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Charge movement in skeletal muscle fibers paralyzed by the calcium-entry blocker D600

(excitation–contraction coupling/nonlinear capacitive current/methoxyverapamil)

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ABSTRACT **We report measurements of nonlinear charge movement in frog skeletal muscle fibers paralyzed by the calcium-entry blocker [Schwartz, A. & Taira, N., eds. (1983) *Circ. Res.* 52, Part II, Number 2, 1–181.] D600 (methoxyverapamil, recently renamed gallopamil). Nonlinear charge movement was not seen in such fibers, suggesting that the drug severs the link between membrane depolarization and the main components of charge movement. This is the only pharmacological agent that blocks the main components of charge movement.**

While current carried by ions across membranes has been studied for many years, nonlinear capacitive currents within membranes have been recorded only in the last decade. Armstrong and Bezanilla (1) measured a nonlinear capacitive current in the membrane of squid axon. Since then, substantial evidence (2) has been gathered supporting the hypothesis that this current arises from the movement of a "voltage sensor," which controls the conformation and, thus, the conductance of the sodium channel. This nonlinear capacitive current is the gating current originally postulated by Hodgkin and Huxley (3). Just before Armstrong and Bezanilla's discovery, Schneider and Chandler (4) measured a nonlinear movement of capacitive charge within the surface or transverse (T) tubular membranes of skeletal muscle fibers. The functional role of this charge movement is not completely understood, although it seems likely to be involved directly in the processes that link membrane depolarization to contraction (4–6). Mathias *et al.* (7) suggested an alternative explanation of charge movement as an ionic current from T tubules to the sarcoplasmic reticulum (SR), but recent findings (8–10) do not support that view. Thus, it seems quite likely that charge movement is a nonlinear capacitive current occurring in the plasma membranes of skeletal muscle, probably in the T tubular membrane.

Eisenberg *et al.* (11) have reported a drug treatment that paralyzes skeletal muscle. In the presence of 30 μM D600 at a temperature of 5°C, single fibers give just one potassium contracture, while whole muscles may give several, probably reflecting the different time course and effectiveness of potassium depolarization in the two preparations. After the paralyzing contracture(s), fibers do not contract in response to electrical stimulation or further application of potassium, although they have a normal resting potential and propagate normal action potentials. Paralyzed fibers contract in response to caffeine, showing that their SR contains and can release a normal amount of calcium. When warmed to some 20°C, paralyzed fibers revive; that is, they regain their contractility and retain it if subsequently recooled.

Here we report a study of the relationship of charge movement and contraction in skeletal muscle. We find that charge movement is absent in paralyzed fibers but present in revived fibers. This result supports the hypothesis that charge movement is responsible for T tubule-to-SR coupling.

MATERIALS AND METHODS

Early experiments were performed on whole sartorius muscles of *Rana temporaria*. In later experiments, semitendinosus muscles were thinned to several layers of fibers, exposing the fiber ends at the tendon and reducing connective tissue interference with solution changes and microelectrode impalement.

The paralyzing treatment of muscle fibers has been described and documented in detail (11). Briefly, a muscle was presoaked in normal Ringer containing 30 μM D600 at about 5°C. A high (190 mM) potassium solution at 5°C also containing 30 μM D600 was applied for 30 sec to depolarize the fibers. The muscle was then soaked in normal Ringer's solution for 10–30 min, and the contractility of surface fibers was checked. If randomly selected surface fibers did not move in response to depolarizing current from an inserted microelectrode, as observed under a compound microscope, the experiment commenced. If the fiber moved in response to depolarization, then a second and sometimes a third contracture was elicited. Surface fibers of these whole muscles were always paralyzed after three applications of potassium, usually after two, and often after one.

Nonlinear charge movement was measured by using the procedures and methods of analysis of Chandler *et al.* (12) and of Gilly and Hui (13, 14). In order to record this nonlinear capacitive current, which is small in magnitude, other currents flowing in parallel pathways must be minimized. Nonlinear ionic currents were reduced by channel blockers, and linear current was removed by subtraction. The following bathing solution minimizes nonlinear ionic currents: 115 mM tetraethylammonium chloride containing 5 mM RbCl, tetrodotoxin (10 μg/ml), 30 μM D600, 11.8 mM $CaCl_2$ (1.8 mM for the experiment in Fig. 2), and 2 mM Pipes. Linear membrane current components were measured in control pulses applied in a potential range more negative than the resting potential, where nonlinear current components are absent. Hence, after subtraction of control traces, the current traces contained only the nonlinear charge movement and a residual K^+ current not completely blocked by tetraethylammonium ion. This latter component had to be removed by subtracting a sloping baseline from the current traces (see ref. 12 for details).

