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Down-regulation of voltage-dependent sodium channels initiated by sodium influx in developing neurons

(astrocyte/Na^+ flux/saxitoxin/scorpion toxin)

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ABSTRACT **To address the issue of whether regulatory feedback exists between the electrical activity of a neuron and ion-channel density, we investigated the effect of Na^+-channel activators (scorpion α toxin, batrachotoxin, and veratridine) on the density of Na^+ channels in fetal rat brain neurons *in vitro*. A partial but rapid ($t_{1/2}$, 15 min) disappearance of surface Na^+ channels was observed as measured by a decrease in the specific binding of [^{3}H]saxitoxin and ^{125}I-labeled scorpion β toxin and a decrease in specific $^{22}Na^+$ uptake. Moreover, the increase in the number of Na^+ channels that normally occurs during neuronal maturation *in vitro* was inhibited by chronic channel activator treatment. The induced disappearance of Na^+ channels was abolished by tetrodotoxin, was found to be dependent on the external Na^+ concentration, and was prevented when either choline (a nonpermeant ion) or Li^+ (a permeant ion) was substituted for Na^+. Amphotericin B, a Na^+ ionophore, and monensin were able to mimick the effect of Na^+-channel activators, while a KCl depolarization failed to do this. This feedback regulation seems to be a neuronal property since Na^+-channel density in cultured astrocytes was not affected by channel activator treatment or by amphotericin B. The present evidence suggests that an increase in intracellular Na^+ concentration, whether elicited by Na^+-channel activators or mediated by a Na^+ ionophore, can induce a decrease in surface Na^+ channels and therefore is involved in down-regulation of Na^+-channel density in fetal rat brain neurons *in vitro*.**

Voltage-sensitive Na^+ channels are responsible for the generation and conduction of action potentials along neuronal membranes. The Na^+ channel purified from rat brain is a large glycoprotein consisting of three subunits, α (250 kDa), β_1 (36 kDa), and β_2 (33 kDa) (1, 2). The α and β_2 subunits are linked by disulfide bonds. The primary structures of three closely related rat brain α subunits have been determined and the expression of mRNAs in *Xenopus* oocytes results in functional Na^+ channels (3–5). At least five different binding sites for neurotoxins have been shown to be present on this ion channel: site 1, tetrodotoxin (TTX) and saxitoxin (STX); site 2, veratridine and batrachotoxin (BTX); site 3, scorpion α toxins (α-ScTX) and sea anemone toxins; site 4, scorpion β toxins (β-ScTX); site 5, brevetoxins (6–8).

During neuronal development, the appearance of electrical excitability is mainly determined by the insertion of Na^+ channels into specific domains of the outgrowing plasma membrane. In brain neurons developing *in vitro*, a large increase in the number of Na^+ channels occurs as detected by measurements of neurotoxin binding capacities (9, 10) or neurotoxin-sensitive $^{22}Na^+$ flux (11). These Na^+ channels are localized predominantly on neurites (12). Studies on biosynthesis and processing of the Na^+ channel in developing neurons have shown that most newly synthesized α subunits are not disulfide linked to β_2 subunits and remain as a metabolically stable pool of intracellular subunits (13). To address the question of whether a regulatory mechanism links the electrical activity of a neuron to the density of channels in its plasma membrane, we investigated the effect of the Na^+-channel activators α-ScTX, BTX, and veratridine in a culture of fetal rat brain neurons.

MATERIALS AND METHODS

Materials. [^{3}H]STX (specific activity, 63 Ci/mmol; 1 Ci = 37 GBq), carrier-free $Na^{125}I$ and $^{22}NaCl$ were purchased from Amersham. Toxin II from the scorpion *Androctonus australis* Hector (α-ScTX) and toxin VI from *Centruroides suffusus suffusus* (β-ScTX) were generous gifts from M. F. Martin-Eauclaire (Laboratoire de Biochimie, Faculté de Médecine-Nord, Marseille, France). BTX was kindly provided by J. Daly (National Institutes of Health, Bethesda, MD). TTX and veratridine were purchased from Sigma; fetal calf serum and dibutyryl cAMP were from Boehringer Mannheim. Monensin was obtained from Calbiochem, amphotericin B was from GIBCO, and synthetic ω-conotoxin was from the Peptide Institute. Protein concentrations were estimated by the Lowry method (14) using bovine serum albumin as a standard.

