1989).

²² J. M. Tang, J. Wang, and R. S. Eisenberg, *Biophys. J.* **57**, 171a (1990).

parts of this chapter closely follow Tang *et al.*²¹ This chapter provides references to only some of the literature on experimentation with lobster SR. Appropriate articles are cited in each section.

Materials and Methods

American lobsters, *Homarus americanus*, are obtained from a commercial fish dealer and maintained in refrigerated (at 12°), recirculating artificial seawater in an aquarium until used, usually within a week. The animals are sacrificed by decapitation, opened by removal of the dorsal part of the carapace, and are cleaned of viscera to expose the remotor muscle of the coxa of the antenna. The muscle of the second antenna is a large, prominent muscle (mass ~ 550 mg each) which has its origin posterolateral to the base of the antenna.⁶ The remotor from both sides of the head are removed intact with the overlying exoskeleton. The muscles are cleaned of blood vessels, connective tissues, and nervous tissues and put in lobster saline (composition given in Table I). Fibers sometimes give spontaneous long contractures or spasms of rapid twitches. Muscle from one side is used immediately, and the other is stored in lobster saline at 4° for up to about 4 hr before use. The rest of the musculature is frozen for later, more conventional use.

Preparation for Patch Clamp Experiments

The preparation is soaked in 460 mM potassium glutamate, so-called relaxing saline (Table I, calculated 100 nM free Ca²⁺), for several minutes. After the initial K contracture, the fibers show no mechanical activity in relaxing saline. A short section of the fiber bundle (5–10 fibers ~ 10 mm in length) is cut from the whole muscle. Single fibers (~ 400 μm in diameter) are isolated with a 27-gauge hypodermic needle and are teased out from the muscle. To obtain a split preparation, one end of a single fiber is split along its longitudinal axis into two pieces. With the pieces held in a pair of fine forceps, the fiber is torn into two strips. The splitting procedure is repeated until a preparation about 50 μm in diameter is left, measured using a Nikon microscope at a total magnification of ×250.

Single skinned fibers are mounted between two balls of grease (Leitz #465) on a Sylgard disk. The disk is pinned down for patch clamp recording with stainless steel insect pins (#00), into a Sylgard-lined acrylic plastic chamber filled with 460 mM potassium glutamate relaxing saline, on a microscopic stage. The split muscle fiber preparations are observed during

²³ J. Wang, J. M. Tang, and R. S. Eisenberg, *Biophys. J.* 55, 207a (1989).

TABLE I
COMPOSITION OF SOLUTIONS FOR LOBSTER SKINNED FIBER PATCH CLAMP EXPERIMENTS

Solution ^a	Concentration (mM)										pCa
	Potassium glutamate	KCl	Sodium glutamate	NaCl	MgATP	CaCl ₂	MgCl ₂	K ₂ EGTA	HEPES	CaCl ₂	
Lobster saline	—	10	—	450	—	16	7	—	25	—	—
Potassium glutamate	460	—	—	—	1	1.2	0.9	5	25	—	7.0
Sodium glutamate	—	—	460	—	1	1.2	0.9	5	25	—	7.0

^a pH 7.4 for lobster saline and pH 7.0 for glutamate solutions.

TABLE II
COMPOSITION OF SOLUTIONS FOR LOBSTER SKINNED FIBER TENSION EXPERIMENTS

Solution ^a	Concentration (mM)										pCa
	Potassium glutamate	KCl	Sodium glutamate	Na ₂ CP	Na ₂ ATP	CaCl ₂	MgCl ₂	K ₂ EGTA	HEPES	CaCl ₂	
Basic	68	87	74	10	5	0	1	0.1	20	—	—
Relaxing	68	87	74	10	5	0.059	1	0.1	20	—	7.3
Loading	68	87	74	10	5	0.011	1	0.1	20	—	6.3

^a pH 7.0 for all solutions.

single-channel experiments using a modified fold-back Nikon (Labophot) Hoffman modulation microscope at a total magnification of $\times 250$.

Single-Channel Recording

Patch pipettes are made from Corning 7052 glass (outside diameter 1.65 mm, inside diameter 1.15 mm, purchased from Garner Glass, Claremont, CA) in a two-stage pulling process, using a patch pipette puller. Immediately before use, the pipettes are coated with Sylgard 184 (Dow Corning, Midland, MI) and heat polished to a nipple shape with a final inside tip diameter of approximately $0.5 \mu\text{m}$. The Sylgard coating is thought to reduce pipette capacitance to the bath, and to prevent creeping of fluid up the shank of the patch pipette. The pipettes, typically filled with 460 mM potassium glutamate relaxing saline, have resistances in the range of 15 to 20 M Ω . Pipette tips are filled by strong backward suction and the shanks backfilled with a fine hypodermic syringe needle. Gigohm seals are obtained using very light suction from a syringe, with seal resistance between 10 and 20 G Ω . In some "better experiments" (about one-fifth), a gigaseal forms without any suction.