RESULTS

Fig. 1*A* shows charge movement records obtained from a control experiment in which a fiber was presoaked in normal Ringer's solution containing D600 at about 5°C, but no conditioning K^+ contracture was applied. Because the fibers

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Abbreviations: T tubular membrane, transverse tubular membrane; SR, sarcoplasmic reticulum.

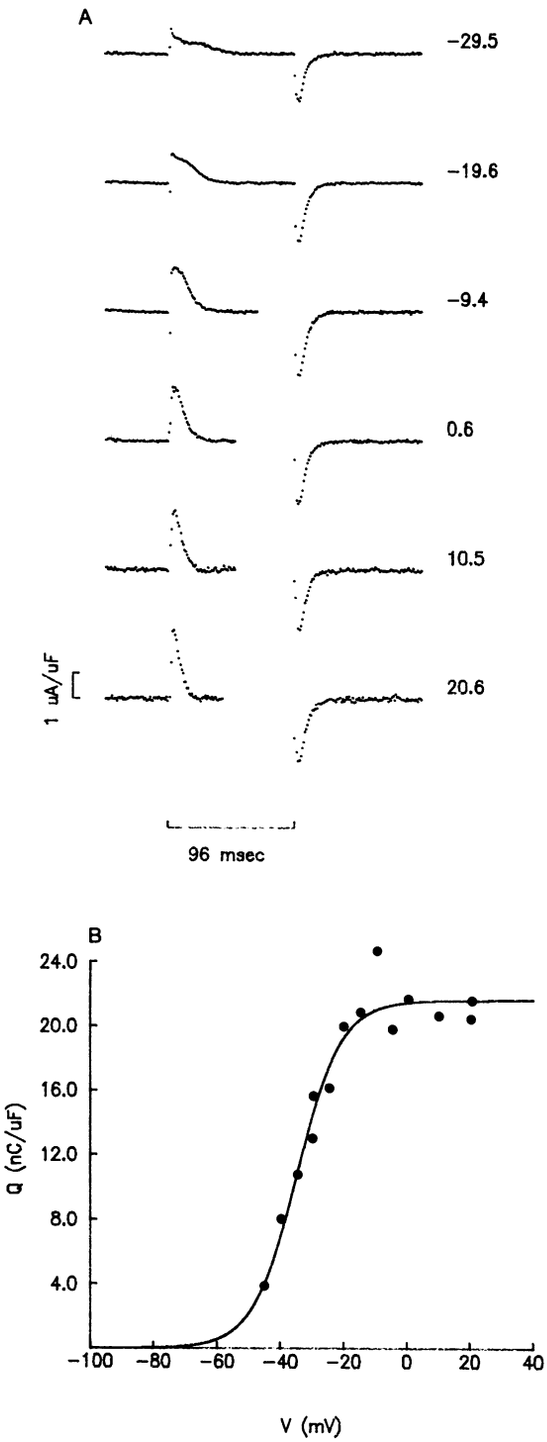

FIG. 1. Charge movement prior to paralysis. Fiber identification, 35031; holding potential, −80 mV; temperature, 4°C. The bathing solution contains all of the ingredients mentioned in *Materials and Methods* and 350 mM sucrose. (*A*) Charge movement traces recorded with the three-microelectrode voltage-clamp technique (15). Electrode spacing: l, 200 μm; l', 40 μm. Some traces are interrupted when the residual K^+ current deviates from a straight line. The first four traces are averages of four sweeps, and the last two traces are averages of two sweeps. The numbers on the right-hand side of each trace indicate the membrane potential (in mV) during the depolarizing test pulse. (*B*) Steady-state Q–V curve. For each of the current traces (some of which are shown in *A*) the time integral of the ON current, Q_{on}, is averaged with the time integral of the OFF current, Q_{off}, and plotted against membrane potential during the test pulse. The smooth curve was fitted to the data by equation 9 of ref. 12, namely, $Q(V) = Q_{max}/[1 + \exp - (V - \bar{V})/k]$. The best-fit parameters are $\bar{V} = -34.7$ mV, $k = 7.0$ mV, $Q_{max} = 21.6$ nC/μF.

were not paralyzed, charge movement had to be measured in a hypertonic solution, which blocked contraction. The kinetics, voltage dependence, and amount of charge movement were similar to those recorded in the absence of D600 (16).