Cell Culture. Primary cultures of rat brain neurons were prepared as described (10). A membrane fraction was prepared from neurons cultured for 10 days. Briefly, cells were harvested and homogenized in 0.32 M sucrose/5 mM Tris·HCl buffer, pH 7.4. Nuclei were eliminated by centrifugation for 10 min at 750 × *g* and membranes were then pelleted at 100,000 × *g* for 40 min. Astrocyte cultures were prepared essentially according to Sensenbrenner (15).

Toxin Binding and $^{22}Na^+$ Uptake Experiments. The binding of [^{3}H]STX was measured in a medium containing 140 mM choline chloride, 5.4 mM KCl, 0.8 mM $MgSO_4$, and 25 mM Tris Hepes (pH 7.4) (standard medium). Cells in multiwell plates were rinsed once with ice-cold standard medium and then incubated with 1 nM [^{3}H]STX for 2 hr at 4°C. Unspecific binding was determined in the presence of 0.3 μM TTX. Unbound [^{3}H]STX was removed by washing three times at 4°C with 1 ml of standard medium containing 0.1% bovine serum albumin (washing buffer). The cells were then dissolved in 0.1 M NaOH and the bound radioactivity was measured by scintillation assay. Since TTX competes with STX, the TTX-treated cells were washed to release bound TTX before conducting the [^{3}H]STX binding assay. The cultures were rinsed three times with 1 ml of washing buffer at 37°C for 5 min each, and once with standard binding buffer

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Abbreviations: TTX, tetrodotoxin; STX, saxitoxin; BTX, batrachotoxin; α-ScTX, scorpion α toxin; β-ScTX, scorpion β toxin.
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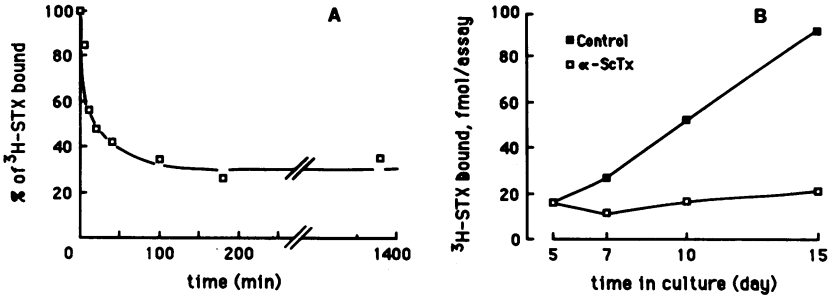

FIG. 1. Effect of α-ScTX treatment on the density of Na^+ channels in cultured neuronal cells. (*A*) Time course of α-ScTX-induced loss of surface Na^+ channels. At 10 days postplating, α-ScTX (0.1 μM) was added to the culture medium. At the indicated times, surface Na^+-channel density was measured by [^{3}H]STX binding as described. Results are expressed as a percentage of the [^{3}H]STX binding capacity in control cells grown in the absence of α-ScTX. (*B*) Effect of α-ScTX treatment on the time course of Na^+-channel appearance during neuronal maturation. Cultured neuronal cells, at 5 days postplating, were grown in the presence (□) or absence (■) of 0.1 μM α-ScTX. At the indicated times, surface Na^+ channels were assayed by [^{3}H]STX binding.

at 4°C. We have verified that, in these conditions, the dissociation of TTX was complete.

Toxin VI of *Centruroides suffusus suffusus* (β-ScTX) was iodinated as described (10). ^{125}I-labeled β-ScTX binding was measured in standard medium supplemented with 10 mM glucose. Typical experiments were conducted for 30 min at 37°C in the absence or the presence of an excess of β-ScTX (10).

Iodination and binding of apamin, ω-conotoxin, and rat insulin on developing neurons were performed as described (16–18).

$^{22}Na^+$ uptake was measured as described (11).