A patch clamp amplifier is used for measuring current. The voltage signals are displayed on a digital oscilloscope and stored on magnetic tapes with the bandwidth dc to 5 kHz for further analysis and graphical display. Data are digitized every 100 μsec after passing through a low-pass 8-pole Bessel filter, -3 dB at 1 kHz. Input resistance and resting potential are measured in some experiments: a voltage pulse of -400 to -500 mV is applied to the pipette to break down the membrane, that is, to remove the impedance of the membrane patch. The input resistance of the SR is measured by applying a 20-mV voltage pulse. For the resting potential measurement, the voltage control circuitry is turned off, the current through the pipette is set to zero, and the resulting "open circuit" voltage is measured. This resting potential is stable for at least 15 min, there being a drift of 2 to 3 mV in that time. Liquid junction potentials and offset currents through the gigaseal undoubtedly limit the precision of our estimates. All experiments are carried out at room temperature of about 20° .

We form seals on the cytoplasmic side of the SR membrane of the split muscle fiber (an on-SR patch), probably the equivalent of the cis side of the reconstituted SR preparations as studied in the laboratories of Miller and Williams.²⁴⁻²⁸ The other side of the on-SR patch is the SR luminal side,

²⁴ C. Miller, in "Current Methods in Cellular Neurobiology" (J. L. Barker and J. F. McKelvy, eds.), Vol. 3, p. 1. Wiley, New York, 1983.

²⁵ C. Miller, J. E. Bell, and A. M. Garcia, in "Current Topics in Membranes and Transport" (F. Bronner and W. D. Stein, eds.), Vol. 21, p. 99. Academic Press, New York, 1984.

probably equivalent to the trans side in experiments on reconstituted systems. Excised patches are formed by pulling the electrode tip away from the SR membrane after the gigaseal is formed. Such "inside-out patches"²⁹ have the SR luminal side exposed to the bath. Our voltage convention places ground (zero potential) on the bath side, and the pipette side could be clamped at a range of voltages relative to virtual ground. Thus, depolarization of the SR membrane, which makes the sarcoplasm more positive, can be produced by (negative) Ca^{2+} current flowing into the sarcoplasm down its concentration gradient across the SR membrane, just as a depolarizing action potential can be produced by (negative) Ca^{2+} current flowing into the sarcoplasm down its concentration gradient across the fiber membrane.

Preparation for Tension Measurement

A small bundle of fibers, several millimeters in length, is cut from the intact muscle, and excess fluid is removed by blotting lightly with Kim-wipes tissue. The small bundle of fibers is placed in light mineral oil. A single fiber is teased out of the bundle and skinned by mechanical removal of the sarcolemma as in patch clamp preparation. The skinned preparation used for tension measurement is about 200 μm in diameter. Skinned fiber less than 100 μm in diameter usually gives little or no tension, perhaps because an outer annulus of 100 μm is damaged in the skinning procedure.

Measurement and Recording of Tension

The preparation is switched to relaxing saline for mounting. Care is taken to keep the preparation under solution during the mounting process. A single skinned fiber is mounted on a force transducer (Cambridge Technology, Watertown, MA, Model 400). One cut end of the skinned fiber is held stationary with a pair of forceps, controlled by a screw clamp. The other cut end of the preparation is held by cyanoacrylate glue (gel form) to the stainless steel rod, which is attached directly to the transducer. The forceps and the transducer are attached to separate manipulators which are positioned beforehand so that the opposing edges of the forceps and the rod are parallel to each other and perpendicular to the fiber axis. The fiber is stretched to about 115% of the slack length (~ 1 mm).

²⁶ B. Tomlins and A. J. Williams, *Pfluegers Arch.* **407**, 341 (1986).

²⁷ B. Tomlins, A. J. Williams, and R. A. P. Montgomery, *J. Membr. Biol.* **80**, 191 (1984).

²⁸ M. A. Gray, R. A. P. Montgomery, and A. J. Williams, *J. Membr. Biol.* **88**, 85 (1985).

²⁹ O. P. Hamill, A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth, *Pfluegers Arch.* **391**, 85 (1981).

The method employed for changing solutions is similar to that of Ashley and Moiescu.³⁰ The bathing salines are contained in 1.5-ml vials (Nalgene cryovial, Halge Company, Rochester, NY) that fit into a series of cylindrical wells drilled out of an aluminum block. This block is assembled so that it could be rotated horizontally about its axis and is attached to a large, adjustable Palmer stand (Harvard Apparatus, South Natick, MA) so that it is easily raised or lowered. All solutions are cooled to 12° and are changed by switching the wells on the mechanical stage. The output from the transducer goes to a chart recorder and to a magnetic tape recorder. Standard protocol starts with a wash in relaxing solution (pCa 7.3) for 30 sec followed by contracture in caffeine solution (20 mM), a wash in relaxing solution for 30 sec, and loading in loading solution (pCa 6.3) for 10 min.

