J. Membrane Biol. 141, 167–175 (1994)

The Journal of
Membrane
Biology

A Voltage-sensitive Cation Channel Present in Clusters in Lobster Skeletal Muscle Membrane

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Received: 3 January 1994/Revised: 30 March 1994

Abstract. The single channel properties of a voltage-sensitive cation channel are described in a study of ion channel activity in enzymatically induced blebs of lobster skeletal muscle membrane. This cation channel, one of several that are spontaneously active in excised patches from bleb membrane, can be distinguished from other channels on the basis of its large single channel conductance (293 pS), voltage-sensitive gating properties, the presence of a subconductance state of the fully open channel, and a weak selectivity for K > Na. At hyperpolarizing voltages, this channel displays flickering or bursting behavior, and a single state of the fully open channel is observed. At depolarizing voltages, the mean channel open time increases and a second longer-lived open state is observed. The voltage dependence of the mean channel open time and the linear *i-V* relation of this channel predict that the macroscopic current carried through this cation channel would be outwardly rectifying.

Channels of this type are infrequently observed in this preparation, but when present in the patch are often present in multiple copies. We describe a statistical test for examining the clustering of ion channels in excised patches of membrane. The result of this test shows that the cation channels appear in clusters in the blebs.

Key words: Ion channels — Lobster — Blebs — Clusters — Muscle

Introduction

Crustacean neural circuits and neuromuscular preparations are well-characterized systems in which the hormonal control of neural activity and behavioral output can be studied at the cellular level (Kravitz, 1988; Harris-Warrick & Marder, 1991; Marder, 1991; Heinzel, Weimann & Marder, 1993). In the lobster *Homarus americanus,* six hormonal substances have been found thus far that influence second messenger systems and/or the physiological activity of nerve-muscle preparations. These include the amines serotonin and octopamine, and the peptides proctolin (RYLPT), peptides F_1 and F_2 (TNRNFLRFamide and SDRNFLRFamide) and peptide G_1 (Evans et al., 1975, 1976; Batelle & Kravitz, 1978; Schwarz et al., 1980; Schwarz et al., 1984; Kravitz et al., 1980; Goy et al., 1987; Kobierski et al., 1987; Trimmer et al., 1987; Mercier, Schiebe & Atwood, 1990). The modulation of neural and muscle activity by these hemolymph-borne substances is an important component of behavioral plasticity in the lobster (Kravitz et al., 1985; Kravitz, 1988).

To establish the cellular mechanisms by which these hormones regulate muscle contractility, it is of interest to identify ion channels in the muscle sarcolemmal membrane that may serve as substrates for hormonal modulation. For this purpose we have used 'bleb' preparations from skeletal muscle, where a number of different ion channels were found (Worden, Rahamimoff and Kravitz 1993). These channels are active in the nominal absence of calcium, metabolites or cytoplasmic factors, and can be distinguished from each other on the basis of single channel conductance, degree of selectivity for K > Na, and gating properties. Other studies have described the single channel properties of glutamate channels (Franke, Hatt & Dudel, 1986; Hatt, Franke & Dudel, 1988), chloride channels (Zufall, Franke & Hatt, 1988, 1989) and calcium channels (Bishop, Krouse & Wine, et al., 1991*a,b*) recorded in cell-attached or excised membrane patches from crustacean skeletal muscle.

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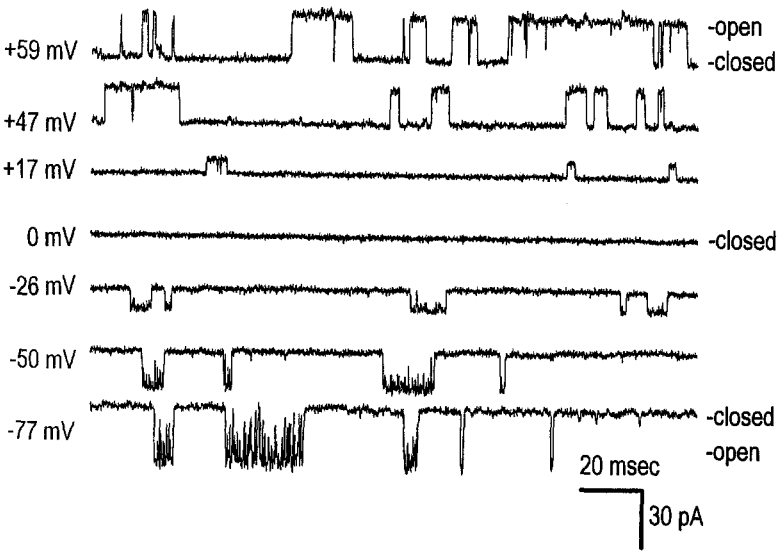

Fig. 1. Single channel currents recorded at a series of DC pipette potentials. The bath solution and pipette solutions are identical (462 mM KCl, 5 mM HEPES, pH 7.8). Traces have been selected to show frequent channel openings and examples of multiple openings, total P_o at all voltages is less than 0.05.