RESULTS

Effect of α-ScTX Treatment on the Number of Surface [^{3}H]STX Binding Sites. To investigate the effect of continuous channel activation on the density of surface Na^+ channels, developing neurons *in vitro* were incubated with α-ScTX, a channel activator that binds to pharmacological site 3 on the channel protein (6). Incubation was carried out at 37°C in the culture medium, which contains 140 mM Na^+—i.e., in conditions in which toxin addition induced Na^+ influx through the channel. At the end of the incubation period, surface Na^+ channels were assayed by using the specific channel ligand [^{3}H]STX, which binds to channel site 1 without any allosteric interaction with site 3 (6). The addition of 0.1 μM α-ScTX, a concentration that at 37°C leads to an almost immediate saturation of the binding sites, induced a decrease in the number of [^{3}H]STX binding sites (Fig. 1*A*). The time course of the effect was rapid with a half-maximum effect obtained 15 min after α-ScTX addition. However, the loss was not complete and a plateau was reached after 90 min. From seven experiments, a decrease of 54% ± 8% in [^{3}H]STX binding capacity was measured after 3 hr of incubation with α-ScTX.

Table 1. Effect of α-ScTX treatment on veratridine-sensitive $^{22}Na^+$ uptake and [^{3}H]STX binding

Cells	$^{22}Na^+$ uptake, nmol per min per assay	[^{3}H]STX bound, fmol per assay
Control	18.5 ± 0.5	63.4 ± 6
α-ScTX treated	7.8 ± 0.3 (42%)	20.3 ± 2 (32%)

Neurons were grown from day 7 to day 10 in the absence or presence of α-ScTX (0.1 μM). At the end of the incubation time (day 10), $^{22}Na^+$ uptake was measured in the presence (total uptake) or absence (nonspecific uptake) of 100 μM veratridine and 0.1 μM α-ScTX. The specific uptake was linear during the first 45 sec and is expressed as nmol of $^{22}Na^+$ per min per assay. [^{3}H]STX binding was carried out as described in *Materials and Methods*. Numbers in parentheses represent % of control.

To exclude a possible loss of STX binding sites due to a conformational change of the Na^+-channel protein upon α-ScTX treatment, we performed the following control experiments. First, [^{3}H]STX binding remained stable when a [^{3}H]STX binding experiment was carried out as described in *Materials and Methods* but in the presence of 0.1 μM α-ScTX. Second, when the preincubation with 0.1 μM α-ScTX was performed on a broken membrane preparation from cultured neurons, no decrease in [^{3}H]STX binding capacity was observed. The effect of α-ScTX therefore requires intact cultured neurons.

We next examined whether the α-ScTX effect was persistent—i.e., if the density in [^{3}H]STX binding sites remains stable at the low level obtained after a 90-min incubation with α-ScTX. As shown in Fig. 1*B*, the large increase in [^{3}H]STX binding sites that normally accompanied neurite outgrowth was completely abolished by α-ScTX treatment. In this experiment, α-ScTX was added at day 5 and the toxin concentration was maintained at 0.1 μM by successive toxin addition on days 8 and 12. No change was detected in the amount of total cellular protein upon α-ScTX treatment, and no significant morphological difference either in neurite surface area or in synapse density was observed by light and electron microscopy as described (10).

The Decrease in [^{3}H]STX Binding Capacity Is Related to Na^+-Channel Disappearance from the Cell Surface. We have used an additional specific ligand of Na^+ channels—i.e., ^{125}I-labeled β-ScTX, which binds to pharmacological site 4—to assay surface Na^+ channels (10). A 58% ± 5% decrease in its binding capacity was observed upon α-ScTX treatment, whereas no competition or allosteric interaction exists between the two toxin binding sites (7, 10). In addition, measurements of $^{22}Na^+$ uptake were performed to correlate a

Table 2. Effect of cations and TTX on the [^{3}H]STX binding loss induced by channel activator treatment

Cation	[^{3}H]STX binding capacity, % of control		
	α-ScTX (0.1 μM)	Veratridine (20 μM)	BTX (0.1 μM)
Na^+	42	39	37
Choline	95	98	99
Li^+	99	ND	ND
Na^+ + TTX (2 μM)	92	95	91

For each condition, test cations (140 mM) were substituted for Na^+ in the culture medium. Then, neurons at 10 days postplating were incubated for 3 hr in the presence or absence of channel activators. [^{3}H]STX-specific binding capacity was measured at the end of the incubation. The data are expressed as % of [^{3}H]STX binding capacity in control cells incubated in 140 mM Na^+ and in the absence of channel activator (α-ScTX, veratridine, or BTX). ND, not determined.

