K+-Selective Channel from Sarcoplasmic Reticulum of Split Lobster Muscle Fibers

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ABSTRACT   The patch clamp technique has been used to study channels in a membrane inside a cell. A single muscle fiber is skinned in relaxing saline (high K^+, low Ca^{2+} with EGTA and ATP), leaving the native sarcoplasmic reticulum (SR) membrane exposed for patching. Fibers are dissected from the second antenna remotor muscles of the American lobster, *Homarus americanus*. Transmission and scanning electron microscopy confirm the large volume fraction of SR (~70%) and absence of sarcolemma in this unusual skinned preparation. The resting potential of the SR was measured after the resistance of the patch of membrane was broken down. It is near 0 mV (-0.4 ± 0.6 mV). The average input resistance of the SR is 842 ± 295 MΩ. Some 25% of patches contain a K^+-selective channel with a mean open time of seconds and the channel displays at least two conducting states. The open probability is weakly voltage dependent, large at zero and positive potentials (cytoplasm minus SR lumen), and decreasing at negative potentials. The maximal conductance of this channel is 200 ± 1 pS and the substate conductance is 170 ± 3 pS in symmetrical 480 mM K^+ solution. The current–voltage relation of the open channel is linear over a range of ± 100 mV. The selectivity is similar to the SR K^+ channel of vertebrates: P_K/P_{Na} is 3.77 ± 0.03, determined from reversal potential measurements, whereas γ_K/γ_{Na} is 3.28 ± 0.06, determined from open-channel conductance measurements in symmetrical 480 mM solutions. Voltage-dependent block in the lobster SR K^+ channel is similar to, but distinct from, that reported for the vertebrate channels. It occurs asymmetrically when hexamethonium is added to both sides of the membrane. The block is more effective from the cytoplasmic side of the channel.

INTRODUCTION

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 sarcoplasmic reticulum [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 Palade, 1988; and Stein et al., 1989), which fill >90% of most muscle fibers (Eisenberg, 1983).

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 (Rosenbluth, 1969; Scales et al., 1982; Villaz et al., 1987), probably because they are synchronous and fast, contracting at >100 Hz (Mendelson, 1969; Young and Josephson, 1984). The remotor muscle of the lobster second antenna was chosen because (a) it has the highest reported content of SR ~70% vol/vol (Rosenbluth, 1969; Villaz et al., 1987), compared with ~34% in synchronous insect muscle (Josephson and Young, 1985), and probably a similar figure in the brain heater muscle of billfish (Block, 1987; Block et al., 1987); and (b) excitation–contraction coupling in crustacean muscle is quite similar to that in vertebrate skeletal muscle (Ashley and Ridgway, 1970; Reuben et al., 1971; Brandt et al., 1972; Lea and Ashley, 1981; Lea, 1986; Timmerman and Ashley, 1986). 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 paper 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 (Endo et al., 1970; Endo and Nakajima, 1973; Endo, 1977; Villaz et al., 1987). Pipettes readily formed gigaseals to this preparation, and thus we could study the behavior of single K^+ channels from the SR membrane.

The existence of a monovalent cation permeability pathway in the SR of mammalian skeletal muscle was first demonstrated using isotope flux measurements. McKinley and Meissner (1977) suggested that this system could act as a charge-compensating mechanism, balancing charge movements associated with the large calcium ion fluxes involved in excitation–contraction coupling. Subsequently, Miller (1978) showed that the monovalent cation permeability pathway of skeletal muscle SR is a voltage-gated K^+-selective channel. This channel has now been extensively investigated in the SR membrane of vertebrate skeletal and cardiac muscle. Macroscopic fluxes have been recorded in isolated SR vesicles using isotope flux techniques (McKinley and Meissner, 1978; Meissner and McKinley, 1982). Single K^+-selective channels have been studied in bilayers after the incorporation of SR vesicles into planar phospholipid bilayers (Coronado and Miller, 1979, 1980, 1982; Coronado et al., 1980; Labarca et al., 1980; Labarca and Miller, 1981; Miller, 1982a, b, c; Tomlins et al., 1984; Cukierman et al., 1985; Gray et al., 1985; Hill et al., 1989), and patch clamp measurements have been made from reconstituted liposomes (Tomlins and Williams, 1986) and the sarcoball preparation (Stein et al., 1989). In this paper, we report the first direct observations of the K^+ channel from the native SR membrane of the split lobster remotor muscle fiber, using the patch clamp technique. We compare its properties with those of the vertebrate muscle SR system studied with different techniques. Some of these results have been presented in abstract (Tang et al., 1987).

MATERIALS AND METHODS

American lobsters, Homarus americanus, were obtained from a commercial fishmonger and maintained in refrigerated (at 12°C), recirculating artificial seawater until used. The animals were opened by removal of the dorsal part of the carapace and the viscera were cleaned out.

The remotor muscle of the second antenna was removed with the overlying exoskeleton and put in lobster saline, the rest of the animal's musculature being frozen for later more conventional use. The preparation was cleaned of blood vessels and connective tissues and soaked in 460 mM K glutamate relaxing saline. Single fibers (≈200 μm in diameter) were isolated with a 27-gauge tuberculin needle from the muscle, which was left attached to the carapace, and a short section of the fiber was cut at one end into two pieces. Holding each piece in fine forceps, the fiber was torn into two strips. The splitting procedure was repeated until a preparation of about 50 μm in diameter was left, measured using a microscope (Nikon Corp., Garden City, NY) at a total magnification of 250.

The muscle fibers (both intact and split) were fixed for electron microscopy (EM) with sodium cacodylate-buffered solution containing 3% glutaraldehyde. The tissues were fixed, stained, dehydrated, and either critical point-dried and sputter-coated (in scanning EM) or embedded and sectioned (in transmission EM) using standard procedures. The muscles were kept at room temperature of ~20°C during all chemical processing of EM.

