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Electrical activity and cytosolic calcium regulate levels of tetrodotoxin-sensitive sodium channels in cultured rat muscle cells

(ion transport/action potentials/nerve–muscle interaction/ionophore)

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ABSTRACT **Pharmacological blockade of the spontaneous electrical activity present in primary cultures of rat myotubes by growth in bupivacaine, tetrodotoxin, or KCl was found to increase the number of voltage-sensitive Na^+ channels 38–83% as measured by the specific binding of [^{3}H]saxitoxin. The inhibition of spontaneous electrical activity and increase in channel density by bupivacaine displayed an identical dose response, with a half-maximal effect at 3.0 μM. Growth of myotubes in the presence of 1 μM A23187, a Ca^{2+}-specific ionophore, resulted in a 30–60% decrease in the number of tetrodotoxin-sensitive channels with no change in affinity for [^{3}H]saxitoxin. A23187 was able to overcome the increase in channel density produced by bupivacaine. These results suggest the presence of a Ca^{2+}-mediated negative feedback system in which electrical excitability may be regulated by altering the number of tetrodotoxin-sensitive Na^+ channels.**

Voltage-sensitive sodium channels are responsible for the rapid depolarization during the action potential of electrically excitable tissues. In mammalian skeletal muscle there are two types of voltage-sensitive sodium channels, which may be distinguished by their affinity for the neurotoxins tetrodotoxin (TTX) and saxitoxin (STX). These small water-soluble toxins bind to a common receptor site, where they block ionic flux (1–6). In innervated adult muscle, the action potential is produced by TTX-sensitive sodium channels in which ionic flux may be blocked by nanomolar concentrations of TTX or STX (1–3). In uninnervated fetal muscle (4) or denervated adult muscle (5, 6), there are TTX-insensitive sodium channels that require a 200-fold greater concentration of TTX or STX to block ionic flux. The presence of the TTX-insensitive channel is related to the absence of motor neuron innervation (5–10). We have recently demonstrated that the developmental appearance of the TTX-sensitive channel is also regulated by innervation but in a biphasic manner (11). The first phase of development occurs in the absence of innervation and is actually accelerated upon denervation, whereas the expression of the second phase of development requires continuing innervation.

Primary cultures of skeletal muscle undergo many of the same developmental processes that occur *in vivo* (12–14) and thus provide a useful experimental system for the study of the developmental regulation of sarcolemmal proteins. Rat myotubes maintained *in vitro* in the absence of innervation contain both TTX-sensitive and TTX-insensitive sodium channels (15). The developmental time course of the TTX-sensitive channel is remarkably similar to the innervation-independent phase of development observed *in vivo* in muscle denervated shortly after birth (15). The TTX-sensitive channels in primary cultures muscle cells are functional and are capable of generating action potentials that give rise to spontaneous contractile activity that is easily observed and quantitated (15). The effects of innervation on TTX-sensitive sodium channels might be mediated by neurally imposed electrical activity or neurally released chemical factors (11). In this report we have investigated the role of spontaneous electrical activity and cellular Ca^{2+} permeability in the regulation of the TTX-sensitive sodium channel of cultured myotubes and compared these effects to those of denervation *in vivo*.

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MATERIALS AND METHODS

Preparation of Primary Cultured Muscle. Myocytes were obtained from the forelimbs of 20-day rat fetuses by enzymatic dissociation in 0.25% trypsin as described (16). The myocytes were seeded at a density of 7.5×10^6 cells per 150-mm dish and maintained in a growth medium consisting of 85% Dulbecco's modified Eagle's medium, 10% horse serum, 5% newborn calf serum, streptomycin at 10 mg/liter, and penicillin G at 30 mg/liter. The sera were heat inactivated by incubation at 56°C for 30 min. The growth medium was replaced every 4 days (30 ml per dish). From days 4 to 8, the growth medium was supplemented with 10 μM cytosine arabinonucleoside to inhibit fibroblast proliferation.

