

# Calcium Currents in Embryonic and Neonatal Mammalian Skeletal Muscle

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**ABSTRACT** The whole-cell patch-clamp technique was used to study the properties of inward ionic currents found in primary cultures of rat and mouse skeletal myotubes and in freshly dissociated fibers of the flexor digitorum brevis muscle of rats. In each of these cell types, test depolarizations from the holding potential ( $-80$  or  $-90$  mV) elicited three distinct inward currents: a sodium current ( $I_{Na}$ ) and two calcium currents.  $I_{Na}$  was the dominant inward current: under physiological conditions, the maximum inward  $I_{Na}$  was estimated to be at least 30-fold larger than either of the calcium currents. The two calcium currents have been termed  $I_{fast}$  and  $I_{slow}$ , corresponding to their relative rates of activation.  $I_{fast}$  was activated by test depolarizations to around  $-40$  mV and above, peaked in 10–20 ms, and decayed to baseline in 50–100 ms.  $I_{slow}$  was activated by depolarizations to  $\sim 0$  mV and above, peaked in 50–150 ms, and decayed little during a 200-ms test pulse.  $I_{fast}$  was inactivated by brief, moderate depolarizations; for a 1-s change in holding potential, half-inactivation occurred at  $-55$  to  $-45$  mV and complete inactivation occurred at  $-40$  to  $-30$  mV. Similar changes in holding potential had no effect on  $I_{slow}$ .  $I_{slow}$  was, however, inactivated by brief, strong depolarizations (e.g., 0 mV for 2 s) or maintained, moderate depolarizations (e.g.,  $-40$  mV for 60 s). Substitution of barium for calcium had little effect on the magnitude or time course of either  $I_{fast}$  or  $I_{slow}$ . The same substitution shifted the activation curve for  $I_{slow}$   $\sim 10$  mV in the hyperpolarizing direction without affecting the activation of  $I_{fast}$ . At low concentrations (50  $\mu$ M), cadmium preferentially blocked  $I_{slow}$  compared with  $I_{fast}$ , while at high concentrations (1 mM), it blocked both  $I_{fast}$  and  $I_{slow}$  completely. The dihydropyridine calcium channel antagonist (+)-PN 200-110 (1  $\mu$ M) caused a nearly complete block of  $I_{slow}$  without affecting  $I_{fast}$ . At a holding potential of  $-80$  mV, the half-maximal blocking concentration ( $K_{0.5}$ ) for the block of  $I_{slow}$  by (+)-PN 200-110 was 182 nM. At depolarized holding potentials that inactivated  $I_{slow}$  by 35–65%,  $K_{0.5}$  decreased to 5.5 nM.

## INTRODUCTION

From microelectrode recordings, it has been known for a number of years that the electrophysiological properties of embryonic and neonatal skeletal muscle differ

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qualitatively from those of adult skeletal muscle. As one example, the action potential in fetal and neonatal rat diaphragm muscle is tetrodotoxin (TTX) resistant, whereas that in adult muscle is TTX sensitive (Harris and Marshall, 1973). As another example, myotubes of embryonic rat skeletal muscle grown in primary culture display spontaneous action potential discharge (e.g., Barrett et al., 1981), whereas innervated adult muscle is electrically quiescent in the absence of motoneuronal input. Understanding the differences in ionic currents that are responsible for these qualitative differences requires voltage-clamp analysis. Although several different techniques have been applied to adult skeletal muscle, only with the recent advent of the patch-clamp technique (Hamill et al., 1981) has it become possible to voltage-clamp developing skeletal muscle.

Using the patch-clamp technique, Weiss and Horn (1986) demonstrated that rat myotubes and myoblasts (the precursor cells that form myotubes) possess two classes of sodium channels. The two kinds of channels differ in their sensitivity to TTX and in other functional properties, with the TTX-sensitive channel representing a higher proportion of sodium channels in myotubes than in myoblasts. Thus, these *in vitro* studies suggest that the *in vivo* transformation of the action potential from TTX insensitive to TTX sensitive involves the replacement of one kind of sodium channel with another. Patch-clamp techniques have also been used to measure calcium currents in embryonic and neonatal rat skeletal muscle (Beam et al., 1986; Cognard et al., 1986a). These measurements have revealed the presence of two distinct kinds of calcium currents, termed  $I_{fast}$  and  $I_{slow}$  (Beam et al., 1986) or dihydropyridine insensitive and dihydropyridine sensitive (Cognard et al., 1986a). A current resembling the  $I_{slow}$ /dihydropyridine-sensitive current has been extensively documented in adult muscle (Beatty and Stefani, 1976; Stanfield, 1977; Sanchez and Stefani, 1978; Almers and Palade, 1981; Donaldson and Beam, 1983; Walsh et al., 1986), but a current has not been reported in adult muscle that is like the  $I_{fast}$ /dihydropyridine-insensitive current.

The purpose of this article is to present a detailed characterization of calcium currents in embryonic and neonatal mammalian skeletal muscle. At present, most of the conclusions about developmental changes in muscle electrophysiology are based on comparisons of measurements of adult muscle with measurements on myotubes. The significance of this comparison is limited by a lack of knowledge about the maturational state of myotubes compared with that of muscle developing *in vivo*. Thus, for our work, we used two kinds of cells, primary cultures of embryonic rat and mouse skeletal myotubes, and acutely dissociated fibers of the flexor digitorum brevis (FDB) muscle of neonatal rats. We have found that three inward ionic currents,  $I_{Na}$  and the calcium currents  $I_{fast}$  and  $I_{slow}$ , are present and have similar properties in myotubes and acutely dissociated FDB fibers of neonatal rats.

Preliminary accounts (Beam and Knudson, 1985; Beam et al., 1985, 1986) of some of the results presented here have appeared previously.

## METHODS

### *Voltage Clamp*

Currents were recorded with the whole-cell variant of the patch-clamp technique (Hamill et al., 1981). Pipettes were fabricated from borosilicate glass and filled with internal solution, which

contained (millimolar): 140 Cs-aspartate, 5 Mg-aspartate<sub>2</sub> or 5 MgCl<sub>2</sub>, 10 Cs<sub>2</sub>EGTA, 10 HEPES, pH 7.4 with CsOH. The resistance of the pipettes varied from 1.5 to 2.5 MΩ. For each cell, the linear capacitive and leakage currents were measured for a depolarizing or hyperpolarizing "control pulse" of 10–20 mV from the holding potential. The area beneath the capacitive transient and the time constant of the transient's decay were used to calculate the cell's linear capacitance and the series resistance associated with the pipette (Matteson and Armstrong, 1984). Electronic compensation was used to reduce the effective series resistance, generally to a value of ~1 MΩ. The control current described above was used to digitally correct test currents for linear components of capacitive and leakage currents. To allow comparison of test currents recorded from different cells, the current recorded from each cell was normalized by that cell's linear capacitance (current expressed as picoamperes per picofarad). In some of the illustrated traces, the first four data points after a change of potential have been blanked. Data were sampled at either 10 (sodium currents) or 1 kHz (calcium currents). The external solutions used are summarized in Table I. Experiments were carried out at room temperature (~20°C). Average numerical values are presented as means ± SD.

