The Journal of Neuroscience, October 1989, 9(10): 3443–3453

Ca^{2+} and Na^+ Currents in Developing Skeletal Myoblasts Are Expressed in a Sequential Program: Reversible Suppression by Transforming Growth Factor Beta-1, an Inhibitor of the Myogenic Pathway

John M. Caffrey,[1,a] Arthur M. Brown,[1] and Michael D. Schneider[1,2,3]

[1]Department of Physiology and Molecular Biophysics, [2]Molecular Cardiology Unit, and [3]Departments of Medicine and Cell Biology, Baylor College of Medicine, Houston, Texas 77030

We have analyzed the biophysical and developmental properties of Ca^{2+} and Na^+ currents in C2 muscle cells, whose morphological and biochemical phenotype closely resembles differentiated skeletal muscle. Both fused and unfused C2 myocytes possessed: (1) membrane capacitance consistent with the presence of complex sarcotubular invaginations, (2) tetrodotoxin-sensitive Na^+ channels, and (3) "fast" and "slow" Ca^{2+} channels that inactivated at holding potentials of −40 and −20 mV, respectively. Thus, the passive electrical properties, Na^+ currents, and Ca^{2+} currents expressed in C2 cells each differed from those found in the nonfusing muscle cell line, BC_3H1, and corresponded more precisely to characteristic findings observed in skeletal muscle fibers. In further contrast to BC_3H1 cells, C2 muscle also expressed "transient" Ca^{2+} channels similar to those reported in embryonic or neonatal skeletal muscle, which were detected within 12–24 hr of mitogen withdrawal, up to 60 hr before appearance of "fast" and "slow" currents. Na^+ channels also were induced 12–24 hr after mitogen withdrawal. Unlike the "fast" and "slow" Ca^{2+} currents, which were maximally expressed at 8–14 d of serum withdrawal, "transient" Ca^{2+} channels became down-regulated upon prolonged differentiation (as found in postnatal skeletal muscle *in vivo*) and were no longer expressed at 14 d. Despite their divergent kinetic and developmental properties, all components of Ca^{2+} and Na^+ current in C2 myocytes were suppressed reversibly in the presence of transforming growth factor β-1, a purified growth factor that inhibits the myogenic phenotype. The results indicate that fusion is not essential for skeletal myoblasts to produce developmentally regulated voltage-gated channels that resemble those of intact muscle and demonstrate that the formation of diverse Ca^{2+} and Na^+ channels can be mediated by a single peptide that affects the myogenic pathway.

The exact developmental mechanisms that determine the induction and tissue-specific expression of voltage-gated ion channels are presently not understood. In skeletal muscle cells an increasing number of extra- and intracellular signals now are known to control the formation of gene products associated with differentiation (Emerson et al., 1986; Schneider and Olson, 1989). The establishment of a differentiated state in muscle appears to require exit from the cell cycle and can be suppressed by serum components (Nadal-Ginard, 1978), individual growth factors (Linkhart et al., 1981; Massague et al., 1986; Olson et al., 1986; Clegg et al., 1987), and certain cellular or viral oncogenes (Falcone et al., 1985; Olson et al., 1987; Payne et al., 1987; Schneider et al., 1987; Schneider and Olson, 1989). However, the interrelationship between the biochemical and biophysical phenotype of myogenic cells is not yet clear, and the transduction pathways that control the onset of differentiation remain controversial (Clegg et al., 1987; Endo and Nadal-Ginard, 1987). We recently observed that the nonfusing muscle cell line, BC_3H1, expresses voltage-gated "fast" and "slow" Ca^{2+} currents whose ion-permeation properties, pharmacology, and kinetics closely resemble those observed in skeletal muscle (Caffrey et al., 1987). Ca^{2+} and Na^+ channels were not expressed during proliferative growth and were induced following mitogen withdrawal and growth arrest. Transfection with an activated c-H-*ras* gene that can block the induction of muscle-specific genes prevented the appearance of Ca^{2+} and Na^+ channels (Caffrey et al., 1987; Payne et al., 1987). In contrast, the appearance of K^+ channels was independent of mitogenic signals, not correlated with the differentiated phenotype, and the activated *ras* gene had no effect on their expression (Caffrey et al., 1987).

These initial results suggested the tentative hypothesis that the ontogeny of Ca^{2+} and Na^+ channels during myogenesis may involve regulatory pathways in common with those that control formation of other muscle-specific gene products, such as the nicotinic ACh receptor (Mishina et al., 1986; Buonanno and Merlie, 1987; Evans et al., 1987), sarcomeric proteins (Emerson et al., 1986), and muscle creatine kinase (Jaynes et al., 1986; Olson et al., 1986, 1987; Payne et al., 1987). To obtain a similar understanding of the transcriptional and posttranscriptional mechanisms that determine channel density and channel type,

Received Mar. 21, 1988; revised Mar. 6, 1989; accepted Mar. 11, 1989.

We are grateful to K. Angelides, M. B. Perryman, and E. N. Olson for their suggestions. M.D.S. thanks R. Roberts for encouragement and support. This investigation was supported by grants to M.D.S. from the National Science Foundation, DCB-8711313, the National Institutes of Health, HL 39141, and the American Heart Association, Texas Affiliate, 85G-223 and 87R-179; grants to A.M.B. from NIH, HL37044 and HL36475; and a National Research Service Award AR07546, and AHA, Texas Affiliate, grant 88G-218, to J.M.C. The Molecular Cardiology Unit is supported by the American Heart Association-Bugher Foundation Center for Molecular Biology of the Cardiovascular System. M.D.S. is an Established Investigator of the American Heart Association.

Correspondence should be addressed to Dr. Michael D. Schneider, Molecular Cardiology Unit, Baylor College of Medicine, One Baylor Plaza, Room 506C, Houston, TX 77030.

[a] Present address: Department of Neurology, Yale University School of Medicine, New Haven, CT 06510.

a model system is required whose biophysical and developmental properties both correspond to skeletal muscle *in vivo*. Furthermore, to elucidate the precise steps involved in the complex pathway leading to channel synthesis and to examine the relationships among ion channels and other differentiation products, defined reagents that impinge on myogenesis are required, as an alternative to fetal sera (cf. Jaynes et al., 1986; Massague et al., 1986; Olson et al., 1986; Endo and Nadal-Ginard, 1987).

Although the Ca^{2+} channels of BC_3H1 cells in other respects resemble those of adult skeletal muscle, important differences were observed in the voltage-dependence of "slow" current inactivation (Caffrey et al., 1987). Since BC_3H1 cells cannot irreversibly lose their proliferative capacity, fuse, assemble striated myofibrils, or form complex transverse tubules, a contrasting myogenic cell line such as C2 might be expected to express ion channels whose properties more closely approximate those of skeletal muscle fibers (Schubert et al., 1974; Yaffe and Saxel, 1977). Furthermore, unlike other myogenic systems, C2 muscle cells are known to induce and deinduce, in an appropriate temporal pattern, isoforms of myosin heavy chain, myosin light chain, and α-actin (Minty et al., 1986; Silberstein et al., 1986; Donoghue et al., 1987; Weydert et al., 1987), which are developmentally regulated *in vivo* (Whalen et al., 1981; Minty et al., 1982; Periasamy et al., 1984; Weydert et al., 1987). Therefore, C2 muscle cells might also be expected to possess specific developmental transitions among ion channel subtypes that occur *in vivo* (Beam and Knudson, 1988b) but not in BC_3H1 cells (Caffrey et al., 1987). Finally, although myogenic cell lines differ in their responsiveness to particular inhibitors of differentiation (Clegg et al., 1987), the serum peptide transforming growth factor β (TGFβ) reversibly blocks the morphological and biochemical differentiation of C2 cells (Olson et al., 1986; cf. Massague et al., 1986). Thus, TGFβ can be used to overcome 2 limitations confounding the interpretation of serum effects on ion channel expression: the coexistence of multiple serum constituents, which in principle might account for the observed findings, and concurrent actions of serum mitogens on DNA synthesis and cell division.

In this report, we show that both Ca^{2+} and Na^+ currents in C2 muscle cells more closely resemble those in adult skeletal muscle than do those found in the BC_3H1 cell line. We demonstrate that C2 muscle cells sequentially express 3 types of Ca^{2+} current with distinct biophysical and developmental properties, following serum withdrawal, and that the progression of Ca^{2+} channel types can occur in the absence of fusion and interaction with neurons. To dissociate the suppressive effects of mitogenic medium on Ca^{2+} channel formation from the possible indirect consequences of cell proliferation, Ca^{2+} and Na^+ currents also were analyzed in C2 cells treated with TGFβ. Expression of all 3 components of Ca^{2+} current was coordinately and reversibly blocked in the presence of nanomolar concentrations of TGFβ, in agreement with its known effects on gene products whose formation is contingent on muscle differentiation.

Materials and Methods

Cell culture. Undifferentiated C2 cells were inoculated onto poly-D-lysine-treated glass coverslips at 3×10^3 cells·cm^{-2} and were maintained in Ham's medium F12 containing 20% fetal bovine serum (FBS; Pinset and Whalen, 1984). At 30% confluency, differentiation was induced for 1–15 d in Dulbecco's modified Eagle's medium containing 2% horse serum (HS). To prevent the formation of myotubes, which was necessary to ensure adequate control of the transmembrane voltage (see below), mitogen step-down was performed at subconfluent density. To induce differentiation of BC_3H1 muscle cells, cells at 30% confluency were fed medium with FBS reduced from 20 to 0.5% (Caffrey et al., 1987). All media contained 50 μg/ml gentamicin. The homodimeric form of TGFβ-1 isolated from porcine platelets was obtained from R&D Systems (Minneapolis, MN).