In this article we describe the single channel properties of a novel voltage-dependent cationic channel found in lobster muscle bleb membranes. This ionic channel type is encountered infrequently in excised patches from bleb membrane. However, when present in a patch, the channel is frequently present in multiple copies, indicating a possible clustering of identical channels. We report here the methodology used to confirm statistically that channels of this type occur in clusters in bleb membranes.

Materials and Methods

Single channel recordings from bleb membranes of the accessory flexor muscle of the walking leg of lobsters (*H. americanus*) were made as previously described (Worden et al., 1993). Briefly, muscles were dissected in physiological lobster saline which approximates the ionic composition of lobster hemolymph (mM: 462 NaCl, 16 KCl, 26 $CaCl_2$, 8 $MgCl_2$, 5 HEPES, 11 glucose, pH 7.4; 1,200 mOsmol). The muscles then were rinsed in a saline solution to which no calcium was added (mM: 462 NaCl, 16 KCl, 34 $MgCl_2$, 5 HEPES, 11 glucose, pH 7.4; 1,200 mOsmol) and transferred to an enzyme solution containing 100 units/ml collagenase (Type 1A; Sigma) in 462 mM KCl buffered with 5 mM HEPES (pH 7.8; 1,100 mOsmol). Treatment with the enzyme solution induced the formation of clear spherical blebs on the surface of the muscle fiber. Following bleb formation, the enzyme solution was rinsed and replaced with an enzyme free solution (462 mM KCl, 5 mM HEPES, pH 7.8).

Pipettes were coated with Sylgard and fire-polished, with typical pipette resistances ranging from 5–10 MΩ. The internal pipette solution contained 462 mM KCl, 5 mM HEPES, pH 7.8. Seals formed readily between the pipette tip and the surface of the bleb when negative pressure was applied to the pipette. Recordings of single channel activity were made from excised patches in an inside-out configuration as described by Hamill et al. (1981); the external face of the bleb was in contact with the internal pipette solution. Channel activity was observed in nearly all membrane patches when a depolarizing or hyperpolarizing potential was applied. Solutions were applied to the intracellular face of the patch using a horizontal array of microcapillary tubes as described by Friel and Bean (1988). Changes of solution were effected by moving the patch to the mouth of a different tube. Alterations in single channel activity occurred within a second with each change of solution, suggesting that the excised patches were open membranes and not closed vesicles.

Currents were recorded using a List EPC-7 amplifier, stored on tape (Hewlett-Packard, model 3964A) and subsequently digitized at a sampling rate of 42 kHz using a NEC computer. Data were filtered at 3 kHz and analyzed using the pCLAMP software (Axon Instruments, versions 4.0 to 5.5) to detect and analyze single channel openings.

The recordings analyzed in this study include eight patches in which the cation channel described below was active. In 78 other patches, the cation channel was not observed, but other types of channels were recorded, as reported previously (Worden et al., 1993).

Results

Basic Channel Properties

Figure 1 illustrates typical recordings of a large conductance channel that exhibits spontaneous activity in excised inside-out patches of lobster muscle bleb membranes under bi-ionic recording conditions (462 mM KCl in bath; 462 mM KCl in pipette). This channel, one of several that are recorded in skeletal muscle bleb membranes (Worden et al., 1993), can be distinguished from others by the large amplitude of single channel currents and the flickering or bursting behavior at hyperpolarizing voltages (*see* −77 mV, Fig. 1). The probability of the channel being in the open state is less than 5% of the total recording time at all voltages tested. The channel also shows evidence of a substate with a conductance approximately 75% of that of the fully open state (Fig. 2). The subconductance state of the channel was observed infrequently and has not been analyzed any further.

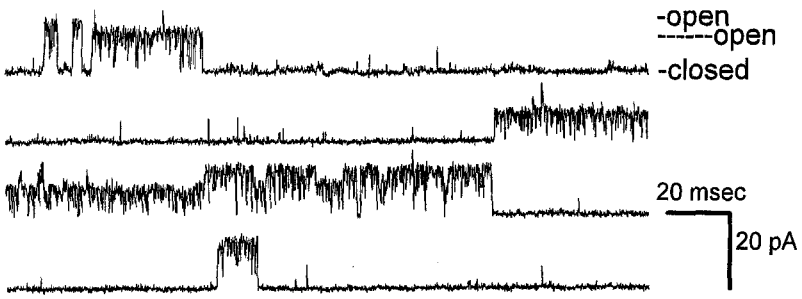

Fig. 2. Single channel current fluctuations showing a subconductance state of the open channel. Upward deflections of the trace show openings from a closed channel state to a noisy subconductance state as well as to the fully open state. The traces are sequential recordings at a $-V_{pipette}$ potential of +50 mV. Traces have been selected to show channel activity.

