Effects of D-600 on Intramembrane Charge Movement of Polarized and Depolarized Frog Muscle Fibers

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ABSTRACT Intramembrane charge movement has been measured in frog cut skeletal muscle fibers using the triple vaseline gap voltage-clamp technique. Ionic currents were reduced using an external solution prepared with tetraethylammonium to block potassium currents, and O sodium + tetrodotoxin to abolish sodium currents. The internal solution contained 10 mM EGTA to prevent contractions. Both the internal and external solutions were prepared with impermeant anions. Linear capacitive currents were subtracted using the P-P/4 procedure, with the control pulses being subtracted either at very negative potentials, for the case of polarized fibers, or at positive potentials, for the case of depolarized fibers. In 63 polarized fibers dissected from *Rana pipiens* or *Leptodactylus insularis* frogs the following values were obtained for charge movement parameters: Q_{max} = 39 nC/μF, $\bar{V}$ = 36 mV, k = 18.5 mV. After depolarization we found that the total amount of movable charge was not appreciably reduced, while the voltage sensitivity was much changed. For 10 fibers, in which charge movement was measured at −100 and at 0 mV, Q_{max} changed from 46 to 41 nC/μF, while $\bar{V}$ changed from −41 to −103 mV and k changed from 20.5 to 30 mV. Thus membrane depolarization to 0 mV produces a shift of >50 mV in the Q-V relationship and a decrease of the slope. Membrane depolarization to −20 and −30 mV, caused a smaller shift of the Q-V relationship. In normally polarized fibers addition of D-600 at concentrations of 50–100 μM, does not cause important changes in charge movement parameters. However, the drug appears to have a use-dependent effect after depolarization. Thus in depolarized fibers, total charge is reduced by ~20%. D-600 causes no further changes in the voltage sensitivity of charge movement in fibers depolarized to 0 mV, while in fibers depolarized to −20 and −30 mV it causes the same effects as that obtained with depolarization to 0 mV. These results are compatible with the idea that after depolarization charge 1 is transformed into charge 2. D-600 appears to favor the conversion of charge 1 into charge 2. Since D-600 also favors contractile inactivation, charge 2 could represent the state of the voltage sensor for excitation-contraction coupling in the inactivated state.

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INTRODUCTION

Intramembrane charge movement in skeletal muscle fibers is thought to represent the coupling mechanism between the T tubule membrane depolarization and calcium release from the sarcoplasmic reticulum (SR) during contractile activation (Schneider and Chandler, 1973). Since the initial proposal for such a role, based mainly on the similarity of the voltage dependency of charge movement (Schneider and Chandler, 1973; Adrian and Almers, 1976; Chandler et al., 1976*a*), the similarity of its reduction by sustained depolarization and glycerol treatment, and of its repriming characteristics (Adrian et al., 1976; Chandler et al., 1976*b*) with corresponding contractile parameters, this hypothesis has been enriched by measurements of charge movement at contractile threshold (Horowicz and Schneider, 1981*b*), simultaneous measurements of charge movement and calcium signals (Rakowski et al., 1985; Melzer et al., 1986), and pharmacological studies of charge movement (Huang, 1982, 1986; Hui, 1983; Vergara and Caputo, 1983).

Recently, some authors have focused their attention on the pharmacology of charge movement in depolarized fibers. When a muscle fiber is depolarized for a prolonged period, it enters a state of contractile refractoriness or inactivation (Hodgkin and Horowicz, 1960), and the intramembrane charge that normally moves in the potential range between -100 and $+40$ mV, and that has been associated with contractile activation, is reduced or abolished (charge 1 inactivation or immobilization) (Adrian and Almers, 1976; Chandler et al., 1976*b*; Rakowski, 1981). A second type of charge, charge 2 has been observed in depolarized fibers, with a different voltage sensitivity, moving at hyperpolarized membrane potentials (Adrian and Almers, 1976). Recently Brum and Rios (1987) have studied this charge and have proposed that in depolarized fibers charge 1 is transformed into charge 2, and that this interconversion could be associated with the processes of contractile inactivation and repriming.

Since the work of Eisenberg et al. (1983), who showed that D-600, a well known calcium antagonist, causes muscle fiber paralysis after a full-sized K contracture has been induced, numerous other works have appeared confirming that this drug maintains the fiber in a state of contractile refractoriness or inactivation, probably by interfering with the repriming process (Berwe et al., 1987; Caputo and Bolaños, 1987). Recently it has been reported that D-600 may also interfere with charge movement. Hui et al. (1984) and Hui and Milton (1987) have reported that D-600 suppresses charge movement after prolonged depolarization, while Melzer and Pohl (1987) found that D-600 increases charge 2 in polarized fibers. Furthermore, Rios and Brum (1987) have reported that nifedipine, another calcium antagonist, reduces charge movement in depolarized fibers. All these results point to an involvement of calcium channel–like dihydropyridine receptors in excitation-contraction coupling (ECC), with the suggestion of Brum and Rios (1987) that these receptors could act as voltage sensors for ECC.

In the present work we have studied charge movement in polarized and depolarized fibers, and the effects of D-600 on charge movement under these conditions, to complement the information existing on the effects of this compound on contractile inactivation and repriming parameters (Berwe et al., 1987; Caputo and Bolaños, 1987). We have used the triple vaseline gap voltage-clamp technique with

the cut muscle fiber preparation as originally developed by Hille and Campbell (1976) to study charge movement in muscle fibers from *Rana pipiens* and *Leptodactylus insularis*. Our results are consistent with the idea that when a muscle fiber is depolarized charge movement changes its voltage sensitivity, in agreement with the conclusions of Brum and Rios (1987), and with the previous findings of Bezanilla et al., (1982*a*), who studied the effect of depolarization on the sodium gating currents in squid axons. Furthermore we report that in the presence of D-600, charge movement is diminished more in depolarized than polarized fibers, but while in the latter case, the voltage sensitivity is not affected, in partially depolarized fibers the voltage sensitivity may be further changed. Short communications of these results have been published (Caputo and Bolaños, 1988*a*, *b*).

METHODS

The triple vaseline gap voltage-clamp technique (Hille and Campbell, 1976) as modified by Vergara et al. (1978), was used to study charge movement in single skeletal muscle fiber segments, dissected from the semitendinosus muscle of either *R. pipiens* or *L. insularis*. *R. pipiens* specimens were obtained either from West Jersey Biological Supply (Wenonah, NJ) (leopard frogs) or from Carolina Biological Supply Co., Burlington, NC (grass frogs). *Leptodactylus* specimens were obtained locally. Intact single fibers were dissected in normal Ringer's solution, whose composition was in millimolar: 115 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 10 buffer Tris pH 7.3. After dissection, one tendon of the fiber was pinned in a small trough lined with Sylgard (Dow Corning Corp., Midland, MI), while the other tendon was held with a dissecting tweezer at slack length, and the loading solution (see Table I) was flushed in the trough, causing a contracture that could be observed under the dissecting microscope. After relaxation, which occurred within few seconds after the solution change, the fiber was transferred to the experimental chamber flooded with the loading solution, and positioned across the chamber partitions, which had been previously lightly coated with Glisseal vacuum grease (Borer Chemie AG, Switzerland; which was a kind gift of Dr. Mike Delay, UCLA, CA).