The split muscle fiber preparations were observed during single-channel experiments using a modified fold-back Nikon (Labophot) Hoffman modulation microscope at a total magnification of 250. In early experiments the preparation was mounted (with the SR membranes facing upwards) by gluing the ends onto small pieces of aluminum foil with cyanoacrylate Super glue and then pinning the foil to a Sylgard disk (Dow Corning Corp., Midland, MI) with stainless steel insect pins (#00). In later experiments the fiber was held directly on the Sylgard disk with grease no. 465 (E. Leitz, Rockleigh, NJ). The disk was pinned down (in all experiments) in a Sylgard-lined acrylic plastic chamber filled with 460 mM K glutamate relaxing saline. Patch pipettes were made from glass (no. 7052, Corning Glass Works, Corning, NY; outside diameter 1.65 mm, inside diameter 1.15 mm, purchased from Garner Glass, Claremont, CA) in a two-stage pulling process, using a vertical Kopf puller (model 750, David Kopf, Inc., Tujunga, CA) with a home-built circuit providing constant power to the heating coil, independent of variations in coil impedance. The pipettes were coated with Sylgard 184 (Dow Corning Corp.) and heat-polished immediately before use to a nipple shape with a final inside tip diameter of ≈0.5 μm. The electrodes, typically filled with 460 mM K glutamate relaxing saline, had resistance in the range of 15–20 MΩ. Electrode tips were filled by strong backward suction and the shanks back filled with a fine hypodermic syringe needle. Gigaohm seals were obtained using very light suction from a syringe in 69% of 512 total attempts, with seal resistance between 10 and 20 GΩ. In some "better experiments" (about one-fifth), a gigaseal formed without any suction. A patch clamp amplifier (model EPC-7, Medical Systems Corp., Greenvale, NY) was used for measuring current. The voltage signals were displayed on a digital oscilloscope (model PM 3305, Phillips Electronic Instruments, Inc., Mahwah, NJ) and stored on analog tapes with the bandwidth DC – 5 kHz (model Store 4DS, Racal Recorders, Inc., Sarasota, FL) for further analysis and graphical display. Data were digitized every 100 μs after passing through a low-pass eightpole Bessel filter (model 902LPF, Frequency Devices, Inc., Haverhill, MA), –3 dB at 1 kHz. Input resistance and resting potential were measured in some experiments: a voltage pulse of –400 to –500 mV was applied to the pipette to break down the membrane, i.e., to remove the impedance of the membrane patch. Input resistance of the SR was measured by applying a 20-mV voltage pulse. For the resting potential measurement, the voltage control circuitry was turned off, the current through the pipette was set to zero, and the resulting "open circuit" voltage was measured. This resting potential was stable for at least 15 min, there being a drift of 2–3 mV in that time. Liquid junction potentials and offset currents through the gigaseal undoubtedly limited the precision of our estimates. All experiments were carried out at a room temperature of ~20°C.

Lobster saline (modified from DeRosa and Govind, 1978), contained 450 mM NaCl, 10 mM KCl, 16 mM CaCl₂, 7 mM MgCl₂, and 25 mM N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid (HEPES), 942 mosmol/(kg H₂O), adjusted to pH 7.4 by adding NaOH, typically

11 mM. K glutamate relaxing saline contained 460 mM K glutamate, 5 mM ethylene glycol-bis-(β-aminoethyl ether)N,N,N',N'-tetraacetic acid (K_2EGTA), 1.2 mM CaCl$_2$, 1 mM MgATP, 0.9 mM MgCl$_2$, and 25 mM HEPES, with 100 nM free Ca^{2+} and 1 mM free Mg^{2+}, calculated from the apparent dissociation constants (Martell and Smith, 1977; Fabiato and Fabiato, 1979; Tsien and Rink, 1980; Fabiato, 1988), 922 mosmol/(kg H$_2$O), adjusted to pH 7.0 by adding KOH, typically 7.5 mM, with a total K$^+$ concentration ([K$^+$]) of $\approx$480 mM. Na glutamate relaxing saline contained 460 mM Na glutamate, 5 mM Na$_2$EGTA, 1.2 mM CaCl$_2$, 1 mM MgATP, 0.9 mM MgCl$_2$, and 25 mM HEPES, 907 mosmol/(kg H$_2$O), adjusted to pH 7.0 by adding NaOH, typically 10 mM. The osmolality of the solutions were routinely monitored with a high precision osmometer (model 3 MO, Advanced Instruments Inc., Needham, MA). The relaxing solutions were made hypo-osmotic, thus the SR membrane had an osmotic gradient across it such that the cytoplasmic side was hypo-osmotic with respect to the SR lumenal side. This condition has been shown by Hamill et al. (1981) to increase the frequency of gigaseal formation. Gigaseals were stable and well behaved: no irregular bursts of fast current transients were observed.

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 by Miller's laboratory and Williams' laboratory (Miller, 1983; Miller et al., 1984). The other side of the on-SR patch is the SR lumenal side, probably equivalent to the *trans* side in experiments on reconstituted systems. Excised patches were formed by pulling the electrode tip away from the membrane. Such "inside-out patches" (Hamill et al., 1981) have the SR lumenal 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.

RESULTS

Structure

Intact muscle fibers (the remotor of the second antenna of lobster) were fixed under our experimental conditions and examined in transverse and longitudinal sections (Fig. 1, *A* and *B*). Fibers had been dissected in 460 mM K glutamate relaxing saline and processed as described in Materials and Methods. The huge amount of SR surrounding the few myofibrils is apparent in survey (low-power) transmission electron micrographs. The SR consists of innumerable sacs in close apposition to one another, the profiles of which are either elongated or circular. Their pattern is consistent with the appearance shown by others working at room temperature (Rosenbluth, 1969; Villaz et al., 1987), but differs from the images seen by Scales et al (1982) when they worked in the cold, ~4°C. The intact fibers show some signs of damage (similar to those of Villaz et al., 1987), though almost all nuclei, mitochondria, and myofibrils were intact. It is not easy to tell whether the observed damage reflects the native structure of the fiber: no one knows how to fix these unusual lobster fibers reliably at room temperature (Rosenbluth, 1969; Scales et al., 1982) or in the slightly hypotonic solutions (Villaz et al., 1987) used here to favor gigaseal formation. Fixation of split fibers was even more of a problem. Split fibers, fixed in solutions designed for intact fibers, showed substantial vesiculation of the SR. No

signs of sarcoballs (Stein and Palade, 1988) were seen in the light microscope, scanning, or transmission electron microscope. The anatomy of skinned fibers was not further studied because we do not know how to assay the structure of the SR in native (i.e., unfixed) split fibers and thus could not determine how much of the vesiculation seen in the electron micrographs was caused by fixation damage and how much was actually present in the split preparations that we studied with the patch clamp.