The large conductance cation channel is observed rarely in bleb-attached and excised membrane patches from lobster bleb membranes. In 86 excised patches with channel activity it was recorded 8 times, for a frequency of detection of about 10%. No obvious correlation was noted between channel detection and the time of year or the morphological region of the muscle fiber from which the patch was excised. In most experiments in which the channel was detected, multiple openings were observed, suggesting that these channels may be clustered in the membrane.

Single Channel Conductance

To estimate the single channel conductance, recordings of channel activity were made at a series of different DC potentials which were imposed on the membrane patch for a period of tens of seconds to minutes. The amplitudes of the single channel currents recorded at several pipette potentials are shown in Fig. 3, with the data at each holding potential being fit by a Gaussian distribution. Figure 3*E* illustrates the *i-V* relation for the channel shown in the experiment of Fig. 1. The conductance of the fully open channel was determined from the slope of the *i-V* relation to be 310 pS. The *i-V* profile of this channel is linear over the voltage range −80 to +60 mV. In eight experiments, the average conductance of the channel was 293.6 ± 20.5 pS (mean ± SEM).

Channel Selectivity

The channel has a reversal potential close to zero mV under symmetrical recording conditions where no ionic gradients exist (Fig. 3*E*). When the bath solution is exchanged for NaCl (462 mM), the *i-V* curve shifts to the right (Fig. 4), indicating that the channel is a cation channel with a weak selectivity for K > Na. In two experiments, the shift of the reversal potential measured +12.5 and +15 mV, with average P_K/P_{Na} of 1.72:1.

Channel activity is absent when cesium is substituted for potassium in the bath solution corresponding to the cytoplasmic face of the patch, suggesting that cesium does not permeate the channel (Fig. 5).

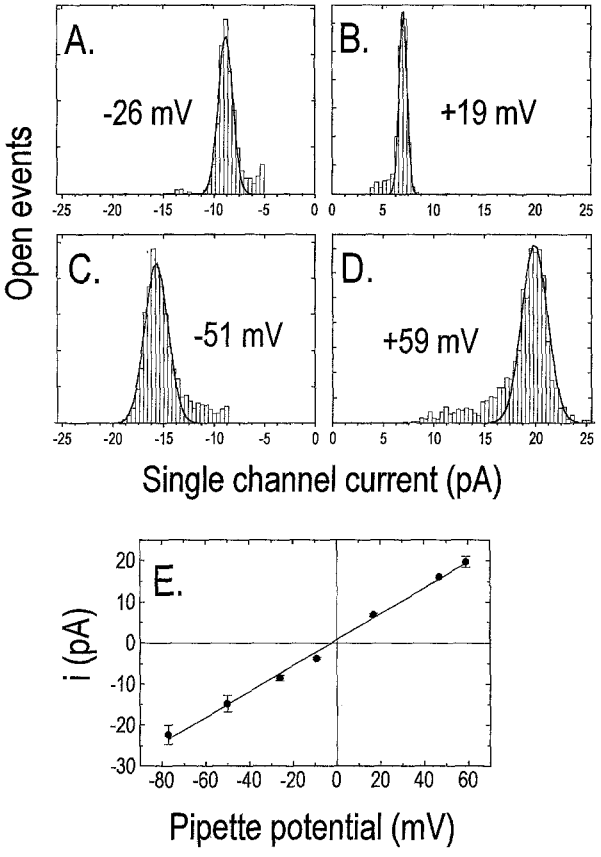

Fig. 3. (*A–D*) Measurement of the amplitudes of the mean single channel current (*i*) at a series of $-V_{pipette}$ potentials. The mean channel current amplitude is determined by a Gaussian fit to the data at each potential. (*E*) *i-V* relation for the bleb channel in symmetrical 462 mM KCl. All data points represent means (±SD) of single current amplitude measurements from >690 open events at each pipette potential. The reversal potential in symmetrical KCl is 0 mV. In some cases, the error bars are not larger than the symbol. (Data are from the experiment shown in Fig. 1).

Steady-State Gating Properties

The steady-state gating of this channel at maintained DC holding potentials shows a strong dependence on membrane potential. At hyperpolarizing potentials, the channel shows large open channel noise and frequent transitions to the closed state. These rapid transient closures

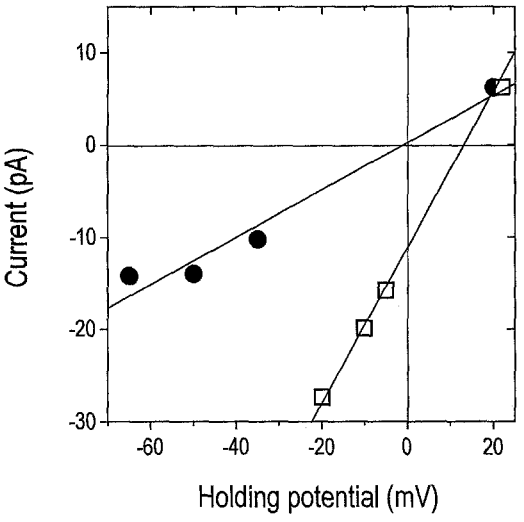

Fig. 4. *i-V* relation for a single channel recorded in bath solutions containing 462 mM KCL (circles) and 462 mM NaCl (squares). All data points represent the mean of single channel currents measured from >320 open events at each pipette potential. In the presence of NaCl, the reversal potential shifts to +15 mV.