The nonlinear current response to a depolarizing step in potential (the ON current) is an initial rise followed by a decay to baseline. This decay has a complex (i.e., nonexponential) time course, which has been resolved into an early and a late component, Q_β and Q_γ, respectively (17). The ON current resulting from a small depolarization contains a hump Q_γ several tens of milliseconds after the peak of the early component Q_β. The ON current resulting from a larger depolarization usually does not show such a clear separation of the two components; rather, Q_γ appears as a "plateau" in the decay of the early component Q_β. Although the functional role of Q_γ is not known with certainty, its steep voltage dependence and pharmacological sensitivity (16, 18) have led workers to suggest that this component of charge movement is somehow associated with calcium release from the SR (16, 18–20). The inward current that occurs when the fiber is repolarized to the holding potential (the OFF current) declines nearly exponentially at all voltages. The total charge carried by the ON current (Q_{on}) is approximately equal in magnitude to that carried by the OFF current (Q_{off}). Fig. 1*B* shows the relationship between the mean of Q_{on} and Q_{off} and the membrane potential during the test pulse. The activation of charge movement had a steep voltage dependence in the potential range near the contraction threshold. The main point of Fig. 1 is that presoaking a fiber in D600 has no effect on the properties of charge movement.

Three conditions need to be satisfied in order to paralyze a fiber: the fiber must be exposed to D600; the fiber must be cold; and the fiber must be depolarized, here by a solution containing a high concentration of potassium. In the experiment of Fig. 1, only the first two conditions were satisfied. Thus, the fibers contracted and had normal charge movement. In the experiment of Fig. 2, all three conditions were satisfied. Thus, the fibers were paralyzed and charge movement was greatly reduced. We studied 30 paralyzed fibers in 11 muscles. In 15 fibers the residue of charge movement was too small to measure, whereas, in the remaining fibers, a small amount of Q_β could be observed, ranging from 2–7 nC/μF. This small charge movement could be a residue of the normal Q_β, but it also could be a distorted image of sodium gating current or a current of unknown origin. Q_γ was not present in normal amounts. If Q_γ were 25% of Q_β in paralyzed fibers, as it is in normal fibers, we could not have resolved it.

Revived single fibers have been shown to give quite normal potassium contractures under warm conditions (11) and when recooled (B. A. Curtis, R. S. Eisenberg, and R. T. McCarthy, personal communication). We warmed paralyzed muscles to 20°C for several min to revive contractility and then cooled the muscle to some 4°C before checking contractility in surface fibers. Such fibers usually contracted in response to depolarization; occasionally the muscle had to be warmed once again before contractility was restored. Fig. 3 documents measurements from such a revived fiber placed in hypertonic Ringer's solution. The charge movement recorded from these fibers was not quite normal. Q_γ was not detectable, and the amount of Q_β was reduced. The extent of this reduction varied considerably from fiber to fiber. Q_{max} in revived fibers varied from 7 to 17 nC/μF compared to 18–25 nC/μF in control fibers. This reduction, and the occasional need for a second warming period, may be due to the short duration of warming we used in these experiments.

DISCUSSION

These results show that one action of D600 is to block charge movement, probably by severing the link between the elec-

tric field in the T membrane and a resulting conformational change in a membrane protein directly involved in T–SR coupling. This action of D600 requires peculiar conditions, namely low temperature and prolonged depolarization. The same conditions are needed for the drug to paralyze muscle fibers (11) and to block a component of calcium influx (B. A. Curtis, R. S. Eisenberg, and R. T. McCarthy, personal communication). These three actions of D600 (paralysis, blockage of charge movement, and blockage of calcium influx) could be explained by a single molecular action of the drug if charge movement were a gating current for a calcium channel in the T membrane—the flux through which induced calcium release from the SR (21–23). However, that explanation has to be reconciled with the well-known finding that skeletal muscle fibers maintain their ability to twitch in solutions containing large amounts of calcium chelators [thus, negligible amounts of free calcium (24, 25)], possibly by invoking a pool of inaccessible calcium in a restricted space (26, 27). Alternatively, our data and that of our colleagues (11) are consistent with other mechanisms of T–SR coupling. For example, if D600 on the one hand blocks a calcium channel responsible for calcium influx and on the other hand blocks the movement of a rigid rod linking T membrane depolarization to SR calcium release (4, 5), then all of the actions of D600 would be explained.