TABLE I  
*External Solutions*

Designation	Na <sup>+</sup>	Ca <sup>++</sup>	Ba <sup>++</sup>	TEA <sup>+</sup>	Cl <sup>-</sup>	Br <sup>-</sup>
20 Na/10 Ca	20	10	—	125	165	—
10 Na/10 Ca	10	10	—	135	165	—
10 Ba	—	—	10	145	165	—
10 Ca	—	10	—	145	20	145
50 Ca	—	50	—	85	185	—

Concentrations are in millimolar units. All solutions were buffered with 10 mM HEPES, pH adjusted to 7.4 with NaOH (20 Na/10 Ca and 10 Na/10 Na) or with CsOH (10 Ba, 10 Ca, 50 Ca).

### *Cell Culture*

Primary cultures of myoblasts were prepared from fore- and hindlimbs of late-term fetal or newborn rats and mice. The limb muscles were finely minced and then incubated at 37°C for 40 min in rodent Ringer (146 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 11 mM glucose, 10 mM HEPES, pH adjusted to 7.4 with NaOH) containing collagenase (2 mg/ml; type I, Sigma Chemical Co., St. Louis, MO). After filtration and centrifugation to remove large debris, the cell suspension was preplated for 1 h in glass to remove rapidly adhering cells and then plated onto 35-mm Falcon Primaria dishes in plating medium containing (vol/vol) 80% Dulbecco's modified Eagle's medium with 4.5 g/liter glucose (DMEM), 10% horse serum, and 10% calf serum. After 4 d, the plating medium was replaced with maintenance medium: 90% DMEM and 10% horse serum. All culture media contained penicillin (100 U/ml) and streptomycin (100 μg/ml). Cultures were maintained at 37°C in a 95% air/5% CO<sub>2</sub>, water-saturated atmosphere.

Currents were recorded from myotubes 1–4 wk after the initial plating. Myotubes selected for study had a compact, nonbranching geometry. Cell dimensions were not routinely recorded, but diameters were typically 8–30 μm and lengths were 200–500 μm. In the cells for which dimensions were measured, the ratio of linear capacitance to cell surface area (calculated under the assumption of cylindrical geometry) averaged  $1.55 \pm 0.08 \mu\text{F}/\text{cm}^2$ , a value similar to that for FDB fibers from newborn rats (Beam and Knudson, 1988).

### *Isolation of Muscle Fibers*

Single muscle fibers were dissociated from the FDB, a fast-twitch (Carlsen et al., 1985) skeletal muscle of the rat. The procedure was modified from that described by Bekoff and Betz (1977). The muscle was dissected from the animal, cleaned of its surface connective tissue, and placed in a small vial containing 2 mg/ml collagenase (type I, Sigma Chemical Co.) and 1 mg/ml bovine serum albumin (fraction V, Sigma Chemical Co.) dissolved in dissociation solution (155 Cs-aspartate, 5 Mg-aspartate, 10 HEPES, pH 7.4 with CsOH). The vial was incubated for 1 h (embryonic and neonatal) or 2.5 h (adult) in a shaking water bath at 37°C. At the end of the collagenase treatment, the muscle was transferred to a small dish containing the same internal solution used for filling the patch pipettes (composition given above) and triturated with Pasteur pipettes whose tips had been fire-polished to produce openings of successively decreasing size. The dissociated fibers were stored in internal solution until they were to be used for measurement of currents, and only then were they exposed to external solutions (see below) of experimental interest. Fibers stored in internal solution remained experimentally viable for several hours.

### *Application of Drugs*

Concentrated (1 or 10 mM) stock solutions of (+)-PN 200-110 (kindly provided by Drs. A. Lindenmann and E. Rissi of Sandoz Ltd., Basel, Switzerland) and nitrendipine (kindly supplied by Dr. Alexander Scriabine, Miles Laboratories, Inc., New Haven, CT) were prepared by dissolving the drugs in ethanol. The stock solutions were stored in the dark at -20°C. Test concentrations of drugs were prepared just before use by dilution with the external saline used for current measurements. Care was taken to minimize exposure of the drug solutions to light. When the bath was changed during drug application, the total volume of the new solution washed through was at least 10 times the bath volume. Durations of drug exposure of at least 7 min were used before the degree of drug block was assessed. Because the dihydropyridines are very hydrophobic, the experimental chamber and solution-changing apparatus were washed extensively with ethanol between experiments.

## RESULTS

The results given below were obtained from enzymatically dissociated FDB fibers from rats aged  $\leq 14$  d and from rat and mouse myotubes. Unless noted, no systematic differences were observed in the properties of the currents measured in these three preparations.

### *Three Inward Ionic Currents*

Fig. 1 illustrates the presence of three distinct inward ionic currents in freshly dissociated FDB muscle fibers of neonatal rats. To record these currents, outward currents were blocked by the combined effects of TEA<sup>+</sup> in the bath and Cs<sup>+</sup> in the pipette. The fastest and largest of the three inward currents is sodium current,  $I_{Na}$  (Fig. 1 A, upper panel). The identity of this current as sodium current is based both on its dependence on external sodium (not shown) and on its susceptibility to block by TTX. The block of  $I_{Na}$  by 1  $\mu$ M TTX is illustrated in the lower panel of Fig. 1 A (same fiber as in the upper panel; note the slower time base). Fig. 1 B illustrates, on a much slower time scale, that in addition to  $I_{Na}$ , slower inward currents are also present in neonatal muscle fibers. For a test pulse to 0 mV,  $I_{Na}$  is followed by a slower inward current that inactivates completely ( $I_{fast}$ ), whereas for a test pulse to +40 mV,  $I_{Na}$  is

followed by a maintained inward current ( $I_{\text{slow}}$ ).  $I_{\text{slow}}$  accounts for the maintained current not blocked by TTX in the lower panel of Fig. 1 A. Both  $I_{\text{fast}}$  and  $I_{\text{slow}}$  are calcium currents, since they are eliminated if bath  $\text{Ca}^{++}$  is replaced by  $\text{Mg}^{++}$ . The properties of  $I_{\text{fast}}$  and  $I_{\text{slow}}$  resemble, respectively, those of the calcium currents