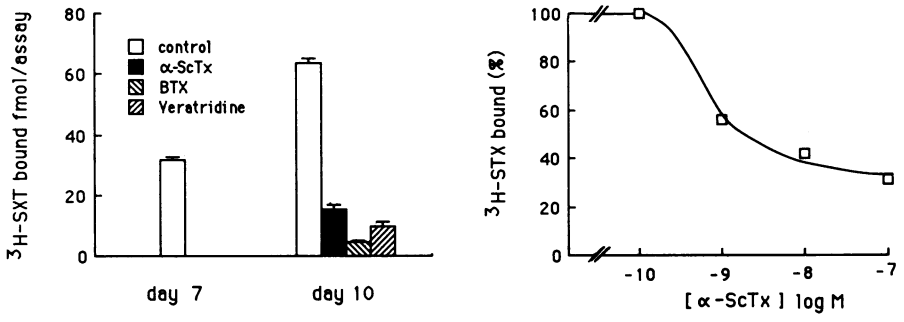

FIG. 2. Loss of surface [^{3}H]STX binding sites induced by Na^+-channel activators in cultured neuronal cells. (*A*) α-ScTX (0.1 μM), BTX (1 μM), and veratridine (20 μM) were added to the culture medium at day 7 and [^{3}H]STX-specific binding sites were assayed before (day 7) and 3 days after (day 10) toxin addition. (*B*) Dose–response curve of the effect of α-ScTX. From day 7, cultured neurons were incubated with the indicated concentrations of α-ScTX for 3 days. At the end of the incubation time (day 10), [^{3}H]STX-specific binding capacity was measured. Results are expressed as a percentage of the [^{3}H]STX binding capacity in control cells grown in the absence of toxin.

decrease in binding capacity with a loss of channel activity. Neurons cultured from day 7 to day 10 in the presence of α-ScTX, showed a 58% decrease in veratridine-sensitive $^{22}Na^+$ uptake, which was associated with a 68% decrease in [^{3}H]STX binding capacity (Table 1). Thus, a loss in Na^+-channel activity was found to be associated with the decrease in [^{3}H]STX and ^{125}I-labeled β-ScTX binding capacities. All together these observations indicate that α-ScTX treatment induced a disappearance of surface Na^+ channels.

The Disappearance of Na^+ Channels Induced by α-ScTX Treatment Is Mimicked by BTX and Veratridine and Inhibited by TTX. As shown in Table 2 and in Fig. 2*A*, a similar effect to that of α-ScTX was induced by BTX and veratridine, which upon binding to pharmacological site 2 induce persistent activation of sodium permeability (6). BTX was found to be more potent than veratridine and α-ScTX, in good agreement with their respective potency in activating voltage-sensitive Na^+ channels (6). The α-ScTX effect was found to be dose dependent (Fig. 2*B*) and the concentration giving the half-maximum inhibition (0.7 nM) is in good agreement with the dissociation constant (0.2 nM) calculated from binding experiments (10). Finally, the time course effect of veratridine was characterized by a $t_{1/2}$ of 15 min as found for α-ScTX (data not shown).

Tetrodotoxin (TTX) is a specific Na^+-channel blocker (6). When cultured neuronal cells were exposed to a mixture of α-ScTX and TTX or to TTX alone from day 7 to day 10, no decrease in the number of [^{3}H]STX binding sites was observed (Fig. 3*A*). In addition, a decrease in channel number induced by preincubation with α-ScTX was reversed by addition of TTX (Fig. 3*B*).

