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REGULATION OF CELL SURFACE EXPRESSION OF VOLTAGE-DEPENDENT $Na_v1.7$ SODIUM CHANNELS: mRNA STABILITY AND POSTTRANSCRIPTIONAL CONTROL IN ADRENAL CHROMAFFIN CELLS

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1. ABSTRACT

Regulated expression of Na^+ channels is indispensable to physiological events, whereas dysregulated expression of otherwise silent or even normal Na^+ channel isoforms causes Na^+ channelopathies; however, the regulatory mechanisms remain unknown. In quiescent cultured bovine adrenal chromaffin cells, constitutive phosphorylation/activation of extracellular signal-regulated kinase-1 (ERK1) and ERK2 destabilized $Na_v1.7$ Na^+ channel alpha-subunit mRNA and decreased its level without altering alpha-subunit gene transcription, thus negatively regulating steady-state level of Na^+ channels. Activation of protein kinase C (PKC) down-regulated Na^+ channels via PKC isoform-specific mechanisms; conventional PKC-alpha promoted endocytic internalization of Na^+ channels, whereas novel PKC-epsilon destabilized alpha-subunit mRNA without altering its gene transcription. Long-lasting (but not short-term) increase of cytoplasmic Ca^{2+} down-regulated Na^+ channels; a slowly-developing moderate increase of Ca^{2+} activated PKC-alpha and calpain, promoting internalization of Na^+ channels, whereas an immediate monophasic and salient plateau increase of Ca^{2+} lowered alpha- and $beta_1$-subunit mRNA levels. Calcineurin, or FK506 binding protein- and rapamycin-associated protein (FRAP), a serine/threonine protein kinase, down-regulated, whereas insulin receptor tyrosine kinase or protein kinase A (PKA) up-regulated, Na^+ channels via modulating Na^+ channel internalization, and/or Na^+ channel externalization from the *trans*-Golgi network. Neuroprotective, antiepiletic, antipsychotic, and local anesthetic drugs up-regulated Na^+ channels via transcriptional/translational events.

2. INTRODUCTION

Density of cell surface voltage-dependent Na^+ channels, a major determinant for the activity of Na^+ channels, accommodates genotypic and phenotypic responses of cells to direct cell fate (1), thus being finely regulated via as yet unknown mechanisms to meet development, differentiation, and survival of cells (2). Dysregulated up- and down-regulations of Na^+ channels are responsible for abnormal excitability of cells in hypoxia/ischemia-induced neuronal injury (reviewed in 3), seizure (4, 5), intolerable pain, and neurodegenerative diseases (e.g. multiple sclerosis) (reviewed in 6). In peripheral nerve injury, aberrant properties of Na^+ currents in dorsal root ganglion neurons are attributed not only to the pathological down-regulation of normal Na^+ channel isoform proteins, but also to the dysregulated de-repression of otherwise silent Na^+ channel isoform genes (reviewed in 6). Much remains, however, 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.

Na^+ channels consist of the principal alpha-subunit (~ 260 kDa), which may be associated with a noncovalently-attached $beta_1$-subunit (~ 36 kDa), and a disulfide-linked $beta_2$-subunit (~ 33 kDa) in some tissues and species (reviewed in 7-9). The alpha-subunit is

composed of four homologous domains (I-IV), each containing six transmembrane segments (Sl-S6), and forms the ion-pore and the toxin binding sites [e.g. site 1 for tetrodotoxin (TTX)/saxitoxin (STX), site 2 for veratridine, site 3 for alpha-scorpion toxin, site 4 for beta-scorpion toxin, and site 5 for *Ptychodiscus brevis* toxin-3 (PbTx-3)] (reviewed in 10). The alpha- and beta-subunits arise from, at least, nine and three different genes/their alternative splicing, respectively (reviewed in 5, 7-9). Either beta-subunit is type 1 transmembrane protein containing a single membrane-spanning segment, and a single immunoglobulin V-set fold structure in its extracellular domain (reviewed in 5). The beta-subunit regulates gating and cell surface expression of Na^+ channels, like auxiliary subunits of other classes of voltage- and ligand-gated ion channels (reviewed in 5, 8). Most distinctively, however, the extracellular V-set fold structure of Na^+ channel beta-subunit is homologous to that of immunoglobulin superfamily of cell adhesion molecules, thus enabling Na^+ channel beta-subunit to interact with cell adhesion molecules (e.g. neurofascin) and extracellular matrix proteins (e.g. tenascin), as well as intracellular scaffold proteins (e.g. ankyrin) (reviewed in 5, 8). The $beta_1$ and $beta_3$ are more homologous structurally to each other, compared with the $beta_2$; also, $beta_1$ and $beta_3$ are widely expressed in central and peripheral tissues with a complementary abundance in several brain regions, whereas $beta_2$ is expressed only in brain.

In adrenal chromaffin cells (embryologically derived from the neural crest), alpha-subunit of Na^+ channels is the TTX/STX-sensitive human neuroendocrine type Na^+ channel alpha-subunit (hNE-Na) (reviewed in 8). hNE-Na is the human homolog (~ 93% identity of amino acid sequence) of rat peripheral nerve type 1 Na^+ channel alpha-subunit (11), and of rabbit Schwann cell Na^+ channel alpha-subunit (12); they belong to the same alpha-subunit subfamily termed $Na_v1.7$, which is encoded by the gene SCN9A (reviewed in 8). In adrenal chromaffin cells, veratridine-induced Na^+ influx via Na^+ channels is the prerequisite for veratridine-induced Ca^{2+} influx via voltage-dependent Ca^{2+} channels, thus triggering Ca^{2+}-dependent exocytic secretion (13, 14) and synthesis (15, 16) of catecholamines. Here, we summarize our previous studies that phosphorylation/dephosphorylation events, and therapeutic drugs cause up- and down-regulations of functional $Na_v1.7$ Na^+ channels via regulating mRNA stability and intracellular trafficking of Na^+ channels (17-30).