Solutions

The dissected intact lobster muscle is kept in lobster saline,³¹ containing 450 mM NaCl, 10 mM KCl, 16 mM CaCl₂, 7 mM MgCl₂, and 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 942 mOsm/(kg H₂O), adjusted to pH 7.4 by adding NaOH, typically 11 mM.

In patch clamp experiments, potassium glutamate relaxing saline (Table I) contains 460 mM potassium glutamate, 5 mM ethylene glycol bis (β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (K₂EGTA), 1.2 mM CaCl₂, 1 mM MgATP, 0.9 mM MgCl₂, and 25 mM HEPES, with 100 nM free Ca²⁺ and 1 mM free Mg²⁺, calculated from the apparent dissociation constants,³²⁻³⁵ with osmolality 922 mOsm/(kg H₂O), adjusted to pH 7.0 by adding KOH, typically 7.5 mM, with a total K⁺ concentration ($[K^+]$) of approximately 480 mM. Sodium glutamate relaxing saline contains 460 mM sodium glutamate, 5 mM Na₂EGTA, 1.2 mM CaCl₂, 1 mM MgATP, 0.9 mM MgCl₂, and 25 mM HEPES, with osmolality 907 mOsm/(kg H₂O), adjusted to pH 7.0 by adding NaOH, typically 10 mM. The osmolality of the solution is routinely monitored with a high precision osmometer (Model 3 MO, Advanced Instruments, Needham, MA). The relaxing solutions are kept hypoosmotic, presumably swelling the SR lumen and making gigaseals easier to form.²⁹ Gigaseals are stable and well behaved: no irregular bursts of fast current transients are observed.

Three different solutions are used in tension experiments with skinned muscle fibers: relaxing, loading, and releasing. All solutions (Table II) are

³⁰ C. C. Ashley and D. G. Moiescu, *J. Physiol. (London)* **270**, 627 (1977).

³¹ R. A. DeRosa and C. K. Govind, *Nature (London)* **273**, 676 (1978).

³² A. E. Martell and R. M. Smith, "Critical Stability Constants." Plenum, New York, 1977.

³³ A. Fabiato and F. Fabiato, *J. Physiol. (Paris)* **75**, 463 (1979).

³⁴ R. Y. Tsien and T. J. Rink, *Biochim. Biophys. Acta* **599**, 623 (1980).

³⁵ A. Fabiato, this series, Vol. 157 [31].

designed to mimic intracellular conditions and have composition and pH close to lobster muscle.^{36,37} The relaxing solution is designed to mimic resting intracellular conditions. The loading solution is designed to facilitate accumulation of calcium in the SR. The release solution is designed to induce calcium release from the SR and is made by adding caffeine as the dry powder to a volume of basic solution. The actual salts and their concentrations are shown in Table II.

Conclusion

The utility of this preparation and these procedures is documented by the results obtained.^{20-23,38} The skinned lobster remotor muscle seems as viable as most skinned preparations as judged by usual criteria, namely, the tension generated by caffeine: responses are vigorous, and the SR can be reloaded many times. In addition, single K^+ and Ca^{2+} channels can be studied in their native membrane by the patch clamp technique. The skinned lobster remotor preparation can be studied with an unusually powerful combination of techniques and so perhaps can yield some unusual information.

³⁶ P. B. Dunham and H. Gainer, *Biochim. Biophys. Acta* **150**, 488 (1968).

³⁷ J. D. Robertson, *J. Exp. Biol.* **38**, 707 (1961).

³⁸ J. M. Tang, Ph.D thesis (1991).

[49] Planar Bilayer Recording of Ryanodine Receptors of Sarcoplasmic Reticulum

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Introduction

Three situations in ion channel analysis require a cell-free recording or planar bilayer technique: (1) in the case of channels confined to regions of the cell which are largely inaccessible to patch electrodes such as narrow tubules and intracellular organelles^{1,2}; (2) to control solutions on both faces of a channel with analytical precision³; and (3) to test the ionophoric

¹ H. Valdivia and R. Coronado, *J. Gen. Physiol.* **95**, 1 (1990).

² C. Valdivia, H. Valdivia, B. V. L. Potter, and R. Coronado, *Biophys. J.* **57**, 1233 (1990).

³ J. S. Smith, T. Imagawa, J. Ma, M. Fill, K. P. Campbell, and R. Coronado, *J. Gen. Physiol.* **92**, 1 (1988).