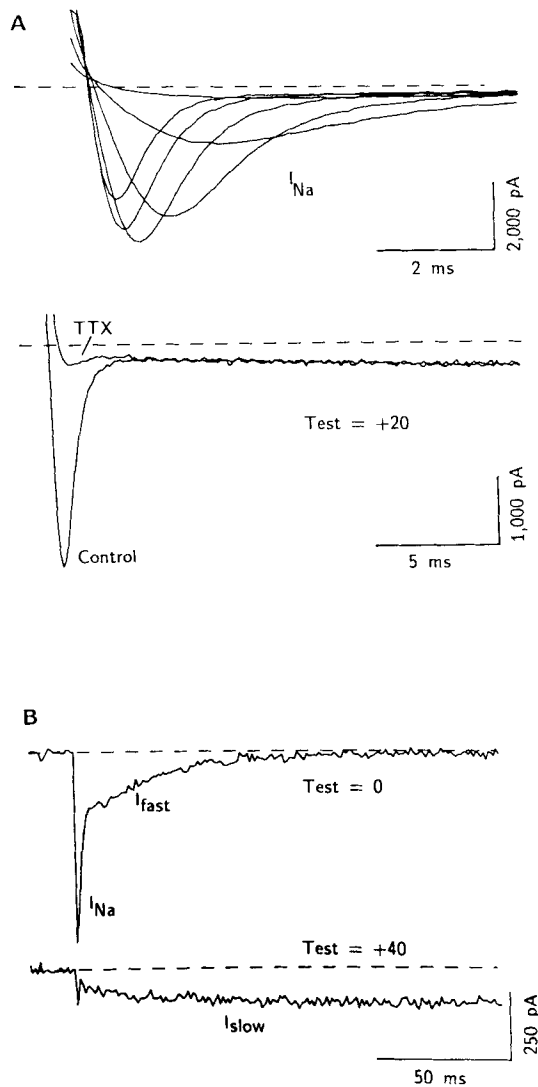


FIGURE 1. Developing muscle possesses three distinct inward ionic currents. (A) Upper panel: sodium currents in a neonatal muscle fiber (test depolarizations of  $-30$  to  $+20$  mV in  $10$ -mV increments). Lower panel: block of  $I_{\text{Na}}$  by TTX reveals a smaller, longer-lasting, inward calcium current. The currents shown were elicited by a test pulse to  $+20$  mV before and after the addition of  $1 \mu\text{M}$  TTX. (For this test pulse to  $+20$  mV, the apparent block by TTX is exaggerated because of contamination of  $I_{\text{Na}}$  by unsubtracted capacity current. For a test pulse to  $-10$  mV,  $1 \mu\text{M}$  TTX reduced peak  $I_{\text{Na}}$  to  $19\%$  of its control value.) (B) Comparison of sodium and calcium currents on a slower time base. A test pulse to  $0$  mV elicits both  $I_{\text{Na}}$  and a transient calcium current ( $I_{\text{fast}}$ ); a test pulse to  $+40$  mV elicits a maintained calcium current ( $I_{\text{slow}}$ ). (A) Fiber F23; 4-d FDB;  $20 \text{ Na}/10 \text{ Ca}$  solution; holding potential (HP) =  $-80$ ;  $C = 191 \text{ pF}$ . For purposes of illustration, the first millisecond of the capacity transient has been blanked in both the upper and the lower panel. (B) Fiber E40; 2-d FDB;  $10 \text{ Na}/10 \text{ Ca}$  bath solution; HP =  $-80$ ;  $C = 102 \text{ pF}$ .

termed "T" current and "L" current by Nowycky et al. (1985). The properties of  $I_{\text{fast}}$  and  $I_{\text{slow}}$  are described at length below.

Large cell size (typically several hundred picofarads linear capacitance), together with the appreciable series resistance of the patch pipette, prevented a detailed analysis of  $I_{\text{Na}}$  in neonatal FDB fibers. This series resistance meant that  $I_{\text{Na}}$  escaped

from control with physiological extracellular  $\text{Na}^+$ . For this reason, sodium currents were measured with reduced extracellular  $\text{Na}^+$ . Even so, peak  $I_{\text{Na}}$  was quite sensitive to the amount of series resistance compensation. Moreover, this reduced sodium current was often contaminated by incompletely subtracted capacitive current (which has a fast time course that partially overlaps that of the sodium current). Nonetheless, it was clear that compared with  $I_{\text{Na}}$  in adult muscle,  $I_{\text{Na}}$  in neonatal muscle is resistant to block by TTX. For example, in FDB fibers of rats  $\leq 10$  d old, 100 nM TTX reduced peak  $I_{\text{Na}}$  to  $39 \pm 9\%$  ( $N = 5$ ) of control, while 1  $\mu\text{M}$  TTX reduced the peak current to  $34 \pm 22\%$  ( $N = 4$ ) of control. For comparison, the  $K_d$  for toxin block of  $I_{\text{Na}}$  in innervated adult rat muscle is  $\sim 5$  nM (Pappone, 1980), so the same toxin concentrations would have reduced  $I_{\text{Na}}$  in adult muscle to 5 and 0.5% of control, respectively.

In adult rat skeletal muscle, it is well established that  $I_{\text{Na}}$  is the dominant inward current. The same appears to be the case in neonatal rat muscle. Indeed, the disparity in size between  $I_{\text{Na}}$  and the calcium currents is larger than it might appear from the records shown in Fig. 1, since these were obtained with 10 or 20 mM external  $\text{Na}^+$  (compared to the physiological value of  $\sim 150$  mM) and 10 mM external  $\text{Ca}^{++}$  (compared to the physiological value of  $\sim 2$  mM). In fibers of animals  $\leq 10$  d old, peak  $I_{\text{Na}}$  (measured in 20 mM external  $\text{Na}^+$ ) averaged  $17.7 \pm 7.2$  (mean  $\pm$  SD,  $N = 17$ ) pA/pF, peak  $I_{\text{fast}}$  averaged  $0.7 \pm 0.6$  pA/pF ( $N = 15$ ), and peak  $I_{\text{slow}}$  averaged  $4.6 \pm 4.3$  pA/pF (both calcium currents were measured in 10 mM external  $\text{Ca}^{++}$ ). If one assumes that peak  $I_{\text{Na}}$  scales linearly with external  $\text{Na}^+$  and that peak calcium currents are similar in 2 and 10 mM, then the peak  $I_{\text{Na}}$  in neonatal muscle is  $\sim 30$ -fold larger than either of the two calcium currents. A similar situation was observed in myotubes. In 20 mM  $\text{Na}^+$ , peak  $I_{\text{Na}}$  was  $34 \pm 18$  pA/pF ( $N = 15$ ), compared to  $0.7 \pm 0.6$  ( $N = 20$ ) and  $8.2 \pm 4.9$  ( $N = 20$ ) pA/pF, respectively, for  $I_{\text{fast}}$  and  $I_{\text{slow}}$  in 10 mM  $\text{Ca}^{++}$ .

#### *Voltage Dependence of the Two Calcium Currents*

The removal of external  $\text{Na}^+$ , usually together with the addition of 10  $\mu\text{M}$  TTX, was used to measure calcium channel currents free of contamination by  $I_{\text{Na}}$ . Fig. 2 A illustrates currents recorded under these conditions from a mouse myotube bathed in 10 mM  $\text{Ba}^{++}$ . Test depolarizations from  $-30$  to  $-10$  mV selectively activated the transient,  $I_{\text{fast}}$  current, which inactivated completely within  $< 50$  ms. Depolarizations to  $\geq 0$  mV additionally activated the maintained,  $I_{\text{slow}}$  current, which decayed little in 200 ms.  $I_{\text{slow}}$  masks the presence of  $I_{\text{fast}}$  for test pulses to  $+20$  mV and above. The presence of distinct  $I_{\text{fast}}$  and  $I_{\text{slow}}$  components of current is reflected in the biphasic shape of the peak current-voltage relationship (Fig. 2 B). The illustrated relationship was typical of many cells, but in other cells, the threshold and the potential required to elicit the maximum inward current for both  $I_{\text{fast}}$  and  $I_{\text{slow}}$  varied by as much as  $\pm 10$  mV compared with the cell illustrated in Fig. 2 B.