3. MATERIALS AND METHODS

3.1. Materials

Eagle's minimum essential medium was from Nissui Seiyaku (Tokyo, Japan). Calf serum was from Nacalai Tesque (Kyoto, Japan). Actinomycin D, brefeldin A, cycloheximide, cyclosporin A, cytosine arabinoside, 12-O-tetradecanoylphorbol 13-acetate, TTX, veratridine, alpha-scorpion venom (*Leiurus quinquestriatus quinquestriatus*), beta-scorpion venom (*Centruroides sculpturatus*), thapsigargin, BAPTA-AM, and EDTA were from Sigma-Aldrich (St. Louis, MO). Calpastatin peptide, Gö6976, H7, thymeleatoxin, PD98059, and rapamycin were from Calbiochem-Novabiochem (San Diego, CA). 2,5-di-(t-butyl)-1,4,-benzohydroquinone (DBHQ) was from BIOMOL (Plymouth Meeting, PA). A23187, PbTx-3, and insulin were from Wako (Tokyo), Latoxan (Westbury, NY), and Eli Lilly (Kobe, Japan), respectively. Fura-2/AM was from Dojindo (Kumamoto, Japan). FK506 and 3-(3-pyridyl)-1-propyl (2S)-1-(3, 3-dimethyl-l, 2-dioxopentyl)-2-pyrrolidinecarboxylate (GPI-1046) were kindly donated from Fujisawa Pharmaceutical (Osaka, Japan) and Amgen (Thousand Oaks, CA), respectively. Rabbit polyclonal antibodies against either ERK, p38 mitogen-activated protein kinase, or *c-Jun* N-terminal kinase, and mouse monoclonal anti-phosphotyrosine ERK antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-phospho-tyrosine/serine/threonine antibodies against p38 mitogen-activated protein kinase or *c-Jun* N-terminal kinase, U0126, RQ1 RNase-free DNase, proteinase K, and serine/threonine phosphatase assay system were from Promega (Madison, WI). TRIzol reagent was from Invitrogen (Carlsbad, CA). BcaBEST labeling kit and Noninterfering protein assay kit were from Takara (Kyoto). [^{3}H]STX (20-40 Ci/mmol), ^{125}I-labeled donkey anti-rabbit IgG, ^{125}I-labeled sheep anti-mouse IgG, [alpha-^{32}P]dCTP (>3000 Ci/mmol), and [alpha-^{32}P]UTP (800 Ci/mmol) were from PerkinElmer Life Sciences (Boston, MA). The Rapid-hyb buffer was from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). cDNA for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from BD Biosciences Clontech (Palo Alto, CA). Plasmid Bluescript II (pBII) was from Stratagene (La Jolla, CA). Plasmids containing hNE-Na cDNA, and rat brain Na^+ channel $beta_1$-subunit cDNA were generously donated by Dr. F. Hofmann (Technischen Universität München), and Dr. Y. Oh (University of Alabama), respectively.

3.2. Primary culture of adrenal chromaffin cells and test treatment

Isolated bovine adrenal chromaffin cells were cultured (4 x 10^6/dish, Falcon; 35 mm in diameter) under 5% CO_2/95% air in a CO_2 incubator in Eagle's minimum essential medium containing 10% calf serum and 3 μM cytosine arabinoside to suppress the proliferation of nonchromaffin cells. When chromaffin cells were further purified by differential plating, experimental results were comparable between conventional and purified chromaffin cells (17, 20, 23-30). Three-day-old cells were exposed to normal fresh medium or serum-free fresh medium (serum deprivation treatment), or treated without or with test compound in normal fresh medium for up to 96h (17-30). Test compounds (e.g. PD98059, U0126, and phorbol esters) were dissolved in dimethyl sulfoxide (DMSO), the final concentration (~0.2%) of DMSO in the test medium being without effect on [^{3}H]STX binding, Northern blot, and immunoblot analysis (23, 24, 26, 30).

3.3. [^{3}H]STX binding

Cells were washed with ice-cold Krebs-Ringer phosphate (KRP) buffer (mM) (154 NaCl, 5.6 KCl, 1.1 $MgSO_4$, 2.2 $CaCl_2$, 0.85 NaH_2PO_4, 2.15 Na_2HPO_4, 5 glucose, and 0.5% bovine serum albumin, pH 7.4), and

incubated with 1-25 nM [^{3}H]STX in 1 ml KRP buffer at 4°C for 15 min in the absence (total binding) and presence (nonspecific binding) of 1 μM TTX (31). The cells were washed, solubilized in 10% Triton X-100, and counted for radioactivity. Specific binding was calculated as the total binding minus nonspecific binding.

3.4. $^{22}Na^+$ influx

$^{22}Na^+$ influx was measured by incubating the cells with 2 μCi ^{22}NaCl at 37°C for 5 min in 1 ml KRP buffer in the absence or presence of veratridine, alpha- or beta-scorpion venom, or PbTx-3 (22, 27, 28, 32). The cells were washed with ice-cold KRP buffer, solubilized in 10% Triton X-100, and counted for radioactivity (13, 14, 31, 32).

3.5. Immunoblot

Cells were washed with ice-cold Ca^{2+}-free phosphate-buffered saline (PBS), and solubilized at 95°C for 3 min in 500 μl of 2x sodium dodecyl sulfate (SDS) electrophoresis sample buffer. Total quantity of cellular proteins was measured by Noninterfering protein assay kit. The same amount of protein (10 μg/lane) was separated by SDS-12% polyacrylamide gel electrophoresis, and transferred onto a nitrocellulose membrane. The membrane was preincubated at room temperature with 5% dry milk in Tris-buffered saline, then reacted for 15h with antibodies raised against either ERK, p38 mitogen-activated protein kinase, or *c-Jun* N-terminal kinase. After repeated washings, the immunoreactive bands were labeled with ^{125}I-anti-mouse IgG (1/1000) or ^{125}I-anti-rabbit IgG (1/1000), and analyzed by a bioimage analyzer BAS 2000 (Fuji Film, Tokyo).

3.6. mRNA isolation and electrophoresis

Total cellular RNA was isolated from the cells by acid guanidine thiocyanate phenol-chloroform extraction using TRIzol reagent. Poly $(A)^+$ RNA was purified by Oligotex-dT30<Super>, electrophoresed on 1% agarose gel containing 6.3% formaldehyde in the buffer [40 mM 3-(N-morpholino) propanesulfonic acid, pH 7.2, 0.5 mM EDTA, and 5 mM sodium citrate], transferred to a nylon membrane (Hybond-N^+, Amersham) in 20 x saline-sodium citrate (SSC; 1 x SSC = 0.15 M NaCl and 0.015 M sodium citrate) overnight, and cross-linked using a UV cross-linker (Funakoshi, Tokyo).

3.7. Northern blot

Plasmids containing hNE-Na cDNA, and $beta_1$-subunit cDNA were digested, respectively, with *Mun*I, and with *Sac*II plus *Hind*III, to obtain nucleotide (nt) fragments for alpha-subunit (nt 1365-2948), and $beta_1$-subunit (nt 457-790). These cDNA fragments and GAPDH cDNA (1.1 kbp) were labeled with [alpha-^{32}P]dCTP using BcaBEST labeling kit. The membrane was prehybridized, and then hybridized with hNE-Na probe at 65°C for 4h in the Rapid-hyb buffer. It was washed in 0.2 x SSC containing 0.1% SDS for 30 min twice, and subjected to autoradiography. The same membrane was successively hybridized with probes for $beta_1$-subunit, and then GAPDH, after being washed with 0.1% SDS at 100°C to remove the former probe. Autoradiogram was quantified by a bioimage analyzer BAS 2000. Relative level of alpha-subunit major mRNA (~9.4 kb) or $beta_1$-subunit mRNA against GAPDH mRNA was calculated.