have the appearance of bursting channel activity (*see* Fig. 1: −77 mV). The bursting activity becomes less pronounced as $V_{pipette}$ approaches zero mV and is not observed in the depolarizing voltage range. Since pipette potentials were imposed in a random order, the absence of bursting activity at depolarizing voltages is not due to progressive channel block (or unblock) over the course of the experiment. In addition, little time-dependent channel inactivation is observed at any pipette potential, the frequency of channel opening appears constant over a time period >25 sec at all membrane voltages. Figure 6 illustrates the frequency of channel opening during the initial 25 sec of steady-state recording at four pipette potentials.

Mean channel open times were determined by measuring the duration of channel opening each time the current exceeded 50% of the mean amplitude of the single channel current. Measurements of the mean channel open time (mcot) as a function of voltage are shown in Fig. 7*A*, and indicate that the open time of this channel is voltage dependent in the range of potentials from −80 to 0 mV, increasing from a mcot of less than 1 msec at −80 mV to a value approximately 2.5- to 4-fold greater at voltages more depolarized than zero mV. The mcot shows no voltage dependence in the depolarizing voltage range from 0 to +80 mV. The probability of finding the channel in the open state (P_o) at extreme hyperpolarizing voltages (−80 mV) is approximately twice as high as the probability of finding the channel open at voltages greater than 0 mV (Fig. 7*B*).

The higher probability of opening at hyperpolarized voltages is significant, given that the mean channel open time in this range is small (Fig. 7*A*). Since P_o depends on the mcot and on the number of channel openings, it is of interest to examine whether the frequency of channel openings depends on voltage. The frequency of opening over the entire recording period at each voltage is shown in Fig. 7*C*. The frequency of opening at the most hyperpolarized voltage is approximately five times as high as the opening frequency at all other voltages, and therefore contributes to the high probability of finding the channel in the open state at hyperpolarized potentials. The charge carried through the open channel is estimated from the product of the mcot and the single channel current (i) and is illustrated in Fig. 7*D*. Under the recording conditions in these experiments, the channel is similar to outwardly rectifying potassium channels in that the ionic flux through the open channel is larger over the depolarizing voltage range.

The flickering or bursting behavior of the channel at hyperpolarized voltages is a general feature of this ion channel type. In Fig. 8, the measurements of mean channel open times at a series of pipette potentials in four experiments are shown. The open time is brief and voltage dependent in the hyperpolarizing voltage range, and voltage independent in the voltage range from 0 to +80 mV.

Open States of the Channel

To examine whether the gating of the channel shows voltage dependence, the distribution of channel open times was analyzed as a function of voltage. Figure 9*A* and *B* illustrates the probability density functions for histograms of open times recorded at the extremes of the voltage range in this experiment. At $-V_{pipette} = -77$ mV (Fig. 9*A*), a single exponential term is sufficient to fit all of the data. In contrast, at $-V_{pipette} = +60$ mV (Fig. 9*B*), the data are better fit by two exponential terms and many longer openings are observed.

Two exponential terms were required to fit the data at all holding potentials, with the exception of the data at the extreme hyperpolarizing voltage (−77 mV). The time constants corresponding to the exponential fits of all open time data as a function of voltage are shown in Table 1. The data suggest that the channel has at least two open states, and that the probability of entering the longer-lived state is favored at depolarizing voltage potentials.

Analysis of channel closed times is complicated by the presence of more than one channel of this type in each recording. However, the channel clearly appears to have at least two closed states, corresponding to the closed state within a burst, and the closed states between bursts. The probability density histogram for channel closed states at $-V_{pipette}$ potential of −77 mV is shown