Biophysical methods. We measured Ca^{2+} currents by standard whole-cell patch-clamp methods (detailed in Sakmann and Neher, 1983; Caffrey et al., 1987), applying voltage steps from a holding potential (V_h) of −90 mV to the test potentials (V_t) shown. Experiments were performed at room temperature (~20°C). Patch pipettes were prepared from thick-wall borosilicate glass (Type 7052, Garner Glass Co., Claremont, CA) using a tip diameter of 2–4 μm. Tip resistances ranged from 1 to 5 MΩ, and seal resistances were >10 GΩ. Ion currents were recorded using a List EPC-7 voltage clamp, filtered at 1–5 kHz with a 4-pole Bessel filter (Wavetek-Rockland, Inc., Rockleigh, NJ), digitized with a PDP 11/23 computer at 4 times the Nyquist frequency, and stored on a video tape recorder (HR-D725U, JVC Corporation, Elmwood Park, NJ) for later analysis. The linear components of capacity and leakage currents were subtracted digitally using scaled, averaged currents elicited by hyperpolarizing voltage steps of 10–30 mV. To correct for the effects of membrane area on current amplitude, current density (mean ± SEM) is shown as current amplitude normalized to membrane area on the basis of membrane capacitance, assuming 1 μF·cm^{-2}.

Ionic conditions. Na^+ currents were elicited in a modified Tyrode's external solution (mM): 130 Na^+, 5 Ca^{2+}, 4 K^+, 10 Hepes (pH 7.3 with NaOH), 4 glucose. To isolate Ca^{2+} currents, the extracellular solution contained (mM): 20 Ba^{2+} as the divalent charge carrier, 110 *N*-methyl-D-glucamine (replacing Na^+ as an impermeant substitute), 5 glucose, and 10 Hepes (pH 7.3 with CsOH). Glutamate was used as an impermeant Cl^- substitute in both external solutions. To eliminate delayed and inwardly rectifying K^+ currents, the cells were dialyzed internally with (mM): 130 cesium aspartate, 10 CsF, 5 EGTA, 10 Hepes (pH 7.3 with CsOH).

Measurements of membrane capacitance. Total input capacitance (C_m) was estimated in Tyrode's external solution, using a voltage command of 1 V·sec^{-1}, which sets the amplitude of the current discontinuity to 1 pF·pA^{-1}. The capacity transient following a 10 mV step was recorded at a bandwidth of 10 kHz and was digitized at 25 μsec/point. The records were fit using linear least-squares routines, by one or more exponential components (Eisenberg, 1983).

Results

Membrane capacitance of mononucleated C2 myocytes after mitogen withdrawal is comparable to that of skeletal muscle fibers

Biochemically differentiated BC_3H1 and C2 muscle cells were cultured as described in Materials and Methods and were analyzed by standard whole-cell clamp methods (Sakmann and Neher, 1983; Caffrey et al., 1987). Developmental changes in the total input capacitance of skeletal muscle, an indirect measurement of membrane area, have demonstrated that the area of the transverse tubular system increases relative to that of the fiber surface as a function of postnatal age (reviewed in Beam and Knudson, 1988b). The input capacitance of BC_3H1 muscle cells was 1.3 ± 0.4 μF·cm^{-2} (n = 37), similar to that found in neonatal rat skeletal muscle, and was well fit by a single-exponential component (Fig. 1*A*). The linear capacitance is similar to that anticipated in skeletal muscle fibers on the basis of surface membrane area alone (1.35 μF·cm^{-2}; Beam and Knudson, 1988b), and is consistent with the known ultrastructural features of BC_3H1 cells, that is, the presence of occasional membrane invaginations and the absence of transverse tubules (Schubert et al., 1974).

Although the input capacitance in proliferating C2 cells was similar to that observed in BC_3H1 cells (1.5 ± 0.6 μF·cm^{-2}), both the amplitude and complexity of the capacity transient in C2 myocytes increased following mitogen withdrawal, even in unfused cells (Fig. 1*B*). Total input capacitance was 3.3 ± 1.2

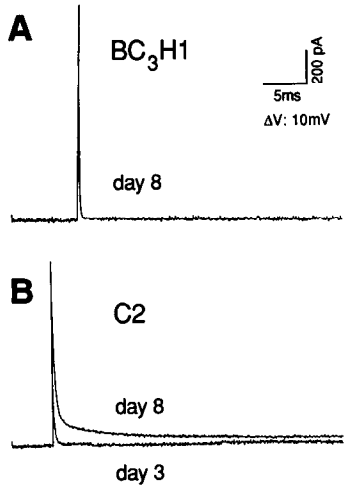

Figure 1. Passive electrical properties of BC_3H1 (*A*) and C2 (*B*) muscle cells. Cell were subjected to mitogen withdrawal for 3–8 d as shown. The capacity transient was recorded following a 10 mV voltage step (see Materials and Methods).

$\mu F \cdot cm^{-2}$ in mononucleated C2 cells examined 5 d or more after mitogen withdrawal ($n = 23$), similar to that found in rat skeletal muscle fibers 4–10 weeks after birth (Beam and Knudson, 1988b). Furthermore, decay of the capacity transient at ≥5 d contained multiple exponential components, as observed previously in skeletal muscle fibers possessing extensive transverse tubular membranes. Thus, the passive electrical properties of mature C2 myocytes correspond to intact skeletal muscle more closely than do those in BC_3H1 muscle cells. These complex passive electrical properties of differentiated C2 cells impaired the temporal control of transmembrane voltage even in fused C2 cells containing as few as 2 nuclei. Therefore, the macroscopic Na^+ and Ca^{2+} currents in C2 cells were assessed quantitatively in unfused, mononucleate myocytes (using mitogen withdrawal at low cell density) but were verified at least qualitatively in short multinucleate myotubes.

Unfused C2 muscle cells express functional tetrodotoxin-sensitive Na^+ channels

To examine voltage-gated Na^+ channels formed in BC_3H1 and C2 myocytes, cells were analyzed in the absence or presence of

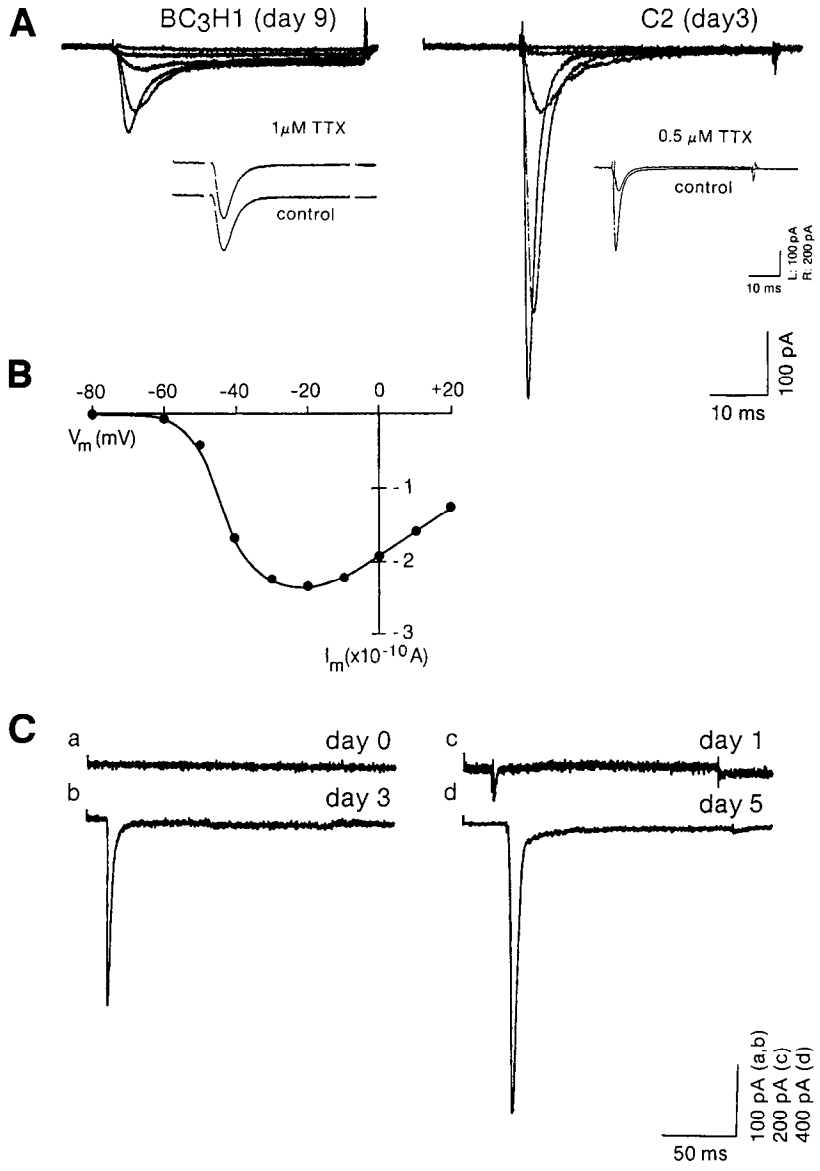

Figure 2. A, Voltage-gated Na^+ currents in BC_3H1 and C2 muscle cells were elicited by step depolarization to −60, −50, −40, −30, or −20 mV, at 9 or 3 d of serum withdrawal, respectively. *Inserts,* Na^+ currents were elicited in the absence (*lower traces*) or presence (*upper traces*) of TTX at the concentrations shown. BC_3H1 cells were differentiated for 14 d and C2 cells for 5 d. *B, I-V* relations for Na^+ current in unfused C2 myocytes. *C,* Developmental regulation of I_{Na} in unfused C2 myocytes following mitogen withdrawal.