3.8. Nuclear run-on assay

Cells were washed twice with ice-cold PBS, dislodged, and centrifuged at 500 x g for 5 min. Cell pellets were suspended in buffer A (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.4% Nonidet P-40), treated on ice for 5 min, and centrifuged at 500 x g for 5 min. Nuclear pellets were washed with buffer A, and suspended in buffer B (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA). Nuclei (1.2 x 10^7 / 100 μl) were incubated at 30°C for 30 min with 100 μl buffer C (10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 200 mM KCl, 2 mM dithiothreitol, 0.5 mM of each ATP, CTP, GTP, and 200 μCi [alpha-^{32}P]UTP), after which DNA was digested by exposing to 2U RQ1 RNase-Free DNase for 10 min at 30°C. Proteins were digested in 200 μl buffer D (20 mM Tris-HCl, pH 7.4, 10 mM EDTA, 20% SDS, and 200 μg/ml proteinase K) at 50°C for 1h. Newly-transcribed RNAs were extracted by using TRIzol reagent, dissolved in TE (10 mM Tris-HCl, pH 7.5 and 1 mM EDTA), and purified by mini Quick Spin RNA Columns. ^{32}P-Labeled RNAs (5 x $l0^6$ cpm/ml) were hybridized overnight at 70°C in Rapid-hyb buffer with nylon membrane immobilizing 10 μg pBII alone, and pBII containing hNE-Na cDNA or GAPDH cDNA. hNE-Na cDNA fragment (nt 1-2253) was liberated by digesting hNE-Na plasmid with *Kpn*I and *Bgl*II, and subcloned into pBII (23). The membrane was sequentially washed in 2x SSC containing 0.1% SDS at 65°C for 15 min, 2x SSC containing 10 μg/ml RNase A at 37°C for 10 min, 0.2x SSC containing 0.1% SDS at 65°C for 10 min, and then subjected to autoradiography.

3.9. Measurement of $[Ca^{2+}]_i$

Cultured cells were preincubated at 35˚C for 1h in HEPES-buffered solution (mM) (140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgC1_2$, 10 HEPES, 10 glucose, and 0.5% bovine serum albumin, pH 7.4) containing 3 mM fura-2/AM (33).

Fluorescence was measured in single cells with a Ca^{2+}-imaging system equipped with an intensified CCD camera (Quanticell/700, JEOL, Tokyo) in the perfusion chamber; cells were continuously perfused with HEPES-buffered solution at a flow rate of 1.0 ml/min in the absence or presence of A23187, thapsigargin, or DBHQ. $[Ca^{2+}]_i$ was calculated from the ratio of fluorescence intensities obtained at 510 nm with dual excitation at 340 and 380 nm, using the equation of Grynkiewicz et al. (34).

3.10. Statistical methods

[^{3}H]STX binding and $^{22}Na^+$ influx were measured in triplicate, and all experiments were repeated at least three times (mean±S.E.M.). Significance ($p<0.05$) was determined by one-way or two-way analysis of variance with post hoc mean comparison by the Newman-Keuls multiple range test. Student's *t* test was used when two group means were compared.

4. RESULTS

4.1. Steady-state level of Na^+ channels in quiescent cells: constitutive destabilization of Na^+ channel alpha-subunit mRNA by ERK1 and ERK2

As shown in Figure 1A, cells were incubated with or without serum for up to 48h, and [^{3}H]STX binding

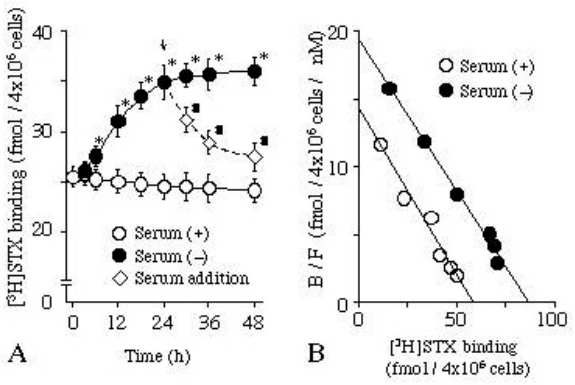

Figure 1. Cell surface [^{3}H]STX binding: serum deprivation-induced increase and serum exposure-induced decrease in cultured adrenal chromaffin cells. **A.** Cells were incubated with or without serum for up to 48h, or initially incubated without serum for the first 24h, then exposed to serum (indicated by arrow); [^{3}H]STX binding was assayed at the indicated times. *p<0.05, compared with serum (+); #p<0.05, compared with serum (-). **B.** Scatchard plot of [^{3}H]STX binding in cells incubated with or without serum for 24h.

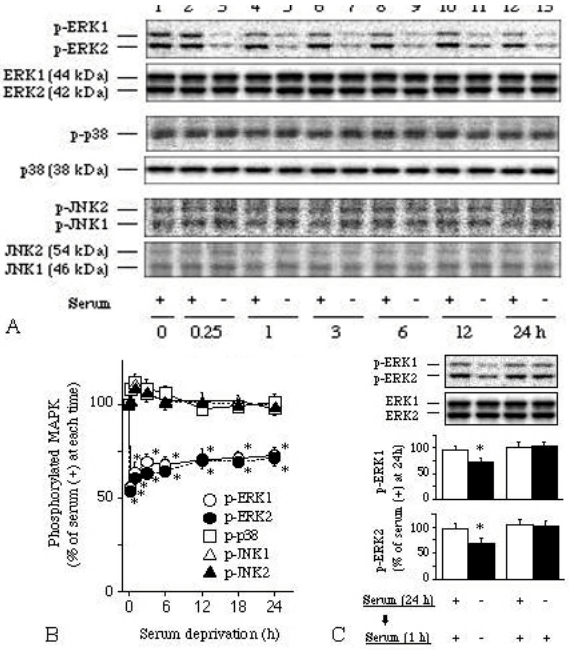

Figure 2. Immunoblot analysis: serum deprivation-induced selective attenuation of constitutive phosphorylation of ERK1 and ERK2, and its rapid restoration after serum exposure. **A.** Cells were incubated with or without serum for 24h and subjected to immunoblot analysis. Phosphorylated ERK (p-ERK), ERK (top panels); p-p38, p38 (middle panels); p-JNK, JNK (bottom panels). **B.** Quantification of p-ERK, p-38, and p-JNK in A. **C.** Cells were incubated with or without serum for 24h; then, serum-nonexposed cells were further incubated without serum or exposed to serum for 1h. *p< 0.05, compared with serum-exposed cells.