The experimental chamber was similar to that described by Vergara et al. (1978). A detailed illustration of the chamber has been presented by Bezanilla et al. (1982*b*). The same reference provides details regarding the voltage-clamp circuit that we have used in this work, and which is schematically shown in Fig. 1, together with the fiber position on the partitions in the experimental chamber. After positioning the fiber, Glisseal seals were placed on top of the fiber at the level of partitions *1*, *2*, and *3*, the electrodes were lowered into the corresponding compartments *E*, *A*, *B*, and *C* (following the nomenclature used by Hille and Campbell, 1976), and then the fluid level was lowered. The electrode assembly consisted of a lucite board with golden pins that were plugged directly into the clamp circuit and connected with silver wire to sintered silver-silver chloride pellets housed in short glass bridges filled with 1% agar in 0.5 M KCl. The cooling device was turned on, to a temperature ~10°C, and the loading solution in pools *E* and *C* was replaced by an internal solution that contained EGTA to prevent contraction and whose composition is shown in Table I. After this the fiber segments in these pools were cut. Normally the fiber stump in pool *E* was 500–800 μm, while that in pool *C* was 200–400 μm. The *A* pool gap width was 150–220 μm and the *B* pool gap was ~300 μm. With the Glisseal threads in place, the width of the partitions separating pools *E* and *A*, *A* and *B*, and *B* and *C* were ~150 μm wide.

After cutting the fiber, it was left to rest for at least 30 min, after which the amplifier A_I was balanced and its gain was raised to maximum and the solution in pool *A* was exchanged with a Ringer solution containing 40 mM sodium (see Table I), this caused the fiber to repolarize between −30 and −60 mV. In some cases normal Ringer solution was placed in pool *A*

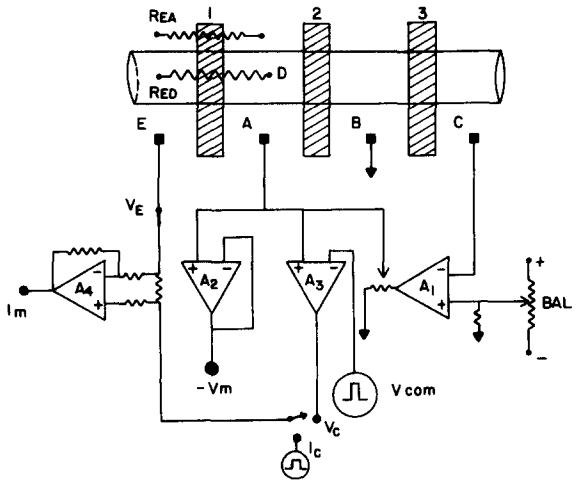

FIGURE 1. Schematic diagram of the cut fiber positioned across the triple vaseline gap chamber. The three vaseline seals, *1*, *2*, and *3*, separate the pools *E*, *A*, *B*, and *C*, which are connected with Ag-AgCl electrodes to the voltage-clamp circuit, which is also shown in a schematic way. See text for further details.

to record membrane action potentials under current-clamp conditions. After the resting period the voltage clamp was turned on and passive electrical properties of the fiber were determined. The relationship between the peak sodium current and the membrane potential, as well as the sodium conductance inactivation curve, were obtained so that an estimate of the holding potential could be determined for each fiber (Adrian et al., 1970; Vergara et al., 1978). Microelectrode measurements of a few fibers confirmed that the estimates thus obtained were within a few millivolts (<4) of the measured membrane potentials. Membrane current was measured as the voltage drop across the resistor (3 kΩ) in series with the electrode in pool *E*. This current represents the total current output of amplifier A_3 and deviates from the current through the membrane, if the resistance across seal *1* between pools *E* and *A* (R_{EA}) allows the flow of substantial amounts of leakage current. This happens if the value of the resistance R_{EA} is not much larger than the value of the internal resistance of the fiber segment R_{ED}.

TABLE I

Ionic Composition of Solutions

Internal solutions	Na	K	Cs	Mg	Tris	Aspartate	SO_4	ATP	EGTA
1. 10EGTA-K	10	120	—	3	10	120*	3	5	10
2. 10EGTA-Cs	10	—	120	3	10	120*	3	5	10

External solutions	Na	K	Ca	Rb	Mg	TEA	NMG	Tris	Cl	SO_4	Aspartate	HEPES	MOPS	Glucose
3. Loading	—	140	1	—	5	—	—	10	12	—	140*	10	—	—
4. Ringer 40 Na	40	2.5	1.8	—	—	—	100	—	46.1	—	—	—	100	—
5. TEA-SO_4, TTX‡	—	—	8	6	5	145§	—	—	—	86§	—	—	5	20

TEA, tetraethylammonium ion; NMG, *N*-methyl-D-glucamine; EGTA, ethylene glycol-bis-(β-aminoethyl ether) *N,N'*-tetraacetic acid; MOPS, morpholinopropanesulfonate; TTX, tetrodotoxin. All concentrations are in millimolar, pH adjusted to 7.2, 245 mosmol.

*The concentration of aspartate given is only a nominal value. Potassium or cesium aspartate was made by neutralization to pH 7.2 of known amounts of KOH or CsOH with D-aspartic acid.

‡TTX was added to a final concentration of 10^{-7} M.

§TEA sulfate was made by neutralization to pH 7.2 of known amounts of TEA hydroxide with sulfuric acid. Care was taken to keep solutions ice cold during neutralization.

The seal resistance R_{EA} was measured in several preparations (following the procedure described by Hille and Campbell, 1976) by eliciting a membrane action potential with the fiber under current-clamp conditions, with the cut extremes in the relaxing solution and the middle pools containing normal Ringer's solution. In five fibers thus tested, the value of R_{EA} ranged between 2.5 and 7 MΩ and was 25- to 30-fold larger than the value of R_{ED}. With some fibers exposed to the 40 mM Na Ringer solution, measurements were taken also of the potential value in pool E and V_E to calculate the value of the ratio V_E/I_E, which represents the parallel value of resistors R_{ED} and R_{EA}. Since independent measurements of R_{EA} gave values between 2.5 and 7.5 MΩ, the mean value of V_E/I_E of 152 kΩ represents approximately the value of the resistance R_{ED}, which is in good agreement with the value of 132 kΩ obtained by Hille and Campbell (1976).

The current (which was supposedly linear) that passed through the resistor R_{EA}, i.e., through the vaseline seal between pools E and A, was linearly subtracted, together with the linear leakage current that passed through the fiber membrane in pool A and the linear capacitive current, by the leak-capacitor subtractor and by the P/4 procedure. Other control experiments were carried out with microelectrodes in the chamber or in the fiber in pool A under voltage-clamp conditions. In these tests the electrode corresponded to the clamp potential within 96% of the applied pulse when it was in the chamber and within 93% when it was in the fiber, in these latter cases the reference electrode was in pool A. These tests were not routinely carried out with each fiber, since with practice it became evident that with poor seals charge movement signals could not be recorded. In some experiments the current was measured by recording the voltage in pool E and calculating the voltage drop across the R_{ED} resistance. However, in the absence of routine independent measurements of R_{EA} and R_{ED} this method depends on the accuracy of the fiber dimension measurements, which could introduce appreciable errors.

This led us to choose an alternate measurement of I_m. I_m current was fed to one differential input of a leak-capacitor subtractor, and from this to the input of a preamplifier (model 113; EG&G PARC, Princeton, NJ) with the high filter roll-off set at 3 kHz. The output of the amplifier was filtered again with a four-pole Bessel filter set at 2 kHz, and then fed to the input of the data acquisition system, which was part of the data acquisition, pulse generation, and analysis UCLA-WAD (waveform generation acquisition and display) system designed by Professor Bezanilla and now commercially available through Axon Instruments Inc. (Burlingame, CA). In the system used in this work the A/D converter was an Analogic ultralinear MP2735 15-bit converter (Analogic, Wakefield, MA), while the D/A converter was a Datel fast 12-bit deglitched model DAC-DG12B2 converter (Datel Systems Inc., Mansfield, MA). With our system signals could be sampled at 7-μs intervals with 1,200 samples per record. The subtracted traces were stored in diskettes for later analysis. The UCLA-WAD analysis program allows you to subtract different traces, subtract the baseline, fit the baseline, integrate between positioned cursors, etc. The results of these operations could be displayed on the oscilloscope.