[^{3}H]STX Binding Assay. STX was obtained from the National Institutes of Health. [^{3}H]STX was prepared by the ^3H_2O exchange technique of Ritchie *et al.* (17). Purification and characterization was performed as described by Catterall *et al.* (16). The preparation of [^{3}H]STX used had a specific activity of 12.2 Ci/mmol (1 Ci = 37 GB_q) and a radiochemical purity of 85%. The specific binding of [^{3}H]STX was determined by rapid filtration over glass fiber filters as described (15). [^{3}H]STX binding was routinely measured at 15 nM with nonspecific binding determined in the presence of 2 μM TTX. Protein content was measured by first diluting an aliquot of each homogenate 1:25 in a solution of 0.2% sodium dodecyl sulfate in 0.4 M NaOH and then assaying for protein content by the method of Lowry *et al.* (18) with bovine serum albumin as the standard.

Measurement of Spontaneous Contractile Activity. Spontaneous contractile activity was determined by visual observation of the cultures at 200-fold magnification, using phase-contrast optics. The number of myotubes displaying contractile activity in each field was noted and divided by the total number of myotubes in that field. At least 25 fields were examined in each culture dish. The determinations were performed without knowledge of the experimental status of individual cultures.

Growth of Cells Under Experimental Conditions. Experimental agents were added directly to the growth medium as concentrated stock solutions. Bupivacaine was added from a 4 mM aqueous solution. This local anesthetic was chosen because of its potency and long duration of action. TTX was added from a 1 mM aqueous solution. This agent was found to be somewhat unstable under tissue culture conditions and replacement of the growth medium every 48 hr was required.

Abbreviations: TTX, tetrodotoxin; STX, saxitoxin.

In addition, TTX interferes with the [^{3}H]STX binding and special precautions were necessary to prevent carryover into the binding assay. Thus, before harvesting, the cells were washed extensively with a solution composed of 140 mM NaCl, 5.5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 50 mM Hepes (adjusted to pH 7.4 at 37°C with Tris base), and 5 mM glucose. Complete removal of the TTX was ascertained by visually noting the return of spontaneous contractile activity. The cells were then processed for determination of the STX receptor density as described (17). Growth of cells in 50 mM KCl was carried out in Dulbecco's modified Eagle's medium in which the 45 mM $KHCO_3$ was substituted for an equimolar portion of $NaHCO_3$. A23187, a Ca^{2+}-specific ionophore, was added to the growth medium from a 1 mM ethanolic solution. Ethanol was added to some of the control cultures at the same final concentration and was found not to influence the development of TTX-sensitive channels.

Materials. Sprague–Dawley rats mated at known times were purchased from Tyler Laboratories (Bellevue, WA). Dulbecco's modified Eagle's medium was from GIBCO. Sera were from either Flow Laboratories or KC Biological. TTX and A23187 were from Calbiochem. Bupivacaine was obtained from Bertil Takman (Astra Pharmaceuticals, Södertälje, Sweden). Veratridine was purchased from Aldrich. Other chemicals were purchased from Sigma.

RESULTS

The number of TTX-sensitive sodium channels in rat muscle cells was quantitated by measuring the specific binding of [^{3}H]STX to muscle tissue homogenates. This has been shown to be a reliable method by a number of investigators (1, 2).

Fusion of myoblasts to form multinucleate myotubes was complete after 4 days in culture. Spontaneous contractile activity appeared shortly after fusion was complete and reached a maximum after 8–10 days in culture. The development of TTX-sensitive sodium channels exhibits a complex time course (15). The number of channels increases from 0 at the time of plating to a maximum value of 60–80 fmol/mg of protein between days 10 and 12 of maintenance in culture. After this peak is reached, the number of TTX-sensitive sodium channels declines over the next 4–6 days to reach a new plateau value that is approximately 40% of the peak density. This decline is not a nonspecific effect due to cell senescence, since the number of TTX-insensitive sodium channels remains relatively constant at days 10–20 in culture (15).