was assayed. Serum deprivation increased [^{3}H]STX binding by ~58% in a time-dependent manner ($t_{1/2}$=12.5h). When the cells were initially incubated without serum for the first 24h, then exposed to serum (Figure 1A, arrow), [^{3}H]STX binding returned gradually toward the control level of nontreated cells. Scatchard plot analysis (Figure 1B) shows that serum deprivation for 24h increased the B_{max} of [^{3}H]STX binding by 52%, with no change in the K_d value. Immunoblot analysis of mitogen-activated protein kinase (MAPK) family (Figure 2A) shows the phosphorylation levels (upper parts of each panel) and cellular levels (lower parts of each panel) of ERK (top panel), p38 MAPK (p38, middle panel), and *c-Jun* N-terminal kinase (JNK, bottom panel). In control cells incubated in serum-containing medium, ERK1 and ERK2, p38, as well as JNK1 and JNK2 were constitutively phosphorylated throughout 24h (lanes 1, 2, 4, 6, 8, 10, and 12). Serum deprivation caused a rapid (<15 min) and sustained (>24h) decrease (~ 50%) in the phosphorylation of ERK1 and ERK2 (but not p38, JNK1 and JNK2) (Figure 2B), with no change in the cellular levels of ERK1 and ERK2 proteins (Figure 2A). Figure 2C shows that the attenuated phosphorylations of ERK1 and ERK2 during 24h-serum deprivation were completely returned to the control levels within 1h after serum addition, when [^{3}H]STX binding was not yet restored by serum addition (Figure 1A). Thus, constitutive phosphorylations of ERK1 and ERK2 in quiescent chromaffin cells produce continuous negative regulatory effects to determine steady-state level of Na^+ channels.

Treatment of chromaffin cells with PD98059 or U0126, a drug that blocks phosphorylation/activation of ERK1 and ERK2 by MAPK/ERK kinase (MEK), attenuated constitutive phosphorylations of ERK1 and ERK2, while increasing [^{3}H]STX binding capacity with no change in the K_d value (30). In addition, concentration-response curves of PD98059 and U0126 showed that the attenuated extents of constitutive phosphorylations of ERK1 and ERK2 were inversely related to the increased extent of [^{3}H]STX binding, thus the phosphorylation levels of ERK1 and ERK2 tightly linking with Na^+ channel density in a quantitative manner. Concurrent treatment of serum deprivation with PD98059 or U0126, however, did not produce additional increasing effect on [^{3}H]STX binding, compared with either treatment alone (30). These results suggest that serum deprivation-induced up-regulation of Na^+ channels is largely attributed to the attenuation of constitutive phosphorylations of ERK1 and ERK2.

Northern blot analysis (Figure 3) shows that serum deprivation, PD98059, or U0126 raised Na^+ channel alpha- (but not $beta_1$-) subunit mRNA level by ~15% as early as 3h, causing the maximum plateau increase of ~53% between 12 and 24h ($t_{1/2}$=6.1h). Steady-state level of mRNA is dependent on gene transcription, processing of heterogeneous nuclear RNA to mRNA, and mRNA stability; these processes are regulated by constitutively-expressed or stimuli-inducible *trans*-acting nucleotide binding proteins that shuttle between nucleus and cytoplasm (reviewed in 35-37). In the present study, we

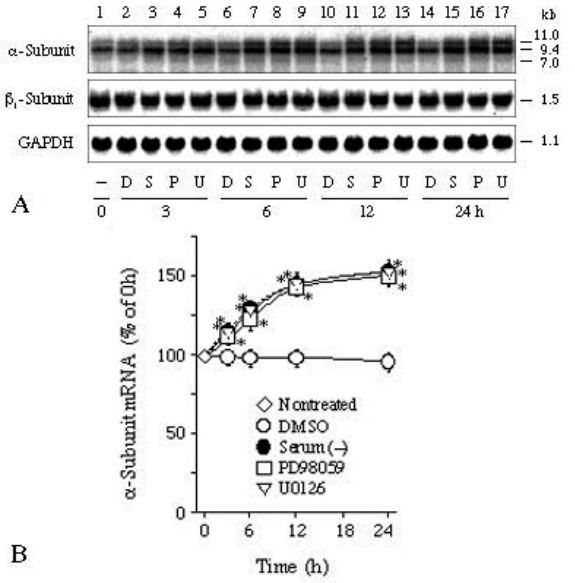

Figure 3. Northern blot analysis: up-regulation of Na^+ channel alpha- but not $beta_1$-subunit mRNA level in cells treated with serum deprivation, PD98059, and U0126. **A.** Cells were treated without (-) or with DMSO (D), serum deprivation (S), 50 μM PD98059 (P), or 10 μM U0126 (U) for up to 24h, and subjected to Northern blot analysis. **B.** Relative level of alpha-subunit mRNA/GAPDH mRNA in A. *p<0.05, compared with DMSO.

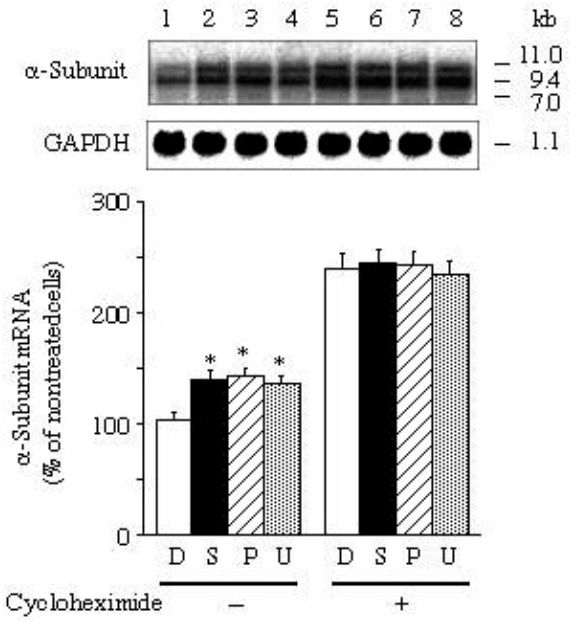

Figure 4. Up-regulation by cycloheximide of Na^+ channel alpha-subunit mRNA level: no additional increase by serum deprivation, PD98059, and U0126. In the absence or presence of cycloheximide, cells were treated without or with DMSO (D), serum deprivation (S), 50 μM PD98059 (P), or 10 μM U0126 (U) for 12h. *p< 0.05, compared with DMSO.