Current–Voltage Relations

Single-channel openings (i.e., stochastic steps of current) were seen after formation of a gigaseal in 91 out of 354 patches. These channels appear in clusters. Approxi-
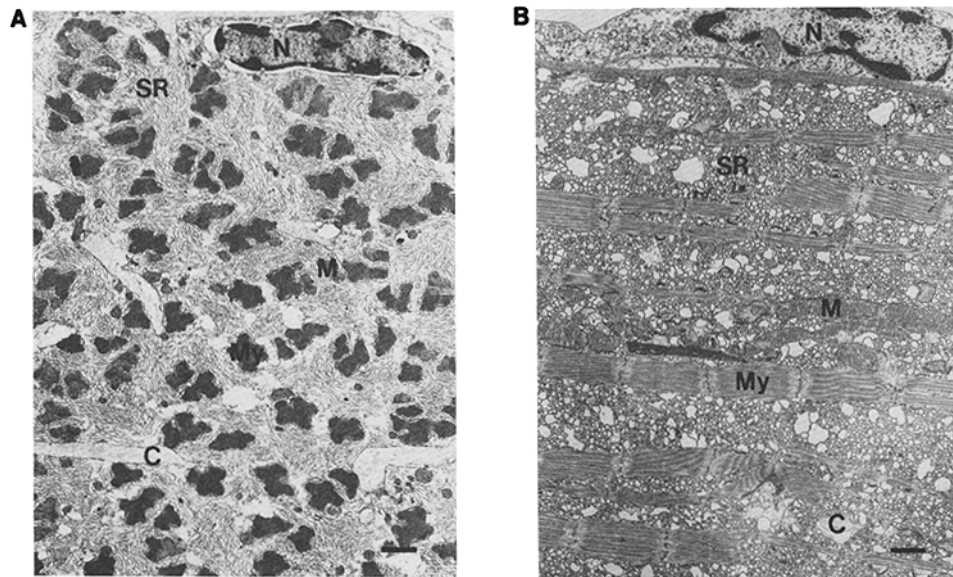

FIGURE 1. Survey transmission electron micrograph of remotor muscle of the lobster second antenna. (A) Transverse section of the muscle fiber with a very high content of sarcoplasmic reticulum (SR). The sarcoplasm surrounding the myofibrils (My) is filled with innumerable SR cisternae. Part of a nucleus (N) is at the top. Mitochondria (M) and part of a cleft (C) are also visible. Bar, 1 μm. (B) Longitudinal section of the muscle fiber. Here, as in transverse sections, the SR consists of short disconnected profiles rather than elongated cisternae. Note the undulating nature of the myofibrils. Bar, 1 μm.

mately 30% of the patches contain one channel; others have between two and six channels. Fig. 2, taken in 460 mM K glutamate relaxing saline, shows the openings and closings of a three-state channel with a clearly defined closed state (zero conductance) and two open states. The fully open main conductance or β state has a conductance of ~200 pS and the "noisy" subconductance or α state has a conductance of ~170 pS. The α state is considerably more variable than the β state; only 50% of channels, in both on-SR and excised patches, exhibit the α subconductance state, although the noise level of our recording should allow consistent identifica-

tion. The α state is much noisier than the main conductance state, even though its conductance is typically ~85% that of the β state. The SR channel appears to be able to enter or leave the α state from either the closed or the β state: transitions from β to α, and vice versa, are commonly seen without intervening closures as illustrated in Fig. 2, taken at -80 mV (because the α state is more probable when the membrane potentials are negative). Openings to a small open level, equal to β minus α are never seen, so it is natural to interpret the data as a subconductance and main conductance state, not as two different independent channels. Of course, different channels with coupled gating could explain these results, and many others as well. The channel activity displayed here is qualitatively similar to that observed when
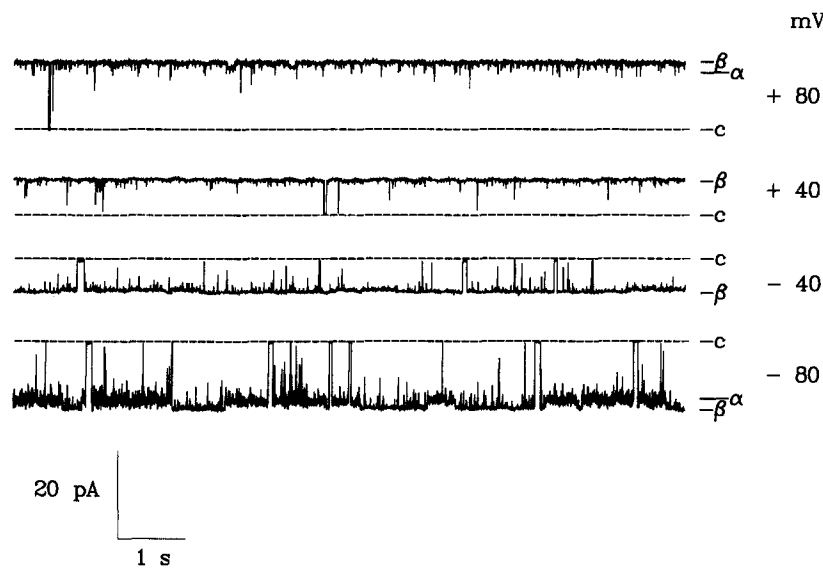

FIGURE 2. Single lobster SR K^+ channel current fluctuations showing three conductance states; Closed (c), "noisy" subconductance (α), and fully open main conductance (β) states. Recording with symmetrical solutions of 460 mM K glutamate relaxing saline, in an on-SR patch configuration. mV, the voltage across the patch membrane, i.e., the pipette voltage minus the SR lumen resting voltage (-0.4 ± 0.6 mV, range from -3.1 to 2.4 mV, 12 direct measurements). An upward deflection of the trace indicates an outward (positive) current, i.e., cytoplasm to SR lumen. These records were filtered at 1 kHz.

native channels are reconstituted into planar bilayers (Miller, 1978; Tomlins et al., 1984; Bell, 1985; Fox, 1985, 1987; Gray et al., 1985) or liposomes (Tomlins and Williams, 1986).