The analysis of each trace was done by first subtracting the baseline: this was done by fitting a straight line to the last points of the trace, before the OFF transient, and when necessary forcing it to be a straight line with no slope. The charge was computed as the numerical integral 20–30 ms of the current trace. Membrane resistance and total membrane capacitance were measured by applying hyperpolarizing pulses and then by measuring the steady current and the time integral of the ON and OFF capacitive transients. For each fiber the measurements were carried out first with 40 Na Ringer and later with the tetraethylammonium (TEA) sulfate solution in the A pool. The mean values for membrane resistance (R_m) and membrane capacity (C_m) obtained in the TEA sulfate solution are shown in Table II. They were considerably larger than those obtained in the 40 Na solution.

TABLE II

Charge Movement Parameters of Muscle Fibers

A: Rana pipiens

	ϕ	R_m	C_m	k	Q_{max}	$\bar{V}$
	μm	$K\Omega \cdot cm^2$	$\mu F/cm^2$	mV	$nc/\mu F$	mV
OC0968	100	2.74	11.0	16.8	22	−36
OC156A	100	1.31	14.0	18.0	22	−20
OC1768	100	2.21	13.0	18.0	29	−40
OC276A	110	2.51	12.0	19.1	41	−25
OC286B	90	4.67	7.0	15.7	33	−40
OC296B	80	3.16	7.5	15.8	28	−34
OC296C	90	2.21	14.6	14.6	48	−34
NV106B	110	1.83	15.3	15.7	41	−41
NV256A	100	1.47	10.7	18.0	44	−32
EN127A	110	1.25	12.6	19.1	32	−40
EN137A	110	1.45	17.6	13.5	24	−49
FE207B	100	1.55	16.8	16.2	34	−26
FE237A	90	1.34	14.4	19.7	56	−20
FE237B	100	1.96	10.0	17.2	38	−35
FE237C	90	1.62	13.5	20.3	52	−22
FE257B1	100	1.15	15.0	20.3	46	−28
FE257B2	100	1.24	11.3	15.8	42	−38
FE277A	80	1.50	15.3	16.9	25	−40
FE277B	100	1.93	9.3	16.9	27	−48
AB037B	110	3.73	13.0	19.1	26	−30
AB067A	100	1.92	11.0	18.0	39	−33
AB077A	90	1.74	7.0	15.3	39	−39
AB077B	100	2.27	7.0	15.7	42	−38
AB097A	90	2.42	12.2	15.8	38	−34
AG057B	90	3.30	12.3	22.5	53	−34
AG067B	110	2.40	12.2	19.7	45	−42
AG177A	90	3.04	12.7	19.7	36	−36
AG187A	100	1.15	13.9	22.5	60	−35
AG197A	90	2.30	12.6	22.5	56	−35
SE027A	110	1.40	15.0	20.8	49	−45
SE027B	90	3.03	11.8	17.4	27	−58
SE027C	100	1.30	16.0	27.0	44	−45
OC217B	90	5.24	7.6	22.5	32	−32
OC227A	100	3.62	6.3	18.0	40	−38
DC047A	80	2.41	7.5	26.6	29	−46
$\bar{X}$	97.14	2.24	11.97	18.6	38.3	−36.1
± SE	1.51	0.17	0.52	0.5	1.8	1.4

The increase in membrane capacity is most probably due to improved potential control at the level of the T tubules caused by the increase in membrane resistance. The survival time of the fibers used in these experiments was not determined for each fiber. However, in 13 fibers the experimental period was 75 ± 5.3 min (mean ± SEM). During this period no deterioration signs were detected; the value of the R_m, C_m, and other parameters, as well as the fiber appearance, remained unchanged. In some experiments the addition of compounds like lanthanum, cadmium, or cobalt, caused no further increase, indicating that the length constant of the T tubule membrane was sufficiently large that it was able to make most of the fiber

TABLE II

Charge Movement Parameters of Muscle Fibers
B: Leptodactylus insularis

	ϕ	R_m	C_m	k	Q_{max}	$\bar{V}$
	μm	$K\Omega \cdot cm^2$	$\mu F/cm^2$	mV	$nc/\mu F$	mV
SE186A	120	1.21	18.3	15.8	46	−36
SE196A	120	1.96	13.6	18.1	35	−28
SE236A	90	2.3	11.7	16.8	55	−30
SE296B	110	2.1	12.0	20.3	39	−35
OC016A	100	1.3	12.0	18.0	25	−34
OC026A	130	1.52	16.0	21.4	37	−28
OC026B	120	1.77	15.0	20.3	25	−34
OC066A	120	1.24	15.0	18.6	55	−31
OC106A	130	1.39	13.0	19.1	34	−30
DC096A	120	1.57	18.0	14.0	26	−45
DC096B	90	2.74	9.0	14.1	30	−54
DC096C	100	2.92	12.0	18.0	40	−40
DC106B	110	1.25	14.0	16.9	45	−48
DC106C	120	1.77	12.5	15.8	33	−50
OC166B	120	1.40	15.6	16.8	38	−42
EN297A	100	1.85	13.0	18.6	33	−44
AB147B	120	1.20	18.3	18.0	48	−28
MY127B	90	1.84	10.9	20.0	48	−26
MY207A	120	1.92	10.3	15.7	40	−38
MY217A	120	1.10	15.0	18.0	37	−36
JN027A	100	1.79	8.1	20.3	50	−26
OC307C	100	2.52	14.2	15.7	49	−50
NV047B	100	1.20	15.5	25.3	62	−25
NV047C	90	2.43	9.6	20.8	46	−38
NV057A	90	2.60	12.6	19.1	46	−32
NV057B	95	1.10	15.4	18.5	51	−42
NV057C	100	2.0	16.7	18.0	32	−53
DC097A	120	1.97	12.7	23.4	66	−21
$\bar{X}$	108.8	1.78	13.6	18.4	41.8	−36.6
± SE	2.5	0.10	0.5	0.5	2.0	1.7

tubule membranes electrically accessible. We did not record the holding current during the experiments; however, the value of this parameter was always <0.15 μA at the moment when the fibers were clamped in the 40 Na solution; in the TEA sulfate solution it was reduced by about half. The holding current value did not change during the experiment and when it started changing it usually did so conspicuously and irreversibly, signifying the end of the experiment.

RESULTS

Intramembrane Charge Movement in Polarized Fibers

To measure charge movement in normally polarized fibers, the P-P/-4 procedure was followed (Bezanilla et al., 1982*a*), with the pulses being subtracted at hyperpolarized membrane potential values (subtracting holding potential, SH). P/-4 indi-

cates that the subtracting pulses were given in the opposite direction to the test pulse, when the SH was negative with respect to the holding potential, while P/4 means that the subtracting pulses were given in the same direction as the test pulse, when the SH was positive with respect to the holding potential (Bezanilla et al., 1982*a*).