examined whether up-regulation of alpha-subunit mRNA caused by ERK pathway inactivation may require protein synthesis by using cycloheximide at 10 μg/ml, a concentration at which cycloheximide inhibits almost completely *de novo* synthesis of proteins in adrenal chromaffin cells (38). In Figure 4, treatment with 10 μg/ml cycloheximide for 12h increased per se alpha-subunit mRNA level by ~2.4-fold. In the presence of cycloheximide, however, serum deprivation, PD98059, or U0126 failed to exert additional increasing effect on alpha-subunit mRNA level, compared with cycloheximide alone. Nuclear run-on assay (Figure 5A) shows that serum deprivation, PD98059, or U0126 did not alter transcriptional rate of alpha-subunit gene. To measure degradation rate of alpha-subunit mRNA (Figure 5B), cells were treated without or with serum deprivation, PD98059, or U0126 for the first 6h, then exposed to actinomycin D, an inhibitor of RNA synthesis, in the continuous absence or presence of either test treatment, and subjected to Northern blot analysis at the indicated times; either treatment elongated half-life ($t_{1/2}$) of alpha-subunit mRNA (~9.4 kb) from 17.5 to ~26.3h.

4.2. Down-regulation of Na^+ channels by multiple signalings

4.2.1. PKC isoform-specific mechanisms: PKC-epsilon-induced destabilization of alpha-subunit mRNA, and PKC-alpha-induced internalization of Na^+ channels

Our previous immunoblot analysis showed that, among eleven PKC isoforms, adrenal chromaffin cells contained only conventional PKC-alpha, novel PKC-epsilon, and atypical PKC-zeta (24). Treatment of chromaffin cells with 1-1000 nM 12-O-tetradecanoylphorbol 13-acetate (TPA) caused a rapid (<15 min) and sustained (>15h) translocation of PKC-alpha and –epsilon (but not –zeta) from cytoplasm to membranes, a hallmark of PKC activation; in contrast, 100 nM thymeleatoxin (TMX) caused the similar, but selective membrane translocation of only PKC-alpha between 15 min and 15h, with the potency of TMX being indistinguishable from that of TPA at 100 nM (24). Figure 6A shows that either 100 nM TPA or 100 nM TMX produced a time-dependent reduction of [^{3}H]STX binding; the potencies of TPA and TMX were similar until 12h, but TPA decreased [^{3}H]STX binding to a greater extent than TMX at 18 and 24h. As shown in Figure 6B, Gö6976, a compound that inhibits PKC-alpha (but not -epsilon), completely prevented TMX-induced decrease of [^{3}H]STX binding at 15h; however, Gö6976 opposed TPA-induced event by only ~57%, in striking contrast to the complete prevention by H7, an inhibitor of PKC isoforms (18). Because only TPA (but not TMX) activated PKC-epsilon, these correlative results suggest that TPA-induced activation of PKC-epsilon contributed to TPA-induced down-regulation of Na^+ channels, which lasted for a longer period, compared with TMX-induced, PKC-alpha-mediated, down-regulation of Na^+ channels.

Northern blot analysis (Figure 7A) shows that 100 nM TPA lowered alpha-subunit mRNA level by 52% at 12h in a monophasic manner, while increasing $beta_1$-subunit mRNA level in a time-dependent manner. In

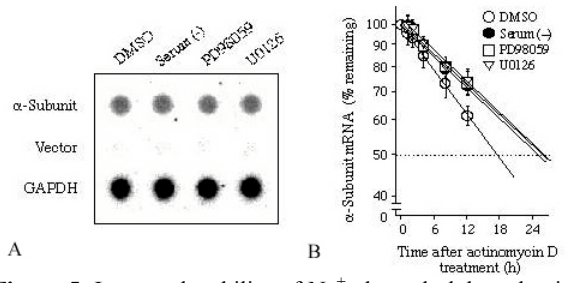

Figure 5. Increased stability of Na^+ channel alpha-subunit mRNA in cells treated with serum deprivation, PD98059, and U0126: no effect on alpha-subunit gene transcription. **A.** Cells were treated with DMSO, serum deprivation, 50 μM PD98059, or 10 μM U0126 for 12h, and subjected to in vitro nuclear run-on assay. pBII alone (Vector), pBII containing hNE-Na cDNA or GAPDH cDNA. **B.** Cells were pretreated with DMSO, serum deprivation, 50 μM PD98059, or 10 μM U0126 for 6h (see Figure 3B), and incubated with 10 μg/ml actinomycin D in the continuous absence or presence of either test treatment. At the indicated times, alpha-subunit mRNA level was measured by Northern blot analysis.

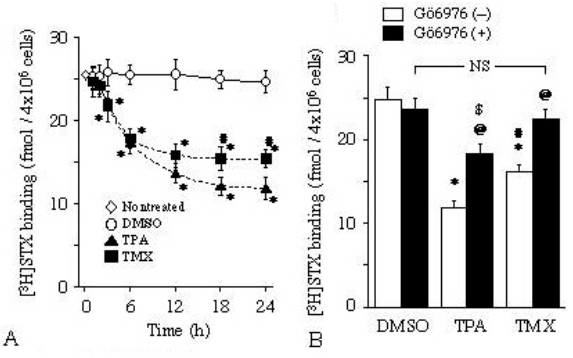

Figure 6. Down-regulation of [^{3}H]STX binding by TPA and TMX: distinct time-course and differential prevention by Gö6976. A. Cells were treated without or with DMSO, 100 nM TPA, or 100 nM TMX for up to 24h, and subjected to [^{3}H]STX binding assay. **B.** In the absence or presence of 1 μM Gö6976, cells were treated with DMSO, 100 nM TPA, or 100 nM TMX for 15h. *p<0.05, compared with DMSO; #p<0.05, compared between TPA and TMX; @P<0.05, compared with TPA or TMX alone; $p< 0.05, compared with Gö6976 alone; NS, no significance.

contrast, 100 nM TMX for up to 24h did not alter alpha- and $beta_1$-subunit mRNA levels. In addition, Gö6976 failed to block TPA-induced decrease of alpha-subunit mRNA level (Figure 7B, lanes 2 and 5), in contrast to the complete prevention by H7 (23). We examined whether translational event may be involved in the TPA-induced, PKC-epsilon-mediated, alterations in alpha- and $beta_1$-subunit mRNA levels (Figure 8). Cycloheximide raised per se alpha-subunit mRNA level in a time-dependent manner, and completely prevented decreasing effect of TPA on alpha-subunit mRNA level. In contrast, cycloheximide lowered per se $beta_1$-subunit mRNA level, and nullified increasing effect of TPA on $beta_1$-subunit mRNA level. TPA (100 nM for ~6h) did not alter transcriptional rate of alpha-subunit gene, but shortened the $t_{1/2}$ of alpha-subunit mRNA from 18.8 to 3.7h (23). Thus, PKC-epsilon destabilized alpha-subunit mRNA and decreased its level, contributing to the prolongation of down-regulation of Na^+ channels.