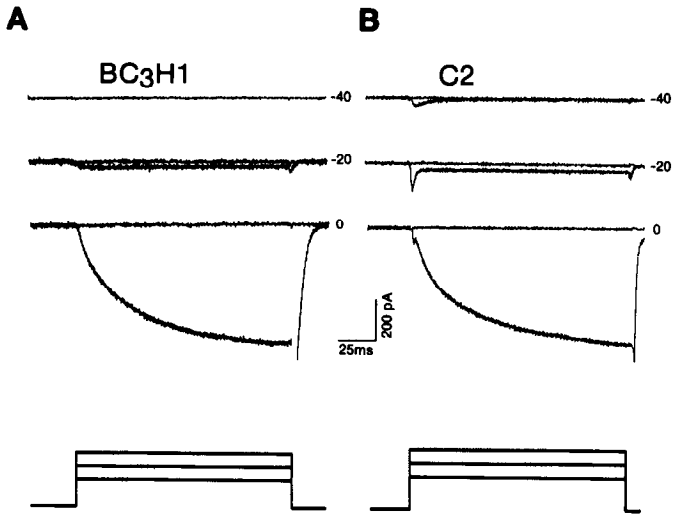

Figure 3. Comparison of voltage-gated Ca^{2+} currents in differentiated BC_3H1 and C2 myocytes. *A*, "Fast" and "slow" inward Ba^{2+} currents are shown in BC_3H1 muscle cells differentiated for 9 d. Test potentials are indicated. *B*, "Transient," "fast," and "slow" Ba^{2+} currents coexist in unfused C2 muscle cells differentiated for 3 d. Test potentials are shown. $V_h = -90$ mV. The transient current alone is evoked at -40 mV.

tetrodotoxin (TTX; Fig. 2). The concentrations of toxin were 100- and 50-fold greater, respectively, than the apparent dissociation constant for binding in differentiated mammalian skeletal muscle (10 nM: Gonoi et al., 1985; Weiss and Horn, 1986). In physiologic Tyrode's solution, peak Na^+ current density was 2.4 $\mu A \cdot cm^{-2}$ in BC_3H1 cells and 9.6 $\mu A \cdot cm^{-2}$ in C2 myocytes. Whereas Na^+ channels in BC_3H1 cells were resistant to TTX, even unfused C2 muscle cells also expressed functional TTX-sensitive Na^+ channels (see Frelin et al., 1984). The proportion of Na^+ current carried by TTX-sensitive channels (~70%) was similar to that previously observed in whole-cell patch-clamp studies of rat myoblasts (Weiss and Horn, 1986) and was ~2.5-fold greater than the proportion of TTX-sensitive channels found in myotubes converted to myoballs with colchicine (Gonoi et al., 1985). Neither class of Na^+ channel was found in proliferating C2 cells in mitogenic medium (Fig. 2*C*). Na^+ currents first appeared within 12–24 hr of serum withdrawal, whereas 4–5 d were required for the induction of Na^+ channels in BC_3H1 muscle cells (Caffrey et al., 1987).

Three voltage-gated Ca^{2+} currents coexist in differentiated C2 muscle cells

BC_3H1 muscle cells expressed 2 components of voltage-gated Ca^{2+} current, corresponding to the "fast" and "slow" currents found in T-tubules of skeletal muscle (Fig. 3*A*; Cota and Stefani, 1986; Caffrey et al., 1987; cf. Beam and Knudson, 1988a). Voltage steps from a holding potential (V_h) of -80 mV to test potentials (V_t) between -40 and -20 mV evoked a "fast," rapidly activating Ca^{2+} current ($\tau \sim 10$ msec) that persisted without decay for >400 msec. Inactivation of the "fast" current was 10- to 50-fold slower than inactivation of low-threshold (T) channels in cells other than adult skeletal muscle (e.g., Bean, 1985; Caffrey et al., 1986; Carbone and Lux, 1987; Fox et al., 1987; Yatani et al., 1987; Rorsman, 1988). At test potentials greater than -20 mV, a Ca^{2+} current was evoked that reached maximal activation only after 50–200 msec, that is, 10-fold more slowly than activation of high-threshold (L) channels in cells apart from skeletal muscle (see the references cited above; Beam et al., 1986; Cognard et al., 1986; Cota and Stefani, 1986; Beam and Knudson, 1988a, b). In BC_3H1 cells that had been differentiated for 9 d and were analyzed in 20 mM external Ba^{2+}, the "fast" and "slow" components of current, respectively, were 0.5 ± 0.2 and 5.5 ± 1.2 $\mu A \cdot cm^{-2}$ ($n = 31$). Previous analysis of BC_3H1 cells at intervals of mitogen withdrawal up to 21 d also detected only these "fast" and "slow" inward Ca^{2+} currents (Caffrey et al., 1987).

The components of Ca^{2+} current expressed by C2 muscle cells first were determined in unfused C2 cells that had been differentiated for 3 d. Unlike BC_3H1 cells, C2 muscle cells expressed a "transient" current evoked by voltage steps to -40 mV, sim-

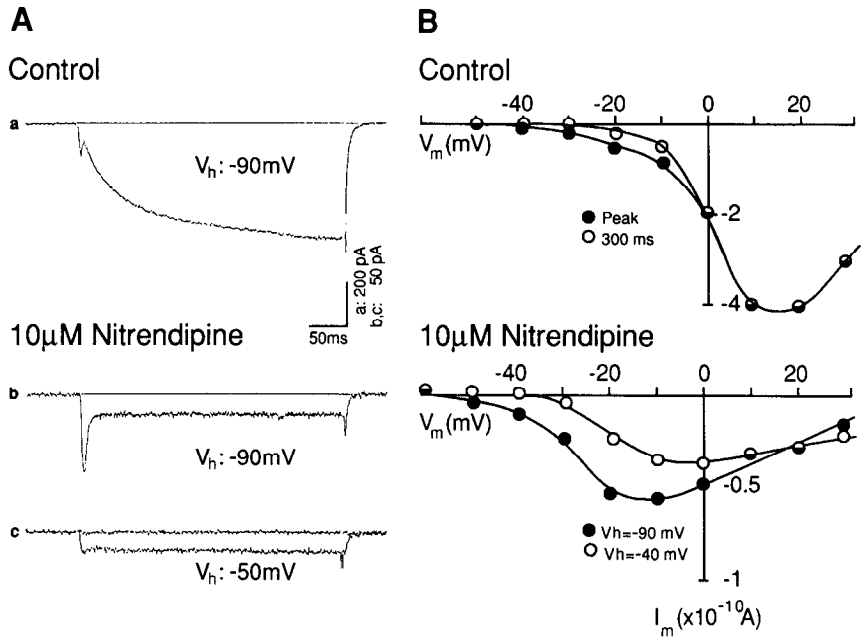

Figure 4. Separation of "transient," "fast," and "slow" components of Ca^{2+} current in C2 myocytes. *A*, Currents were evoked by voltage steps to $V_t = +10$ mV from $V_h = -90$ (*traces a, b*) or -50 mV (*trace c*), in the absence (*trace a*) or presence (*traces b, c*) of 10 μM nitrendipine. *B*: *Upper panel*, Current–voltage relationships are shown for the peak (●) and 300 msec (○: "slow") components of Ba^{2+} current in C2 muscle cells. $V_h = -90$ mV. *Lower panel*, Current–voltage relationships are shown for Ba^{2+} currents evoked in the presence of 10 μM nitrendipine from a $V_h = -90$ (●: "transient") or -40 mV (○: "fast").

Table 1. Properties of calcium currents in C2 cells and other skeletal myocytes

Muscle cell	Channel type	Gating kinetics				V_h	DHP sensitivity	References
		V_{th}	V_p	T_{act} (msec)	T_{inact}			
C2	Transient	−40	−10	1–5	5–10 msec	−70	−	This report
	Fast	−20	−10	5	1 sec	−60	−	This report
	Slow	0	+10	55–60	4 sec	−50	+	This report
BC_3H1	Fast	−40	−10	5	1 sec	−20	−	Caffrey et al., 1987
	Slow	−20	+10	55–60	4 sec	−70	+	Caffrey et al., 1987
Mouse								
Neonatal	T	−50	−10	3–5	30–40 msec	−70	n.t.	Gonoi and Hasegawa, 1988
	Slow	−20	+20	20–40	>200 msec	−40	n.t.	Gonoi and Hasegawa, 1988
>17 d	Slow	−10	+20	17.5	>200 msec	−40	n.t.	Gonoi and Hasegawa, 1988
Rat								
Neonatal	T	−30	−10	1–5	5–10 msec	−50	−	Beam and Knudson, 1988a
	Slow	0	+20	10–50	>200 msec	−30	+	Beam and Knudson, 1988a
>25 d	Slow	0	+20	10–50	>200 msec	n.t.	+	Beam and Knudson, 1988b

V_{th}, threshold potential for activation; V_p, potential at which inward current is maximal; T_{act}, T_{inact}, time to half-maximal activation or inactivation; V_h, holding potential at which steady-state inactivation is half-maximal; n.t., not tested.

ilar to the low-threshold current found in immature skeletal muscle fibers and the T-type current in other cell types (Fig. 3*B*). Step depolarizations to −20 and 0 mV, respectively, evoked a "fast," noninactivating Ca^{2+} current and "slow" Ca^{2+} current similar to those observed in BC_3H1 cells and adult skeletal muscle. However, at relatively depolarized test potentials the 3 apparent components of Ca^{2+} current coexist.