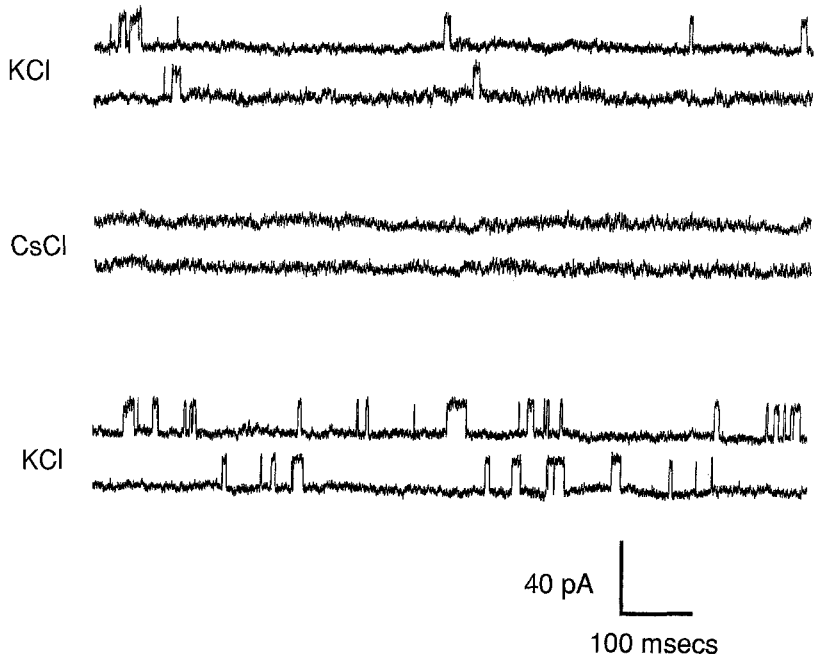

Fig. 5. Block of single channel openings by cesium. The top pair of traces show channel openings under recording conditions where the intracellular face of the patch is exposed to a bath solutions of KCl (462 mM); the middle pair of traces show no channel openings recorded from the same membrane patch when KCl has been replaced by equimolar CsCl. The bottom pair of traces show channel activity recorded from the same membrane patch when KCl bath solution is restored. All pairs of traces are sequential records at $-V_{pipette}$ potential of +35 mV. Traces have been selected to show channel activity.

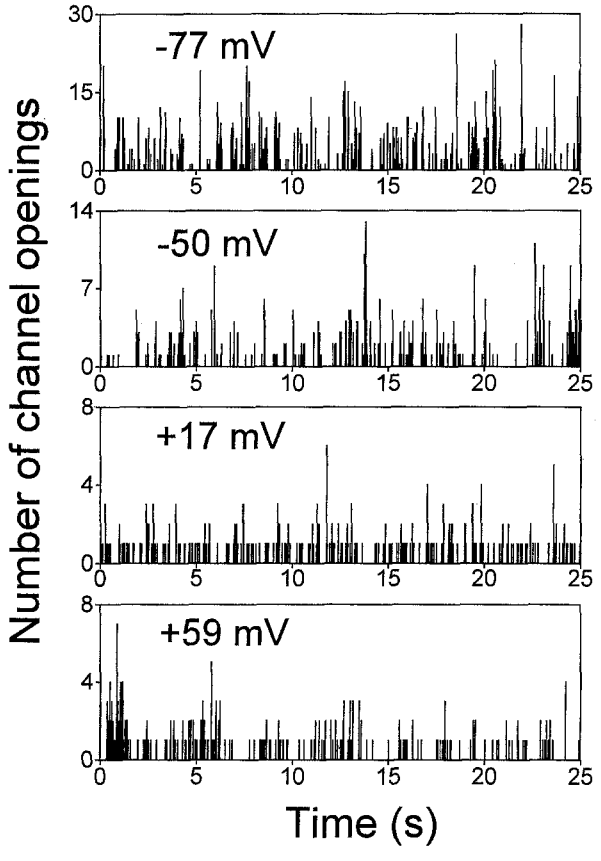

Fig. 6. Frequency of channel opening. The bin width is 0.02 sec. Two channels of the same type were present in this patch. Note that the Y axis is different in the upper two graphs.

in Fig. 10*A*. Most of the channel closures are brief (less than 0.5 sec; Fig. 10*B*), although long closures lasting tens of seconds also are observed.

Channel Clustering

The probability of observing single channel openings of this cationic channel is rare in two respects. First, openings of the channel were observed in only 8 of 86 patches in which single channel activity was detected. Second, when this channel is detected in a membrane patch, its openings occupy only a small fraction of the total recording time. However, when the channel is present, it usually is observed to be present in more than one copy. To quantitatively test for evidence that these cation channels are clustered in bleb membranes, we examined whether copies of this channel were distributed randomly among the membrane patches from which recordings were made. Because of the low probability of observing the channel in bleb membrane patches, we scored the number of cation channels observed in each experiment and examined whether the Poisson distribution accurately described the data. (If the rate of detection of the channel in different patches had been higher, the binomial distribution would have been more appropriate.)