Fig. 3 shows how single-channel current varies with membrane potential. The relationship between open-state current and membrane potential is nearly linear over the range ± 100 mV for both α and β states with mean conductances of the α subconductance and β main conductance states of 170 ± 3 pS (mean $\pm$ SEM, $n = 6$) and 200 ± 1 pS ($n = 12$) respectively, in 460 mM K glutamate relaxing saline. These values were calculated from 12 different membrane patches at membrane potentials in the range of ± 100 mV. At membrane potentials outside ± 100 mV, slight recti-

fication becomes apparent (see Fig. 3), a result found previously in negatively charged bilayers (Tomlins et al., 1984; Gray et al., 1985). We find no difference in current–voltage relation between on-SR and excised patches ($n = 20$).

Ionic Selectivity

One measure of the selectivity of the lobster SR K⁺ channel is the single-channel reversal potential in the presence of bi-ionic conditions, i.e., with 480 mM K⁺ in the bath (SR lumenal) side and 480 mM Na⁺ in the pipette (cytoplasmic) side (Fig. 4). The measured reversal potential of the fully open β state was 32.4 ± 0.2 mV ($n = 7$), which corresponds to $P_K/P_{Na} = 3.77 ± 0.03$, for permeability ratios calculated using the equation in the caption of Fig. 4, allowing differences in activity coefficient.
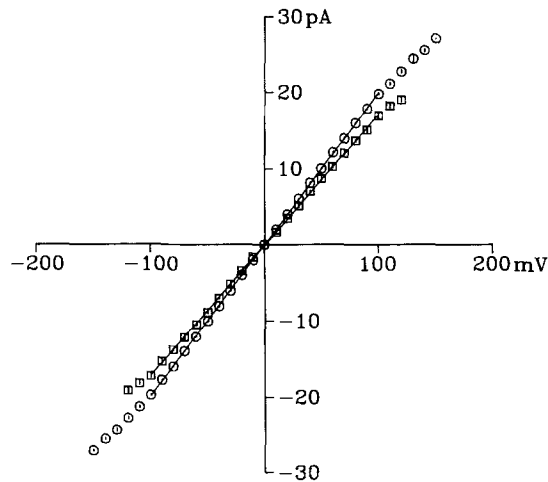

FIGURE 3. Single-channel current-voltage relation for both α (subconductance) (□) and β (main conductance) (O) states. Recording from on-SR patches with symmetrical solutions of 460 mM K glutamate relaxing saline. Data points are mean ± standard error of the mean (SEM) from 12 different membrane patches and all symbols have an SEM smaller than the size of the symbol. The solid lines are drawn by least-squares regression. *mV*, the voltage across the patch membrane, i.e., the pipette voltage minus the SR lumen resting voltage (0.1 ± 0.1 mV, range from −0.4 to 0.6 mV, 12 estimates from linear regression of the current-voltage curves). *pA*, the single-channel current; a positive current indicates an outward current, i.e., cytoplasm to SR lumen. α state single-channel conductance = 170 ± 3 pS (range from 164 to 178 pS: six experiments). β state single-channel conductance = 200 ± 1 pS (range from 194 to 204 pS: 12 experiments). Note that single channels show ohmic behavior for both α and β open states only over a membrane potential range of ±100 mV.

The conductance of the fully open state of the channel was measured in the presence of 480 mM K⁺ on both sides of the membrane or in the presence of 480 mM Na⁺ on both sides (Table I). Open states are more selective for K⁺, with conductance ratios $\gamma_K/\gamma_{Na} = 3.28 ± 0.06$ as are channels reconstituted from skeletal (Coronado et al., 1980; Tomlins and Williams, 1986) or cardiac (Tomlins et al., 1984, Gray et al., 1985) muscle.

Permeability to Ca²⁺

The Ca²⁺ permeability of the SR K⁺ channel is of interest because the SR functions as the Ca²⁺ regulatory system in skeletal muscle. Previous work by Miller (1978) showed that Ca²⁺ appears to block the K⁺ conductance from the cis (cytoplasmic)

-120 -80 -40 40 80 $120\,\mathrm{mV}$
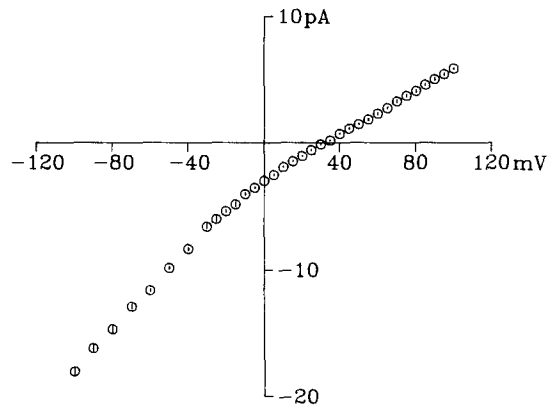
$10\,\mathrm{pA}$

-10

-20

FIGURE 4. Single-channel current–voltage relation under bi-ionic conditions. The curve for the open channels was measured with 460 mM Na glutamate relaxing saline on the cytoplasmic side (pipette) and 460 mM K glutamate relaxing saline on the SR lumenal side (bath) of excised membrane patches. Data points are mean ± SEM from seven membrane patches and all symbols have a SEM smaller than the size of the symbol. Permeability ratios were calculated for Na^+ using $P_{Na}/P_K = (a_K/a_{Na})\exp(-FV/RT)$, where a_K and a_{Na} are ion activities of K^+ and Na^+ (computed from concentration and Robinson and Stokes, 1965), F is the Faraday constant, V is the reversal potential, R is the gas constant, and T is absolute temperature. The mean reversal potential was 32.4 ± 0.2 mV (range from 31.7 to 33.0 mV) (seven experiments), which corresponds to $P_K/P_{Na} = 3.77 \pm 0.03$ (range from 3.67 to 3.86) when activity coefficient differences are considered.

side of the channel, but not from the trans (SR lumenal) side. Coronado et al. (1980), and Stein et al. (1989) reported that this channel is ideally selective for K^+ over Ca^{2+}.