Fig. 1 shows the effect of blocking spontaneous contractile activity on the time course of development of TTX-sensitive sodium channels. After 4 days in culture, cells were grown in the presence of 1 μM TTX, 40 μM bupivacaine, or 50 mM KCl. TTX and the local anesthetic bupivacaine eliminate electrical activity directly by blocking ionic flux through the voltage-sensitive sodium channel, while maintenance in 50 mM KCl abolishes spontaneous electrical activity indirectly by chronically depolarizing the cells and inactivating the sodium channels. Each of these treatments produced a significant increase in the number of TTX-sensitive sodium channels measured at days 8, 12 and 16 (Fig. 1). The treated cells still showed a decline in channel number after day 12 that paralleled the decrease observed in the controls. Thus, the regulatory effect produced by blocking spontaneous electrical activity is apparently superimposed upon whatever regulatory process is responsible for the biphasic nature of the developmental time course observed in the control cultures. In addition to the above methods of eliminating spontaneous electrical activity, we also examined the effects of treating cells with veratridine (10 μM), which blocks action potential production by causing chronic depolarization due to increased influx of extracellular Na^+ (3). At day 12, veratridine-treated cells had 2.1-fold more TTX-sensitive channels than controls ($P < 0.005$; Student's t test).

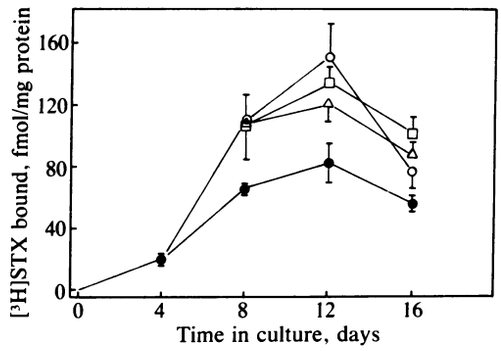

FIG. 1. Pharmacological blockade of spontaneous contractile activity increases the density of TTX-sensitive sodium channels. After 4 days in culture, myotubes were maintained for the indicated period of time in growth medium containing 40 μM bupivacaine (○), 1 μM TTX (□), 50 mM KCl (△), or no additions (●). Binding of [^{3}H]STX was determined; each point represents the mean of at least three separate experiments and the error bars indicate the SEM. In all cases the myotubes in the experimental groups had significantly higher channel densities compared to control cells ($P < 0.005$ for bupivacaine-treated cells on day 16, $P < 0.001$ for all other groups; Student's t test).

Since the increase in sodium channel number is caused by indirect blockade of electrical activity as well as by agents that act directly on the voltage-sensitive sodium channel at different receptor sites (1–3, 19), it seems unlikely that this effect simply results from receptor–ligand interaction. Rather, the common mechanism must be related to the loss of spontaneous electrical activity. Block of spontaneous electrical activity in skeletal muscle prevents the phasic increase in cytosolic Ca^{2+} that accompanies each contraction. To evaluate the role of Ca^{2+} in regulation of sodium channel density, we examined the effect of the Ca^{2+} ionophore A23187. This ionophore specifically increases the permeability of both sarcolemmal and sarcoplasmic reticulum membranes to Ca^{2+} (19, 20) and causes both an increase in cytosolic Ca^{2+} and a tonic contracture in intact muscle (20, 21). Fig. 2 shows that growth of cells in the presence of 1 μM A23187 nearly abolishes the further development of TTX-sensitive sodium channels from day 4 to day 10 *in vitro*. Growth of cells in 40 μM bupivacaine leads to a 5-fold in-

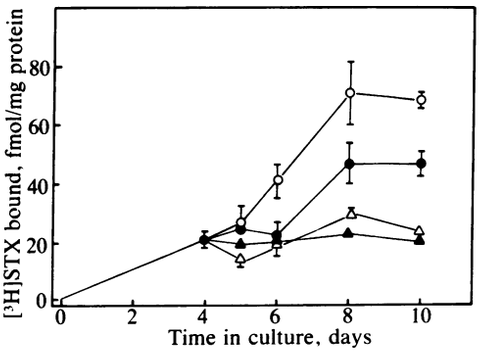

FIG. 2. Effect of A23187 on the development of TTX-sensitive Na^+ channels. After 4 days in culture, myotubes were maintained for the indicated period of time in growth media containing 1 μM A23187 (▲), 40 μM bupivacaine (○), 1 μM A23187 + 40 μM bupivacaine (△), or with no additions (●). The density of TTX-sensitive sodium channels was determined; each point represents the mean of at least three separate experiments, and error bars denote the SEM. Differences between the A23187-treated and control groups on days 8 and 10 were highly significant ($P < 0.001$; Student's t test).

crease in the number of TTX-sensitive sodium channels as compared to growth under conditions of high Ca^{2+} permeability (1 μM A23187). The sodium channel density of cells grown in the presence of bupivacaine plus A23187 is not significantly different from that of cells grown in A23187 alone (Fig. 2). This result is expected, because the tonically increased Ca^{2+} influx mediated by the A23187 should override the loss of phasic Ca^{2+} influx produced by blockade of electrical activity. These results are consistent with the hypothesis that cytosolic Ca^{2+} levels modulate the levels of TTX-sensitive sodium channels in response to changes in electrical activity.