In a Poisson distribution, the number of patches with x copies of the channel (N_x) would be given by

$$N_x = N_T[\exp(-m)m^x/x!]. \quad (1)$$

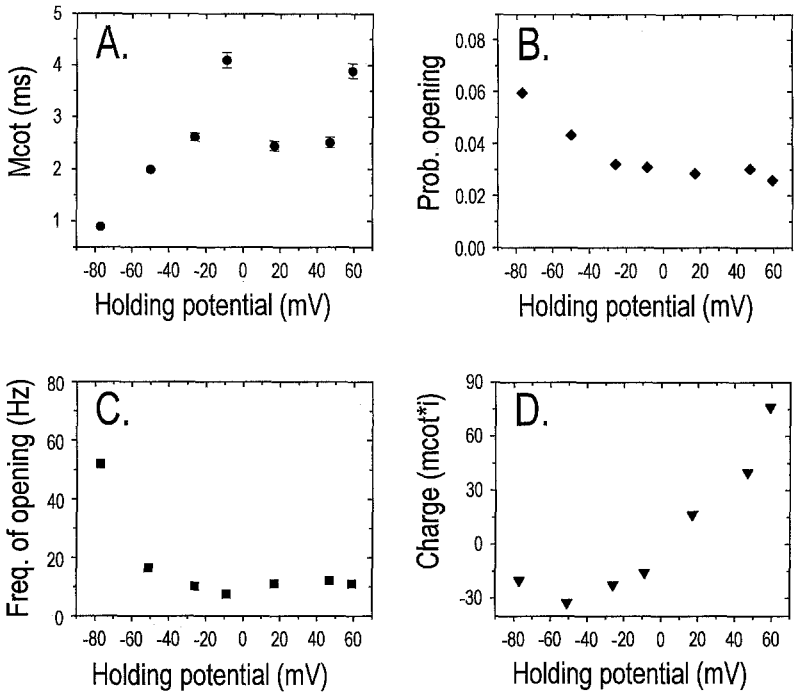

Fig. 7. Steady-state gating properties of the channel. (*A*) Mean channel open time (mean = ±SE) as a function of pipette potential. In some cases, the error bars are not larger than the symbols. Number of open events >690 at all potentials. (*B*) Probability of the channel being in the open state as a function of pipette potential. (*C*) Frequency of channel opening as a function of pipette potential. (*D*) Predicted charge carried through the open channel. Recording at each pipette potential exceeds 50 sec. Two channels of the same type were present in this patch.

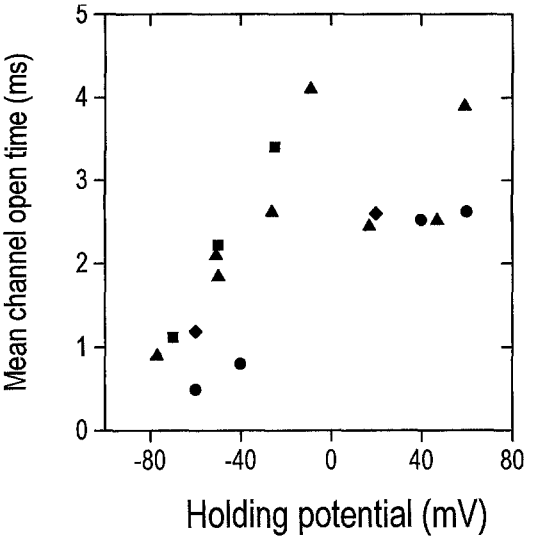

Fig. 8. Mean channel open times measured at different $-V_{\text{pipette}}$ potentials in four experiments. Each experiment is represented by a different symbol.

where N_T is the total number of successful patches from which recordings were made under experimental conditions that permit observation of these channels and m is the mean number of channel copies/patch. The notation is similar to that used for quantal analysis by del Castillo and Katz (1954) and to that used in the analysis of the bursting behavior of potassium channels at the presynaptic nerve terminal membrane (Edry-Schiller, Ginsburg & Rahamimoff, 1991).

The number of patches with no channels of this type N_0 will be given by

$$N_0 = N_T e^{-m}. \tag{2}$$

By knowing the total number of patches and the number of patches in which the channel does not appear (N_0), it is possible to estimate the expected mean number of copies/patch ($m = \ln(N_T/N_0)$). Since this channel has a low rate of opening even when present in a patch, a precaution must be taken to avoid "false failures." Therefore, a patch was scored as containing no cation channels only when no openings were observed and when the recording was of a sufficient duration for at least 300 openings of this channel to have occurred.

In 8 of the 86 patches recorded in this study at least one copy of the cation channel was observed. The other 78 patches were "failures" in which either the cation channel was absent or inactive (although in all cases, other types of ion channels were active). According to Eq. (2), $m = 0.0976$ channels/patch. The expected distribution of channels according to the Poisson equation and the experimentally observed distribution are given in Table 2. A chi-square test shows that the expected distribution of channels is significantly different from the observed distribution, indicating that copies of the cation channel are present in a nonrandom distribution among the membrane patches from which we recorded. In addition, the number of copies of the channel predicted by the Poisson equation ($n = 8$) is significantly different from the experimentally observed