We studied the effect of Ca^{2+}, in physiological concentrations (free $[Ca^{2+}]$ of 100 nM in cytoplasmic side and 1.2 mM in SR lumenal side), on the conductance and the reversal potential of the SR K^+ channels in the presence of K^+ on both sides of the membrane (Table II). The single-channel conductance and reversal potential of the channel are not different from those determined by symmetrical 460 mM K glutamate relaxing saline in which the free $[Ca^{2+}]$ is 100 nM on both sides of the channel.

Voltage Dependence

The open probability of reconstituted vertebrate skeletal and cardiac muscle SR K^+ channels is known to depend on voltage; i.e., the channels show voltage-dependent

TABLE I

Selectivity Parameters for Sodium Ion Measured from Single-Channel Opening of the Lobster SR K^+ Channel

Ion X	Conductance γ	γ_K/γ_X	P_K/P_X
	pS		
K	200 ± 1 (range 194–204, n = 12)	1.00	1.00
Na	61 ± 2 (range 56–67, n = 6)	3.28	3.77

Open-channel conductances were calculated from single-channel events occurring at membrane potentials between ±100 mV with symmetrical relaxing saline of the indicated ion. Permeability ratios were determined under bi-ionic conditions as described in Fig. 4.

gating (Labarca et al., 1980; Tomlins et al., 1986). We too find that the probability of the channel being in the open state varies with potential, although the conductance of both the α and β open state remains virtually unchanged. When a holding potential is applied so the cytoplasmic side is positive with respect to the SR lumenal side (i.e., the channels are depolarized), the channel tends to open (Fig. 5, *A* and *B*). When the SR membrane is hyperpolarized (i.e., when the cytoplasmic side is held negative with respect to the SR lumenal side), the channel tends to close.

Fig. 5 *A* shows the effect of membrane potential on the open probability of the channel. It is a continuous (2 min) recording of channel activity from a patch of SR membrane, with a membrane potential of +40 mV (upper trace) or −40 mV (lower trace) made with a pipette filled with 460 mM K glutamate relaxing saline. The long closed periods at negative (hyperpolarized) membrane potentials seen in the lower trace were found in ~50% of both on-SR and excised patches. Fig. 5 *B* summarizes the potential dependence in two separate experiments. Open probability was estimated only from complete closings. Brief, apparently incomplete closings are too

TABLE II

Permeability Parameters for Calcium Ion Measured from Single-Channel Opening
of the Lobster SR K⁺ Channel

Solutions	Conductance	Reversal potential
	pS	*mV*
Control	200 ± 1 (range 194–204, n = 12)	0.1 ± 0.1 (range −0.4–0.6, n = 12)
Experiment	197 ± 2 (range 194–205, n = 6)	0.1 ± 0.2 (range −0.5–0.6, n = 6)

Open-channel conductances and reversal potentials were calculated from single-channel events occurring at membrane potentials between ±100 mV with symmetrical 460 mM K glutamate relaxing saline (with 100 nM free [Ca²⁺] on both sides) for control as described in Fig. 3 and Table I. For experiment, measurements were done with 460 mM K glutamate relaxing saline in the pipette, and the bath was perfused with the same K glutamate relaxing saline except 5 mM K₂EGTA was substituted by 10 mM K glutamate (with a total [K⁺] of ≈480 and 1.2 mM free [Ca²⁺]). Equilibrium potentials computed with the above solutions are E_K = 0 mV and E_{Ca} = 119 mV.

rare to significantly affect our results. Only one channel was present within the pipette in both experiments, judging by the complete absence of "double" openings over a period of two minutes at voltages where the channel open probability was near unity. Records were taken for 2 min at each voltage separated by rest periods of 2 min at 0 mV potential, hoping to avoid hysteretic complexities akin to desensitization and slow inactivation. The relationship of open probability to voltage is consistent within the same experiment, but is *not* reproducible between experiments: some channels lack long closed periods at hyperpolarized voltages.

Resting Potential and Input Resistance of the SR

The resting potential of the SR was measured directly at the end of 12 experiments, as described in Materials and Methods, averaging −0.4 ± 0.6 mV (range from −3.1 to 2.4 mV). The resting potential could also be measured indirectly in on-SR patches, from the reversal potential of the SR K⁺ channel measured in sarcoplasmic solutions thought to mimic the [K⁺] of the SR lumen. This estimate was 0.1 ± 0.1

mV (range from −0.4 to 0.6 mV, $n = 12$). The measured input resistance of the SR was 842 ± 295 MΩ (range from 3.3 GΩ to 25 MΩ, $n = 15$).

Block by Cholinergic Drugs

K^+ permeation through reconstituted (vertebrate) SR K^+ channels is blocked asymmetrically by *bis*-quaternary ammonium compounds such as hexamethonium, decamethonium, and succinyl choline (Coronado and Miller, 1980; Miller, 1982b; Tom-
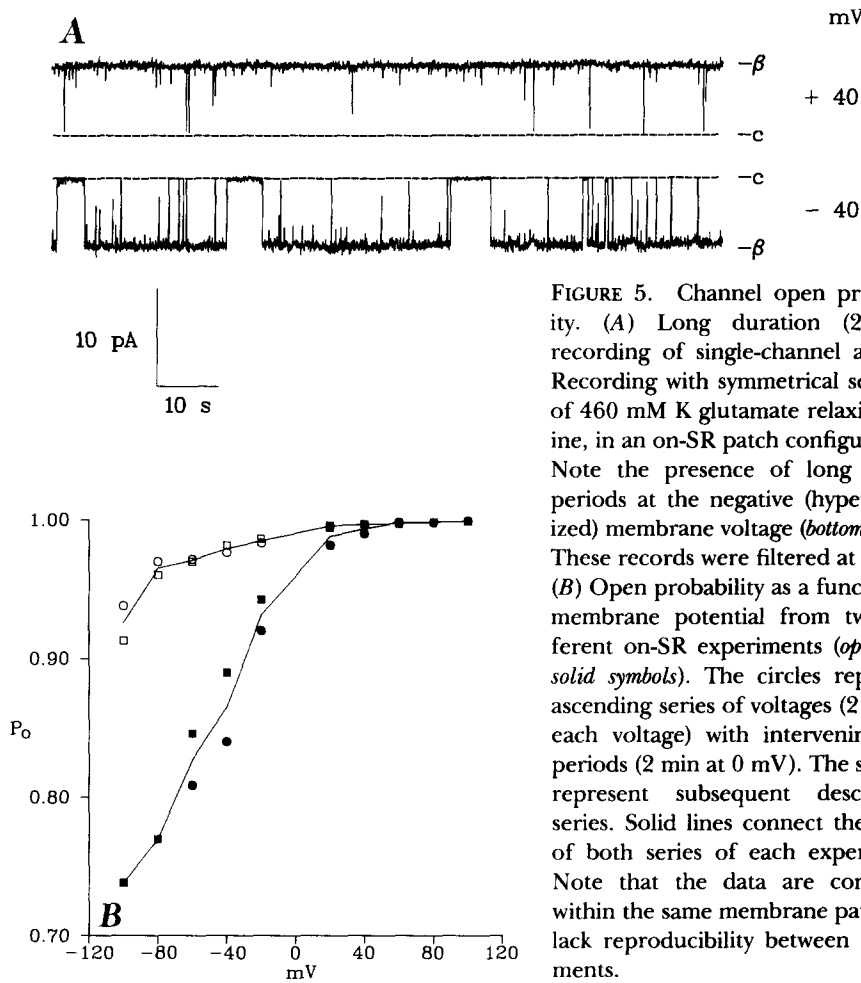