In our present study, the reduction of [^{3}H]STX binding caused by TMX or TPA was rapid (<3h) in onset, further growing with $t_{1/2}$ of 4.5h (Figure 6A). Such a rapid onset of Na^+ channel down-regulation may involve an increased endocytic internalization of cell surface Na^+ channels. Brefeldin A (BFA), an inhibitor of guanine nucleotide exchange protein of ADP-ribosylation factor 1, a monomeric GTPase, has been used to measure internalization rate of cell surface ion channels/receptors, because BFA blocks cell surface externalization from the *trans*-Golgi network of various newly-synthesized proteins, with no effect on their internalization. Figure 9 shows that a mono treatment with either TPA, TMX, or BFA did not yet lower [^{3}H]STX binding at 1h, whereas the concurrent treatment of BFA with TPA or TMX lowered [^{3}H]STX binding. At 3h, concurrent treatment decreased [^{3}H]STX binding to a greater extent than either treatment alone. Because TPA and TMX exhibited equipotent capacity to decrease [^{3}H]STX binding for ~12h (Figure 6A), these results suggest that PKC-alpha promoted endocytic internalization of Na channels, causing down-regulation of Na^+ channels.

4.2.2. Heterogeneous increases of cytoplasmic Ca^{2+}: distinct effects on internalization of Na^+ channels, and Na^+ channel alpha- and $beta_1$-subunit mRNA levels

Treatment of chromaffin cells with 1 μM A23187, a Ca^{2+}-ionophore, produced an immediate monophasic and salient plateau increase of cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) (Figure 10), which lasted for, at least, 96h (26). Thapsigargin and DBHQ are two inhibitors of sarco(endo)plasmic Ca^{2+}-ATPase, but not of the plasma membrane Ca^{2+}-ATPase. Thapsigargin (100 nM) induced a slowly-developing monophasic rise of $[Ca^{2+}]_i$, followed by the persistent (>48h) plateau increase (26); in contrast, DBHQ (100 nM) robustly increased $[Ca^{2+}]_i$, which gradually waned at 60 min. A23187 produced long-lasting (>96h) time-dependent gradual decrease of [^{3}H]STX binding by 66% at 96h, whereas thapsigargin-induced reduction of [^{3}H]STX binding leveled off by 35% at 48h (26). In striking contrast, DBHQ did not alter [^{3}H]STX binding at 12, 24, 48, and 96h (26). A23187 (1 μM for 48h) or thapsigargin (100 nM for 48h) decreased B_{max} value of [^{3}H]STX binding by 56 or 34%, with no change in the K_d value. A23187- or thapsigargin-induced decrease of [^{3}H]STX binding required long-lasting continuous increase of $[Ca^{2+}]$i, because addition of BAPTA-AM, a cell membrane-permeable Ca^{2+} chelator, at 24h abolished subsequent long-lasting decreasing effect of A23187 or thapsigargin on [^{3}H]STX binding at 48h (26). The decreasing effect of A23187 or thapsigargin was partially prevented by ~62% by the 24h-concurrent treatment with either calpastatin, an inhibitor of calpain, or Gö6976. Internalization rate of Na^+ channels, as measured in the presence of BFA, was accelerated by A23187 or TG (26). In addition, A23187 (but not thapsigargin) lowered Na^+

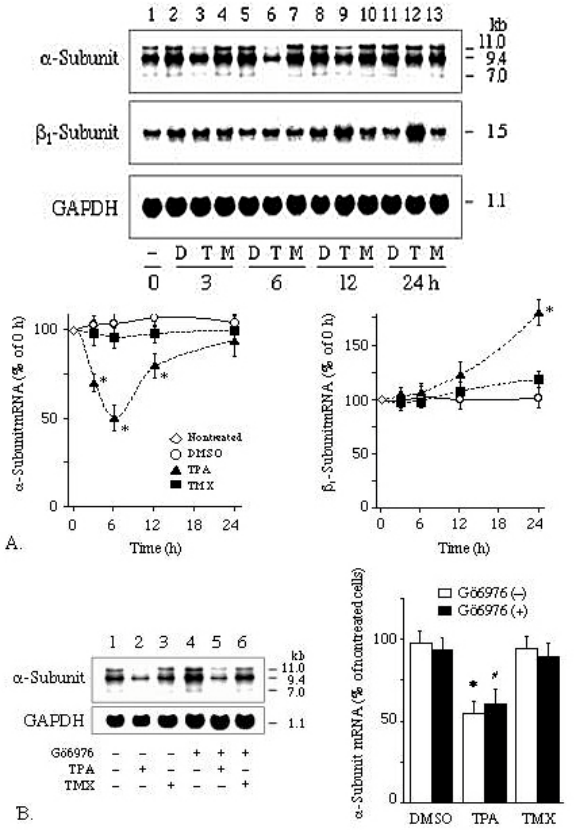

Figure 7. Decrease of alpha-subunit mRNA level and increase of $beta_1$-subunit mRNA level caused by TPA but not by TMX: no prevention by Gö6976. **A.** Cells were treated without (-) or with DMSO (D), 100 nM TPA (T), or 100 nM TMX (M) for up to 24h. **B.** In the absence or presence of 1 μM Gö6976, cells were treated without or with 100 nM TPA or 100 nM TMX for 6h. *p<0.05, compared with DMSO; #p<0.05, compared with Gö6976 alone.

channel alpha-and $beta_1$-subunit mRNA levels by ~48 and ~50% between 3 and 48h (26), consistent with the observation that A23187-induced down-regulation of Na^+ channels lasted for a much longer period, compared with thapsigargin-induced Na^+ channel down-regulation.

4.2.3. Calcineurin and FRAP (also, known as mTOR): internalization and externalization of Na^+ channels

We examined whether calcineurin and FRAP could modulate cell surface expression of Na^+ channels (27). Treatment of chromaffin cells with cyclosporin A followed by the *in vitro* assay in cell lysate showed that cyclosporin A inhibited calcineurin activity between 1 and 100 μM (IC_{50}=0.6 μM). At 1-100 μM, cyclosporin A increased [^{3}H]STX binding by ~56% at 24h, with no change in the K_d value. FK506, an inhibitor of calcineurin, raised [^{3}H]STX binding by 36%, whereas GPI-1046, a FK506 analog incapable to inhibit calcineurin, had no effect. The increasing effect of cyclosporin A was time ($t_{1/2}$=15.2h)- and concentration (EC_{50}=2.9 μM)-dependent. In addition, rapamycin, an inhibitor of FRAP, elevated [^{3}H]STX binding by 25%. Cyclosporin A or rapamycin accelerated cell surface externalization of Na^+ channels from the *trans*-Golgi network; in contrast, cyclosporin A (but not rapamycin) also retarded internalization rate of cell surface Na^+ channels. Cyclosporin A, however, did not change Na^+ channel alpha- and $beta_1$-subunit mRNA levels.

