Multiple intracellular regulatory mechanisms of cell surface expression of sodium channels: therapeutic implications.

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Regulated expression of cell surface voltage-dependent Na^+ channels ensures development and differentiation of excitable cells; dysregulated expression of normal Na^+ channel isoforms or otherwise silent Na^+ channel isoforms causes the Na^+ channelopathies. In bovine adrenal chromaffin cells, (1) constitutive phosphorylation of extracellular signal-regulated kinase destabilized Na^+ channel □-subunit mRNA, thus negatively regulating steady-state level of Na^+ channels. (2) Protein kinase C (PKC)-□ down-regulated Na^+ channels via destabilization of □-subunit mRNA. (3) Cytoplasmic Ca^{2+} activated PKC-□ and calpain, and down-regulated Na^+ channels by promoting internalization of Na^+ channels. (4) Protein kinase A or insulin receptor tyrosine kinase up-regulated Na^+ channels via translational events. (5) Neuroprotective, antiepiletic, antipsychotic, and local anesthetic drugs up-regulated Na^+ channels via translational events, without or with elevating □- subunit mRNA levels. Thus, constitutive and inducible cellular signals, as well as therapeutic drugs up- and down-regulate cell surface Na^+ channel expression largely via regulating mRNA stability and intracellular trafficking of Na^+ channels.

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Regulated up- and down-regulations of voltage-dependent Na^+ channels play pivotal roles in the short- and long-term regulation of various cellular events (e.g. neuronal development, synaptogenesis and neuronal plasticity)[1]. Dysregulated up- and down-regulations of Na^+ channels are responsible for hypoxia/ischemia-induced neuronal injury[2], seizure, intolerable pain, and neurodegenerative diseases[3]. Although the molecular structures and biophysical properties of Na^+ channels have been well characterized, much remains unknown about the extracellular signals and intracellular mechanisms that regulate cell surface expression of Na^+ channels in these physiological and pathological states. In addition, Na^+ channels are molecular target for a growing number of therapeutic drugs (e.g. neuroprotective, antiepileptic, antipsychotic, and local anesthetic drugs). However, little is known whether therapeutic drugs could affect regulated expression of cell surface Na^+ channels. In this paper, we summarize our previous studies that multiple intracellular signals and therapeutic drugs cause up- and down-regulations of functional Na^+ channels via regulating mRNA stability and intracellular trafficking of Na^+ channels[4-17].

RESULTS AND DISCUSSION

Regulation of steady-state level of Na^+ channels: constitutive destabilization of Na^+ channel □-subunit mRNA by ERK. Serum deprivation caused time-dependent ($t_{1/2}$=12 h) increase (~52%) in cell surface [^{3}H]saxitoxin (STX) binding with no change in the Kd value; the increase was reversed after the addition of serum. Immunoblot analysis of mitogen-activated protein kinase (MAPK) family revealed that extracellular signal-regulated kinase (ERK), p38 MAPK (p38), c-Jun N-terminal protein kinase (JNK) were constitutively phosphorylated in quiescent chromaffin cells; however, serum deprivation rapidly (<15 min) lowered (~50%) the phosphorylation of ERK1 and ERK2 (but not p38, JNK1 and JNK2) without changing protein levels of MAPK molecules[4]. Treatment with PD98059 or U0126, a selective inhibitor of ERK kinase, lowered the phosphorylation of ERK1 and ERK2 (but not p38, JNK1 and JNK2), and increased cell surface [^{3}H]STX binding; the reduced extent of the former was inversely related to the increased extent of the latter. Cotreatment of serum deprivation with PD98059 or U0126 did not

further increase [^{3}H]STX binding, compared with either treatment alone. Serum deprivation, PD98059 or U0126 increased (~50%) Na^+ channel α- (but not α_1-) subunit mRNA level between 3 and 24 h; the half-life of α-subunit mRNA was prolonged from 17.5 to ~26 h, with no change in the α-subunit gene transcription[4]. Cycloheximide, an inhibitor of protein synthesis, increased α-subunit mRNA level, and nullified additional increasing effect of either treatment on α-subunit mRNA level.

Thus, constitutively phosphorylated/activated ERK destabilizes Na^+ channel α-subunit mRNA via translational event(s), which negatively regulates steady-state level of α-subunit mRNA and cell surface expression of functional Na^+ channels. Down-regulation of Na^+ channels by PKC: PKC-α-induced destabilization of α-subunit mRNA, and PKC-ε-induced internalization of Na^+ channels

Down-regulation of Na^+ channels by PKC: PKC$_\alpha$-induced destabilization of α-subunit mRNA, and PKC$_\varepsilon$-induced internalization of Na^+ channels. In adrenal chromaffin cells expressing only conventional protein kinase C (PKC)-α, novel PKC-ε, and atypical PKC-ζ [5], treatment with 1-1000 nM 12-O-tetradecanoylphorbol 13-acetate (TPA) caused a rapid (<15 min) and sustained (>15 h) translocation of PKC-α and -ε (but not -ζ) from cytoplasm to membranes; in contrast, 100 nM thymeleatoxin (TMX) caused the similar, but selective membrane translocation of only PKC-ε [5].