Most myotubes and neonatal FDB fibers displayed both  $I_{\text{fast}}$  and  $I_{\text{slow}}$ , although the relative magnitude of these two currents varied considerably from one cell to another, especially in myotubes. In addition to displaying cell-to-cell variability, the relative magnitude of  $I_{\text{fast}}$  and  $I_{\text{slow}}$  in FDB fibers shows a dependence on postnatal age (Beam and Knudson, 1988). Both  $I_{\text{fast}}$  and  $I_{\text{slow}}$  activate quite slowly compared with  $I_{\text{Na}}$ . For test pulses in the range  $-20$  to 0 mV,  $I_{\text{fast}}$  typically peaked within 10–15 ms.

For a test pulse to +20 mV,  $I_{\text{slow}}$  typically peaked in ~50 ms (e.g., Figs. 2 A and 3 B), but in some cells the time to peak was much longer (~150 ms for the myotubes in Figs. 3 C and 5 A).

Fig. 3 illustrates the difference in the voltage dependence of inactivation of  $I_{\text{fast}}$  and

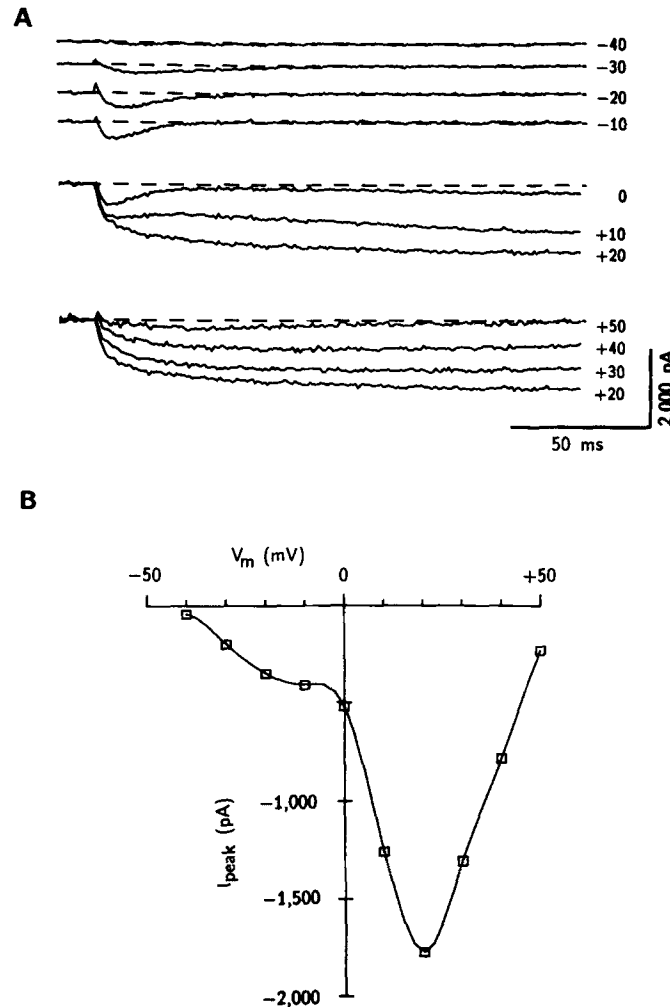


FIGURE 2. Activation of transient ( $I_{\text{fast}}$ ) and maintained ( $I_{\text{slow}}$ ) calcium channel currents. (A) Currents elicited by the test potentials indicated to the right of each trace. Test potentials of less than or equal to -10 mV activated  $I_{\text{fast}}$  selectively. The test pulses to 0 and +10 mV activated both  $I_{\text{fast}}$  and  $I_{\text{slow}}$ . For test potentials of  $\geq 20$  mV, only  $I_{\text{slow}}$  is evident. (B) Peak  $I$ - $V$  plot for the currents shown in A. Cell F32; mouse myotube 11 d in culture; HP = -80;  $C = 375$  pF; 10 Ba solution + 10  $\mu$ M TTX.

$I_{\text{slow}}$ . Brief prepulses to moderately depolarized levels caused the inactivation of  $I_{\text{fast}}$ . For the cell in Fig. 3 A, a 1-s prepulse to -45 mV caused half-inactivation and a prepulse to -30 mV caused complete inactivation. In other cells, half-inactivation of  $I_{\text{fast}}$  was observed for a 1-s prepulse to potentials in the range from -55 to -45 mV.

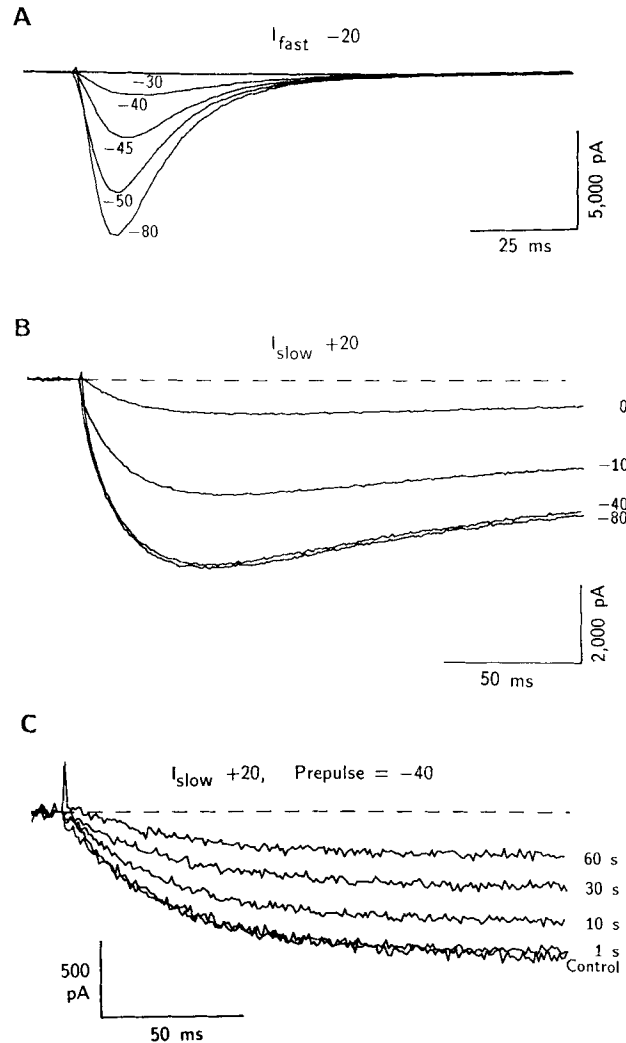


FIGURE 3. Inactivation of  $I_{fast}$  and  $I_{slow}$  with holding potential. (A) Test currents were elicited by a constant test depolarization to  $-20$  mV. The holding potential was changed from  $-80$  mV to the indicated holding potential for 1 s before application of the test depolarization. After the test depolarization, the cell was returned to the original  $-80$  mV holding potential, where it was held for a sufficient period to allow complete recovery of the test current. Cell F35; rat myotube 26 d in culture; HP =  $-80$  mV;  $C = 660$  pF;  $10$  Ca +  $10$   $\mu$ M TTX. (B) The holding potential was changed to the indicated potential for 2 s before application of the test potential of  $+20$  mV. The record for the  $-80$  mV holding potential is the average of two sweeps, one taken before and the other after the other traces illustrated. Fiber D24; 14-d FDB;  $C = 360$  pF;  $10$  Ca +  $10$   $\mu$ M TTX. (C) Before application of the test potential, the holding potential was changed to  $-40$  mV for the durations indicated next to each trace. Cell F12; rat myotube 21 d in culture;  $C = 353$  pF;  $10$  Ca solution.



