ACTIVATION AND INHIBITION OF THE ACTION POTENTIAL Na^+ IONOPHORE OF CULTURED RAT MUSCLE CELLS BY NEUROTOXINS

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Summary

Both myoblasts and myotubes in cultures of clonal rat muscle cells have action potential Na^+ ionophore activity. The ionophore is activated by batrachotoxin ($K_{0.5}$ = 3 to 5 x 10^{-7} M) and veratridine ($K_{0.5}$ = 4 to 6 x 10^{-5} M) which compete for the same activation site. As in denervated rat muscle, the ionophore of cultured muscle is 100 fold more resistant to inhibition by tetrodotoxin ($K_{0.5}$ = 0.6 to 2 x 10^{-6} M) and 20 fold more resistant to inhibition by saxitoxin ($K_{0.5}$ = 1.5 to 3 x 10^{-7} M) than in nerve, innervated muscle, or cultured neuroblastoma cells.

Clonal lines derived from rat skeletal muscle (1) fuse into multinucleate myotubes *in vitro* and express a number of differentiated properties characteristic of adult muscle including electrical excitability (2,3).

The neurotoxic alkaloids veratridine and batrachotoxin activate the action potential Na^+ ionophore of nerve (4,5), muscle (4,5), and cultured neuroblastoma cells (6,7) while the neurotoxins saxitoxin and tetrodotoxin are specific inhibitors of the Na^+ ionophore activated by either depolarization (8,9) or neurotoxin treatment (4-7). In this communication, I present experiments which describe the activation and inhibition of the action potential Na^+ ionophore of cultured rat muscle cells by these 4 neurotoxins.

Methods

Stock cultures of rat muscle clonal lines L5 and L8 (1), kindly provided by Drs. D. Yaffee and M. Nirenberg, were maintained in 5% fetal calf serum in the Dulbecco-Vogt modification of Eagle's minimal essential medium (DMEM, Gibco) in a humidified atmosphere of 10% CO_2/90% air. Stock cultures were not allowed to fuse. Experimental cultures were prepared by suspending cells in growth medium after treatment with .02% trypsin (Worthington Biochemical) in Ca^{++} and Mg^{++} free Dulbecco's phosphate buffered saline. Cells were seeded at a density of 0.75 to 1.0 x 10^4 cells/ cm^2 in multiwell plates (1.6 cm diameter). Growth medium was replaced every 2 or 3 days. Under these conditions, fusion of myoblasts into myotubes begins 5 or 6 days after seeding and continues for several days. In cultures older than 9 days, 70 to 80% of the cell nuclei are in myotubes.

For measurements of $^{22}Na^+$ uptake, cells were treated with toxins for 30 min at 36° in a Na^+-free medium consisting of 50 mM HEPES adjusted to pH 7.4 with Tris

base, 0.8 mM $MgCl_2$, 5.5 mM glucose, 5.4 mM KCl, and either 130 mM tetraethylammonium Cl or 156 mM Tris Cl, pH 7.4 to allow equilibration of the cells with toxin without loss of the Na^+ gradient. Uptake measurements were initiated by removal of this medium and addition of assay medium containing the components listed above plus 1 to 10 μCi/ml $^{22}Na^+$, 10 mM ouabain, and 10 mM NaCl replacing an osmotically equivalent amount of Tris Cl or tetraethylammonium Cl. Uptake was terminated following a 1 min incubation at 36° by removing the radioactive assay medium and washing at 0° as described previously. This procedure is a slight modification of previously published methods (6,7). Protein was determined by the method of Lowry et al (10). Uptake is expressed as nmoles $^{22}Na^+$ taken up per minute per mg cell protein.

Batrachotoxin was kindly provided by Drs. J. Daly and B. Witkop and saxitoxin by Dr. E. Schantz. Other chemicals were obtained commercially as previously (6,7).

Results

Veratridine and batrachotoxin increase the initial rate of $^{22}Na^+$ uptake of 10 day old cultures of L5 having greater than 70% of the cell nuclei in myotubes (Fig. 1A) and also of 4 day old cultures containing only myoblasts (Fig. 1B). Titration experiments in which either 4 day (Fig. 2A and B) or 10 day (Fig. 2C and D) cultures of L5 were incubated with various concentrations of veratridine or batrachotoxin and then assayed for $^{22}Na^+$ uptake show that, as in neuroblastoma cells (7), batrachotoxin acts at a lower concentration and induces a greater rate of Na^+ uptake than veratridine. Addition of veratridine in the presence of an effective concentration of batrachotoxin (1.3 μM, Fig. 2B and D) causes reduction of the high rate of $^{22}Na^+$ uptake observed in the presence of batrachotoxin alone to the lower rate characteristic of veratridine-treated cells. The concentration

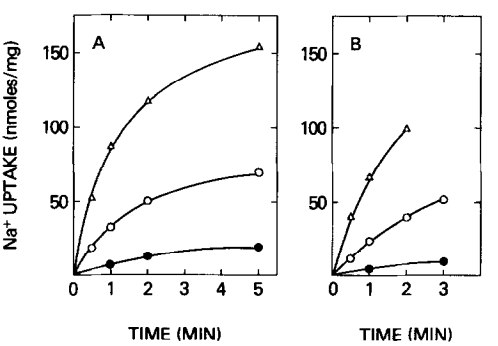

Figure 1. Stimulation of $^{22}Na^+$ uptake by veratridine and batrachotoxin. Cultures of L5 grown for 4 days (B) or 10 days (A) were incubated with control medium (O), 200 μM veratridine (O), or 1 μM batrachotoxin (Δ) and then assayed for the indicated times with the same toxin concentration.