We characterized pharmacological properties of Na^+ channels in nontreated and cyclosporin A-treated cells, because allosteric modulation of Na^+ channels by distinct classes of toxins occurs in a Na^+ channel isoform-specific manner (reviewed in 10). In cyclosporin A-treated cells, $^{22}Na^+$ influx caused by veratridine, a toxin acting at site 2 in segment 6 of domain I (S6I) of Na^+ channel alpha-subunit, was augmented by 30% with no change in the EC_{50} of veratridine, compared with nontreated cells. Either alpha-scorpion venom, which binds to site 3 between S3IV and S4IV, or beta-scorpion venom, which interacts with site 4, or PbTx-3, which binds site 5 between S5IV and S6I, had little effect per se, but potentiated veratridine-induced $^{22}Na^+$ influx in a more than additive manner by 2.2-, or 2.4-, or 3.6-fold in cyclosporin A-treated cells, as in nontreated cells. PbTx-3 in combination with alpha- or beta-scorpron venom further potentiated veratridine-induced $^{22}Na^+$ influx by 6.2- or 6.0-fold in both nontreated and cyslosporin A-treated cells. In cyclosporin A-treated cells, increased density and activity of Na^+ channels enhanced veratridine-induced $^{45}Ca^{2+}$ influx via voltage-dependent Ca^{2+} channels and exocytic secretion of catecholamines by 21 and 27% without changing EC_{50} values of veratridine, compared with nontreated cells.

4.3. Up-regulation of Na^+ channels by insulin receptor tyrosine kinase and PKA: externalization of Na^+ channels

Chronic treatment of chromaffin cells with insulin (~24h) or dbcAMP (~12h) increased [^{3}H]STX binding by ~49 or ~50%, with no change in the K_d value (17, 19, 21). Although the effect of insulin or dbcAMP was blocked by actinomycin D or cycloheximide, insulin or dbcAMP did not alter Na^+ channel alpha- and $beta_1$-subunit mRNA levels (17, 21). In cells treated with insulin or dbcAMP, veratridine-induced $^{22}Na^+$ influx, $^{45}Ca^{2+}$ influx, and catecholamine secretion were potentiated by ~56, ~60, and ~45%, compared with nontreated cells.

4.4. Up-regulation of Na^+ channels by therapeutic drugs: chronic treatment with neuroprotective, antiepileptic, antipsychotic, and local anesthetic drugs

Valproic acid, originally identified as an antiepileptic drug, has attracted widespread attention, because of its potential multiple therapeutic effectiveness, which may be related to its capacity to inhibit proapoptotic glycogen synthase kinase-3, and histone deacetylase, an enzyme that represses gene transcription (reviewed in 39, 40). In adrenal chromaffin cells, acute treatment with therapeutic concentration of valproic acid did not inhibit veratridine-induced $^{22}Na^+$ influx; however, its chronic (~24h) treatment increased Na^+ channel alpha- and $beta_1$-subunit mRNA levels by ~74 and ~83%, and caused up-regulation of [^{3}H]STX binding by ~40%, with no change in the K_d value (20). The up-regulation of Na^+ channels resulted in the enhancement of veratridine-induced $^{22}Na^+$

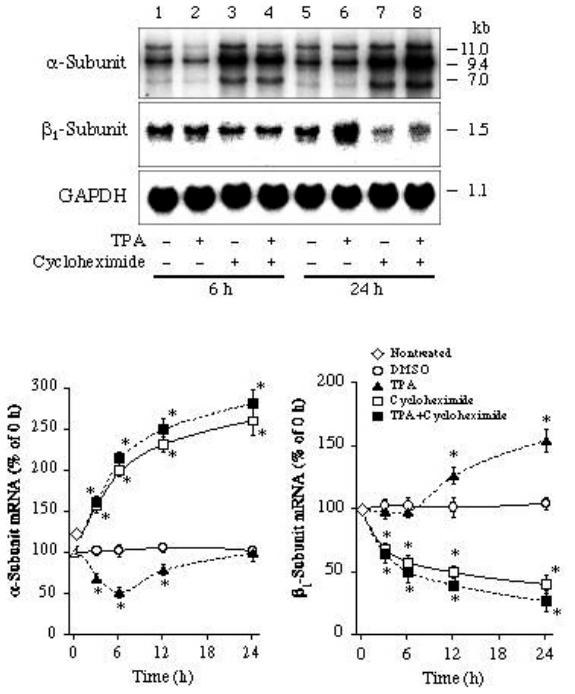

Figure 8. Increase of alpha-subunit mRNA level and decrease of beta$_1$-subunit mRNA level caused by cycloheximide: complete reversion by cycloheximide of TPA-induced opposite changes in alpha- and beta$_1$-subunit mRNA levels. Cells were incubated with DMSO or 100 nM TPA in the absence or presence of cycloheximide for up to 24h. Typical immunoreactive bands obtained at 6 and 24h incubation periods are shown. *p< 0.05, compared with DMSO.

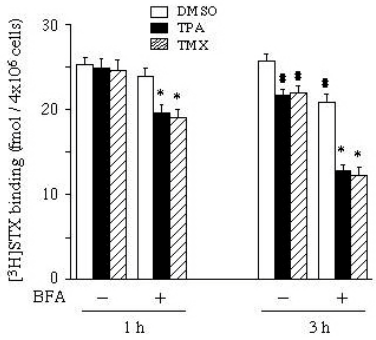

Figure 9. Reduction of cell surface Na$^+$ channels due to endocytic internalization: promotion by TPA and TMX. In the absence or presence of 10 μg/ml BFA, cells were treated for 1 or 3h with DMSO, 100 nM TPA, or 100 nM TMX, and the remaining cell surface Na$^+$ channels were measured by [^{3}H]STX binding assay. *p<0.05, compared with BFA alone; #p<0.05, compared with DMSO alone.

influx, thus augmenting veratridine-induced $^{45}Ca^{2+}$ influx and catecholamine secretion.