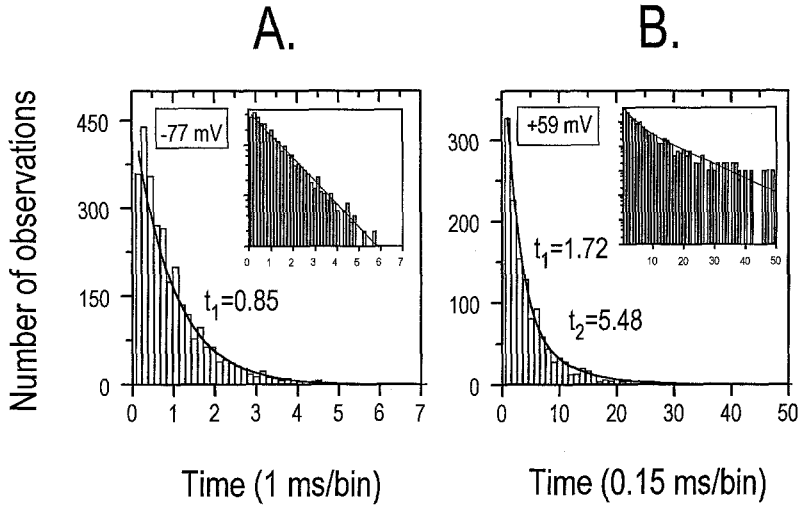

Fig. 9. Probability density histograms for channel open state. (*A*) Open events recorded at $-V_{pipette}$ potential of −77 mV. Data are fit by a single exponential term with a time constant of 0.85 msec (n = 2,885 open events). Inset shows the same data on log scale. (*B*) Open events recorded at +59 mV. Data are fit by the sum of two exponential terms with time constants 1.72 and 5.48 msec (n = 1,342 open events). Inset shows the same data on a log scale.

Table 1. Time constants of channel open state

$-V_{pipette}$ potential (mV)	tau1	tau2
−77	0.85	
−51	1.01	2.78
−26	0.68	3.37
17	1.38	3.22
47	0.78	3.25
59	1.72	5.48

Time constants were determined by fitting the histograms of open time distributions with exponential curvefits. Data were fit by two exponential terms at all pipette potentials with the exception of the data at −77 mV, where a single exponential term was sufficient.

number of channel copies (n = 15). These results support the conclusion that these cation channels are present in clusters in bleb membranes.

Discussion

The single channel characteristics of a cation-selective channel identified in bleb membranes from lobster skeletal muscle are reported in this communication. Like other channels described in these bleb membranes (Worden et al., 1993), the cation channel is active in excised patches at DC holding potentials in the nominal absence of calcium or second messengers. In addition, the analysis of channel activity over a range of voltages predicts that the macroscopic current carried through the cation channel would be outwardly rectifying. However, this channel differs from other potassium-selective channels in this preparation in five ways: the single channel conductance is large (293 pS); subconductance states of the open channel exist; the gating of the channel shows significant voltage dependence; the channel is weakly selective for potassium over sodium; and channels of this type appear to be clustered in bleb membrane.

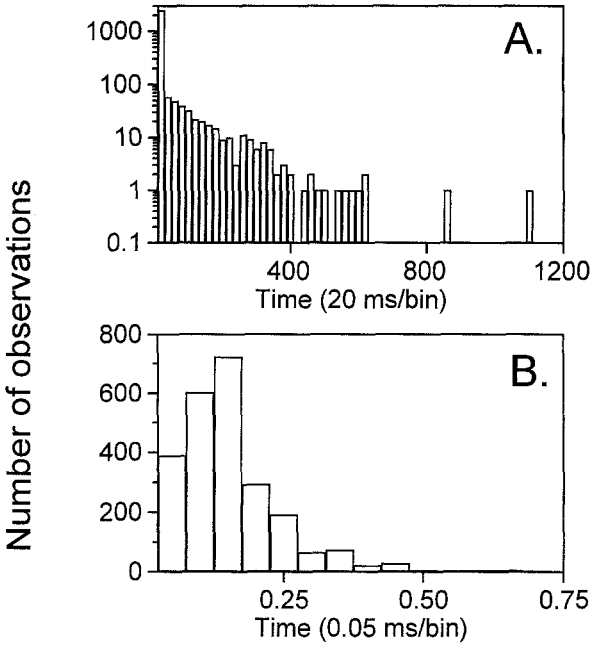

Fig. 10. (*A*) Probability density histogram for closed events at a $-V_{pipette}$ potential of −77 mV. Y axis is displayed on a log scale to enable visualization of longest events (n = 2,838 closed events). (*B*) Same histogram on a linear scale with expansion of x axis from 0–0.75 sec (n = 2,414 closed events).