TPA reduced [^{3}H]STX binding to a greater extent than did TMX [5,6]; TMX (100 nM)-induced reduction of [^{3}H]STX binding reached the maximum fall of 38% at 12 h, whereas TPA (100 nM)-induced reduction of [^{3}H]STX binding further developed into the almost maximum fall of 53% at 18 h. Gö6976, an inhibitor of PKC-α (but not -ζ), completely reversed the TMX-induced event; in contrast, TPA-induced event was partially prevented by Gö6976, but abrogated by H7, an inhibitor of PKC family.

Northern blot analysis revealed that 100 nM TPA (but not 100 nM TMX) lowered α-subunit mRNA level by 52% at 12 h in a monophasic manner, while increased α_1-subunit mRNA level in a time-dependent

manner. H7 (but not Gö6976) completely prevented TPA-induced reduction of □-subunit mRNA level [5,7]. TPA shortened the half-life of □-subunit mRNA from 18.8 to 3.7 h, without changing □-subunit gene transcription.

Table 1. Multiple mechanisms of Na^+ channel up- and down-regulations

Intracellular signals & Therapeutic drugs	[^{3}H]STX Binding	Subunit mRNA □	Subunit mRNA □$_i$	Mechanism	Ref.
Down-regulation					
ERK	↓	↓	→	□-Subunit mRNA stability ↓	4
PKC-□	↓	↓	↑	□-Subunit mRNA stability ↓	5,6,7
PKC-□	↓	→	→	Internalization ↑	5,7
$[Ca^{2+}]_i$ ↑	→				8
$[Ca^{2+}]_i$ ↑ ↑	↓	→	→	Internalization ↑	8
$[Ca^{2+}]_i$ ↑ ↑ ↑	↓ ↓	↓	↓	Internalization ↑	
				& □-, □$_i$-Subunit mRNA ↓	8
Calcineurin	↓	→	→	Internalization ↑	
				& Externalization ↓	9
Up-regulation					
PKA	↑	→	→	Intracellular trafficking	10
Insulin	↑	→	→	Intracellular trafficking	11
Therapeutic drugs					
Valploic acid	↑	↑	↑	□-, □$_i$□Subunit mRNA ↑	12
Riluzole	→				13
NS-7	↑	→	→	Intracellular trafficking	14
Carvedilol	↑	→	→	Intracellular trafficking	15
Bupivacaine	↑	→	→	Intracellular trafficking	16
Ropivacaine	↑				16
Lidocaine	→				16

Thus, TPA-induced, PKC-□-mediated, destabilization of □-subunit mRNA contributes to the elongation of Na^+ channel down-regulation, compared with TMX-induced, PKC-□-mediated, Na^+ channel down-regulation. Treatment (24 h) with cycloheximide progressively in-

creased α-subunit mRNA level, while continuously decreasing β_1-subunit mRNA level; in addition, cycloheximide completely reversed TPA-induced decrease of α-subunit mRNA level and increase of β_1-subunit mRNA level.

Because the reduction of [^{3}H]STX binding caused by TMX or TPA was rapid (<3 h) in onset, we measured internalization rate of cell surface Na^+ channels by using brefeldin A (BFA), an inhibitor of guanine nucleotide exchange protein of ADP-ribosylation factor 1, a monomeric GTPase. Concurrent treatment of BFA with TPA or TMX decreased [^{3}H]STX binding to a greater extent, compared with either treatment alone. Thus, PKC-α promotes endocytic internalization rate of cell surface Na^+ channels, causing down-regulation of Na^+ channels[5].