To resolve these components of Ca^{2+} current, the selective effects of Ca^{2+} channel antagonists and holding potential were utilized. As shown in Figure 4, only the "slow" current was sensitive to dihydropyridines (DHPs: 10 μM nitrendipine). Although both "transient" and "fast" Ca^{2+} currents were resistant to DHPs (V_h = −90 mV), "transient" current was inactivated at a holding potential of −50 mV. Thus, "transient," "fast," and "slow" Ca^{2+} currents in C2 muscle cells could be readily distinguished by standard procedures. For the "transient" Ca^{2+} current, the time to peak inward current was 20–25 msec at the threshold for activation, decreasing to 1–5 msec at more positive potentials. Time constants for decay decreased from 30–50 msec at threshold to 5–10 msec at −10 mV. The time constant for inactivation of this "transient" component of Ca^{2+} current was 2–3 orders of magnitude faster than decay of the "fast" and "slow" currents in C2 muscle cells (Table 1). In 20 mM Ba^{2+}, the peak "transient" current was 1.16 ± 0.23 $\mu A \cdot cm^{-2}$ (n = 8). The "fast" and "slow" currents were 0.35 ± 0.05 and 1.35 ± 0.20 $\mu A \cdot cm^{-2}$ (n = 8), similar in both relative and absolute density to those measured in BC_3H1 cells. Thus, a third, kinetically distinct component of calcium current coexists in differentiated C2 muscle cells, in addition to "fast" and "slow" currents similar to those in BC_3H1 cells. Although adult skeletal muscle fibers do not possess a comparable, rapidly inactivating component of current contributed by T-type Ca^{2+} channels (Cota and Stefani, 1986; Beam and Knudson, 1988b), a similar "transient" current has been found both in embryonic and neonatal skeletal muscle cells (Beam et al., 1986; Cognard et al., 1986; Beam and Knudson, 1988a; Garcia and Stefani, 1988; Gonoi and Hasegawa, 1988).

Effects of holding potential on steady-state inactivation distinguish between the "slow" Ca^{2+} currents of C2 and BC_3H1 muscle cells

Although the activation kinetics and voltage dependence of "fast" and "slow" Ca^{2+} currents were virtually identical in BC_3H1 and C2 muscle cells, their steady-state inactivation properties were unexpectedly different. The "slow" current of C2 muscle cells, in its voltage-dependence for steady-state inactivation, resembled the "slow" current of adult skeletal muscle closely, whereas the "slow" current of BC_3H1 cells did not (Fig. 5*A–C*; Caffrey et al., 1987). At a holding potential of −30 mV, the residual current elicited by voltage pulses to +10 mV in C2 cells was predominantly the high-threshold, "slow," DHP-sensitive type. In other systems, the analogous high-threshold "L" channels are only slightly inactivated at such holding potentials, whereas "T" and "N" channels are almost completely inactivated (e.g., Carbone and Lux, 1987; Fox et al., 1987). However, in BC_3H1 cells, the higher-threshold "slow" current inactivated completely between holding potentials of −90 and −40 mV, isolating the lower-threshold "fast" current at higher V_h. Thus, the "slow" current in BC_3H1 cells became inactivated at a holding potential 20–30 mV more negative than in C2 muscle cells or in adult skeletal muscle fibers (Almers et al., 1981; Cota et al., 1984).

Expression of "transient," "fast," and "slow" Ca^{2+} currents in C2 muscle cells occurs in a developmental sequence

To determine if all components of inward current in C2 muscle cells were equally dependent on mitogen withdrawal during myogenesis, proliferating C2 cells in 20% FBS were compared with cells cultured for 1–14 d in 2% HS (Fig. 6). At test potentials ranging from −80 to +50 mV, none of the 3 components of inward Ca^{2+} current was detected in proliferating C2 cells. Under the conditions tested, Ca^{2+} currents of ≤10 pA could be resolved. Thus, the absence of Ca^{2+} channels shown previously in proliferating BC_3H1 muscle cells (Caffrey et al., 1987) is not merely due to anomalous sensitivity of BC_3H1 cells to serum

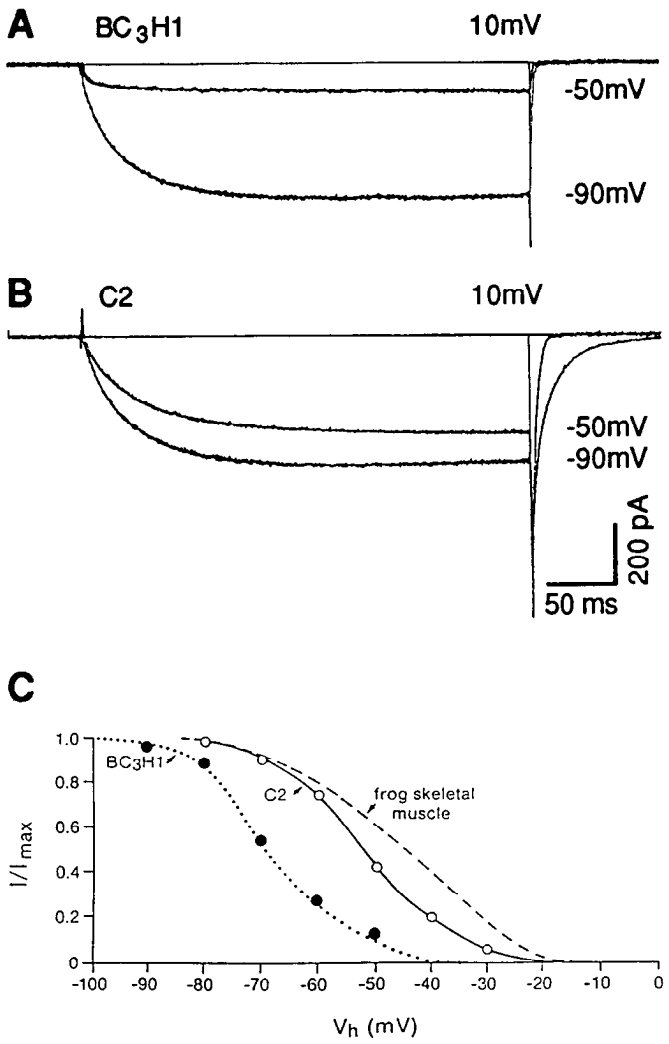

Figure 5. Divergent effects of holding potential on steady-state inactivation of the high-threshold "slow" Ba^{2+} current in C2 and BC_3H1 myocytes. Whole-cell currents in BC_3H1 (*A*) and C2 (*B*) cells stepped to +10 mV from each holding potential shown. *C*, The residual "slow" currents shown in *A* and *B* were normalized to current evoked from −80 mV. BC_3H1 (•) and C2 (○) cells were differentiated as in Figure 3. Data for inactivation of the "slow" current in frog skeletal muscle are from Cota and Stefani (1986).

constituents. In partial agreement with the ontogeny of "fast" and "slow" currents, the "transient" Ca^{2+} current also was developmentally regulated during myogenic differentiation. However, induction of "transient" Ca^{2+} current was detected in C2 cells within 12–24 hr of mitogen withdrawal, similar to results shown above for Na^+ channels, whereas neither the "fast" nor "slow" current was found until 3 d or more in differentiating medium.

In 20 mM Ba^{2+}, the mean peak "transient" current was 0.31 ± 0.073 $\mu A \cdot cm^{-2}$ at 24 hr ($n = 4$), 0.676 ± 0.176 $\mu A \cdot cm^{-2}$ at 2 d ($n = 5$), and 1.38 ± 0.281 $\mu A \cdot cm^{-2}$ at 4 d of serum withdrawal ($n = 4$; Fig. 6, *A*, *B*). Interestingly, the "transient" current was subsequently diminished (0.512 ± 0.109 $\mu A \cdot cm^{-2}$ at day 8; $p < 0.05$; $n = 5$), and no "transient" current was detected in C2 muscle cells that had been differentiated for 14 d ($n = 5$). Taking into account a threshold for current detection of ~10 pA, the percentage of C2 cells that possessed the "transient" component of Ca^{2+} current increased from 0% in proliferation medium to 37.5 and 71.4% at 1 and 2 d of mitogen withdrawal, respectively (Fig. 6*C*). Although all C2 cells examined on day 4 were found to express the "transient" current, the proportion of cells in which the current was detected declined with time in differentiation medium, to 61.5, 26.7, and 0%, at 5, 8, and 14 d, respectively. In contrast, the "fast" and "slow" Ca^{2+} currents first were detected after 3 d of mitogen withdrawal (see above). Unlike the "transient" current, the amplitude of the "fast" and "slow" components of current continued to increase, and reached a maximum at 8 d of mitogen withdrawal. The "fast" current was 0.96 ± 0.077 $mA \cdot cm^{-2}$ in 20 mM Ba^{2+}, and the "slow" current was 6.86 ± 0.15 $\mu A \cdot cm^{-2}$ ($n = 15$). Whereas the "transient" current could not be elicited after 14 d, expression of the "fast" and "slow" currents was stably maintained (1.38 ± 0.208 $\mu A \cdot cm^{-2}$ and 6.89 ± 0.485 $\mu A \cdot cm^{-2}$; $n = 5$; Fig. 6, *A*, *B*), and, similarly, the percentage of C2 cells that possessed the "fast" and "slow" currents did not decline (Fig. 6*C*). Thus, in C2 cells the expression of "transient," "fast," and "slow" Ca^{2+} currents are regulated throughout myogenic differentiation in a developmental program.