There did not appear to be appreciable steady state inactivation at  $-80$  mV (the standard holding potential) since the magnitude of  $I_{\text{fast}}$  was not increased by changing to a more negative holding potential. The inactivation of  $I_{\text{slow}}$  required stronger (e.g.,  $0$  mV for 2 s; Fig. 3 B) or longer (e.g.,  $-40$  mV for 60 s; Fig. 3 C) depolarizations than the inactivation of  $I_{\text{fast}}$ . Depending on the cell in question, half-inactivation of  $I_{\text{slow}}$  by prolonged depolarizations ( $\geq 60$  s) was observed for potentials in the range  $-40$  to  $-20$  mV.

#### *Comparison of Calcium and Barium Currents*

Fig. 4 compares  $I_{\text{fast}}$  and  $I_{\text{slow}}$  with either barium or calcium as the charge carrier. Changing from  $10$  mM  $\text{Ba}^{++}$  to  $10$  mM  $\text{Ca}^{++}$  had little effect on the kinetics or magnitude of  $I_{\text{fast}}$ .  $I_{\text{slow}}$  was also relatively unaffected by changing from barium to calcium. For the fiber illustrated in Fig. 4,  $I_{\text{slow}}$  was  $\sim 30\%$  larger in  $10$  mM  $\text{Ba}^{++}$  than in  $10$  mM  $\text{Ca}^{++}$ , but in some cells the current in barium was smaller than in calcium.

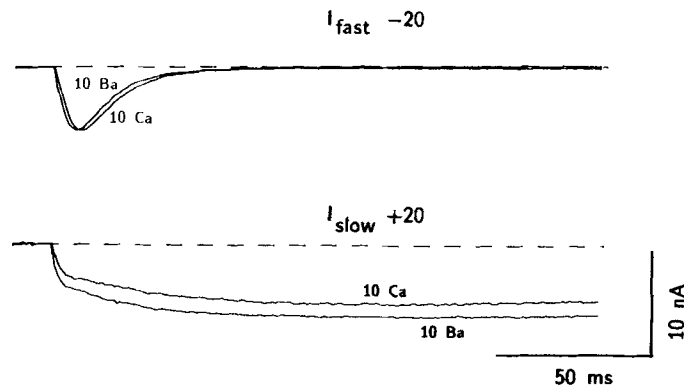


FIGURE 4. Effect of exchanging Ca for Ba on  $I_{\text{fast}}$  and  $I_{\text{slow}}$ . Cell F35; rat myotube 26 d in culture; HP =  $-80$  mV;  $C = 660$  pF;  $10 \mu\text{M}$  TTX was present in both the  $10 \text{ Ba}$  and  $10 \text{ Ca}$  solutions.

Altogether, the maximum peak  $I_{\text{slow}}$  was measured in 10 cells before and after exchanging  $10$  mM  $\text{Ca}^{++}$  for  $10$  mM  $\text{Ba}^{++}$  (or  $10$  mM  $\text{Ba}^{++}$  for  $0$  mM  $\text{Ca}^{++}$ ). For these 10 cells, the ratio of maximum peak  $I_{\text{slow}}$  in barium to that in calcium was  $108 \pm 41\%$ . Thus, the available data suggest that the slow calcium channel of developing rodent skeletal muscle is about equally permeable to barium and calcium, a property also displayed by the slow calcium channel of adult rodent muscle (Donaldson and Beam, 1983). Independent of changes in current magnitude, exchanging barium for calcium caused a hyperpolarizing shift in the activation of  $I_{\text{slow}}$ ;  $\bar{V}$ , the potential for half-maximal activation of  $I_{\text{slow}}$ , was  $9.1 \pm 2.4$  mV hyperpolarized in  $10$  mM  $\text{Ba}^{++}$  as compared with  $10$  mM  $\text{Ca}^{++}$  (the method for determining  $\bar{V}$  is described in Beam and Knudson, 1988). By contrast, the voltage dependence of activation of  $I_{\text{fast}}$  was the same in barium and calcium.

#### *Calcium Channel Blockers*

As described for the comparable currents in other cell types,  $I_{\text{fast}}$  and  $I_{\text{slow}}$  can be distinguished on the basis of calcium channel antagonists. Thus,  $50 \mu\text{M}$   $\text{Cd}^{++}$  reduced

$I_{fast}$  by only about one-third but produced a  $\sim 75\%$  block of  $I_{slow}$  (Fig. 5 A). Higher concentrations of cadmium (1.0 mM) caused a nearly complete block of both  $I_{fast}$  and  $I_{slow}$ . Dihydropyridine calcium channel antagonists also distinguished between  $I_{fast}$  and  $I_{slow}$ . At a concentration of 1  $\mu\text{M}$ , (+)-PN 200-110 blocked  $I_{slow}$  by  $\sim 85\%$  without

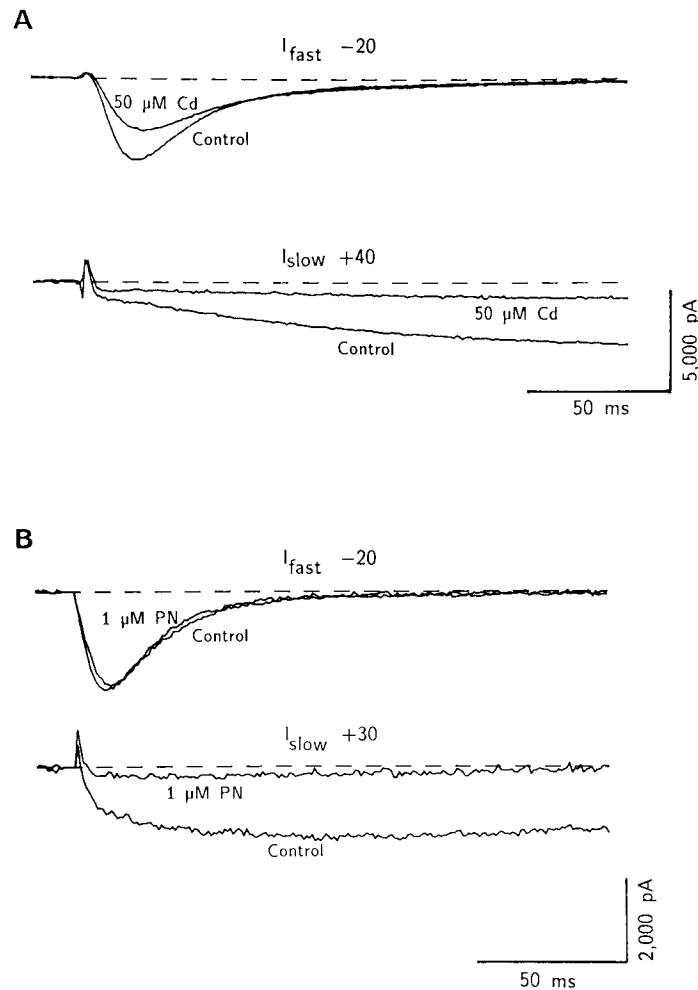


FIGURE 5. Differential sensitivity of  $I_{fast}$  and  $I_{slow}$  to calcium channel blockers. (A) Currents were measured in the absence (control) and presence of 50  $\mu\text{M}$  Cd. Cell F94; mouse myotube 12 d in culture; HP =  $-80$ ;  $C = 416$  pF; 10 Ca + 10  $\mu\text{M}$  TTX. (B) Currents were measured in the absence (control) and presence of 1  $\mu\text{M}$  (+)-PN 200-110. Cell F35; rat myotube 26 d in culture; HP =  $-80$  mV;  $C = 660$  pF; 10 Ca + 10  $\mu\text{M}$  TTX.