The data presented in Fig. 1 indicate that manipulations which affect the spontaneous electrical activity of cultured myotubes have significant regulatory effects on the density of voltage-sensitive sodium channels. It is possible to obtain an index of the extent to which a pharmacological agent blocks voltage-sensitive sodium channels by visually observing and tabulating the degree of spontaneous contractile activity that is present. If growth of cells in the presence of bupivacaine leads to an increased level of channels by virtue of its specific effect on voltage-sensitive sodium channels, then the dose–response curves for inhibition of spontaneous contractile activity and increase of STX receptor density should overlap. Fig. 3 shows the effect of bupivacaine on STX receptor density and spontaneous contractile activity in cells treated with the concentrations indicated from day 4 to 10. The degree of spontaneous contractile activity was tabulated as the fraction of the total myotubes displaying rhythmic contractions observable at ×200 magnification. Both of these parameters are expressed as a percentage of the maximally achieved effect. The data shown in Fig. 3 represent the mean of three separate experiments in which the maximally effective concentration of bupivacaine increased TTX-sensitive sodium channel number 86%, from 59 ± 6.1 to 110 ± 15.1 fmol/mg of protein. Blockade of spontaneous contractile activity by bupivacaine was 100% effective at concentrations greater than 30 μM. The dose response of blockade of spontaneous contractile activity and increase in STX receptor number fall on single curve with a $K_{0.5}$ of 3.3 μM. This value is in good agreement with the results of studies in which the K_d for bupivacaine was found to be 2.6 μM (22) in neurotoxin binding experiments. This result is consistent with the conclusion that bupivacaine acts by blocking spontaneous electrical activity rather than by a nonspecific membrane effect.

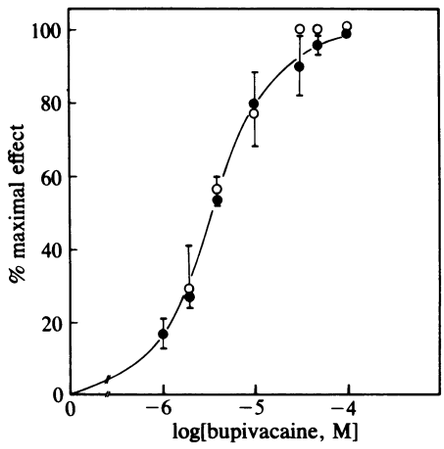

FIG. 3. Dose response of blockade of spontaneous contractile activity and increase in TTX-sensitive channel density produced by bupivacaine. The density of TTX-sensitive sodium channels (●) and the spontaneous contractile activity (○) were quantitated; each point represents the mean of three separate experiments and error bars denote the SEM. The concentration at which the effect is half of the maximum, $K_{0.5}$, = 3.0 μM for both of these effects.

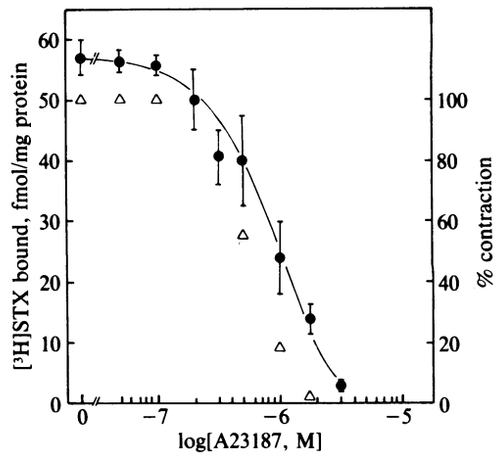

FIG. 4. Dose response of blockade of spontaneous contractile activity and decrease in TTX-sensitive channel density produced by A23187. The density of TTX-sensitive sodium channels (●) and the fraction of myotubes exhibiting spontaneous contractile activity (△) were measured; data points with error bars represent the mean and SEM of three separate experiments. The $K_{0.5}$ = 0.9 μM for both spontaneous contractile activity and sodium channel density.