TGFβ reversibly blocks the induction of all three calcium currents in C2 muscle cells

To address the issue of whether Ca^{2+} channel expression was merely dependent upon exit from the cell cycle and to examine the possibility that a single peptide inhibitor of myogenic differentiation could prevent the induction of one or more Ca^{2+} currents, we analyzed whole-cell Ca^{2+} currents in C2 muscle cells subjected to serum withdrawal in the presence of TGFβ, whose effects on the myogenic pathway are independent of DNA synthesis and cell proliferation. In concordance with its previously demonstrated effects on muscle-specific mRNA transcripts and proteins (Florini et al., 1986; Massague et al., 1986; Olson et al., 1986), 1 nM TGFβ was sufficient to block the appearance of all 3 components of Ca^{2+} current (Fig. 7). While the "transient," "fast," and "slow" components all were elicited in control cells at 3–5 d in differentiation medium (day 5: $I_{transient}$ = 0.76 ± 0.16 $\mu A \cdot cm^{-2}$; I_{fast} = 1.01 ± 0.13 $\mu A \cdot cm^{-2}$; I_{slow} = 3.88 ± 0.18 $\mu A \cdot cm^{-2}$; $n = 5$), no inward Ca^{2+} current was expressed in quiescent C2 cells that had been cultured in the presence of 1 nM TGFβ (each Ca^{2+} current was ≤0.1 $\mu A \cdot cm^{-2}$; $n = 10$). Thus, TGFβ inhibited induction of the "transient," "fast," and "slow" Ca^{2+} currents by at least 7.5-, 10-, and 38-fold, respectively, and a single peptide suffices to regulate each of the 3 Ca^{2+} currents associated with the differentiated state. Since TGFβ has no mitogenic activity for C2 cells (Olson et al., 1986), exit from the cell cycle appears to be necessary, but not sufficient, for the expression of these 3 voltage-gated Ca^{2+} currents in developing C2 myoblasts. In contrast, none of the 3 Ca^{2+} currents was altered acutely when TGFβ was added to differentiated C2 cells for up to 10 min (Fig. 8). Conversely, none of the 3 currents was elicited initially, following the removal of TGFβ. Thus, no evidence was found to support the interpretation that TGFβ might modulate Ca^{2+} currents. Whereas proliferating myoblasts required removal of serum for 3 d before "fast" and "slow" channels were expressed (Fig. 6), in contrast, all 3 components of Ca^{2+} current were expressed in myoblasts released from TGFβ for 2 d ($I_{transient}$ = 0.49 ± 0.24 $\mu A \cdot cm^{-2}$; I_{fast} = 0.53 ± 0.28 $\mu A \cdot cm^{-2}$; I_{slow} = 1.04 ± 0.20 $\mu A \cdot cm^{-2}$; $n = 3$; Fig. 7). Each current continued to accumulate following removal of TGFβ for 3 d: $I_{transient}$ = 0.91 ± 0.17 $\mu A \cdot$

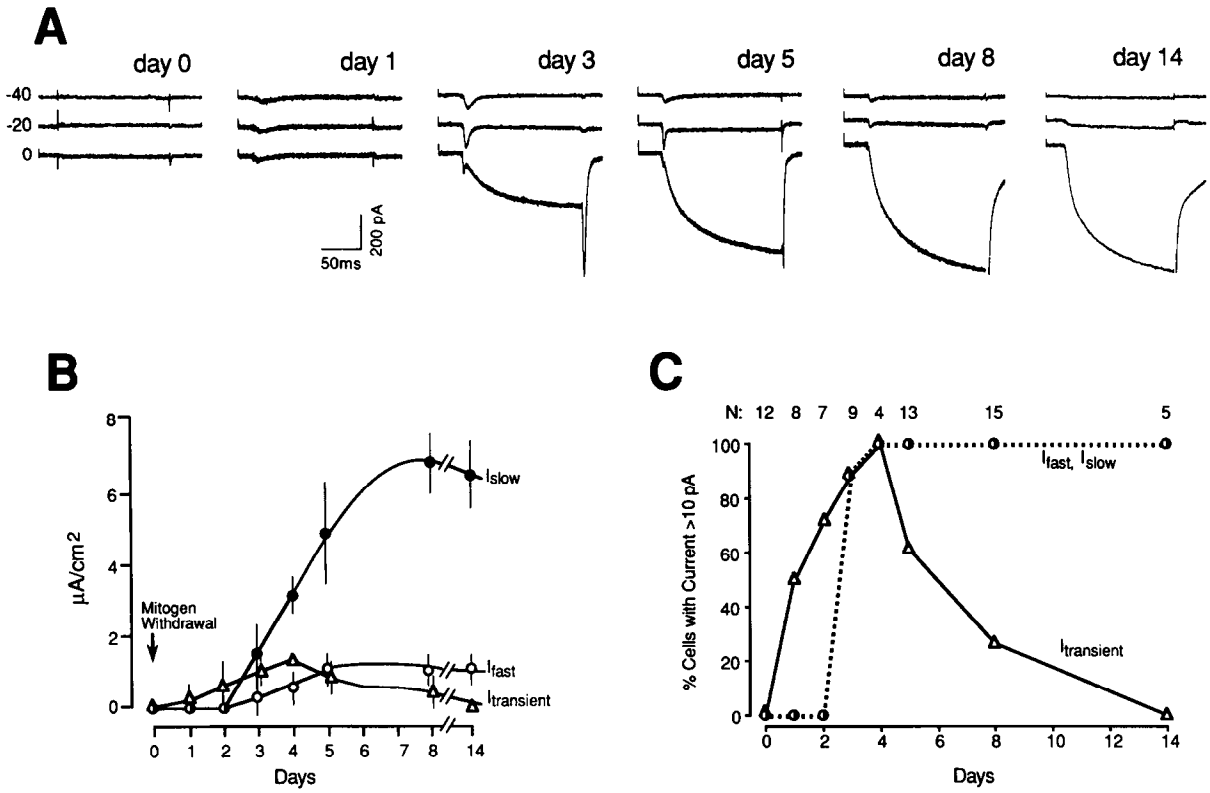

Figure 6. Expression of the 3 components of Ca^{2+} current in C2 muscle cells occurs in a developmental sequence. Medium containing 20% FBS was replaced with 2% HS on day 0. *A*, "Transient," "fast," and "slow" currents were evoked in 20 mM Ba^{2+} by voltage steps from −90 mV to −40, −20, or 0 mV, respectively. At day 1–2, voltage steps elicited only current through "transient" channels. *B*, Expression of the "transient" (△), "fast" (○), and "slow" (●) components of Ca^{2+} current at intervals after mitogen withdrawal was determined as in *A* and is shown as current density ($\mu A \cdot cm^{-2}$; mean ± SEM). *C*, Proportion of C2 cells that express the "transient" (△), "fast" (○), and "slow" (●) components of Ca^{2+} current is shown after 0–14 d of mitogen withdrawal. The number of cells sampled is shown above each time-point. The threshold for detecting each current as present was 10 pA.

cm^{-2}; $I_{fast} = 1.01 \pm 0.16\ \mu A \cdot cm^{-2}$; $I_{slow} = 3.16 \pm 0.02\ \mu A \cdot cm^{-2}$; $n = 3$; Fig. 7). At the concentration tested, TGFβ also reversibly abolished the expression of both TTX-sensitive and -insensitive Na^+ channels (Fig. 9).

Discussion

Attempts to elucidate the molecular events that precisely control the synthesis of tissue-specific ion channels during myogenesis

Table 2. "Transient" calcium channels are induced prior to "fast" and "slow" channels in C2 muscle cells, then are selectively down-regulated

	Current density ($\mu A \cdot cm^{-2}$)			
Day	$I_{transient}$	I_{fast}	I_{slow}	n
0	n.d.[a]	n.d.	n.d.	12
1	0.310 ± 0.073[b]	n.d.	n.d.	4
2	0.676 ± 0.176	n.d.	n.d.	5
3	1.16 ± 0.178	0.345 ± 0.052[c]	1.35 ± 0.202[c]	8
4	1.38 ± 0.281	0.565 ± 0.140[c]	2.91 ± 0.256[c]	4
5	0.602 ± 0.084[b] ($n = 8$)	1.04 ± 0.121	4.93 ± 0.334[c]	13
8	0.512 ± 0.109[b] ($n = 5$)	0.956 ± 0.077	6.86 ± 0.15	15
14	n.d.	1.38 ± 0.208	6.89 ± 0.485	5

Ba^{2+} currents were elicited as described in the legend to Figure 6 and were normalized to membrane area on the basis of membrane capacitance. Results are shown as means ± SE for currents greater than or equal to the detection threshold (10 pA ≈ 0.1 $\mu A \cdot cm^{-2}$). Maximal current densities observed during the time course were used for comparison: $I_{transient}$, day 4; I_{fast}, day 14; I_{slow}, day 14.

[a] n.d., not detected.

[b] $p < 0.05$.

[c] $p < 0.01$.