The Induced Decrease in Surface Na^+ Channels Is Related to Na^+ Influx. Although BTX, veratridine, and α-ScTX do not have the same pharmacological action, they cause an increase in Na^+ influx. Therefore, one could assume that it is the increase in Na^+ inward current resulting from channel activator binding that triggers Na^+-channel disappearance. To test this possibility further, either choline, a nonpermeant ion, or Li^+, which passes through the Na^+ channel (19), was substituted for Na^+ in the culture medium. In these conditions, cells were exposed to channel activator for 3 hr, an incubation time that allows a maximal response without any toxic effects due to the absence of Na^+. As shown in Table 2, the channel activator-induced decrease in [^{3}H]STX binding sites was prevented in both conditions as it was by TTX. Furthermore, we determined the dose-dependence relationship between the disappearance of Na^+ channels and the external Na^+ concentration. As shown in Fig. 4*A*, a linear relationship exists between the effect of α-ScTX treatment and the external Na^+ concentration. All together, these results indicate that the disappearance of Na^+ channels induced by channel activators is dependent on Na^+ influx.

This was confirmed by using Na^+ ionophores such as amphotericin B and monensin. The addition of amphotericin B (5 μg/ml) induced a decrease in [^{3}H]STX binding sites (Fig. 4*B*). The time course of the effect was slower than with α-ScTX, leading to a 55% inhibition of [^{3}H]STX binding after 2 hr of amphotericin B treatment. Monensin (1 μM) was also found to induce a decrease (14%) in [^{3}H]STX binding capacity after 1 hr of incubation.

Finally, membrane depolarization induced by increasing the external KCl concentration (50 mM) did not change the number of [^{3}H]STX binding sites (data not shown).

The Decrease in Surface Na^+ Channels Is Specific to Neuronal Na^+ Channels. It is well established that astrocytes carry a variety of ion-channel types—in particular Na^+ channels, the electrophysiological and biochemical properties of which are slightly different from those of neuronal

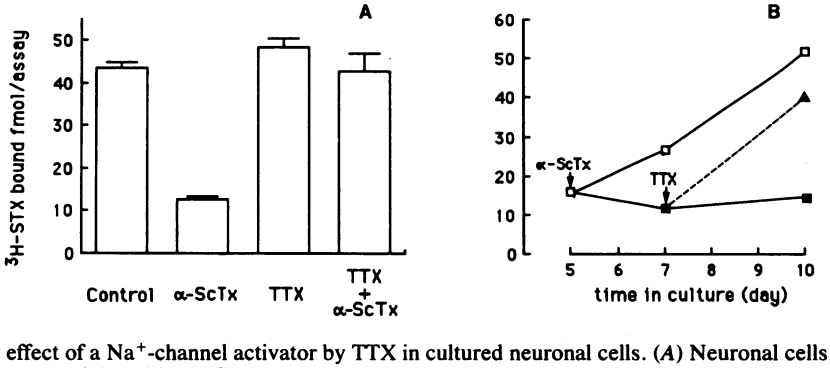

FIG. 3. Inhibition of the effect of a Na^+-channel activator by TTX in cultured neuronal cells. (*A*) Neuronal cells were maintained from day 7 to day 10 in culture medium containing either α-ScTX (0.1 μM) or TTX (0.4 μM) or TTX (0.3 μM) and α-ScTX (0.1 μM). At the end of the incubation time, [^{3}H]STX-specific binding was measured. (*B*) From day 5, neurons were grown in the presence (■, ▲) or absence (□) of 0.1 μM α-ScTX. At day 7, TTX (0.4 μM) was added to the growth medium of treated cells as indicated (dashed line; ▲). At the indicated days, [^{3}H]STX-specific binding was measured.