affecting  $I_{fast}$  (Fig. 5 B). Similarly, 1  $\mu\text{M}$  nitrendipine produced an  $\sim 50\%$  block of  $I_{slow}$  without affecting  $I_{fast}$ .

The block of  $I_{slow}$  by (+)-PN 200-110 was examined in more detail, as illustrated in Fig. 6. Both myotubes and dissociated FDB fibers were used for these experiments.

The dissociated FDB fibers were used in order to permit a more direct comparison with studies others have carried out on the binding of radiolabeled dihydropyridines to the transverse-tubular, subcellular fraction of rodent skeletal muscle (Ferry et al., 1983; Cognard et al., 1986b). Fig. 6 shows that after a 10-min exposure to 200 nM (+)-PN 200-110 (at a holding potential of  $-80$  mV),  $I_{\text{slow}}$  is reduced by  $\sim 60\%$  compared to control. The block of calcium currents by dihydropyridines is very much potentiated at depolarized holding potentials, as if the blocking action of the drug were largely a consequence of binding to, and stabilizing, the inactivated form of the calcium channel (Bean, 1984). Thus, to ensure that the relatively small block shown in Fig. 6 was not a consequence of incomplete equilibration of the drug, the fiber was exposed to holding potentials sufficiently depolarized to reduce the magnitude of  $I_{\text{slow}}$  to nearly zero. Even after these periods of depolarized holding potential, the magnitude of  $I_{\text{slow}}$  (after recovery at a holding potential of  $-80$  mV) was  $\sim 30\%$  of the

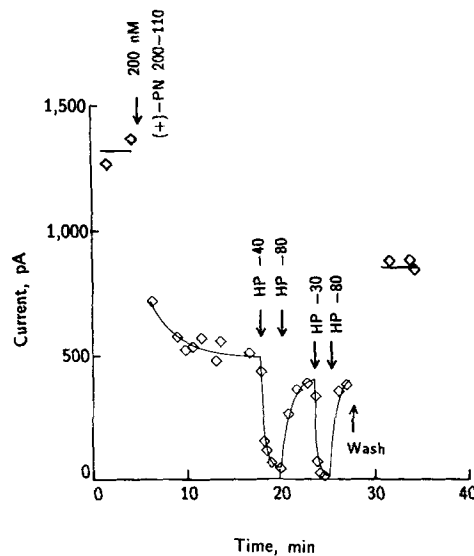


FIGURE 6. Blockade of  $I_{\text{slow}}$  by (+)-PN 200-110. The smooth curves, which were drawn by eye, are interrupted to indicate the period during which solution changes were made. (A) Initial holding potential,  $-80$  mV. Note that substantial  $I_{\text{slow}}$  remains even after a  $>20$ -min exposure to 200 nM (+)-PN 200-110 and periods of depolarized holding potential that reduced the inward current to near zero. Fiber F41; 12-d FDB;  $C = 210$  pF; 10 Ca.

control value. Altogether, the ability of (+)-PN 200-110 (100–1,000 nM) to block  $I_{\text{slow}}$  elicited from a holding potential of  $-80$  mV was examined in three FDB fibers and three myotubes. For these cells, the half-maximal blocking concentration ( $K_{0.5}$ ) was  $182 \pm 71$  nM, calculated under the assumption that the binding of one drug molecule blocks the channel.

#### *Maintained Depolarization Potentiates Dihydropyridine Block of $I_{\text{slow}}$*

In both cardiac muscle (Bean, 1984) and rat myoballs (Cognard et al., 1986b), it has been found that dihydropyridine concentrations that have relatively little effect at hyperpolarized holding potentials produce a significant block of calcium current at depolarized holding potentials. A similar phenomenon was observed in dissociated FDB fibers and myotubes. As shown in Fig. 7 A, exposure to 20 nM (+)-PN 200-110 produced only a small (25%) reduction in  $I_{\text{slow}}$  at a holding potential of  $-80$  mV. 5

min after changing to a holding potential of  $-50$  mV (still in the presence of  $20$  nM (+)-PN 200-110), peak  $I_{\text{slow}}$  was reduced by  $\sim 85\%$ . Fig. 7 *B* shows for the same fiber, after washout of the (+)-PN 200-110, that holding at  $-50$  mV for  $5$  min produced only an  $\sim 50\%$  reduction in  $I_{\text{slow}}$ . After holding the fiber at  $-50$  mV for  $5$  min, appli-