Fig. 2 *A* shows intramembrane charge movement records obtained at different membrane potential values from a holding potential of −100 mV, using a SH value

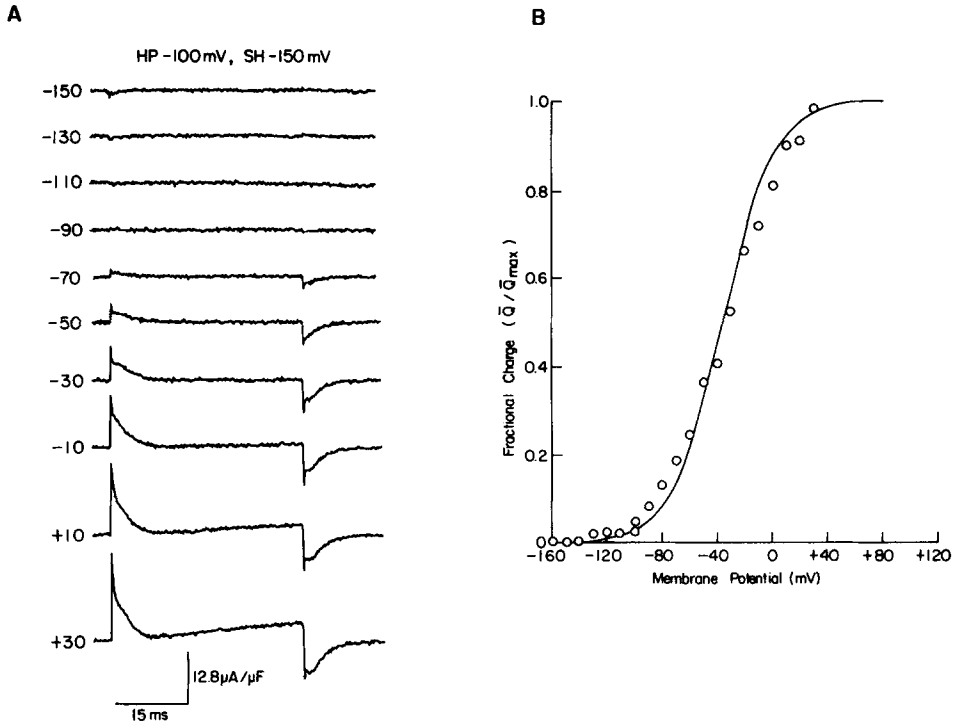

FIGURE 2. Potential dependence of charge movement. *A* shows charge movement records obtained with one fiber from *R. pipiens* (NV047C) held at −100 mV and depolarized with 40-ms pulses to the potentials indicated at the left of each record. The protocol of the pulse sequence for this and all other experiments appears in the inset of Fig. 3. In *B* the normalized averages of the ON and OFF signals from the same experiment have been plotted against the membrane potential value. The continuous curve was calculated from Eq. 1 using the mean values of $\bar{V} = -36.1$ and $k = 18.6$ mV, obtained for *R. pipiens* and shown in Table II.

of −150 mV and the P/-4 procedure. It seems important to notice that small but sizeable charge movement occurs even at hyperpolarized potential values. For the case of this experiment, some residual outward ionic current was observed at positive potentials. In some other cases inward currents were visible, which could be blocked by cobalt. In many cases no residual currents were present and the ON and OFF signals differed by <10%. In the records of Fig. 2 *A* a fast spike-like component appears in the ON transients probably associated with sodium gating currents, as

shall be discussed later. In the graph of Fig. 2 *B* the ON and OFF average value of the time integrals of the nonlinear currents are normalized and plotted against the membrane potential; the continuous line was calculated using the equation for a two-state Boltzmann distribution model (Schneider and Chandler, 1973):

$$Q/Q_{max} = 1/[1 + \exp -(V - \bar{V})/k], \quad (1)$$

in which Q is the charge moved at a given potential V, Q_{max} is the maximum charge moved, $\bar{V}$ is the voltage at which 50% of the charge is moved, and $1/k$ is the steepness factor of the Q-V relationship, with $k = RT/AZF$ (Chandler et al., 1976*a*), where R is the gas constant, T is the absolute temperature, F is the Faraday constant, Z is the valence of the charge molecule, and A is the membrane thickness across which the charge molecule can move. For the case of Fig. 2, Eq. 1 was used with mean values of the parameters obtained with many fibers from *R. pipiens* shown in Table II. In the graph, the values of Q have been normalized with respect to the value of Q_{max} shown in Table IIA. For the case of the other figures the curves were drawn using the parameters $\bar{V}$ and k determined by least-squares fitting of the above equation, to the charge movement data of each fiber.

Table II indicates that there were no significant differences between the fibers of the two species, except for the fiber diameter and the membrane resistance. Table II also shows that the C_m and k values appear to be larger than the values obtained in most works in which the three microelectrode voltage-clamp technique has been used. This is in agreement with the results obtained by other authors who have used the triple vaseline gap technique. For instance, Collins et al. (1982) and De Coursey et al. (1984) have found that membrane capacity measured with this technique is considerably larger than the value obtained with the microelectrode technique (Hodgkin and Nakajima, 1972). They concluded that with the triple vaseline gap the electrically effective membrane area is 1.5–1.9 times larger than the area calculated by geometrical parameters.

This is the reason why in the present work the results are normalized with respect to the effective area. For the case of the parameter k, a similarly higher value has been reported recently by Melzer et al. (1986) who used a double vaseline gap technique. The amount of maximum charge, Q_{max}, measured in these experiments agrees with the values reported by other authors (Brum and Rios, 1987; Brum et al., 1988), and is somewhat larger than the values obtained with the three-microelectrode technique. Using the single vaseline gap, Horowicz and Schneider (1981*a*) have reported that the measured values of Q_{max} were ~15% smaller than the values obtained with the three-microelectrode technique.

Finally, it is worth noticing that in some records from Fig. 2 (as well as from Figs. 5 and 8) a "hump," referred as $Q\gamma$ component, is visible at membrane potentials between −70 and −30 mV. This confirms earlier descriptions of the presence of this component in the cut fiber preparation (Vergara and Caputo, 1983; Hui and Chandler, 1988). Analysis of the figures of this work shows that the $Q\gamma$ component decays during the 40-ms pulse. This contrasts with the results of other authors (Huang, 1982; Hui and Chandler, 1988), according to which, for just suprathreshold depolarizations $Q\gamma$ may last hundreds of milliseconds. We have no explanation for this discrepancy and it should be further investigated.

As it has already been mentioned, Fig. 2 shows that charge movement occurs even at hyperpolarized membrane potentials. This stresses the necessity of carrying out the subtraction of the control pulses at membrane potential values as negative as possible, to diminish the errors due to charge contamination (Adrian and Almers, 1976; Brum and Rios, 1987). This point was further addressed in a few experiments summarized in Fig. 3. The graph shows the dependency of the charge signal measured with a 40-ms pulse from −100 to 0 mV, on the value of the SH. The mean normalized values obtained with six fibers are shown in the graph. The inset in the figure shows the pulse structure used in these experiments for a case in which the SH was −150 mV. It appears that maximum charge is obtained when the SH value is more negative than −150 mV, and that a considerable reduction of the measured charge is observed at values near −100 mV. This observation determined the value of the SH that was used in most experiments in which charge movement was measured in polarized fibers. For the case of measurements of charge in depolarized fibers it was more convenient to use positive values for the SH, as will be shown later.

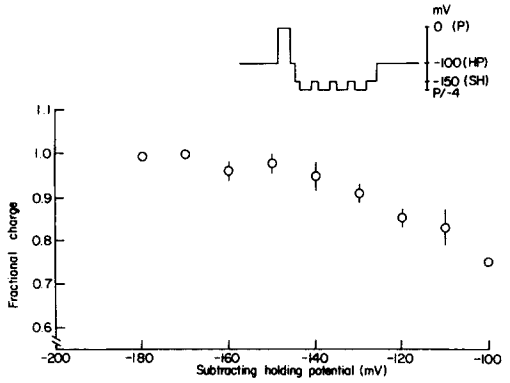

FIGURE 3. Effect of the SH value on intramembrane charge movement from different fibers of both species. The fractional charge obtained with a 40-ms pulse from −100 to 0 mV has been plotted vs. the SH value. In all experiments each test pulse was followed by the control pulses applied from the SH value. The results shown in the graph represent the mean values obtained in six experiments. *P*, pulse.