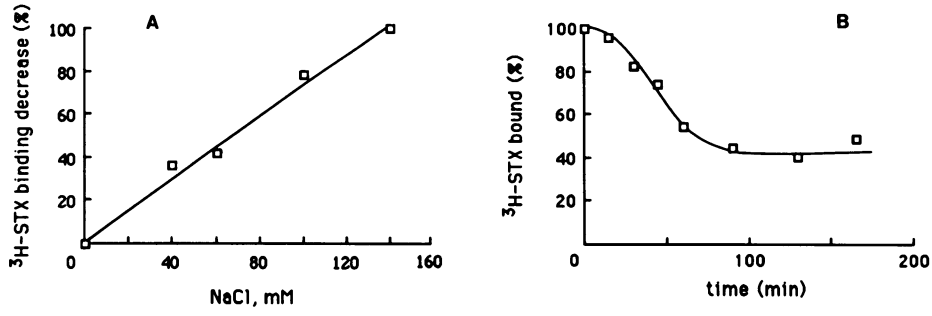

FIG. 4. Role of Na^+ influx in the disappearance of surface Na^+ channels in cultured neuronal cells. (*A*) Effect of external Na^+ concentration on the disappearance of Na^+ channels induced by α-ScTX treatment. At 10 days postplating, the standard culture medium was replaced by a medium containing different Na^+ concentrations ranging from 0 mM to 140 mM and complemented with choline to isotonicity. Then, α-ScTX (0.1 μM) was added for 2 hr. At the end of the incubation time, surface Na^+ channels were assayed by [^{3}H]STX binding. Results are expressed as a percentage of the α-ScTX effect obtained in standard medium (Na^+, 140 mM). (*B*) Effect of amphotericin B on the density of Na^+ channels in cultured neuronal cells. At 10 days postplating, amphotericin B (5 μg/ml) was added to the culture medium. At the indicated times, surface Na^+-channel density was measured by [^{3}H]STX binding. Results are expressed as a percentage of the [^{3}H]STX binding capacity in control cells grown in the absence of amphotericin B.

channels (20, 21). To determine whether the disappearance of Na^+ channels induced by channel activators is specific to neurons, we examined the effect of channel activators on the number of astrocyte Na^+ channels. As illustrated in Table 3, no change in [^{3}H]STX binding capacity was observed when astrocytes were exposed to a mixture of veratridine (50 μM) and α-ScTX (0.1 μM), to TTX alone (1 μM), or to a mixture of veratridine, α-ScTX, and TTX (50, 0.1, and 1 μM, respectively). These concentration ranges have been shown to activate and to block voltage-sensitive Na^+ current in astrocytes (20). In addition, the presence of either monensin or amphotericin B failed to decrease the [^{3}H]STX binding capacity (Table 3).

We have also examined in developing neurons whether the density of other ion channel-associated receptors was affected by α-ScTX treatment. A Ca^{2+}-activated K^+ channel and a voltage-sensitive Ca^{2+} channel have been assayed by using specific iodinated ligands, ^{125}I-labeled apamin and ^{125}I-labeled ω-conotoxin, respectively (16, 17). Similarly ^{125}I-labeled insulin receptors, which are not directly involved in producing electrogenic ion flux, were assayed as an additional control (18). In all cases, no change in the surface density of these receptors was detected after Na^+-channel activation (data not shown).

DISCUSSION

Our results show that a rapid partial disappearance of surface Na^+ channels occurs in brain neurons developing *in vitro*, which is triggered by Na^+ influx into the cell. The effect of channel activators was found to be dependent on the external Na^+ concentration and was prevented when choline or Li^+ was substituted for Na^+. Moreover, it was mimicked by a Na^+ ionophore, amphotericin B. Although it seems clear that the first event is an increase in Na^+ influx, two major questions remain: (*i*) What is the molecular mechanism responsible for the decrease in Na^+ channels at the surface of neurons? (*ii*) What are the transducing events that associate Na^+ entry and the channel disappearance?

Since the observed phenomenon is rapid ($t_{1/2}$, 15 min), it could be postulated that the disappearance of Na^+ channels results from an internalization process rather than a blockade of channel synthesis. This is supported by the finding that the $t_{1/2}$ for Na^+-channel synthesis is 50 hr in developing neurons (13). However, we cannot exclude another molecular mechanism: for instance, one can postulate that a dissociation of α and β_1 subunits occurs upon channel activation and induces the disappearance of STX and β-ScTX binding sites, since it has been shown that high-affinity STX binding requires the association of α and β_1 subunits (22). However, following this hypothesis, the reversal of the phenomenon should have been complete after TTX addition, which was not the case (Fig. 3*B*).