FIGURE 5. Channel open probability. (*A*) Long duration (2 min) recording of single-channel activity. Recording with symmetrical solution of 460 mM K glutamate relaxing saline, in an on-SR patch configuration. Note the presence of long closed periods at the negative (hyperpolarized) membrane voltage (*bottom trace*). These records were filtered at 1 kHz. (*B*) Open probability as a function of membrane potential from two different on-SR experiments (*open* and *solid symbols*). The circles represent ascending series of voltages (2 min at each voltage) with intervening rest periods (2 min at 0 mV). The squares represent subsequent descending series. Solid lines connect the mean of both series of each experiment. Note that the data are consistent within the same membrane patch but lack reproducibility between experiments.

lins et al., 1984; Gray et al., 1985; Tomlins and Williams, 1986; Stein et al., 1989) with a dramatic increase in the flicker of open-channel current in the presence of decamethonium. In general, subconductance states are not visible in the presence of blocker in our preparation, in contrast to the vertebrate channel. We find that decamethonium blocks K^+ conductance but does *not* cause channel flickering; instead it produces a smooth reduction in single channel conductance, yielding a

time-averaged conductance perhaps resulting from unresolved rapid blocking events (Hainsworth et al., 1988). Note, however, that the bandwidth of recording in our patch clamp measurements exceeds the bandwidth of bilayer experiments and so we would expect to see more, not less flicker than earlier experiments (Coronado and Miller, 1980; Miller, 1982b), if the intrinsic behavior of the channels were identical. Fig. 6 shows currents measured after the addition of 7 mM hexamethonium to both sides of a membrane patch: the channel can be seen to undergo a smooth reduction in single-channel current and an increase in open-channel noise, but no flicker is apparent, although flicker is seen in reconstituted channels (Coronado and
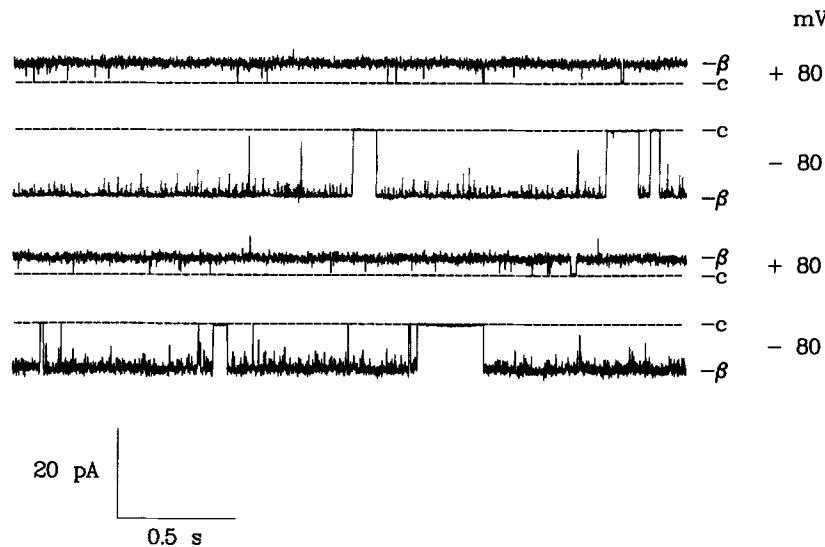

FIGURE 6.   Single-channel current fluctuations showing voltage dependence of hexamethonium block. Top two traces were recorded from an excised membrane patch with symmetrical solutions of 460 mM K glutamate relaxing saline containing 7 mM hexamethonium on the cytoplasmic side (pipette) only. Bottom two traces were recorded after the SR lumenal side (bath) of the same excised patch was perfused with 460 mM K glutamate relaxing saline containing 7 mM hexamethonium. Note the smooth reduction in single-channel current and an increase of open-channel noise in the presence of the blocker. These traces should also be compared with data obtained in the absence of hexamethonium (Fig. 2). These records were filtered at 1 kHz. Note the second trace served as the control before the hexamethonium block of the same channel on the SR lumenal side. In this experiment, no α subconductance state was observed.

Miller, 1980; Miller, 1982b; Tomlins et al., 1984). The decrease of current is greater at positive than negative membrane potentials. Thus, hexamethonium is more effective when acting from the *cytoplasmic* side of the lobster channel even though it (and decamethonium) blocks vertebrate channels less effectively from that side (Coronado and Miller, 1980; Miller, 1982b; Tomlins et al., 1984; Tomlins and Williams, 1986). Some experiments were done with blocker applied to just one side: in general, current leaving the side containing the drug was blocked.