Fig. 4 shows the effect of increasing concentrations of the A23187 on levels of sodium channels and spontaneous contractile activity of cells maintained in the presence of the ionophore from day 4 to day 10. The specific binding of [^{3}H]STX and measurement of spontaneous contractile activity were performed as described for Fig. 3. Concentrations of A23187 greater than 0.3 μM decreased both sodium channel number and spontaneous contractile activity. At 2 μM A23187, the level of TTX-sensitive sodium channels was decreased to 24% of the control value. At 3 μM A23187, toxic effects became apparent upon visual inspection of the cells. A small fraction of the myotubes became detached from the culture dish and increasing numbers of vacuoles were evident in myotubes that remained attached. Thus at 3 μM A23187, a nonspecific toxic effect may have contributed somewhat to the decline in sodium channel level. The data presented in Fig. 4, however, indicate that a significant decline in channel number occurs at A23187 concentrations much lower than 3 μM, without signs of toxicity or any change in the total cellular protein per culture dish (data not shown). Thus, it seems clear that the effect of A23187 represents a specific regulation of channel number rather than a manifestation of toxic effects on the cultures.

Treatment with A23187 also completely blocks observable spontaneous contractile activity, with half-maximal inhibition at 0.5 μM (Fig. 4). This effect probably results from membrane depolarization due to increased Ca^{2+} influx (23). Our results indicate that this increased Ca^{2+} influx also reduces the number of TTX-sensitive sodium channels.

In the experiments so far discussed, the number of STX receptors was estimated by measuring binding in the presence of 15 nM [^{3}H]STX. We have previously shown this concentration to be sufficient to occupy greater than 85% of the receptors present (17). Nevertheless, it is possible that the regulatory effects on STX binding reflect changes in the affinity of the receptor for its ligand as well as changes in the number of sarcolemmal TTX-sensitive channels. To exclude this possibility, we have performed Scatchard analysis of [^{3}H]STX binding to control cells and cells treated with bupivacaine or A23187 for 6 days (Fig. 5). In each of the experimental conditions the data are best fit by straight lines of similar slope, indicating the presence of a single class of re-

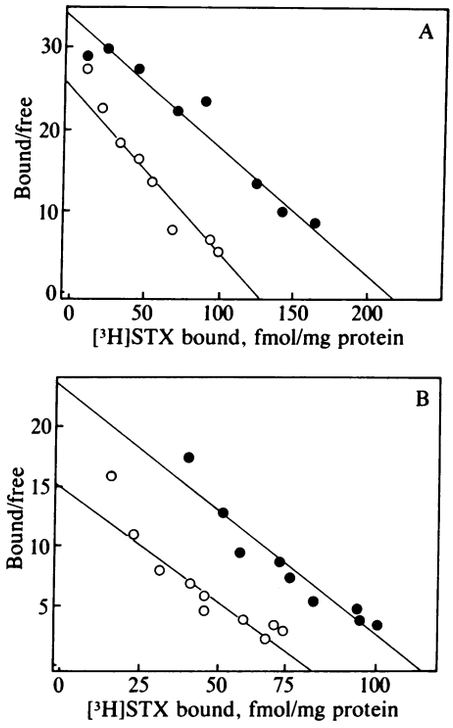

FIG. 5. Scatchard analysis of [^{3}H]STX binding to muscle cells grown in the presence of bupivacaine or A23187. Binding of [^{3}H]STX was measured as the concentration of [^{3}H]STX was varied from 0.5 to 30 nM. The K_d and B_{max} (maximal binding) were determined by a weighted least-squares fit to the data, assuming the presence of a single class of binding sites without cooperative interactions. The units shown on the ordinates are fmol per mg of protein/nM. (*A*) Muscle cells were grown for 6 days in growth medium with no additions (○) or containing 40 μM bupivacaine (●). The binding parameters are as follows: control cells, K_d = 4.98 nM and B_{max} = 125 fmol/mg of protein; bupivacaine-treated cells, K_d = 6.23 nM and B_{max} = 213 fmol/mg of protein. (*B*) Muscle cells were grown for 6 days in growth medium with no additions (●) or containing 1 μM A23187 (○). The binding parameters are as follows: control cells, K_d = 4.81 nM and B_{max} = 120 fmol/mg of protein; A23187-treated cells, K_d = 5.23 nM and B_{max} = 79.8 fmol/mg of protein.