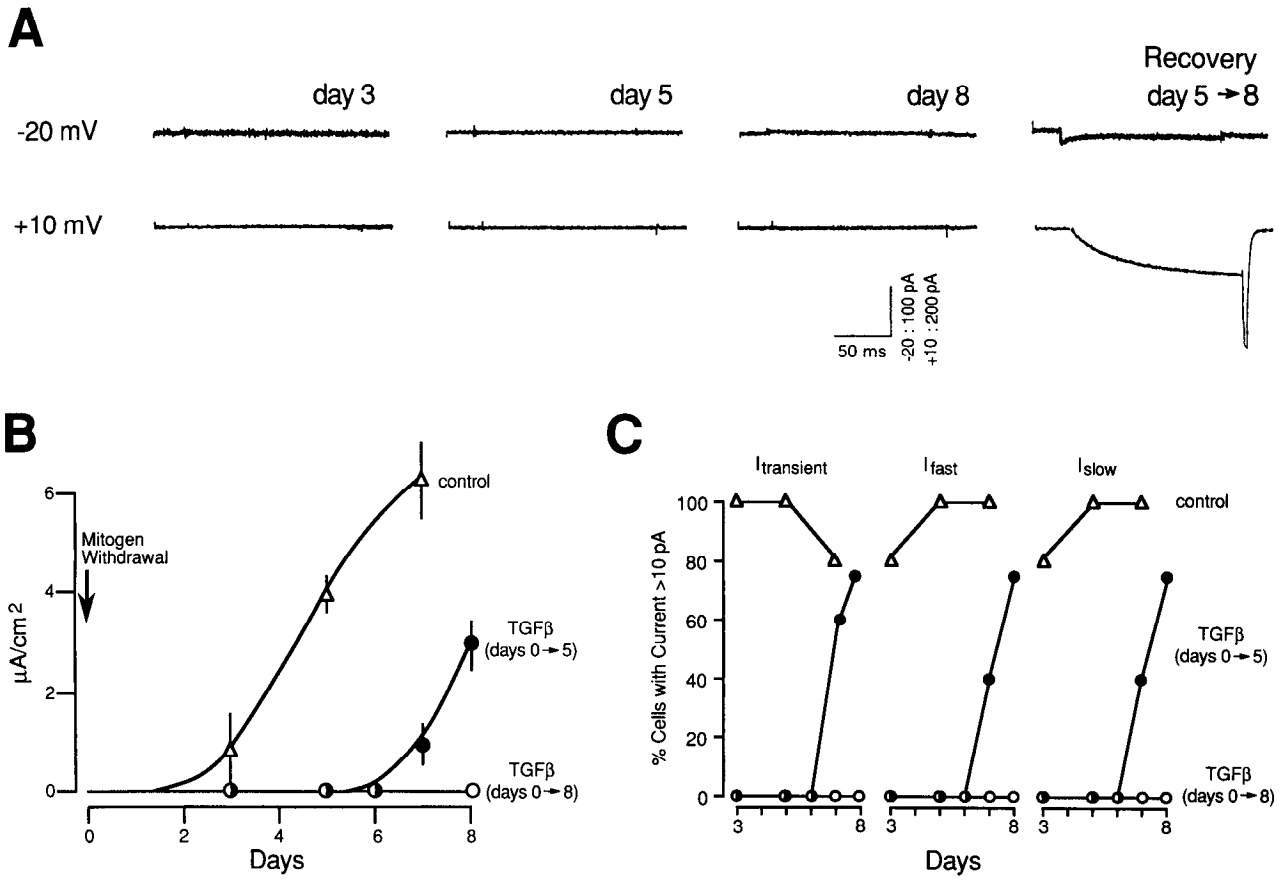

Figure 7. TGFβ reversibly blocks the induction of all 3 voltage-gated Ca^{2+} currents in C2 muscle cells. *A*, Individual currents evoked at −20 and +10 mV are shown following mitogen withdrawal on day 0 in the presence of 1 nM TGFβ. Currents in the fourth column were elicited after recovery from TGFβ for 3 d, beginning on day 5. *B*, Expression of the "slow" current (calculated as in Fig. 3*B*) is shown after mitogen withdrawal in the absence (△) or presence (○) of 1 nM TGFβ, and for C2 myoblasts released from TGFβ on day 5 (●). *C*, The percentage of C2 cells that express the "transient" (*left*), "fast" (*middle*), and "slow" (*right*) components of Ca^{2+} current was determined as in Figure 5. (△) Control; (○) 1 nM TGFβ; (●) TGFβ withdrawn on day 5.

require model myogenic systems whose biophysical and developmental properties accurately reflect those of skeletal muscle *in vivo*, together with reagents that reversibly block steps in the differentiation pathway. In the present study, we have shown that 3 biophysical properties of unfused C2 cells more accurately resemble skeletal muscle fibers than do those of nonfusing BC_3H1 cells: (1) passive electrical properties, reflecting transverse tubular membrane area; (2) TTX sensitivity of Na^+ channels; and (3) steady-state inactivation of "slow" Ca^{2+} channels. In addition, C2 myocytes formed functional "transient" Ca^{2+} channels, like those associated with immature muscle *in vivo*, within 24 hr of mitogen withdrawal (that is, up to 2 d before the earliest appearance of the "fast" and "slow" components of current) and selectively down-regulated the "transient" component of current after prolonged differentiation. All components of Ca^{2+} and Na^+ current were induced even in mononucleated C2 muscle cells. This progression of events in a homogeneous cell line resembles the attenuation of "transient" current in late-embryonic and neonatal myocytes (chick: Cognard et al., 1986; mouse: Beam et al., 1986; rat: Beam and Knudson, 1988b; premetamorphic tadpoles: Garcia and Stefani 1988) and shows that "transient" Ca^{2+} channels in C2 cells possess intrinsic developmental properties distinct from those of the "fast" and "slow" currents. Our results also agree with previous evidence that C2 muscle cells do not require prolonged culture, complex substrates, or innervation for the sequential induction and deinduction of appropriate isoforms (Silberstein et al., 1986; Donoghue et al., 1987; Weydert et al., 1987). Whether the unanticipated steady-state inactivation of "slow" Ca^{2+} channels in nonfusing BC_3H1 cells can be accounted for by local effects such as membrane topography and milieu or inherent differences in Ca^{2+} channel structure is unknown. However, a monoclonal antibody directed against the α_1-subunit of the rabbit

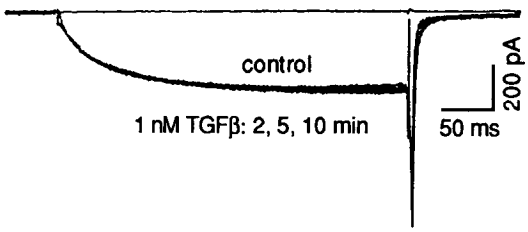

Figure 8. TGFβ does not modulate Ca^{2+} currents. Ba^{2+} currents were elicited by step depolarizations from $V_h = -90$ mV to $V_t = +20$ mV. The cell was retested after treatment with 1 nM TGFβ for 2, 5, or 10 min as shown (cf. Fig. 7).

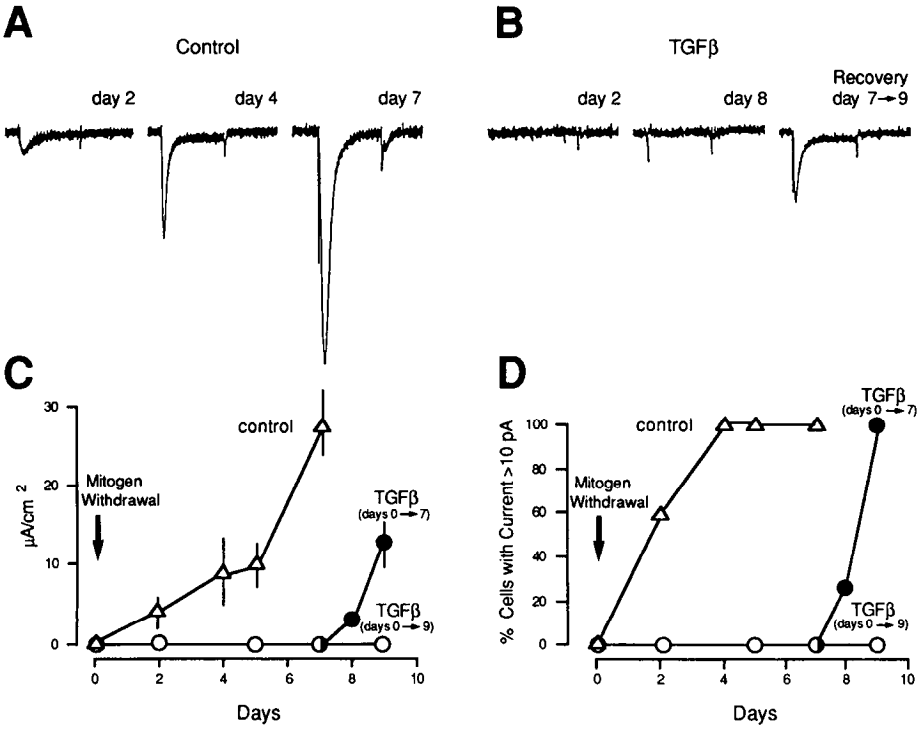

Figure 9. Reversible suppression of Na^+ currents by TGFβ. *A* and *B*, Individual currents evoked at $V_t = -30$ mV are shown following mitogen withdrawal on day 0 in the absence (*A*) or presence (*B*) of 1 nM TGFβ. *C*, Expression of I_{Na} (calculated as in Fig. 4*B*) is shown after mitogen withdrawal in the absence (△) or presence (○) of 1 nM TGFβ, and for C2 myoblasts released from TGFβ on day 7 (●). *D*, The percentage of C2 cells that express I_{Na} was determined as in Figure 6. (△) Control; (○) 1 nM TGFβ; (●) TGFβ withdrawn on day 7.

skeletal muscle DHP receptor inhibits "slow" current and identifies a 210 kDa membrane polypeptide in BC_3H1 cells (Morton et al., 1988).

That neither fusion nor interaction with motor neurons was required for C2 myocytes to express TTX-sensitive Na^+ channels differs from the properties shown by L6 muscle cells [L6 myocytes possess high-affinity TTX-binding sites that reportedly become functional only after fusion (Haimovich et al., 1986)] but agrees with the existence of TTX-sensitive Na^+ channels in short-term muscle cultures (Gonoi et al., 1985; Weiss and Horn, 1986). *In vivo* studies suggest, however, that the absence of TTX-insensitive Na^+ channels from adult skeletal muscle can be ascribed at least in part to innervation (reviewed by Sherman and Catterall, 1982). Similarly, muscle activity may affect "slow" Ca^{2+} current amplitude after birth, as well as the kinetic properties of Ca^{2+} channels (Gonoi and Hasegawa, 1988). Since C2 cells expressed both Na^+ channels and transient Ca^{2+} channels up to 60 hr before the earliest detectable "slow" current, there is no evidence to suggest that Ca^{2+} influx through "slow" channels is required at least for these limited, early aspects of muscle differentiation (see Sherman and Catterall, 1984; Beam et al., 1986).