Because Q_γ seems closely associated with calcium release in normal fibers (16, 18), we were surprised to find it absent in revived fibers. Perhaps, these fibers were not warmed long enough to be restored to a normal state. Perhaps, the absence of Q_γ may represent an action of D600 that persists in warmed fibers even after contractile activity is revived.

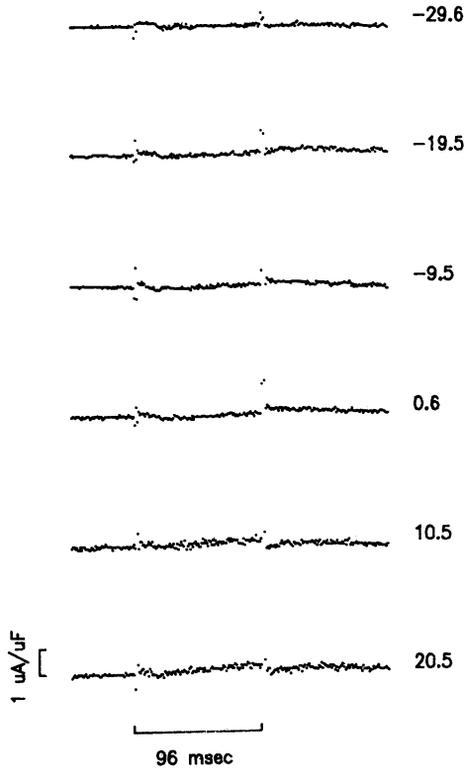

FIG. 2. Charge movement after paralysis. Fiber was paralyzed by the application of two 30-sec K^+ contractures 13 min apart. Fiber identification, 35134; holding potential, −80 mV; temperature, 4°C. Charge movement was measured in isotonic tetraethylammonium Ringer's solution containing 1.8 mM $CaCl_2$. Because the delayed K^+ current was small, no sloping baseline subtraction was required. This is a common finding in paralyzed fibers. The first four traces are averages of eight sweeps, and the last two traces are averages of four sweeps.