ceptors with essentially identical affinities for [^{3}H]STX. The total receptor number, however, is increased 70% by treatment with bupivacaine and reduced 33% by A23187 treatment, indicating that these agents cause a true change in the number of sarcolemmal TTX-sensitive channels. This increase in receptor number per mg of cellular protein is likely to reflect an increase in the sarcolemmal density of TTX-sensitive sodium channels. Neither bupivacaine nor A231897 affects the ratio of mononucleate cells to myotubes in the culture. Treatment with bupivacaine has no effect on total cellular protein, whereas A23187 causes at most a small decrease. This action of A23187 to decrease cellular protein can at most reduce the magnitude of the observed effects. Thus, we can conclude from these results that experimentally induced changes in spontaneous electrical activity and in Ca^{2+} permeability can regulate the number, and probably the sarcolemmal density, of TTX-sensitive Na^+ channels in cultured myotubes.

DISCUSSION

Sarcolemmal electrical activity regulates many properties of skeletal muscle (24, 25), including the density and distribution of acetylcholine receptors (26), the subtype of acetylcholinesterase (27), and the activity of TTX-insensitive sodium channels (26, 28). We now report that electrical activity can regulate the number of TTX-sensitive sodium channels in primary cultures of rat skeletal muscle as determined by the specific binding of [^{3}H]STX, and we present evidence that this regulation is mediated by alterations in the level of cytosolic Ca^{2+}. Blockade of spontaneous electrical activity by a variety of methods leads to a significant increase in the level of sodium channels. Since each action potential leads to a large transient increase in cytosolic Ca^{2+} (29), blockade of spontaneous electrical activity will undoubtedly reduce the time-averaged level of cytosolic Ca^{2+}. Thus, it is an attractive hypothesis that the observed increase in sodium channel number produced by blockade of electrical activity is mediated by changes in cytosolic Ca^{2+}.

The ability of A23187 to decrease TTX-sensitive channel levels gives strong support for this hypothesis. Although we have not directly determined the cytosolic Ca^{2+} levels in these experiments, it seems likely from previous investigations that A23187 increases the level of Ca^{2+} in the cytosolic pool. A23187 markedly increases the permeability of lipid bilayer membranes to Ca^{2+} (30). In skeletal muscle, the cytosolic concentration of Ca^{2+} is three to four orders of magnitude lower than either the extracellular Ca^{2+} concentration or the concentration of Ca^{2+} stored intracellularly in the sarcoplasmic reticulum (29). Thus, any increase in the Ca^{2+} permeability of lipid bilayer membranes would allow Ca^{2+} to flow down its electrochemical gradient into the cytoplasm. A23187 has been shown to increase the Ca^{2+} permeability of both sarcolemmal and sarcoplasmic reticulum membranes of mammalian muscle (19, 20). Furthermore, spectrofluorimetric determination of cytosolic Ca^{2+} levels has shown that A23187 increases the concentration of cytosolic Ca^{2+} in intact barnacle muscle (20). A23187 also causes tonic contracture of intact frog skeletal muscle (21). On the basis of these results, it seems highly likely that cytosolic Ca^{2+} levels of cultured muscle cells grown in the presence of A23187 are chronically elevated. Thus, blockade of spontaneous electrical activity lowers cytosolic Ca^{2+} and increases the number of TTX-sensitive channels. Conversely, growth in A23187, which almost certainly increases cytosolic Ca^{2+}, decreases the number of TTX-sensitive channels.