Some experiments were carried out to explore the possibility that the first spike-like component in the charge signals could be due to gating current for the sodium channels. Conditioning depolarizing prepulses were used to test whether the gating currents could be inactivated (Campbell, 1983). One such experiment is shown in Fig. 4. The three traces show that the charge moved in response to a pulse of 110 mV amplitude, to +10 mV from a holding potential of −100 mV and 40 ms duration. Trace *a* was obtained without a conditioning prepulse, while traces *b* and *c* were obtained with 30-ms prepulses of 20 and 25 mV amplitude, respectively, that were applied immediately before the test pulse. The main effect of the prepulse was a marked reduction, dependent on the prepulse amplitude, of the fast component in the ON transient, without noticeable effects in the OFF transient, as was expected (Campbell, 1983) since inactivation of the gating currents occurs during the pulse duration.

Charge Movement in Depolarized Fibers

From the works of Adrian and Almers (1976) and Chandler et al. (1976*b*) it is known that after membrane depolarization charge 1 is diminished and disappears com-

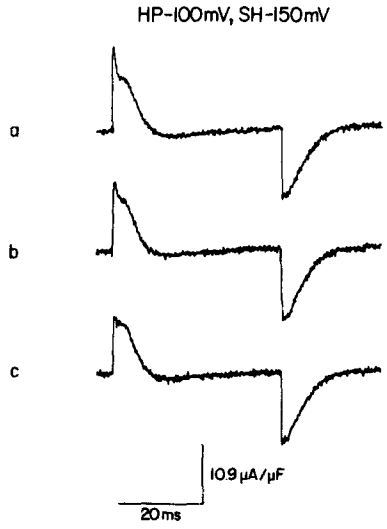

FIGURE 4. Effect of conditioning depolarizing prepulses on the spike-like component in the ON transient of the charge signal from a fiber of *L. insularis* (EN058A) held to −100 mV and depolarized to +10 mV; SH was −150 mV. The figure shows a clear reduction of the spike, dependent on the prepulse amplitude, with no effect on the OFF transient.

pletely, while another type of charge, charge 2, appears, moving in a different potential range. Fig. 5 shows measurements of charge movement, over a wide potential range, carried in the same fiber held at different membrane potentials. The records on the left were obtained at a holding potential of −100 mV using a SH of −150 or −180 mV. The records on the right were obtained while the fiber was depolarized to 0 mV, using a SH of +70 mV. It is clear that in the polarized state, charge movement occurs in the potential range between −80 and +20 mV, while after depolarization, charge movement occurs at potentials more negative than −60 mV. In this experiment, as well as in others, no inward currents were seen

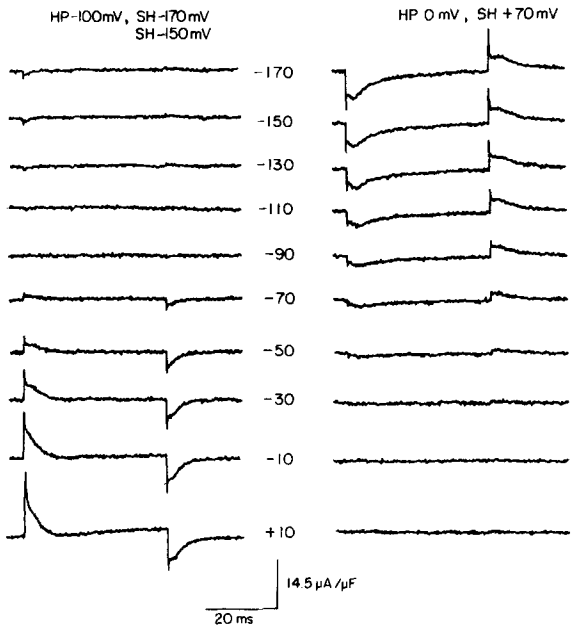

FIGURE 5. Effect of the holding membrane potential value on charge movement signals of one fiber from *L. insularis* (NV047C). The traces show the charge moved during a 40-ms pulse to the potential value indicated between records.

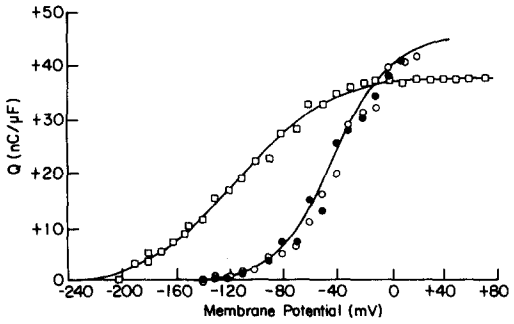

FIGURE 6. Potential dependence of charge movement in a fiber from *R. pipiens* (AG067B). The circles and the squares represent the results obtained with one fiber held at −100 or 0 mV, respectively. The open circles were obtained at the beginning of the experiment, while the filled ones were obtained after recovery from depolarization to 0 mV. The continuous lines were generated as best fits with Eq. 1 fitted to the experimental points. The best fit parameters were Q_{max} = 45 nC/μF, $\bar{V}$ = −42 mV, and k = 19.7 mV for the circles and Q_{max} = 39 nC/μF, $\bar{V}$ = −115 mV, and k = 33.2 mV for the squares. The total charge moved is about the same at −100 and 0 mV, although the steepness and the potential dependency are different.

flowing during the pulses, and the ON and OFF components of the charge signal were rather similar. In some experiments however, such currents were observed, confirming the report of Brum and Rios (1987). In these cases the analysis of the signals was based mainly on the OFF transients. The convenience of using positive values for the SH in the case of depolarized fibers is demonstrated in this figure and the next ones, which show that no charge is moved at positive potentials and that in the positive potential region, the relationship between charge and potential is completely flat.

Fig. 6 shows the Q-V relationships obtained from another experiment. The filled and open circles represent the experimental points obtained before and after depolarization, respectively. The open squares represent the points obtained with the fiber depolarized to 0 mV. Since in this case the time integrals of the intramem-

TABLE III

Voltage Dependence of Charge Movement Parameters

	Holding potential, −100 mV			Holding potential, 0 mV			Q_{max} (0 mV) / Q_{max} (−100 mV)
	Q_{max}	k	$\bar{V}$	Q_{max}	k	$\bar{V}$	
	nc/μF	*mV*	*mV*	*nC/μF*	*mV*	*mV*	
AG057B	53	22.5	−34	42	39.4	−92	0.79
AG067B	45	19.7	−42	39	33.2	−115	0.87
AG187A	60	22.5	−35	54	32.6	−115	0.90
AG197A	56	22.5	−35	61	28.1	−102	1.09
SE027A	49	20.8	−45	40	28.1	−96	0.82
SE027B	27	17.4	−58	30	22.5	−105	1.11
OC217B	32	22.5	−32	30	23.6	−146	0.94
OC307CLi	49	15.7	−50	37	30.9	−73	0.76
NV047CLi	46	20.8	−38	35	35.0	−83	0.76
$\bar{X}$	46.3	20.5	−41	40.9	30.0	−103	0.89
± SE	3.6	0.8	3	3.5	2.0	7	0.04

brane currents had negative values, we used their absolute value. As result of this, the experimental points and the curve fitted to them lay in the same region as the curve obtained with the polarized fiber instead of lying in the negative region. The figure shows that membrane depolarizations mainly affect the voltage dependence of charge movement, causing a shift of the Q-V relationship toward more negative potentials and a decrease in the curve steepness. The figure also indicates that the amount of total charge, irrespectively of its sign, is not much changed after depolarization.