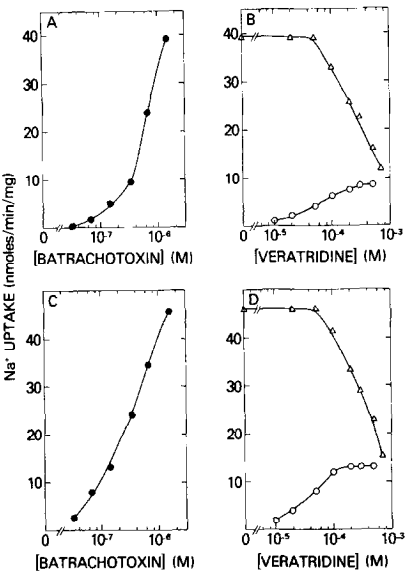

Figure 2. Concentration dependence of activation by veratridine and batrachotoxin. Cultures of L5 grown for 4 days (A and B) or 10 days (C and D) were incubated with the indicated concentrations of batrachotoxin (O) or veratridine (O), or with 1.3 μM batrachotoxin plus the indicated concentrations of veratridine (Δ). Cultures were then assayed in the presence of the same toxin concentrations. Uptake by cultures not treated with toxins has been subtracted.

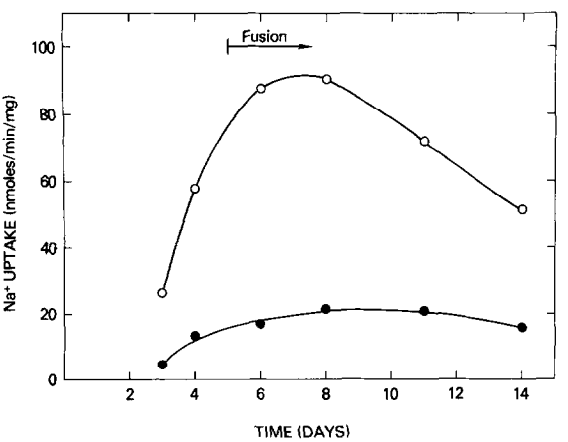

Figure 3. Development of the response to veratridine and batrachotoxin. L5 cells were seeded at 7.5 x 10^3 cells/cm^2 on day zero and the medium was changed every third day thereafter. Myotubes were first observed on day 5 and rapid fusion continued until day 8. Rates of $^{22}Na^+$ uptake were measured on the indicated days after incubation with 200 μM veratridine (O) or 1.1 μM batrachotoxin (O). Uptake by cultures not treated with toxins has been subtracted.

of veratridine required is consistent with competitive inhibition of activation by the less effective activator veratridine. Similar competitive interactions among veratridine, batrachotoxin, and a third neurotoxin, aconitine, have been studied in more detail in neuroblastoma cell cultures and the results are fit well by equations derived assuming that the three toxins compete for a single class of binding sites (11).

Both myotubes and myoblasts respond to veratridine and batrachotoxin. The toxin concentrations required for 50% activation (3 to 5 x 10^{-7} M for batrachotoxin and 4 to 6 x 10^{-5} M for veratridine, Fig. 2A-D) are identical for myotubes and myoblasts and are similar to the concentrations required for 50% activation of neuroblastoma cells (7).

The time course of development of the response to veratridine and batrachotoxin is illustrated in Fig. 3. On the first day after subculture, cells have little if any response to the toxins. On days 2 through 5, a substantial response develops although no myotubes are observed until day 5. The specific activity increases to a plateau during fusion and then in some experiments (as in Fig. 3) decreases. These results are consistent with electrophysiologic experiments in which fast depolarizing responses were observed in myoblasts and myotubes of a similar clonal rat muscle line (2).

Action potential Na^+ responses of nerve and muscle are inhibited by low concentrations ($K_{0.5}$ = 1 to 9 x 10^{-9} M) of tetrodotoxin or saxitoxin (8,9, for a review see reference 12). Binding experiments indicate that these 2 toxins compete for the same binding site (13,14). The response of nerve, muscle, and cultured neuroblastoma cells to veratridine and batrachotoxin is inhibited by low concentrations of saxitoxin or tetrodotoxin ($K_{0.5}$ = 1 to 9 X 10^{-9} M) (4,5,7,15). The observed inhibition is noncompetitive (7) suggesting that the activating and inhibiting toxins act at separate sites associated with the ionophore.