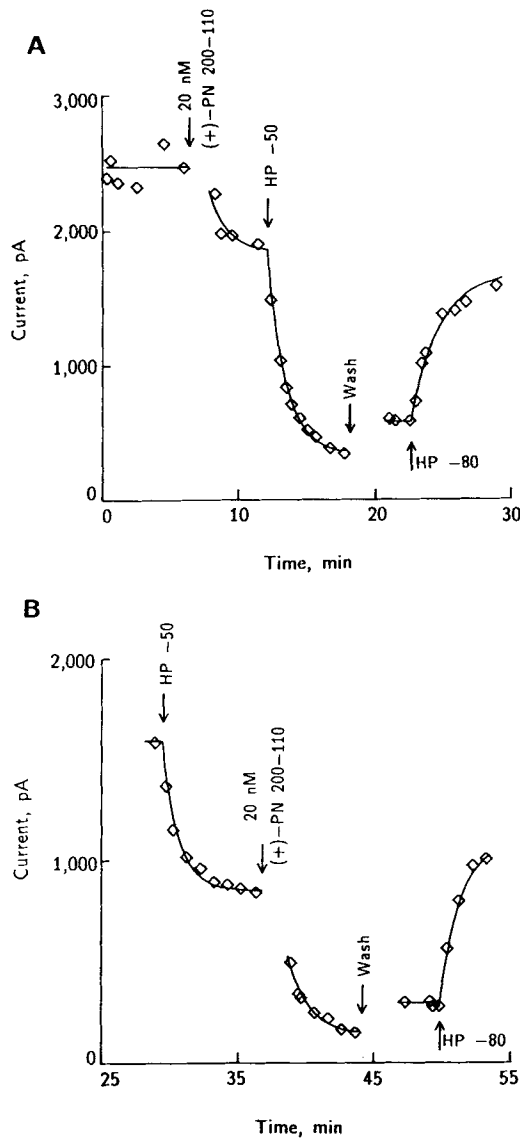


FIGURE 7. (*A* and *B*) Depolarized holding potentials potentiate the (+)-PN 200-110 block of  $I_{\text{slow}}$ . (*A*) At a holding potential of  $-80$  mV, a 5-min exposure to  $20$  nM (+)-PN 200-110 caused a  $<25\%$  reduction in  $I_{\text{slow}}$ . Changing the holding potential to  $-50$  mV (still in the presence of (+)-PN 200-110) caused a further  $80\%$  reduction within  $5$  min. Subsequent washing and restoration of HP =  $-80$  mV caused  $I_{\text{slow}}$  to recover to  $64\%$  of the original control value. (*B*) In the absence of (+)-PN 200-110, changing to HP =  $-50$  mV for  $>5$  min reduced  $I_{\text{slow}}$  by only  $50\%$ , as compared to the  $80\%$  reduction in the presence of  $20$  nM (+)-PN 200-110 (cf. *A*). Subsequent exposure to  $20$  nM (+)-PN 200-110 reduced  $I_{\text{slow}}$  by  $75\%$  after  $5$  min, much more than the  $<25\%$  reduction by the same duration of drug exposure at HP =  $-80$  mV as shown in *A*. The data in *B* represent a continuation of the experiment illustrated in *A*. Fiber F45; 14-d FDB;  $C = 300$  pF;  $10$  Ca.

cation of  $20$  nM (+)-PN 200-110 caused a  $>80\%$  reduction of  $I_{\text{slow}}$ . A similar effect of holding potential on block of  $I_{\text{slow}}$  by  $20$  nM (+)-PN 200-110 was observed in four myotubes on which a similar experiment was carried out. For each myotube,  $I_{\text{slow}}$  was first measured before drug exposure at a holding potential of  $-80$  mV. The holding

potential was then set at a level that inactivated  $I_{\text{slow}}$  by 35–65%; after inactivation of  $I_{\text{slow}}$  had reached a steady state, the cell was exposed to 20 nM (+)-PN 200-110. The average value of  $K_{0.5}$  at these depolarized holding potentials was  $5.5 \pm 2.6$  nM (calculated assuming the channel is blocked by the binding of one drug molecule).

## DISCUSSION

### *Comparison with Calcium Currents in Other Tissues*

The major finding described here is that developing mammalian skeletal muscle possesses three distinct inward ionic currents, a large sodium current and two smaller calcium currents. The two calcium currents,  $I_{\text{fast}}$  and  $I_{\text{slow}}$ , in developing muscle have properties similar to calcium currents that have been measured in a variety of other tissues. These tissues include chick (Carbone and Lux, 1984; Nowycky et al., 1985) and rat (Bossu et al., 1985; Fedulova et al., 1985) sensory neurons, canine atrial cells (Bean, 1985), guinea pig ventricular cells (Nilius et al., 1985), transformed cells (GH<sub>3</sub>) derived from rat pituitary (Armstrong and Matteson, 1985), neuroblastoma cells (Tsunoo et al., 1985), acutely dissociated (Bean et al., 1986; Benham and Tsien, 1987) and primary cultured (Sturek and Hermsmeyer, 1986; Loraind et al., 1986) smooth muscle, and primary cultures of hippocampal neurons (Yaari et al., 1987). In all of these preparations, weak depolarizations selectively activate a transient current (decay time constant of 10–30 ms), whereas stronger depolarizations activate a long-lasting current that decays much more slowly (or not at all). In most of these preparations, it has been shown that the transient current is more resistant to block by cadmium and dihydropyridines than the long-lasting current, and that briefly changing the holding potential to a depolarized level inactivates the transient current without affecting the long-lasting current. The terms “T” and “L” are gaining acceptance for describing, respectively, the transient and long-lasting currents, while “N” is used to describe a third kind of calcium current found in sensory neurons (Nowycky et al., 1985). While the use of a single nomenclature is useful, it is important to note that both the transient and long-lasting calcium currents display tissue-specific properties. For example, the transient current generally activates at more negative potentials in neuronal cells compared with cardiac, smooth, and skeletal muscle. The persistent current shows prominent calcium-dependent inactivation in cardiac cells but not in sensory neurons or skeletal muscle, and the persistent current in skeletal muscle activates ~10 times more slowly in skeletal muscle than in the other tissues. The persistent current in neurons, but not that in muscle cells, is blocked by  $\omega$ -conotoxin (McCleskey et al., 1987).

### *Calcium Currents in Adult Skeletal Muscle*

Adult skeletal muscle fibers of frogs (Beatty and Stefani, 1976; Stanfield, 1977; Sanchez and Stefani, 1978; Almers and Palade, 1981), rats (Donaldson and Beam, 1983), and rabbits (Walsh et al., 1986) possess a slow calcium current with a voltage dependence and kinetics like those of  $I_{\text{slow}}$  in developing muscle. Thus, it seems likely that  $I_{\text{slow}}$  in developing muscle and the slow calcium current in adult muscle represent the functioning of the same, or a closely related, channel protein. Recently, Cota and Stefani (1986) have reported that in addition to the slow calcium current, adult frog

muscle possesses another calcium current, which they termed the fast-activated current. The fast-activated calcium current in adult muscle can be distinguished from the slow calcium current by the speed of its activation (time to peak of ~25 ms at -10 mV compared with 250–400 ms for the slow current) and by its more negative potential range for activation (~30 mV hyperpolarized compared with the slow calcium current). In both these regards, the fast-activated current of adult muscle resembles  $I_{fast}$  in developing muscle. An important difference, however, is that the fast-activated current of adult muscle is maintained without decline for depolarizations of up to 2 s duration, whereas  $I_{fast}$  in developing muscle inactivates completely within 50 ms. On this basis, it seems likely that different channel species give rise to these two currents. This conclusion is strengthened by the observation that  $I_{fast}$  disappears during the first 3 wk of postnatal development (Beam and Knudson, 1988). If  $I_{fast}$  in developing muscle is distinct from the fast-activated current in adult muscle, then the question arises why we failed to observe the fast-activated current. One possibility is that the fast-activated current is absent from developing muscle. Alternatively, because the fast-activated current is quite labile (Cota and Stefani, 1986; Garcia and Stefani, 1987), it is possible that the current rapidly disappears under our recording conditions. We did observe, in a few cells, a current with kinetics like those of the fast-activated current, but the current was not stable enough to allow us to determine whether it was simply a reflection of a nonlinear leak current.