From the analysis of this and other figures it appears that the data points obtained with the polarized fibers continue to rise with increasing depolarizations, and saturation does not appear to be reached. This means that the values of Q_{max} could be underestimated. This could affect the comparison with Q_{max} obtained with depolarized fibers. However, analysis of the figures indicates that underestimation also occurs in the case of the data points obtained with depolarized fibers in the negative end of the potential axis. Measurements carried out in five fibers showed that the

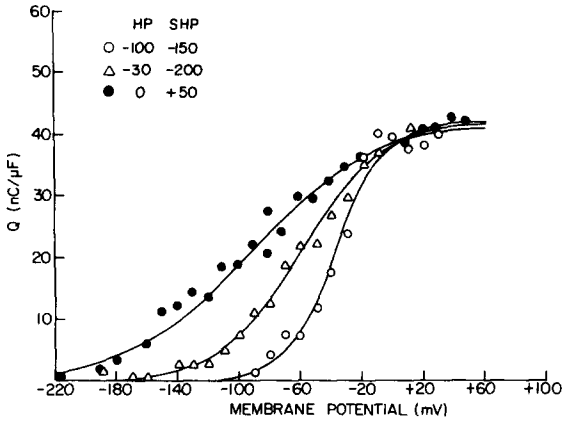

FIGURE 7. Potential dependency of charge movement of one fiber from *R. pipiens* (AB087B) at three different holding potentials: −100 (*open circles*), −30 (*open triangles*), and 0 mV (*filled circles*). The curves obtained in the depolarized states were raised arbitrarily as shown in Fig. 6. The best-fit parameters for the generation of the curves were for the open circles: Q_{max} = 42 nC/μF, $\bar{V}$ = −38 mV, and k = 15.7 mV; for the open triangles: Q_{max} = 42 nC/μF, $\bar{V}$ = −60 mV, and k = 25.9; and for the filled circles: Q_{max} = 42 nC/μF, $\bar{V}$ = −92 mV, and k = 39.4 mV.

membrane capacity was not much affected by changes in the membrane potential from −100 to 0 mV. Table III summarizes the results obtained with nine different fibers subjected to the same experimental procedure. The results indicate that, for the case of these fibers, the parameter V changes from −41 to −103 mV and the k parameter increases from 21 to 30 mV, while Q_{max} is reduced by only 10%.

There is a marked analogy between these results and those obtained by Bezanilla et al. (1982*a*) in the case of sodium gating currents in squid axons. These authors found a shift of charge toward more negative potentials in depolarized axons, but no change in steepness, and they interpreted their results assuming that membrane depolarization causes a change in the state of the gating charge. In agreement with this interpretation Rios and Brum (1987) have presented evidence supporting the idea that depolarization of muscle fibers cause transformation of charge 1 into charge 2. Fig. 7 shows plots of the charge potential relationships obtained in another experiment in which charge movement was measured at three different holding potential values in the same fiber. The open circles and triangles and the

filled circles were obtained at −100, −30, and 0 mV respectively. It appears that the extent of the shift of the *Q-V* relationship along the voltage axis is determined by the value of the holding potential.

Effect of D-600

Fig. 8 shows an experiment in which charge movement signals were obtained in a fiber polarized to −100 mV in the absence (records on the left) and in the presence (records on the right) of 50 μM D-600. For the case of this particular fiber, a small but noticeable decrease in the charge signal occurred, particularly at the more positive potentials. With other fibers the effect was smaller, amounting to <10% as it is

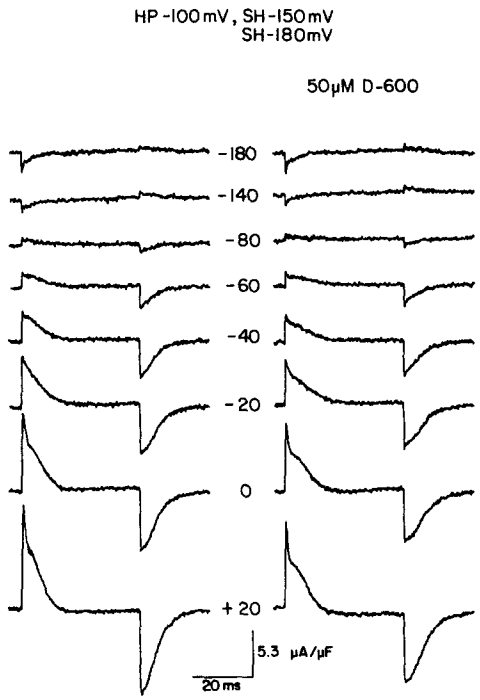

FIGURE 8. Charge movement signals in a fiber from *L. insularis* (NV047B) polarized to −100 mV in the absence (records on the left) and in the presence (records on the right) of 50 μM D-600.

shown in Table IV. The *Q-V* relationship in polarized fibers was not much affected by D-600 as is shown in Fig. 12 (below) and indicated by the parameters presented in Table IV. These results confirm the observations of Hui and Milton (1987), that 30 μM D-600 had no effect on the parameters describing the *Q-V* relationship in polarized fibers that were not subjected to a previous prolonged depolarization.

Although D-600 does not affect charge movement in a resting polarized fiber, we have obtained evidence indicating that the fiber sensitivity to the drug is increased by membrane depolarization. This is in analogy with the results obtained in contractile experiments (Berwe et al., 1987; Caputo and Bolaños, 1987).

Fig. 9 shows the effects of 100 μM D-600 on charge movement in a fiber steadily depolarized to −30 mV. The records on the left were obtained before adding the

TABLE IV

Effect of D-600 on Charge Movement in Polarized and Depolarized Fibers

	Control			D-600		
	Q_{max}	k	$\bar{V}$	Q_{max}	k	$\bar{V}$
	nC/μF	*mV*	*mV*	*nC/μF*	*mV*	*mV*
	Holding potential, −100 mV					
FE277A	25	16.9	−40	25	16.9	−40
NV047B	62	25.3	−25	60	25.8	−30
NV057B	51	18.5	−42	44	18.0	−45
NV057C	32	18.0	−53	31	18.0	−53
	Holding potential, −20, −30 mV					
MY207A	58	39.0	−38	35	39.0	−110
MY217A	45	36.0	−45	34	35.0	−110
DC047A	30	38.4	−58	29	35.0	−81
OC097A	60	36.1	−36	36	45.0	−80
	Holding potential, 0 mV					
NV047B	—	—	—	48	30.0	−87
NV047C	35	35.0	−83	36	26.0	−88

drug, using a SH of −210 mV, and the records on the right were obtained in the presence of D-600, using a SH of +40 mV. It can be observed that without D-600 a substantial amount of charge moves in the positive potential region, thus justifying the value of the SH used here, while after adding the drug all charge movement occurs in the negative potential range. The results of a similar experiment are plotted in Fig. 10. The open circles represent the *Q-V* relationship obtained in the absence of the drug and with the fiber polarized to −100 mV. The filled circles were obtained after depolarizing the fiber to −30 mV. The graph shows the shift of the curve caused by decreased membrane potential. The open triangles represent the data obtained after the addition to D-600. It can be seen that the *Q-V* relation-

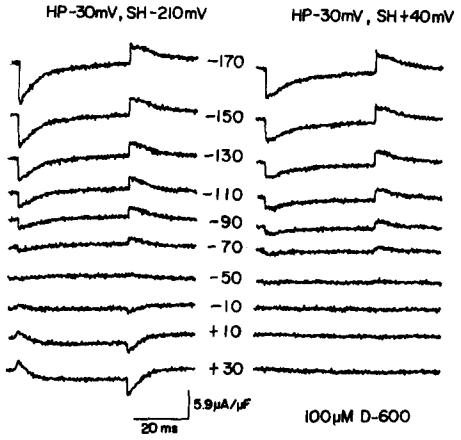

FIGURE 9. Effect of 100 μM D-600 on charge movement in a fiber from *L. insularis* (MY217A) depolarized to −30 mV. The records on the left were obtained in the absence, and those on the right in the presence of the drug. It can be observed that in the presence of D-600 charge movement signals are absent at positive potentials.