Taken together, our findings suggest the presence of a Ca^{2+}-mediated negative feedback loop controlling cellular excitability. Increases in electrical excitability that lead to increased cytosolic Ca^{2+} levels may, in turn, decrease the number of sodium channels and thereby decrease electrical excitability. Conversely, a paucity of electrical activity would lead to an increase in the number of electrically excitable sodium channels. In this way, the cell would be able to maintain a given level of electrical excitability by altering the sodium channel density in response to the frequency of action potentials, using changes in the concentration of cytosolic Ca^{2+} as the feedback signal.

Other recent studies have shown that A23187 treatment is able to mimic motor neuron innervation with regard to the regulation of enzymes involved in intermediary metabolism (31), maintenance of resting membrane potential (32), and acetylcholine receptor density (32, 33). The effects on acetylcholine receptors are particularly relevant since receptor density is regulated by electrical activity both *in vivo* and *in vitro* (25). These other results support the view that cytosolic Ca^{2+} is an important intracellular mediator of the effects of electrical activity on a number of muscle properties, including the density of TTX-sensitive sodium channels.

The physiological relevance of these results, obtained *in vitro* with uninnervated myotubes, to Na^+ channel regulation in adult innervated muscle *in vivo* is uncertain. The similar developmental time courses of TTX-sensitive channels studied *in vivo* and *in vitro* suggest that cultured primary myotubes provide an adequate model of sodium channel regulation in developing skeletal muscle denervated at an early age (15). We have previously shown that the developmental

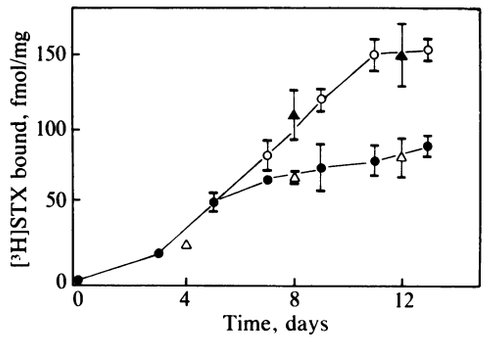

FIG. 6. Comparison of TTX-sensitive channel development *in vivo* and *in vitro*. The developmental time course of channel development *in vivo* in rat triceps surae is shown for normal muscle (●) and muscle denervated at postnatal day 5 (○) (from ref. 11). The data presented in Fig. 1 of this report for control (△) and bupivacaine-treated (▲) cells are shown for comparison. The units on the ordinate are fmol/mg of total cellular protein. Error bars indicate the SEM.

regulation *in vivo* is biphasic (11). The first phase, which occurs from birth until postnatal day 11 and accounts for approximately 60% of the total adult density of TTX-sensitive channels, does not require continuing innervation, and the rate of increase of TTX-sensitive sodium channel density is actually increased upon denervation at day 5 *in vivo* (11). Fig. 6 compares the development of TTX-sensitive channels observed *in vivo* in innervated and denervated muscle (data taken from ref. 11) with the development observed *in vitro* in cultures with and without spontaneous contractile activity (from Fig. 1). There is a close correspondence between sodium channel development in cultured myotubes displaying spontaneous contractile activity—e.g., control cultures—and innervated muscle *in vivo*. Similarly, cultures in which spontaneous contractile activity is blocked by bupivacaine fall on the same curve as denervated muscle *in vivo*. Thus, both *in vivo* and *in vitro*, the reduction of electrical activity leads to similar increases in the number of TTX-sensitive channels. This comparison suggests that the lack of neuronally driven motor activity in muscles denervated at day 5 *in vivo* may account for the acceleration of sodium channel development observed from days 5 to 11. Although denervated muscle develops fibrillations that may be analogous to the spontaneous contractile activity observed *in vitro*, it is likely that this type of muscle activity is of a substantially lower magnitude than the normal neuronally driven activity (24). This view is supported by studies of the acetylcholine receptor in which exogenous electrical activity, but not endogenous fibrillatory activity, could prevent denervation-induced changes (34). Thus, in both denervation *in vivo* and blockade of spontaneous contractile activity *in vitro*, there is a substantial decrease in the level of electrical and contractile activity and an associated increase in the number of TTX-sensitive channels. These results suggest a role for a Ca^{2+}-mediated negative-feedback regulation of the number of TTX-sensitive sodium channels *in vivo* as well as *in vitro*.

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