The response of denervated rat muscle to electrical stimulus or to batrachotoxin treatment is resistant to inhibition by tetrodotoxin and saxitoxin ($K_{0.5}$ > 10^{-7} M (15,16)). Some action potential responses in cultured rat muscle cells

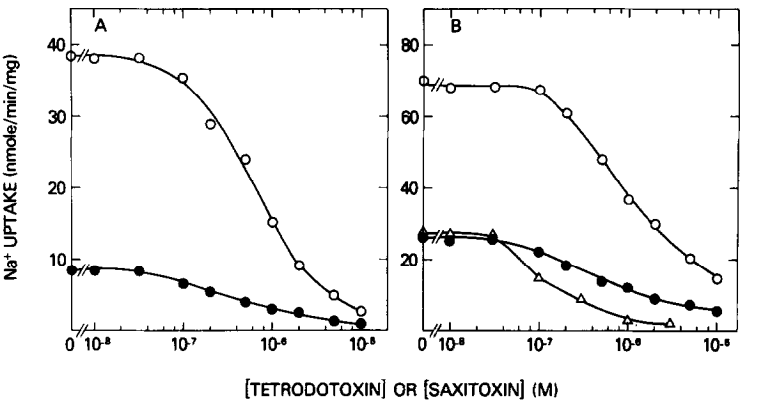

Figure 4. Inhibition of veratridine-dependent and batrachotoxin-dependent $^{22}Na^+$ uptake by tetrodotoxin and saxitoxin. Cultures of L5 grown for 5 days (A) or 11 days (B) were incubated with 200 μM veratridine (O, Δ) or 2 μM batrachotoxin (♀) plus the indicated concentrations of tetrodotoxin (O, O) or saxitoxin (Δ). Na^+ uptake was then measured in the presence of the same toxin concentrations. Uptake by cultures not treated with toxins has been subtracted.

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are not inhibited by 3 x 10^{-7} M tetrodotoxin (2,3). The results of Fig. 4 show that the response of 4 day and 11 day cultures of L5 to veratridine and batrachotoxin is also resistant to inhibition by tetrodotoxin ($K_{0.5}$ = 0.6 to 1.0 x 10^{-6} M) or saxitoxin ($K_{0.5}$ = 1.5 to 3 x 10^{-7} M). Rat muscle clone L8 is also resistant to tetrodotoxin ($K_{0.5}$ = 1 to 2 x 10^{-6} M). These values are in close quantitative agreement with electrophysiologic experiments on denervated rat muscle in which 50% inhibition of the rate of rise of the action potential was obtained at 1 x 10^{-7} M saxitoxin or 9 x 10^{-7} M tetrodotoxin (16).

In contrast, both denervated chick muscle (17) and cultured chick muscle (18) have tetrodotoxin-sensitive action potentials, and toxin stimulated $^{22}Na^+$ uptake by cultured chick muscle has normal sensitivity to tetrodotoxin ($K_{0.5}$ = 1 to 2 x 10^{-8} M, unpublished results).

Discussion

The results described in this communication support our earlier conclusion (6) that veratridine is a specific activator of the action potential Na^+ ionophore since both toxin-dependent Na^+ uptake and active electrical responses of rat muscle cells are similarly insensitive to inhibition by tetrodotoxin and saxitoxin.

The activating toxins veratridine and batrachotoxin appear to compete for the same activation site in muscle cells, as shown previously in neuroblastoma cells (7). This site must be distinct from the site at which the inhibitors tetrodotoxin and saxitoxin interact since the inhibition is noncompetitive (7) and the affinity of the Na^+ ionophore of rat muscle cells for tetrodotoxin and saxitoxin is reduced 100 fold and 20 fold respectively while the affinity for batrachotoxin and veratridine is comparable to that of the other excitable cells (Fig. 4, (15)).

Both cultures containing only myoblasts and cultures containing mainly myotubes respond to veratridine and batrachotoxin. The response increases during growth in culture, however it is uncertain whether this increase is due to differentiation of the cells or to recovery from trypsin treatment during subculture. In either case, L5 myoblasts have significant levels of action potential Na^+ ionophore activity. The Na^+ ionophore is unusual in this respect since most differentiated functions of muscle cells are expressed concomitantly with fusion into myotubes.

The insensitivity of the action potential Na^+ ionophore of clonal rat muscle cells to tetrodotoxin and saxitoxin described in this communication suggests that rat muscle cells that have never been innervated are resistant to inhibition by these toxins, that synapse formation is accompanied by a change in the properties of the ionophore which is reflected in increased affinity for tetrodotoxin and saxitoxin, and that denervation causes a return to toxin resistance. Since clonal rat muscle cells form synapses in culture (19), it will be of interest to determine whether synapse formation *in vitro* increases the affinity of the action potential Na^+ ionophore for these toxins.

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