The degree of channel block at different membrane potentials (Fig. 7 A) can be
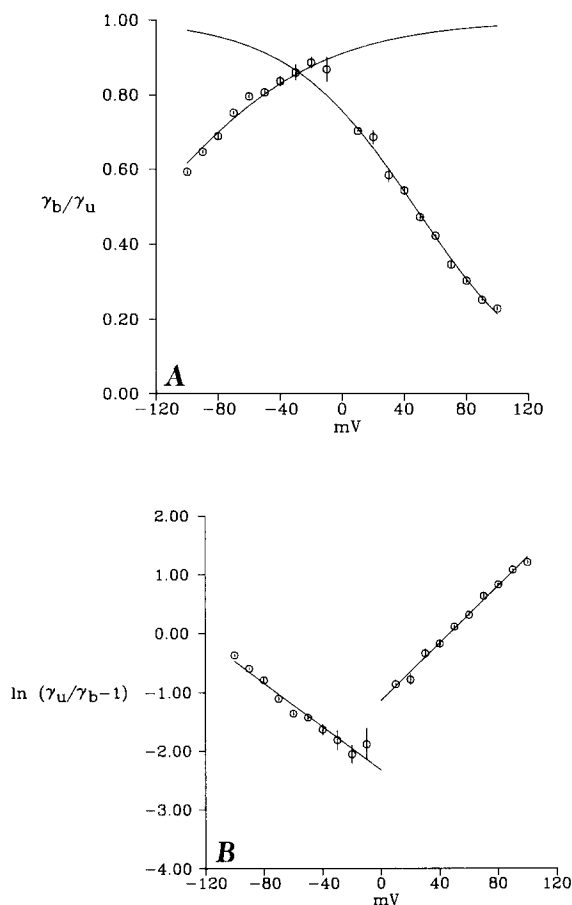
FIGURE 7. Hexamethonium block of K^+ conductance. Plot of blocked relative conductance against membrane potential illustrating the voltage-dependent nature of the block. Experiments were carried out with symmetrical K glutamate relaxing saline containing 7 mM hexamethonium on both sides of the excised membrane patches. Data points are mean ± SEM from five membrane patches. Symbols without error bars have a SEM smaller than the size of the symbol. (A) Blocker access from just one side. The solid lines are drawn according to the equation: $\gamma_b/\gamma_u = [1 + (B/K_0) \exp (z\delta FV/RT)]^{-1}$, where γ_b/γ_u is the blocked conductance/control (unblocked) conductance (200 pS), B is the concentration of the blocker, K_0 is zero-voltage dissociation constant of the blocker; z is the valence of the blocker, δ is the fraction of the total voltage drop across the membrane at the site of block, and V is membrane voltage. K_0 and $z\delta$ are obtained from the intercept on the ordinate and slope of the linearized form of the plot as in Fig. 7 B. Other parameters have the same definition as in the legend of Fig. 4. The use of 200 pS as the control conductance is based on the β state single-channel conductance from Fig. 3 and second trace of Fig. 6, which served as a control of the same channel before the block. (B) Same data as A, plotted in a linearized form. The solid lines are drawn by least-squares regression. Parameters for cytoplasmic block (positive membrane voltages) are $K_0 = 21.9 \pm 1.7$ mM (range from 19.0 to 28.5 mM); $z\delta = 0.717 \pm 0.019$ (range from 0.554 to 0.692); $r = 0.996$ (range from 0.979 to 0.998). For SR lumenal block (negative membrane voltages): $K_0 = 71.5 \pm 11$ mM (range from 54.0 to 114.9 mM); $z\delta = -0.468 \pm 0.035$ (range from -0.399 to -0.612); r = 0.979 (range from 0.871 to 0.999).

used to define an affinity of a channel binding site for the blocker, $K_b(0)$, the dissociation constant at zero voltage, and its "electrical location" δ in certain models of ion permeation and channel structure (Woodhull, 1973; Coronado and Miller, 1980; Miller, 1982b; Tomlins et al., 1984; Gray et al., 1985; Tomlins and Williams, 1986). δ is defined as (the potential drop to the binding site) *divided by* (the potential drop across the membrane). Fig. 7 B shows an analysis of the block of K^+ conductance by hexamethonium, assuming the blocking site is only accessible from one

side, yielding the following blocking parameters: cytoplasmic side, K_0 = 21.9 ± 1.7 mM and $z\delta$ = 0.616 ± 0.019 (n = 5); SR lumenal side, K_0 = 71.5 ± 11 mM and $z\delta$ = −0.468 ± 0.035 (n = 5). The affinity for the blocker from the cytoplasmic side is approximately threefold higher than that obtained for the SR lumenal side, not a surprising result given the asymmetry of the hexamethonium block. Fig. 8 shows an analysis of the data of Fig. 7 *A* assuming the blocking site is accessible from both sides, following Coronado and Miller (1979, Fig. 5 legend; see also Labarca and Miller, 1981, Eq. 1; and Gray et al., 1985, Fig. 4 legend). The failure of the model to fit the data is not too surprising given the nature of its assumptions, particularly (*a*) its use of a theory best suited to describe gas phase chemical reactions (Eyring rate theory) to describe the voltage dependence of a diffusion like process in a liquid
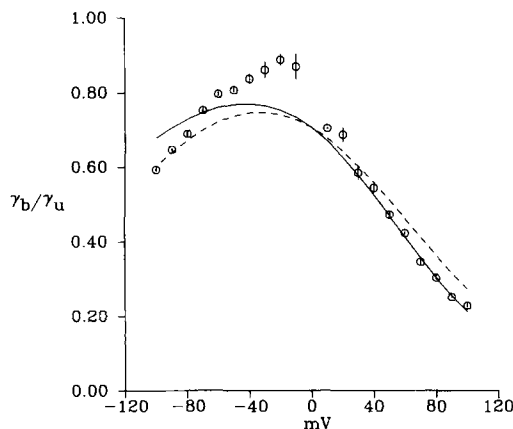

FIGURE 8. Blocker access from two sides. Data and conditions as described in caption to Fig. 7. The solid and dotted lines are drawn according to the following equation (Coronado and Miller, 1979, Fig. 7 legend; see also Labarca and Miller, 1981, Eq. 1; and Gray et al., 1985, Fig. 4) with δ values measured from just the cytoplasmic ($z\delta$ = 0.616, *solid line*) or just lumenal side ($z\delta$ = 0.532, *dotted line*) of the channel: γ_b/γ_u = $[1 + (B_c/K_{0c}) \exp (z\delta FV/RT) + (B_1/K_{01}) \exp (z(\delta - 1))FV/RT]^{-1}$, where γ_b/γ_u is the blocked conductance/control (unblocked) conductance

(200 pS); B_c and B_1 are concentration of the blocker on cytoplasmic and lumenal sides, respectively, K_{0c} and K_{01} are the zero-voltage dissociation constant of the blocker on cytoplasmic and lumenal sides, respectively, z is the valence of the blocker, δ is the fraction of the total voltage drop across the membrane at the site of block, and V is the membrane voltage. K_0 and $z\delta$ are obtained from the intercept on the ordinate and slope of the linearized form of the plot as in Fig. 7 *B*. Other parameters have the same definition as in the legend of Fig. 4.