A channel with similar characteristics has been described previously in excised patches of membrane from sarcoplasmic reticulum (SR) of skinned lobster remoter muscle (Tang, Wang & Eisenberg, 1989). The SR channel has a linear *i-V* relation with a single channel conductance of about 200 pS in symmetrical solutions of K-glutamate (480 mM). Like the channel described above, the SR channel is active in the absence of calcium (≤100 nM), is weakly selective for K > Na, shows a subconductance of the open state and has a probability of opening that depends on voltage. The main dif-

Table 2. Clustering of ion channels

Number of channel copies/patch	Expected	Observed
0	78	78
1	7.61	3
2	0.37	3
3	0.01	2
Total number of patches	85.99	86
Total number of channel copies	8	15

ference between the SR channel and the bleb membrane channel recorded in these studies is in the probability of being in the open state; P_o of the SR channel is substantially higher than that of the bleb channel at all voltages, and approaches 1.0 at the depolarizing end of the voltage range. Although mean channel open time measurements were not reported for the SR channel, the records shown in Tang et al. (1989; Figs. 2 and 5) suggest that the mean channel open time is in the range of seconds, approximately 500–1,000 times longer than that of the bleb channel. We cannot tell whether the differences between the two channel types suggest that these are different channels, or whether the SR and the bleb channel are a single molecular species bearing different structural or regulatory modifications.

The similarities between the SR channel and the channel described in this report raise the question of whether bleb membranes originate, in part, from the sarcoplasmic reticulum and T-tubule system which underly the sarcolemma. Our previous study of channels in bleb membranes presented evidence that the outside of the bleb membranes corresponds to the external face of the sarcolemma, and that the interior of the blebs is in communication with the cytoplasm of the muscle fiber during the period of bleb formation. This does not exclude the possibility that the sarcoplasmic reticulum and T-tubule membranes might fuse with the sarcolemmal membrane during the formation of blebs, with the luminal side of the sarcoplasmic reticulum membrane eventually forming part of the exterior surface of the blebs. One possibility is that the cation channels appear in bleb membranes infrequently because they originate from SR membranes and have a low probability of migrating into the bleb membrane during the period of bleb formation.

The cationic channel described in this report also resembles the nonselective cation channel activated by patch excision from lobster olfactory receptor neurons (McClintock & Ache, 1990). Like the bleb cation channel, the olfactory receptor channel has a linear *i-V* relation with a large single channel conductance (320 pS), shows steady-state activity under DC voltage conditions, is weakly voltage dependent, and is poorly selective for monovalent cations. In addition, the olfactory cation channel is highly permeable to external divalent cations and blocked by internal millimolar concentrations of magnesium. However, openings of the olfactory cation channel in excised patches were observed at a much greater frequency than that seen with the muscle bleb channel. Under almost identical experimental conditions, the olfactory channel was observed frequently in patches excised from olfactory receptor cell somata. At depolarizing voltages (+40 mV), the olfactory channel had a probability of opening of nearly 1, and a mean channel open time of 25 msec. In comparison, the probability of opening of the muscle bleb cation channel was a factor of 50 lower than that of the olfactory cation channel, and the mean channel open time was sixfold smaller. Since both studies were performed on excised patches of membrane under similar ionic conditions, it is unlikely that the differences in P_o of these two channels can be attributed to differences in regulation of the channels by soluble factors. It remains possible, however, that enzymatic induction of bleb formation alters channel activity. Alternatively, the muscle bleb channel may be a distinct form of a weakly selective cation channel present in crustacean olfactory receptors and muscles and in molluscan neurons (Chesnoy-Marchais, 1985).

A Poisson test of ion channel distribution suggests that the cation channels are not randomly distributed in membranes: instead, this type of channel occurs in clusters in bleb membrane. The observation that single kinds of ion channels might aggregate in clusters has been reported in other studies in which loose patch clamp recordings were used to assay the regional distribution of channels (Roberts, Jacobs & Hudspeth, 1990; Frosch & Dichter, 1992). The mechanism by which channel clusters are maintained in bleb membranes is unknown: one possibility is that a cortical cytoskeleton on the interior surface of the bleb membrane holds the channels in place; a second is that a multivalent extracellular molecule might link the channels in clusters; a third is that the clusters represent small islands of contaminating SR membrane within the patches.

In summary, the cation channel described in this study is one of several ion channels that are present in bleb membranes prepared from lobster skeletal muscle. Under physiological conditions, this channel probably conducts outward current and may serve a role in repolarizing the membrane potential. It is important to note that the properties of the channels recorded in lobster membrane blebs may differ from those in the native membrane due to enzymatic treatment and long exposure to solutions nominally free of divalent cations. However, the variety of single channel conductances and gating behaviors detected in the bleb membranes suggests that a diverse family of potassium-conducting channels is present in intact muscle membranes in lob-

ster. Further studies of how the single channel types relate to macroscopic currents should help clarify the physiological roles served by these channels in the regulation of muscle contractility.

The authors gratefully acknowledge the technical assistance of Ms. Nicole Cherbuliez. M.K.W. was the recipient of an NRSA (5F32NS08605) and a Grass Foundation Fellowship (Marine Biological Laboratory, Woods Hole). This investigation was supported by a Program Project Grant (NS25915; E.A.K., Principal Investigator) and by grants from the US–Israel Binational Science Foundation and Muscular Dystrophy Association.

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