Cooperative down-regulation of Na^+ channels by Ca^{2+}-activated signals. Treatment with 1 μM A23187, a Ca^{2+}-ionophore, produced a rapid monophasic rise of cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$), followed by the salient plateau increase lasting for 96 h[8]. Thapsigargin (TG) and DBHQ are two inhibitors of sarco(endo)plasmic Ca^{2+}-ATPase, but not of the plasma membrane Ca^{2+}-ATPase. TG (100 nM) produced a slowly-developing monophasic rise of $[Ca^{2+}]_i$, followed by the persistent (>48 h) plateau increase; in contrast, DBHQ (100 nM) produced only a rapid monophasic rise of $[Ca^{2+}]_i$. A23187 or TG (but not DBHQ) decreased [^{3}H]STX binding, with no change in the Kd value[8]. A23187 produced long-lasting (>96 h) time-dependent gradual decrease of [^{3}H]STX binding by 66% at 96 h, whereas TG-induced reduction of [^{3}H]STX binding leveled off by 35% at 48 h. A23187- or TG-induced event required long-lasting continuous increase of $[Ca^{2+}]_i$, because addition of BAPTA-AM, a cell membrane-permeable Ca^{2+} chelator, at 24 h abolished the subsequent decreasing effect of A23187 or TG at 48 h. The decreasing effect of A23187 or TG was partially prevented by calpastatin, an inhibitor of calpain, or Gö6976, and due to the increased internalization of Na^+ channels, as evidenced by using BFA, suggesting that PKC-α and calpain are involved in the Ca^{2+}-induced acceleration of Na^+ channel internalization. In addition, A23187 (but not TG) lowered Na^+ channel α- and β_1-subunit mRNA levels by ~50% between 3 and 48 h[8].

Thus, heterogeneous increases of $[Ca^{2+}]_i$ caused down-regulation of Na^+ channels via multiple mechanisms, depending on the amplitude and duration of $[Ca^{2+}]_i$ rise. Because down-regulation of Na^+ channels has been assumed to prevent hypoxia-induced neuronal injury [2], $[Ca^{2+}]_i$-induced down-regulation of Na^+ channels may be a defensive event against Ca^{2+} overload.

Up-regulation of Na^+ channels by activation of protein kinase A and insulin receptor tyrosine kinase. Chronic treatment with 1 mM dbcAMP (>12 h) or 100 nM insulin (>24 h) increased [^{3}H]STX binding by ~50%, without altering the Kd value [10,11]. The effect of dbcAMP or insulin was blocked by actinomycin D or cycloheximide, but was not associated with increased levels of Na^+ channel α- and β_1-subunit mRNAs. Thus, activation of PKA or insulin receptor tyrosine kinase up-regulates cell surface Na^+ channels via the de novo synthesis of as yet unidentified protein(s), which may be involved in the regulation of intracellular trafficking of Na^+ channels [5].

Up-regulation of Na^+ channels by antiepileptic, antipsychotic, neuroprotective, and local anesthetic drugs. Chronic treatment (>2 d) with therapeutic concentration of valproic acid, an antiepileptic drug, increased Na^+ channel α- and β_1-subunit mRNA levels by 74 and 83%, respectively, and caused up-regulation (~40%) of [^{3}H]STX binding, with no change in the Kd value [12]; it resulted in the enhancement of veratridine-induced $^{22}Na^+$ influx via Na^+ channels, thus augmenting veratridine-induced $^{45}Ca^{2+}$ influx via voltage-dependent Ca^{2+} channels and catecholamine secretion.

Acute treatment with neuroprotective drug, riluzole [13], NS-7 [14], or carvedilol [15] inhibited veratridine-induced $^{22}Na^+$ influx, $^{45}Ca^{2+}$ influx, and catecholamine secretion. In contrast, chronic treatment (>12 h) with NS-7 or carvedilol (but not riluzole) increased [^{3}H]STX binding by ~86%, with no change in the Kd value. Bupivacaine enantiomers and ropivacaine, a propyl homolog of bupivacaine, are amide-type local anesthetics structurally similar to lidocaine, except that their amine-containing group is a piperidine, instead of a tertiary amine in lidocaine. Acute treatment with either local anesthetic inhibited veratridine-induced $^{22}Na^+$ influx with comparable potency; however, chronic treatment (>3 h) with bupivacaine or ropivacaine (but not

lidocaine) increased (~48%) [^{3}H]STX binding. Up-regulation of cell surface Na^+ channels caused by these Na^+ channel blockers was presumably due to the promotion of cell surface externalization of newly-synthesized protein(s) from the trans-Golgi network, because the increasing effect of either drug was prevented by cycloheximide or BFA, and was not associated with the increased levels of □- and □$_1$-subunit mRNAs.

CONCLUSION

Two major regulatory mechanisms of cell surface expression of Na^+ channels can be drawn from our study: the modulation of (1) □-subunit mRNA stability, and (2) intracellular trafficking, especially modulation of endocytic internalization rate of cell surface Na^+ channels (Table 1). Steady-state level of cell surface Na^+ channels is negatively regulated by constitutive activity of ERK; down-regulation of Na^+ channels by cooperative activation of PKC and Ca^{2+}-activated signals is significant in extent and rapid in onset, and thus may contribute to the neuroprotection[17]. Insulin-induced up-regulation of Na^+ channels may be informative for the down-regulation of Na^+ channels at the node of Ranvier in myelinated axons of insulin-deficient diabetic neuropathy[17]. Up- and down-regulation of cell surface Na^+ channels in our present study may provide a new avenue to understand the crucial roles of Na^+ channel modulation in the physiological and pathological states.

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