(Cooper et al., 1988*a*); and (*b*) its assumption that the position of binding sites is voltage independent (see Fig. 2 of Cooper et al., 1988*b*).

DISCUSSION

We have developed a split fiber preparation of muscle allowing patch clamp of ionic channels of the SR membrane. The preparation allows measurements of channels embedded in a relatively intact SR membrane (i.e., on-SR), of excised channels, and it allows measurements of properties of the whole SR compartment. We are unaware of other preparations that allow all three types of measurements. The sarcoball preparation of Stein and Palade (1988), and Stein et al. (1989) involves the exposure of the sarcoplasm to *extracellular* concentrations of Ca^{2+}. Such concentra-

tions, thousands of times larger than physiological, could well modify channels, stimulate Ca^{2+} pumps, produce volume changes, or fuse membranes of organelles (e.g., SR, surface, and T membrane). Thus control experiments are needed to establish the state and origin of the channels observed in sarcoballs. Channels reconstituted into artificial bilayers would be expected to be modified, with greater disruption of accessory proteins than channels in the skinned lobster remotor preparation. Only the skinned remotor preparation allows measurements of current voltage relations and membrane potential of a compartment of the SR.

SR Preparation

It seems quite clear to us that the channels recorded reside in the membrane of the SR and not the outer membrane of the fiber. No sign of the outer membrane is seen in electron micrographs of split fibers. The outer membrane of the fiber is unlikely to survive a dissection procedure that yields a strip of the fiber, a remnant only a fraction of the diameter of the original fiber. Intact remotor fibers contain clefts of outer membrane and T tubules and it is just possible that gigaseals might occasionally be formed to these in split fibers, despite the overwhelming preponderance of SR membrane: it is always difficult to absolutely rule out unlikely events. We have been unable, however, to form gigaseals to the outer membrane of unskinned fibers bathed in relaxing saline, perhaps because the low concentration of Ca^{2+} destroys the outer membrane (Weisberg et al., 1983).

Formation of Gigaseals

The formation of gigaseals on SR almost certainly requires rearrangement of the normal SR structure of tiny tubules much smaller than the diameter of the patch pipette. We imagine that the SR membrane changes structure (Sommer et al., 1980a, b; Nassar et al., 1986) when the pipette touches the organelle, with its membrane lipid flowing into the pipette to form the mushroom shaped dome of the gigaseal (Hamill et al., 1981). Although little is known of the physics of the gigaseal, it seems safe to assume that the structure has a low free energy: if the structure were not exceedingly tight, i.e., energetically favored, it seems likely that more than a few million ions could traverse its short length (<2 μm) each second out of the $\sim 10^{16}$ in each μl of salt solution. Structures of low free energy tend to form spontaneously and so our guess is that gigaseals form whenever they can, whenever the barrier of activation energy permits rapid enough flow of membrane lipid into the stable dome structure of the gigaseal. The large driving force favoring gigaseal formation probably explains why gigaseals form readily on membranes of SR and on some outer membranes of cells seemingly surrounded by extracellular matrix. The membrane lipid may well evaginate between the fibers of the matrix, forming a bleb extending into the pipette, as it apparently does in big pipettes forming loose patches (Milton and Caldwell, 1986).

Comparison with Reconstituted Systems

The split remotor preparation has certain advantages over reconstituted systems. The preparation is more physiological in the sense that it has been less perturbed by preparative procedures. The proper orientation of the membranes is known. The

recording conditions are better than in most experiments on reconstituted preparations. The membrane area and thus capacitive shunt are much smaller, yielding a larger ratio $i_{channel}/i_{capacitance}$, a better signal to noise ratio and wider bandwidth. The preparation is rapid and easy to prepare (taking <1 h), once it is learned. Compared with reconstituted preparations, the split preparation has both the advantages and disadvantages of being impure, particularly in the on-SR configuration: cofactors needed for normal function are more likely to be present than in reconstituted preparations and so channels in the patch are more likely to function physiologically. For the same reason, however, cofactors are harder to identify in the patch. If they are already present, they do not have such dramatic effects when added back! The split preparation has the disadvantage, in our initial primitive setup, that solution changing is more awkward than in bilayer experiments, the gigaseal being sensitive, especially in the on-SR configuration, to disturbance in the bath. Improvements in technique should remove this problem (see Lapointe and Szabo, 1987); the excised patch should then be particularly helpful in unraveling cofactors controlling the open probability and (perhaps) open channel properties of SR channels.

SR K⁺ Channel

The lobster SR K⁺ channel has properties quite similar to those reported for the vertebrate channel (Coronado and Miller, 1979, 1980, 1982; Coronado et al., 1980; Labarca et al., 1980; Labarca and Miller, 1981; Miller, 1982*a, b, c*; Tomlins et al., 1984; Gray et al., 1985, Tomlins and Williams, 1986) but the lobster channel has quite different subconductance kinetics and responds differently to blockers. Decamethonium blocks without flicker; and hexamethonium, decamethonium, and succinyl choline block from both sides but more from the cytoplasmic side (data not shown). These differences may reflect a modification in channel properties in the patch clamp or reconstitution procedure but it seems more likely to us, given the evolutionary distance between lobsters and vertebrates, that the channels have somewhat different atomic structure.

Resting Potential and Input Resistance of the SR

We were gratified to find a negligible resting potential across the SR membrane, confirming the firm, but indirect conclusions of Somlyo et al (1981), Oetliker (1982), and Kitazawa et al. (1984). Our measurements of membrane resistance demonstrate directly that the preparation is *not* leaky; the 0-mV resting potential is a consequence of the ionic conductances of the preparation, not of a damaged membrane.

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