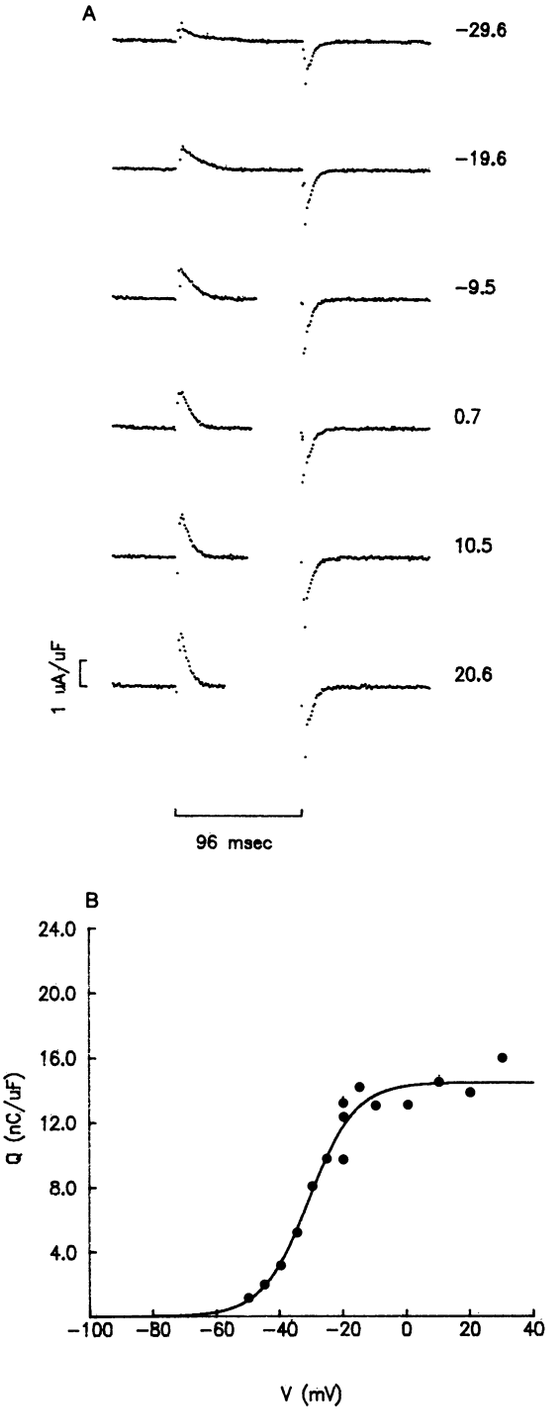

FIG. 3. Charge movement in a revived fiber. Surface fibers in this muscle were found to be paralyzed by the application of one 30-sec K^+ contracture. After paralysis was confirmed, the muscle was warmed twice, each time to about 20°C for several minutes and then slowly recooled to a holding temperature of 3°C. Fiber identification, 2NO48; holding potential, −80 mV. Charge movement was measured in the same hypertonic solution used in the experiment of Fig. 1. (*A*) Charge movement traces after sloping baseline correction. The first two traces are averages of eight sweeps, and the last four traces are averages of four sweeps. (*B*) Steady-state *Q–V* curve. Averages of Q_{on} and Q_{off} are plotted against membrane potential. Least squares fit of equation 9 in ref. 12 yields the best-fit parameters: $\bar{V}$ = −30.7 mV, k = 7.4 mV, Q_{max} = 14.5 nC/μF.

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1. Armstrong, C. M. & Bezanilla, F. (1974) *J. Gen. Physiol.* **63,** 533–552.
2. Armstrong, C. M. (1981) *Physiol. Rev.* **61,** 644–683.
3. Hodgkin, A. L. & Huxley, A. F. (1952) *J. Physiol.* (*London*) **117,** 500–544.
4. Schneider, M. F. & Chandler, W. K. (1973) *Nature* (*London*) **242,** 244–246.
5. Chandler, W. K., Rakowski, R. F. & Schneider, M. F. (1976) *J. Physiol.* (*London*) **254,** 285–316.
6. Schneider, M. F. (1981) *Annu. Rev. Physiol.* **43,** 507–517.
7. Mathias, R. T., Levis, R. A. & Eisenberg, R. S. (1980) *J. Gen. Physiol.* **76,** 1–31.
8. Meissner, G. & McKinley, D. (1982) *J. Biol. Chem.* **257,** 7704–7711.
9. Huang, C. L. (1983) *J. Physiol.* (*London*) **336,** 527–543.
10. Kitazawa, T., Somlyo, A. V., Shuman, H. & Somlyo, A. P. (1983) *Biophys. J.* **41,** 168a (abstr.).
11. Eisenberg, R. S., McCarthy, R. T. & Milton, R. L. (1983) *J. Physiol.* (*London*) **341,** 495–505.
12. Chandler, W. K., Rakowski, R. F. & Schneider, M. F. (1976) *J. Physiol.* (*London*) **254,** 245–283.
13. Gilly, W. F. & Hui, C. S. (1980) *J. Physiol.* (*London*) **301,** 157–173.
14. Gilly, W. F. & Hui, C. S. (1980) *J. Physiol.* (*London*) **301,** 175–190.
15. Adrian, R. H., Chandler, W. K. & Hodgkin, A. L. (1970) *J. Physiol.* (*London*) **208,** 607–644.
16. Hui, C. S. (1983) *J. Physiol.* (*London*) **337,** 509–529.
17. Adrian, R. H. & Peres, A. (1979) *J. Physiol.* (*London*) **289,** 83–97.
18. Hui, C. S. (1983) *J. Physiol.* (*London*) **337,** 531–552.
19. Almers, W. (1978) *Rev. Physiol. Biochem. Pharmacol.* **82,** 97–190.
20. Huang, C. L. (1982) *J. Physiol.* (*London*) **324,** 375–387.
21. Endo, M., Tanaka, M. & Ogawa, Y. (1970) *Nature* (*London*) **228,** 34–36.
22. Ford, L. E. & Podolsky, R. J. (1970) *Science* **167,** 58–59.
23. Fabiato, A. (1982) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41,** 2238–2244.
24. Armstrong, C. M., Bezanilla, F. M. & Horowicz, P. (1972) *Biochim. Biophys. Acta* **267,** 605–608.
25. Spiecker, W., Melzer, W. & Lüttgau, H. C. (1979) *Nature* (*London*) **280,** 158–160.
26. Hodgkin, A. L. & Horowicz, P. (1960) *J. Physiol.* (*London*) **153,** 386–403.
27. Frank, G. B. (1982) *Can. J. Physiol. Pharmacol.* **60,** 427–439.