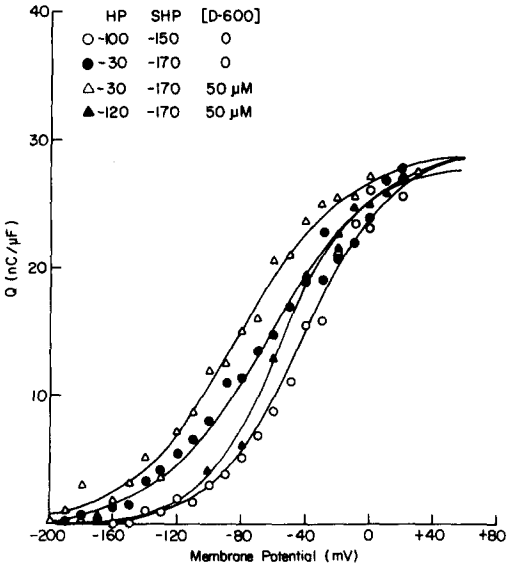

FIGURE 10. Effect of D-600 on the charge vs. voltage relationship in a fiber from *R. pipiens* (DC047A) held at different holding potentials (HP). The open circles represent the experimental results obtained with the fiber polarized to −100 mV, and the curve was obtained with the best-fit parameters Q_{max} = 29 nC/μF, $\bar{V}$ = 42.5 mV, and k = 26.6 mV. The filled circles were obtained after depolarizing the fiber to a holding potential of −30 mV, the best-fit parameters for the curve were Q_{max} = 30 nC/μF, $\bar{V}$ = −62 mV, and k = 35 mV. With the fiber held at −30 mV a further shift of the curve toward more negative potentials is observed after the addition of 50 μM D-600 (*open triangles*). The best-fit parameters for the curve were Q_{max} = 29 nC/μF, $\bar{V}$ = −83 mV, and k = 33 mV. Finally, the figure shows that these effects can be partially reversed after repolarization of the fiber to −120 mV (*filled triangles*), Q_{max} = 28 nC/μF, $\bar{V}$ = 56 mV and k = 24 mV.

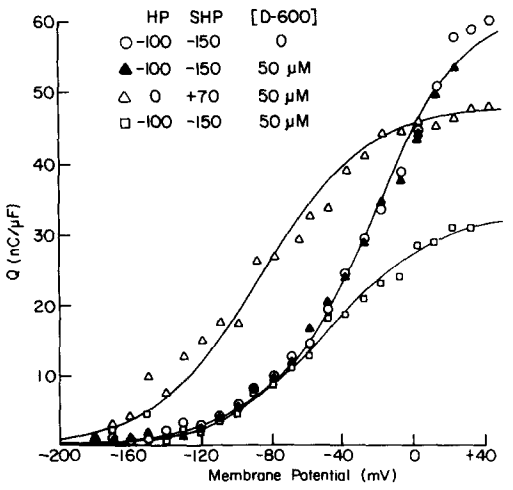

FIGURE 11. Effect of D-600 on charge 1 and 2. The graphs show the charge vs. voltage relationship at different holding potential (*HP*) (NV047B). In this case 50 μM D-600 was added with the fiber polarized to a holding potential of −100 mV. The empty circles and filled triangles were obtained before and after adding D-600, respectively; Q_{max} = 64 nC/μF, $\bar{V}$ = −25 mV, and k = 29 mV. The empty triangles were obtained after depolarization to 0 mV; Q_{max} = 48 nC/μF, $\bar{V}$ = −87 mV, and k = 30 mV. Repolarization of the membrane (*empty squares*) reverses the shift with an additional decrease in Q_{max}; Q_{max} = 33 nC/μF, $\bar{V}$ = −48 mV, and k = 31.5 mV.

ship is further shifted toward more negative potential values, thus mimicking the effects of a larger depolarization. After membrane hyperpolarization to −120 mV, the *Q-V* shift is partially reversed (filled triangles).

Fig. 11 shows an experiment similar to that of Fig. 10, except that the drug was added before the membrane was depolarized to 0 mV, which caused no effects on the *Q-V* relationship obtained at −100 mV. After depolarization of the membrane in the presence of D-600, a large shift of the *Q-V* relationship is obtained, along with a substantial decrease of Q_{max}. Repolarization of the membrane reverses the shift, and causes a further decrease in Q_{max}. Finally, Fig. 12 shows an experiment in which D-600 was added after the membrane was depolarized to 0 mV. In this case the drug caused no further shift of the *Q-V* relationship, as was the case in lesser depolarization. Also in this experiment a substantial reduction of Q_{max} was caused by the

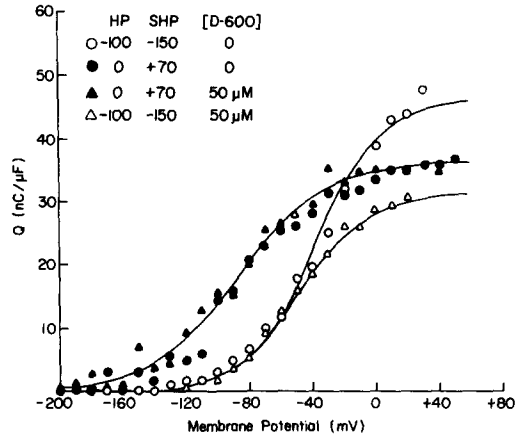

FIGURE 12. Effect of 50 μM D-600 on charge voltage relationships for charge 1 and 2, obtained with one fiber from *L. insularis* (NV047C) under different conditions. The curve represented by the empty circles was obtained before adding the drug at a holding potential of 100 mV; Q_{max} = 46 nC/μF, $\bar{V}$ = −38 mV, and k = 20.8 mV. A large shift of the *Q-V* relationship occurs after depolarization to 0 mV (*filled circles*). Addition of 50 μM D-600 causes no major changes (*filled triangles*); Q_{max} = 36 nC/μF, $\bar{V}$ = −88 mV, and k = 27 mV. The empty triangles show the result obtained after repolarizing the fiber to −100 mV in the presence of the drug; k = 21.4 mV, Q_{max} = 31 nC/μF, $\bar{V}$ = −49 mV.

drug with a further reduction after membrane depolarization. Table IV reviews the results obtained with different fibers, showing the effects of D-600 on the parameters describing the *Q-V* relationship under different conditions.

DISCUSSION

The cut fiber preparation, used with the triple vaseline gap voltage-clamp technique as originally described by Hille and Campbell (1976), has been used in several laboratories to study different phenomena. Starting with the report of Vergara and Cahalan (1978), numerous works have appeared in which the technique (or a variant of it using a double vaseline gap voltage-clamp technique) has been successfully used to study intramembrane charge movement. Advantages of the technique are that it allows the control of membrane potential over a wide range of values and the control of the internal medium.

We have found that after membrane depolarization in skeletal muscle fibers the total amount of charge moved is not considerably reduced, while a large change in the potential sensitivity of charge movement is observed. Furthermore, the parameter k is markedly increased after depolarization. The parameter k is related to the effective valence of the charge movement particle, and an increase in its value could mean either a decrease in the effective valence value or a decrease in the membrane thickness across which the charged particle can move.