With regard to the transducing mechanism, it has been shown by Gusovsky *et al.* (23) with a brain synaptosomal preparation that channel activators induce phosphatidylinositol diphosphate breakdown. Therefore, it is tempting to speculate that this might occur also in developing neurons treated with channel activators. Stimulation of phosphatidylinositol diphosphate breakdown is expected to increase the level of second messengers such as inositol trisphosphate, intracellular calcium, and diacylglycerol and subsequently to activate the kinase system. Since brain Na^+ channel is a good substrate for cAMP-dependent kinase and protein kinase C (24, 25), an increase in intracellular Na^+ concentration might contribute, via phosphatidylinositol diphosphate breakdown, to a change in the degree of phosphorylation of the Na^+ channel and/or of a protein interacting with the channel and modulate channel density. Although a modulation of Na^+ channels by protein kinase C has not yet been reported in neurons, recent electrophysiological studies do support this hypothesis. Na^+ current measured in *Xenopus* oocytes injected with brain mRNA has been shown to be inhibited by a protein kinase C activator (26). However, since it has been recently shown that Na^+ channels expressed in *Xenopus* oocytes are inhibited by a nonhydrolyzable GTP analog (27), the hypothesis that neuronal Na^+ channels might be also modulated by guanine nucleotide-binding regulatory proteins cannot be ruled out.

Table 3. Lack of effect of Na^+-channel activators and Na^+ ionophores on [^{3}H]STX binding in cultured astrocytes

Culture condition	[^{3}H]STX bound, % of control
Veratridine (50 μM) + α-ScTX (0.1 μM)	98 ± 17
TTX (1 μM)	99 ± 22
Veratridine (50 μM) + α-ScTX (0.1 μM) + TTX (1 μM)	87 ± 14
Monensin (1 μM)	98 ± 4
Amphotericin B (5 μg/ml)	98 ± 7

Astrocytes were cultivated for 24 hr in the presence of the indicated concentrations of Na^+-channel activators and [^{3}H]STX-specific binding was measured at the end of the incubation time as described in *Materials and Methods*. For monensin and amphotericin B, the incubation time was only 3 hr to prevent any toxic effect.

Another interesting aspect is that the observed phenomenon is specific to neurons since it has not been observed in astrocytes or in skeletal muscle cells. Although a decrease in surface Na^+ channels has been detected in chicken muscle upon exposure to BTX (28), this latter phenomenon is presumably different from the one we have described in neurons. In chicken muscle, regulation was slower ($t_{1/2}$, 3–6 hr) and did not appear to be directly related to an increase in Na^+ influx. In rat skeletal muscle cells, the level of mRNA encoding the α subunit of TTX-sensitive Na^+ channel is regulated by the electrical activity, cAMP, and cytosolic calcium (29). Subsequently, blockade of spontaneous electrical activity by TTX or KCl depolarization induced an increase in the number of surface Na^+ channels, whereas a decrease was observed upon addition of the calcium ionophore A 23187 (30). Several lines of evidence indicate that the process reported here is different from that observed in rat muscle. (*i*) Neither KCl depolarization, which causes an increase in the cytosolic calcium concentration, nor an activation of adenylate cyclase by forskolin (data not shown) modulates the number of neuronal Na^+ channels. (*ii*) In rat skeletal muscle cells as in chicken muscle, regulation is slow.

On the basis of electrophysiological studies, it has been recently shown that Na^+ channels detected in type 1 astrocytes appeared to differ slightly from neuronal channels (21), showing slower kinetics and more-negative voltage dependence. Our observations indicate that they differ also by the fact that their presence at the cell surface is not modulated by channel-activator treatment.

We have shown that the disappearance of Na^+ channel was never complete even when channels were fully activated by BTX. This observation may indicate that two populations of Na^+ channels are present in these cultured neurons, one being regulated and the other not. Since at least three channel subtypes are expressed in adult brain (3, 4), it is tempting to postulate that the two populations correspond to two of these subtypes. Recent data on the temporal expression pattern in rat central nervous system (31) suggests that subtypes II and III are good candidates since their level of expression is high in the fetal rat brain, whereas subtype I shows a later increase (31, 32). Another possibility is that the two populations correspond to only one channel subtype, which is or is not associated with other membrane proteins or with the cytoskeleton (33, 34).

Is this regulation specific for a particular stage of neuronal development or does it also occur in mature neurons *in vivo*? In the latter case, this modulation could allow neurons to maintain a certain density of Na^+ channels and to adjust this density in response to external stimuli. An alteration of this regulatory mechanism, resulting for instance in an increase in Na^+-channel density, might be involved in generating states of abnormal neuronal hyperexcitability.

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