These investigations also demonstrate that the biophysical phenotype of developing muscle cells can be regulated by a serum peptide that affects morphological and biochemical parameters of differentiation. These findings were anticipated on the basis of our previous evidence that undifferentiated myoblasts lack Na^+ and Ca^{2+} channels (Caffrey et al., 1987), together with the consequences of an exogenous c-H-*ras* gene that disrupts muscle development (Caffrey et al., 1987; Olson et al., 1987; Payne et al., 1987). However, it is inaccurate to assume that myogenic differentiation occurs as an all-or-none process. For example, stable transfection with an activated c-H-*ras* allele can suppress many of the muscle-specific genes that are inhibited by serum factors but has no effect on mRNA encoding the δ subunit of the nicotinic ACh receptor (H.-T. Shih, J. M. Caffrey, H. Bigo, and M. D. Schneider, unpublished observations). In L6E9 myoblasts, EGTA permits the accumulation of muscle-specific mRNAs but prevents their translation (Endo and Nadal-Ginard, 1987). Chick skeletal myocytes cultured in medium containing high K^+ can express the embryonic myosin heavy chain but fail to synthesize the neonatal heavy chain (Cerny and Bandman, 1986). Analogously, EGTA blocks induction of α-skeletal actin, while permitting up-regulation of the α-cardiac actin gene, which encodes the isoform found in fetal skeletal muscle (Haywood et al., 1988). Under these conditions, saxitoxin binding sites appear (Frelin et al., 1981), but no DHP receptor protein is induced (Schmid et al., 1984). In C2 cells themselves, medium containing low Ca^{2+} is permissive for expression of the nicotinic ACh receptor and the embryonic (4-6S) acetylcholinesterase but prevents appearance of the adult (16S) form (Inestrosa et al., 1983). Thus, inhibitory conditions exist that allow certain initial differentiation products to occur, while blocking later differentiation products. Since functional Na^+ channels and "transient" Ca^{2+} channels are detected very early after serum withdrawal, up to 60 hr before "slow" channels, the possibility that all Na^+ and Ca^{2+} channels might be suppressed by TGFβ was not a foregone conclusion and such predictions must be tested empirically. At present it is not yet known whether TGFβ acts through a myogenic "determination gene" such as MyoD1 (Davis et al., 1987). Though MyoD1 itself is repressed by TGFβ, suggesting that the peptide acts on the myogenic pathway by inhibiting this muscle regulatory gene, MyoD1 expression vectors fail to overcome the block to differentiation created by TGFβ (Vaidya et al., 1989) or mitogenic medium (Tapscott et al., 1989).

Moreover, the precise effects of oncogenes and serum factors on ion channel expression vary dramatically with cell type. For example, in 3T3 cells, which express both "T" and "L" Ca^{2+} channels, activated *ras* genes down-regulate only the DHP-in-

sensitive T-type channels (Chen et al., 1988), and *ras* can induce Ca^{2+} channels, together with other markers of differentiation, in PC12 pheochromocytoma cells (Noda et al., 1985; cf. Falcone et al., 1985). Finally, the effects of TGFβ on developing muscle also vary with the stage of development, stimulating the synthesis of muscle proteins during induction of embryonal mesoderm (Kimelman and Kirschner, 1987). Thus, the control of ion channel formation by growth factors and oncogenes is likely to be determined by the specific cellular context in which the signals are transduced. Indeed, "transient" Ca^{2+} channels reappear in postnatal mouse skeletal muscle fibers after 12 d or more in medium containing high concentrations of serum (Gonoi and Hasegawa, 1988), suggesting that serum factors might stimulate the expression of these channels in terminally differentiated myotubes. Some discrepancies in growth factor sensitivity have been observed between particular myogenic systems, and contrasting muscle cell preparations exist for which fibroblast growth factor may be the critical serum component affecting the onset of differentiation (Clegg et al., 1987).

Abundance of the DHP receptor protein associated with "slow" Ca^{2+} channels parallels the changes in "slow" current produced by serum factors and cellular oncogenes (Rampe et al., 1988). No analogous molecular markers presently exist to label "transient" and "fast" Ca^{2+} channels. While TGFβ is not known to act through mechanisms involving translational control or subunit assembly, the tentative hypothesis that growth factors and cellular oncogenes might impinge on expression of the DHP receptor gene can readily be tested. The DHP receptor α_1 cDNA (Tanabe et al., 1987) restores both excitation–contraction coupling and "slow" Ca^{2+} current in dysgenic muscle (Tanabe et al., 1988), supporting the inference that the α_1 subunit encodes the functional "slow" channel, and DHP receptor mRNA can be detected in mouse skeletal muscle using probes derived from the rabbit cDNA (Tanabe et al., 1988). We have recently shown that the formation of "slow" Ca^{2+} channels in mouse skeletal muscle involves control through a pretranslational mechanism *in vitro* and *in vivo*: Both tissue specificity and developmental regulation are conferred at least partly at the level of DHP receptor gene induction (H.-T. Shih, J. M. Caffrey, H. Bigo, and M. D. Schneider, unpublished observations). Further information is necessary to define the precise contribution of transcriptional and posttranscriptional processes to the developmental control and tissue-specific expression of Ca^{2+} channels during myogenesis (Schwarz et al., 1988).

References

Almers, W., F. Fink, and P. T. Palade (1981) Calcium depletion in frog muscle tubules: The decline of calcium current under maintained depolarization. J. Physiol. (Lond.) *312:* 177–207.

Beam, K. G., and C. M. Knudson (1988a) Calcium currents in embryonic and neonatal mammalian skeletal muscle. J. Gen. Physiol. *91:* 781–798.

Beam, K. G., and C. M. Knudson (1988b) Effect of post-natal development on calcium currents and slow charge movement in mammalian skeletal muscle. J. Gen. Physiol. *91:* 799–815.

Beam, K. G., C. M. Knudson, and J. A. Powell (1986) A lethal mutation in mice eliminates the slow calcium current in skeletal muscle cells. Nature *320:* 168–170.

Bean, B. P. (1985) Two kinds of calcium channels in canine atrial cells: Differences in kinetics, selectivity, and pharmacology. J. Gen. Physiol. *86:* 1–30.

Buonanno, A., and J. P. Merlie (1986) Transcriptional regulation of nicotinic acetylcholine receptor genes during muscle development. J. Biol. Chem. *261:* 11452–11455.

Caffrey, J. M., I. R. Josephson, and A. M. Brown (1986) Calcium channels of amphibian stomach and mammalian aorta smooth muscle cells. Biophys. J. *49:* 1237–1242.

Caffrey, J. M., A. M. Brown, and M. D. Schneider (1987) Mitogens and oncogenes can block the formation of specific voltage-gated ion channels. Science *236:* 570–574.

Carbone, E., and H. D. Lux (1987) Single low-voltage-activated calcium channels in chick and rat sensory neurones. J. Physiol. (Lond.) *386:* 571–601.

Cerny, L. C., and E. Bandman (1986) Contractile activity is required for the expression of neonatal myosin heavy chain in embryonic chick pectoralis muscle cultures. J. Cell Biol. *103:* 2153–2161.

Chen, C., M. J. Corbley, T. M. Roberts, and P. Hess (1988) Voltage-sensitive calcium channels in normal and transformed 3T3 fibroblasts. Science *239:* 1024–1026.

Clegg, C. H., T. A. Linkhart, B. B. Olwin, and S. D. Hauschka (1987) Growth factor control of skeletal muscle differentiation: Commitment to terminal differentiation occurs in G1 phase and is repressed by fibroblast growth factor. J. Cell Biol. *105:* 949–955.

Cognard, C., G. Romey, J.-P. Galizzi, M. Fosset, and M. Lazdunski (1986) Different types of Ca^{2+} channels in mammalian skeletal muscle cells in culture. Proc. Natl. Acad. Sci. USA *83:* 517–521.

Cota, G., and E. Stefani (1986) A fast-activated inward calcium current in twitch muscle fibres of the frog (*Rana montezume*). J. Physiol. (Lond.) *370:* 151–163.

Cota, G., L. Nicola Siri, and E. Stefani (1984) Calcium channel inactivation in frog (*Rana pipiens* and *Rana moctezuma*) skeletal muscle fibres. J. Physiol. (Lond.) *354:* 99–108.

Davis, R. L., H. Weintraub, and A. B. Lassar (1987) Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell *51:* 987–1000.

Donoghue, M., H. Ernst, B. Wentworth, B. Nadal-Ginard, and N. Rosenthal (1987) A muscle-specific enhancer is located at the 3′ end of the myosin light chain 1/3 gene locus. Genes Dev. *2:* 1779–1790.

Eisenberg, R. S. (1983) Impedence measurement of the electrical structure of skeletal muscle. In *Handbook of Physiology,* Section 10, pp. 301–323, American Physiological Society, Bethesda, MD.

Emerson, C., D. Fischman, B. Nadal-Ginard, and M. A. Q. Siddiqui, eds. (1986) *Molecular Biology of Muscle Development,* Liss, New York.

Endo, T., and B. Nadal-Ginard (1987) Three types of muscle-specific gene expression in fusion-blocked rat skeletal muscle cells: Translational control in EGTA-treated cells. Cell *49:* 515–528.

Evans, S., D. Goldman, S. Heinemann, and J. Patrick (1987) Muscle acetylcholine receptor biosynthesis: Regulation by transcript availability. J. Biol. Chem. *262:* 4911–4916.

Falcone, G., F. Tato, and S. Alema (1985) Distinctive effects of the viral oncogenes *myc, erb, fps,* and *src* on the differentiation program of quail myogenic cells. Proc. Natl. Acad. Sci. USA *82:* 426–430.

Florini, J. R., A. B. Roberts, D. Z. Ewton, S. L. Falen, K. C. Flanders, and M. B. Sporn (1986) Transforming growth factor-β: A very potent inhibitor of myoblast differentiation, identical to the differentiation inhibitor secreted by Buffalo rat liver cells. J. Biol. Chem. *261:* 16509–16513.

Fox, A. P., M. C. Nowycky, and R. W. Tsien (1987) Single-channel recordings of three types of calcium channels in chick sensory neurones. J. Physiol. (Lond.) *394:* 173–200.

Frelin, C., A. Lombet, P. Vigne, G. Romey, and M. Lazdunski (1981) The appearance of voltage-sensitive Na^+ channels during the in vitro differentiation of embryonic chick skeletal muscle cells. J. Biol. Chem. *256:* 12355–12361.