In the following discussion the terms charge 1 and charge 2 are used to describe charge measured in polarized and depolarized fibers, respectively, without necessarily implying that they are two separate entities. In polarized fibers charge 1 has been measured in the potential range between -140 and $+40$ mV and the parameters that describe the charge-voltage (Q-V relationship according to a two-states Boltzmann distribution, Eq. 1) have the following values: $Q_{max} = 40$ nC/μF, $\bar{V} = -41.3$ mV, $k = 20.5$ mV. These values are similar to those reported in numerous other works, excepting the value of k, which appears to be larger than the values obtained with the three-microelectrode technique, usually ~8–10 mV. Horowicz and Schneider (1981*a*, *b*) and Melzer et al. (1986), using the double vaseline gap technique, have reported similarly higher values of 16 and 21 mV, respectively. After depolarization to 0 mV, charge 2 moves in the potential range between -100 and -200 mV mainly, and for the same group of fibers, the parameters were: $Q_{max} = 41$ nC/μF, $\bar{V} = -103$ mV, $k = 30.0$ mV. The values obtained in depolarized fibers are in fair agreement with those reported by Brum and Rios (1987), which were $Q_{max} = 48$ nC/μF, $\bar{V} = -115$ mV and $k = 20.5$ mV, except the value of the parameter k, which appears to be greater in our work.

It has already been mentioned that there is a large variability in the values reported for the parameter k, which is probably due to the different experimental conditions. It is important to point that in the present experiments the k values of 20.5 and 30 mV were obtained in the same fibers under the same conditions before and after membrane depolarization. This increase appears to be consistent with the earlier results of Adrian et al. (1976) who also found that the values of k for charge 2 were larger than those for charge 1. They also reported that the value of k was larger in a partially reprimed fiber.

The differences observed between charge 1 and 2 can be interpreted in terms of a change of state of a single molecular moiety after membrane depolarization, which is in agreement with the conclusion of Rios and Brum (1987). The possibility of interconversion of charge 1 into charge 2 was originally considered Adrian et al. (1976). Based on the observation that after repolarization of a depolarized fiber, disappearance of charge 2 took more time than necessary for full repriming of charge 1, these authors concluded that the interconversion hypothesis could be ruled out. Thus they proposed that charge 1 and charge 2 are associated with two sets of unrelated molecular moieties with different properties, and that only charge 1 is involved in ECC. However, in these early experiments charge 1 and 2 were not measured simultaneously, nor in the same fibers. More recently, Brum and Rios (1987), have followed simultaneously the disappearance of charge 2 and the reappearance of charge 1 when depolarized fibers were repolarized, and found that these changes followed the same time course. On the basis of these observations

they proposed that charge 1 and 2 may be interconverted into each other when the membrane potential is changed. This conclusion is similar to that reached by Bezanilla et al. (1982*a*) who studied the process of slow inactivation in squid giant axons and also obtained evidence favoring the idea that after membrane depolarization the gating charge for the sodium conductance is not diminished nor abolished but changes its potential dependency. These authors concluded that during maintained depolarization the gating molecules undergo a conformational change occupying a different set of physical states that still allow transitions across the membrane but in a different potential range.

In the present experiments we have not measured the time course of charge modification after membrane depolarization, however, our results can be similarly explained assuming that the molecular moiety responsible for charge movement changes its properties through a conformational change dependent on the membrane potential. Under this scheme the increase in the value of the parameter k could be explained assuming that the change in the physical state of the charge molecule decreases its effective valence. However, a decrease in the effective valence of the charge molecule should be accompanied by a decrease of Q_{max} by a factor of two thirds. This decrease was not observed in our experiments. When fibers are depolarized at intermediate potentials such as -30 or -20 mV, the shift of the Q-V relationship is not complete, suggesting an intermediate state between charge 1 and 2. An alternate possibility, however, is that charge 1 and 2 may be simultaneously present at this potential.

In agreement with previous works (Hui and Milton, 1987; Melzer and Pohl, 1987), we have found that in polarized, resting fibers D-600 does not have much effect on charge movement. Maximum charge is however appreciably decreased after repetitive activity, suggesting a use-dependent mode of action. It is interesting to point out that a decrease in charge movement, observed by Lamb (1986) in two rabbit sternomastoid fibers treated with 30 μM D-600, also appeared to be time- or use-dependent. This mode of action appears to be mediated by an increased affinity for D-600 of the molecular moiety, or part of it associated with charge movement, when the membrane is in a depolarized state since under these conditions charge movement is markedly reduced. This observation parallels the results obtained in contractile experiments (Caputo and Bolaños, 1987; Berwe et al., 1987), favoring the idea that charge movement acts as the voltage sensor for ECC.

Besides the decrease in charge movement observed in fibers depolarized to 0 mV, no other effects are caused by D-600 on charge distribution (Melzer and Pohl, 1987). However, when fibers are moderately depolarized to -30 and -20 mV, another interesting effect of D-600 is observed: the Q-V relationship, which in the absence of the drug occupies an intermediate position between those corresponding to charge 1 and 2, is further shifted toward the position corresponding to charge 2. Thus in the presence of D-600 the state corresponding to charge 2 is favored. It appears that there is a difference in the mode of action of D-600 and nifedipine, since different authors (Lamb, 1986, 1987; Brum and Rios, 1987; Rios and Brum, 1987; Brum et al., 1988) have found that nifedipine decreases charge movement in normally polarized fibers.

In a recent work, Brum et al. (1988) have presented evidence indicating that in

low calcium, charge 1 is decreased and charge 2 is increased. This suggests that external calcium is required for the proper functioning of the sensor for ECC and that in its absence the inactivation state is favored, which supports earlier evidence obtained with contractile experiments (Lüttgau, 1963; Caputo, 1972, 1981; Bolaños et al., 1986; Berwe et al., 1987; Caputo and Bolaños, 1987). On the basis of their results, Rios and Brum (1987) and Brum et al. (1988) have proposed that compounds known as calcium channel blockers, like nifedipine and D-600, could act by lowering the affinity of the voltage sensor for calcium, binding preferentially to inactivated states. The close parallelism of the results obtained with measurements of calcium release and charge movement led them to the conclusion that most of the charge movement signal is involved in the signalling between membrane potential and calcium release. Although, the results obtained with D-600 in this work do not match exactly the results of Brum et al. (1988) that were obtained with low calcium solutions and nifedipine, there is a close similarity in their overall effects. While these differences could be due to variations in the experimental procedure, it is worth recalling that the effects of D-600, low calcium, and nifedipine on contractile inactivation also differ quantitatively (Caputo and Bolaños, 1987).

Thus, in spite of some discrepancies, the main conclusions that can be derived from the present work support the idea of Brum et al. (1988) that charge movement may serve as the voltage sensor for ECC. After membrane depolarization, charge 1 undergoes a change of state through a series of transitions, and is transformed into charge 2. This transformation is associated with the onset and buildup of contractile inactivation. Contractile repriming occurs when charge 2 is retransformed into charge 1 after membrane repolarization. Furthermore, the results presented here agree with the idea that the voltage sensors for ECC are chemically similar to calcium channels. In analogy with the conclusions derived for the gating charge of sodium channels (Bezanilla et al., 1982*a*), the results presented here support the view that the molecular moiety responsible for charge movement may exist in several (at least four) different physical states, and that the occupancy of each state is determined by the steady membrane potential. D-600, as well as other drugs and experimental conditions, may affect this potential-dependent distribution, favoring the distribution obtained in the more depolarized states, and at the same time, favoring contractile inactivation and/or paralysis (Eisenberg et al., 1983).

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