#### *Comparison with Calcium Currents in Rat Myoballs*

Cognard et al. (1986a, b) have presented descriptions of two kinds of calcium currents in cultured rat skeletal muscle (dihydropyridine insensitive and dihydropyridine sensitive, corresponding, respectively, to our  $I_{fast}$  and  $I_{slow}$ ). Our results agree quite well with theirs with respect to the time course of the two currents and the currents' relative sensitivity to block by cadmium and dihydropyridines. Our results and theirs disagree regarding the voltage dependence of both currents. Specifically, the activation of the dihydropyridine-insensitive current is shifted 20–25 mV in the hyperpolarizing direction compared with  $I_{fast}$ , as is the activation of the dihydropyridine-sensitive current compared with  $I_{slow}$ . Similarly, the potentials that cause half-inactivation of the dihydropyridine-insensitive (Cognard et al., 1986b) and dihydropyridine-sensitive (Cognard et al., 1986a) currents are 20–25 mV hyperpolarized compared to the values we found, respectively, for half-inactivation of  $I_{fast}$  and  $I_{slow}$ . These differences in potential dependence may not be very significant, given that the patch-clamp technique provides a relatively imprecise measurement of absolute potential. To the extent that the differences are real, differences in preparation and experimental protocols are probably involved. Thus, we used rat and mouse myotubes, as well as acutely dissociated rat FDB muscle fibers, whereas Cognard et al. (1986a, b) used "myoballs" that were obtained from primary cultures of rat thigh muscle (i.e., one of the preparations we used) by treatment with colchicine. Another experimental difference was that our internal solution was largely (or wholly) chloride free, whereas theirs contained chloride.

#### *Dihydropyridine Block of $I_{slow}$*

We found that high concentrations of both (+)-PN 200-110 (1  $\mu$ M) and nitrendipine (10  $\mu$ M) produce a nearly complete block of  $I_{slow}$  in both myotubes and FDB muscle

fibers of 12–14-d-old rats. This result is similar to that of Cognard et al. (1986*b*), who found a nearly complete block of the dihydropyridine-sensitive calcium current in rat myoballs by 2  $\mu\text{M}$  (+)-PN 200-110. Contradictory results have been reported for dihydropyridine block of the slow calcium current in adult mammalian skeletal muscle. Walsh et al. (1986) found that 10  $\mu\text{M}$  nitrendipine had no effect on the slow calcium current in sternomastoid fibers of adult rabbits, while Lamb and Walsh (1987) found that 10  $\mu\text{M}$  nifedipine completely blocked the slow calcium current in adult rat extensor digitorum longus and soleus muscles.

#### *Blocking Affinity of (+)-PN 200-110*

Although our results and those of Cognard et al. (1986*b*) are in agreement for high concentrations of (+)-PN 200-110, they differ with respect to  $K_{0.5}$ , the concentration of (+)-PN 200-110 that half-maximally blocks  $I_{\text{slow}}$ . At a negative holding potential ( $-80$  mV), we determined a value of  $182 \pm 71$  nM for  $K_{0.5}$ . This value is similar to the  $K_{0.5}$  of 430 nM found for block of the slow calcium current ( $-90$  mV holding potential) by racemic PN 200-110 in frog skeletal muscle (Schwartz et al., 1985). By contrast, Cognard et al. (1986*b*) found a  $K_{0.5}$  of 13 nM for the block by (+)-PN 200-110 ( $-90$  mV holding potential). We observed that, at depolarized holding potentials that inactivated  $I_{\text{slow}}$  by 35–65%,  $K_{0.5}$  decreased to  $5.5 \pm 2.6$  nM. Cognard et al. (1986*b*) found a value of 0.15 nM at a holding potential of  $-60$  mV, which according to their Fig. 2 should have inactivated the dihydropyridine-sensitive current by  $\sim 70\%$ . The comparable amount of inactivation in the two sets of studies means that the values for  $K_{0.5}$  should also have been comparable, under the assumption that (+)-PN 200-110 binds with high affinity only to the inactivated state of the channel (Bean, 1984). Differences in the identity or concentration of the divalent charge carrier do not seem to provide an explanation for the discrepancy either, since these do not affect the potency of nitrendipine block of cardiac calcium currents (Bean, 1984).

Skeletal muscle, cardiac muscle, and brain have all been shown to possess high-affinity receptors for dihydropyridines. Of these tissues, skeletal muscle possesses the highest density of dihydropyridine receptors by a factor of 50–500 (Fosset et al., 1983). Given the ability of dihydropyridines to block calcium currents, it is often assumed that the dihydropyridine receptor protein is in fact the calcium channel or a component thereof. In skeletal muscle, it is uncertain whether this assumption is correct. Specifically, there are discrepancies in both the number and affinity of skeletal muscle dihydropyridine receptors when compared with slow calcium channels. Thus, Schwartz et al. (1985) found there to be a large number of high-affinity binding sites ( $K_d = 0.93$  nM) for (+)-PN 200-110 in frog sartorius muscle fibers having a resting potential of  $-88$  mV. At a comparable holding potential, the  $K_{0.5}$  for (+)-PN 200-110 block of calcium current is  $\sim 430$  nM. Additionally, Schwartz et al. (1985) found that there are 35–50 times more high-affinity (+)-PN 200-110 binding sites in frog sartorius muscle than slow calcium channels (based on specific assumptions about both the single-channel calcium current and the maximum probability that a channel is open). The discrepancy in binding and blocking affinities reported by Schwartz et al. was not observed by Cognard et al. (1986*b*), who found affinities of 0.22 and 0.15 nM for the binding and blocking (at depolarized potentials), respectively. However, as pointed out in the preceding paragraph, our value for the blocking affinity at depolarized potentials (5.5 nM) is much larger than theirs.

*Electrical Excitability of Neonatal Muscle*

Microelectrode studies of the electrical excitability of chick skeletal muscle, developing either in vivo (Kano, 1975) or in vitro (Kano et al., 1972; Spector and Prives, 1977), have led to the conclusion that the action potential undergoes a change in these cells from predominantly calcium-based early in development to predominantly sodium-based later in development (cf. Spitzer, 1979). A different sort of transition seems to occur in rodent skeletal muscle. The action potential in rat diaphragm fibers, which is resistant to TTX early in development (fetal day 17), becomes increasingly sensitive to TTX block during the first 20 d postnatal (Harris and Marshall, 1973). The ionic basis of the TTX-resistant responses in diaphragm was not established, but the ionic basis of action potential generation has been studied using L6 cells, a clonal cell line derived from rat leg muscle. As in primary cultures of myoblasts, the L6 myoblasts fuse in vitro to produce multinucleate myotubes. The action potential in L6 myotubes has two components, an initial, rapid, sodium-dependent component, followed by a slower, calcium-dependent plateau (Land et al., 1973; Kidokoro, 1975). The plateau becomes shorter, but does not disappear, as the multinucleate myotubes undergo subsequent in vitro development; the action potential in adult rat skeletal muscle lacks a calcium-dependent component (e.g., Kidokoro, 1975). These measurements of action potentials seem entirely consistent with our measurements of sodium and calcium currents in developing rodent muscle. Specifically, it seems quite certain that  $I_{Na}$ , the fastest and largest inward current, is responsible for the initial, rapid spike and that  $I_{fast}$  and/or  $I_{slow}$  are responsible for the secondary plateau. As mentioned, the secondary plateau is absent in adult muscle. Since adult muscle possesses a slow calcium current but lacks  $I_{fast}$  (Beam and Knudson, 1988), it seems likely that  $I_{fast}$  is necessary (and perhaps sufficient) for generation of the secondary plateau in developing muscle. The following article (Beam and Knudson, 1988) describes the effect of postnatal development on  $I_{fast}$  and  $I_{slow}$  in rat FDB fibers and speculates on the possible roles of these two currents.

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