Frelin, C., H. P. M. Vijverberg, G. Romey, P. Vigne, and M. Lazdunski (1984) Different functional states of tetrodotoxin sensitive and tetrodotoxin resistant Na^+ channels occur during the in vitro development of rat skeletal muscle. Pfluegers Arch. *402:* 121–128.

Garcia, J., and E. Stefani (1988) Changes in calcium currents of striated muscle during tadpole metamorphosis. Biophys. J. *53:* 553a.

Gonoi, T., and S. Hasegawa (1988) Post-natal disappearance of transient calcium channels in mouse skeletal muscle: Effects of denervation and culture. J. Physiol. (Lond.) *401:* 617–637.

Gonoi, T., S. J. Sherman, and W. A. Catterall (1985) Voltage-clamp analysis of tetrodotoxin-sensitive and -insensitive sodium channels in rat muscle cells developing *in vitro.* J. Neurosci. *5:* 2559–2564.

Haimovich, B., J. C. Tanaka, and R. L. Barchi (1986) Developmental appearance of sodium channel subtypes in rat skeletal muscle cultures. J. Neurochem. *47:* 1148–1153.

Hayward, L. J., Y. Y. Zhu, and R. J. Schwartz (1988) Cellular localization of muscle and non-muscle actin mRNAs in chicken primary myogenic cultures: The induction of α-skeletal actin mRNA is regulated independently of α-cardiac actin gene expression. J. Cell Biol. *106:* 2077–2086.

Inestrosa, N. C., J. B. Miller, L. Silberstein, L. Ziskind-Conhaim, and Z. W. Hall (1983) Developmental regulation of 16S acetylcholinesterase and acetylcholine receptors in a mouse muscle cell line. Exp. Cell Res. *147:* 393–405.

Jaynes, J. B., J. S. Chamberlain, J. N. Buskin, J. E. Johnson, and S. D. Hauschka (1986) Transcriptional regulation of the muscle creatine kinase gene and regulated expression in transfected mouse myoblasts. Mol. Cell. Biol. *6:* 2855–2864.

Kimelman, D., and M. Kirschner (1987) Synergistic induction of mesoderm by FGF and TGF-β and the identification of an mRNA coding for FGF in the early Xenopus embryo. Cell *51:* 869–877.

Linkhart, T. A., C. H. Clegg, and S. D. Hauschka (1981) Myogenic differentiation in permanent clonal mouse myoblast cell lines: Regulation by macromolecular growth factors in the culture medium. Dev. Biol. *86:* 19–30.

Massague, J., T. Cheifetz, S. Endo, and B. Nadal-Ginard (1986) Type β transforming growth factor is an inhibitor of myogenic differentiation. Proc. Natl. Acad. Sci. USA *83:* 8206–8210.

Minty, A. J., S. Alonso, M. Caravatti, and M. E. Buckingham (1982) A fetal skeletal muscle actin mRNA in the mouse and its identity with cardiac actin mRNA. Cell *30:* 185–192.

Minty, A., H. Blau, and L. Kedes (1986) Upstream regions of the human cardiac actin gene that modulate its transcription in muscle cells: Presence of an evolutionarily conserved repeated motif. Mol. Cell. Biol. *6:* 2125–2136.

Mishina, M., T. Takai, K. Imoto, M. Noda, T. Takahashi, S. Numa, C. Methfessel, and B. Sakmann (1986) Molecular distinctions between fetal and adult forms of muscle acetylcholine receptor. Nature *321:* 406–411.

Morton, M. E., J. M. Caffrey, A. M. Brown, and S. C. Froehner (1988) Monoclonal antibody to the α_1-subunit of the dihydropyridine-binding complex inhibits calcium currents in BC_3H1 myocytes. J. Biol. Chem. *263:* 613–616.

Nadal-Ginard, B. (1978) Commitment, fusion and biochemical differentiation of a myogenic cell line in the absence of DNA synthesis. Cell *15:* 855–864.

Noda, M., M. Ko, A. Ogura, D. Liu, T. Amano, T. Takano, and Y. Ikawa (1985) Sarcoma viruses carrying *ras* oncogenes induce differentiation-associated properties in a neuronal cell line. Nature *318:* 73–75.

Olson, E. N., E. Sternberg, J. S. Hu, G. Spizz, and C. Wilcox (1986) Regulation of myogenic differentiation by type b transforming growth factor. J. Cell Biol. *103:* 1799–1805.

Olson, E. N., G. Spizz, and M. T. Tainsky (1987) The oncogenic forms of N-*ras* or H-*ras* prevent skeletal myoblast differentiation. Mol. Cell. Biol. *7:* 2104–2111.

Payne, P. A., E. N. Olson, P. Hsiau, R. Roberts, M. B. Perryman, and M. D. Schneider (1987) An activated c-Ha-*ras* allele blocks the induction of muscle-specific genes whose expression is contingent on mitogen withdrawal. Proc. Natl. Acad. Sci. USA *84:* 8956–8960.

Periasamy, M., E. E. Strehler, L. I. Garfinkel, R. M. Gubits, N. Ruiz-Opazo, and B. Nadal-Ginard (1984) Fast skeletal myosin light chains 1 and 3 are encoded by a single gene with novel structural organization. J. Biol. Chem. *259:* 13595–13604.

Pinset, C., and R. G. Whalen (1984) Manipulation of medium conditions and differentiation in the rat myogenic cell line L6. Dev. Biol. *102:* 269–277.

Rampe, D. E., J. M. Caffrey, M. D. Schneider, and A. M. Brown (1988) Control of expression of the 1,4-dihydropyridine receptor in BC_3H1 cells. Biochem. Biophys. Res. Comm. *152:* 769–775.

Rorsman, P. (1988) Two types of Ca^{2+} currents with different sensitivities to organic Ca^{2+} channel antagonists in guinea pig pancreatic α_2 cells. J. Gen. Physiol. *91:* 243–254.

Sakmann, B., and E. Neher, eds. (1983) *Single Channel Recording,* Plenum, New York.

Schmid, A., J.-F. Renaud, M. Fosset, J.-P. Meaux, and M. Lazdunski (1984) The nitrendipine-sensitive Ca^{2+} channel in chick muscle cells and its appearance during myogenesis in vitro and in vivo. J. Biol. Chem. *259:* 11366–11372.

Schneider, M. D., and E. N. Olson (1989) Control of myogenic differentiation by cellular oncogenes. Mol. Neurobiol. (in press).

Schneider, M. D., M. B. Perryman, P. A. Payne, G. Spizz, R. Roberts, and E. N. Olson (1987) Autonomous expression of c-*myc* in BC_3H1 cells partially inhibits but does not prevent myogenic differentiation. Mol. Cell. Biol. *7:* 1973–1977.

Schubert, D., A. J. Harris, C. E. Devine, and S. Heinemann (1974) Characterization of a unique muscle cell line. J. Cell Biol. *61:* 398–413.

Schwarz, T. L., B. L. Tempel, D. M. Papazian, Y. N. Jan, and L. Y. Jan (1988) Multiple potassium-channel components are produced by alternative splicing at the *Shaker* locus in *Drosophila.* Nature *331:* 137–142.

Sherman, S. J., and W. A. Catterall (1982) Biphasic regulation of development of the high-affinity saxitoxin receptor by innervation in rat skeletal muscle. J. Gen. Physiol. *80:* 753–768.

Sherman, S. J., and W. A. Catterall (1984) Electrical activity and cytosolic calcium regulate levels of tetrodotoxin-sensitive sodium channels in cultured rat muscle cells. Proc. Natl. Acad. Sci. USA *81:* 262–266.

Silberstein, L., S. G. Webster, M. Travis, and H. M. Blau (1986) Developmental progression of myosin gene expression in cultured muscle cells. Cell *46:* 1075–1081.

Tanabe, T., K. G. Beam, J. A. Powell, and S. Numa (1988) Restoration of excitation-contraction coupling and slow calcium current in dysgenic muscle by dihydropyridine receptor complementary DNA. Nature *336:* 134–139.

Tanabe, T., H. Takeshima, A. Mikami, V. Flockerzi, H. Takahashi, K. Kangawa, M. Kojima, H. Matsuo, T. Hirose, and S. Numa (1987) Primary structure of the receptor for calcium channel blockers from skeletal muscle. Nature *328:* 313–318.

Tapscott, S. J., A. B. Lassar, R. L. Davis, and H. Weintraub (1989) 5-Bromo-2′-deoxyuridine blocks myogenesis by extinguishing expression of MyoD1. Science *245:* 532–536.

Vaidya, T. B., S. J. Rhodes, E. J. Taparowsky, and S. F. Konieczny (1989) Fibroblast growth factors and transforming growth factor β repress transcription of the myogenic regulatory gene MyoD1. Mol. Cell. Biol. *9:* 3576–3579.

Weiss, R. E., and R. Horn (1986) Functional differences between two classes of sodium channels in developing rat skeletal muscle. Science *233:* 361–364.

Weydert, A., P. Barton, A. J. Harris, C. Pinset, and M. Buckingham (1987) Developmental pattern of mouse skeletal myosin heavy chain gene transcripts in vivo and in vitro. Cell *49:* 121–129.

Whalen, R. G., S. M. Sell, G. Butler-Browne, K. Schwartz, P. Bouveret, and I. Pinset-Harstrom (1981) Three myosin heavy chain isozymes appear sequentially in rat muscle development. Nature *292:* 805–809.

Yaffe, D., and O. Saxel (1977) Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. Nature *270:* 725–727.

Yatani, A., C. L. Seidel, J. Allen, and A. M. Brown (1987) Whole-cell and single-channel calcium currents of isolated smooth muscle cells from saphenous vein. Circ. Res. *60:* 523–533.