Acute treatment with neuroprotective drug riluzole (22), NS-7 (25), or carvedilol (28) inhibited veratridine-induced $^{22}Na^+$ influx, $^{45}Ca^{2+}$ influx, and catecholamine secretion. In contrast, chronic (~12h) treatment with NS-7 or carvedilol (but not riluzole) increased [^{3}H]STX binding by ~86%, with no change in the K_d value; this event was presumably due to the promotion of cell surface externalization of newly-synthesized protein(s) from the *trans*-Golgi network, because [^{3}H]STX rise was prevented by cycloheximide or BFA, and was not associated with the increased levels of alpha- and beta$_1$-subunit mRNAs.

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 (~3h) treatment with bupivacaine or ropivacaine (but not lidocaine) increased [^{3}H]STX binding by ~48% by a mechanism similar to that of NS-7 or carvedilol (29).

5. DISCUSSION

5.1. Mechanisms of Na$^+$ channel up- and down-regulations

5.1.1. Na$^+$ channel subunit mRNA stability

Although regulation of gene transcription by extracellular signals is believed to be the major mechanism that regulates genetic information from DNA to protein synthesis, it has become increasingly evident that extracellular signals can regulate degradation rate of mRNAs, rapidly orchestrating expression levels of gene products in a spatiotemporal-specific manner (reviewed in 35-37). Constitutively-expressed and external stimuli-inducible *trans*-acting nucleotide-binding proteins, which shuttle between nucleus and cytoplasm, bind to specific nucleotide *cis*-elements at the 3'- and 5'-untranslated regions, as well as coding region, thereby causing stabilization and destabilization of mRNAs (reviewed in 35-37). In several mRNAs (e.g. beta-tubulin mRNA and Bcl-2 mRNA), nucleotide-binding proteins are encoded in their target mRNA, and exert translation-dependent mRNA destabilization, thus negative autoregulation of mRNA level (reviewed in 36, 37). Evidence has emerged that nucleotide-binding proteins, including a particular family of signal transduction and activation of RNA (STAR) proteins, recruit additional, as yet, not fully-defined *trans*-acting factors (e.g. heat shock protein-70 kDa) to their target *cis*-elements, and the functions and nucleocytoplasmic distribution of these multimeric complexes are regulated by their phosphorylation in response to extracellular signals, thus directing stabilization and destabilization of mRNAs (reviewed in 36, 37, 41). However, the *cis*- and *trans*-elements, as well as the regulatory mechanisms involved in the stabilization and destabilization of most of individual mRNAs have only begun to be specified.

In our present study, treatment of quiescent adrenal chromaffin cells with cycloheximide alone caused a rapid (<3h) and sustained time-dependent increase of alpha-subunit mRNA level, coincident with the similar

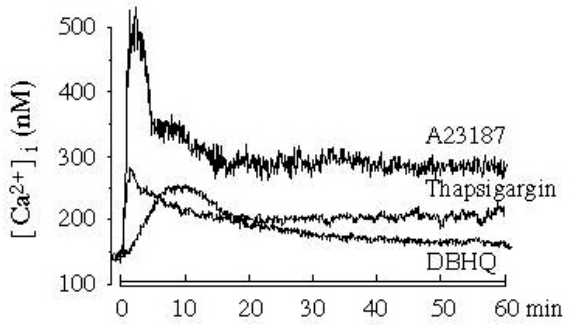

Figure 10. Heterogeneous patterns of $[Ca^{2+}]_i$ increase caused by A23187, thapsigargin, and DBHQ. Cells preloaded with fura-2/AM were treated with 1 μM A23187, 100 nM thapsigargin, or 100 nM DBHQ for up to 60 min. Each recording was obtained in a single cell and is typical of independent multiple experiments (n= > 68) with similar results.

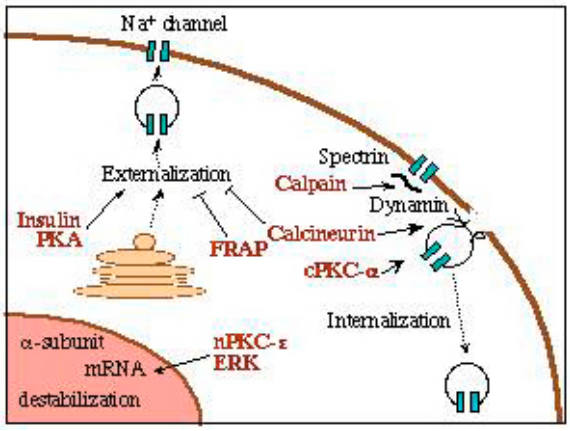

Figure 11. Multiple mechanisms of Na^+ channel up- and down-regulations. ERK and novel PKC-epsilon destabilize alpha-subunit mRNA. Calpain, calcineurin, and conventional PKC-alpha cooperatively promote internalization of Na^+ channels. Insulin and PKA promote, whereas calcineurin and FRAP retard, cell surface externalization of Na^+ channels from the *trans*-Golgi network.

rapid and sustained decrease of $beta_1$-subunit mRNA level. It seems that constitutively-expressed nucleotide-binding protein(s) with a short half-life destabilized alpha-subunit mRNA, while stabilizing $beta_1$-subunit mRNA, thereby determining steady-state levels of alpha- and $beta_1$-subunit mRNAs in a opposite direction in quiescent chromaffin cells. In quiescent chromaffin cells, ERK1 and ERK2 were constitutively phosphorylated; the attenuation of constitutive phosphorylations of ERK1 and ERK2 caused by serum deprivation, PD98059, or U0126 elongated half-life of alpha-subunit mRNA from 17.5 to 26.3h, but had no effect on alpha-subunit gene transcription and steady-state level of $beta_1$-subunit mRNA. Concentration-response curves of PD98059 and U0126 showed that the phosphorylation levels of ERK1 and ERK2 were inversely related to the [^{3}H]STX binding capacity, thus the strength of ERK signaling pathway being tightly linked to the stability of alpha-subunit mRNA in a quantitative manner. Therefore, the surveillance for the stability of alpha-subunit mRNA is the major checkpoint to determine steady-state levels of alpha-subunit mRNA and cell surface Na^+ channels. In the presence of cycloheximide, however, attenuated phosphorylations of ERKl and ERK2 failed to increase alpha-subunit mRNA level over the level attained with cycloheximide alone. In addition, activation of novel PKC-epsilon shortened half-life of alpha-subunit mRNA from 18.8 to 3.7h, and decreased its level without changing the alpha-subunit gene transcription, while increasing $beta_1$-subunit mRNA level. Thus, PKC-epsilon-induced down-regulation of Na^+ channels was due to the destabilization of alpha-subunit mRNA. However, PKC-epsilon-induced decrease of alpha-subunit mRNA level was completely prevented, and instead, reversed as early as 3h after the concurrent treatment with cycloheximide. Most straightforward interpretation of these results may be that destabilization of alpha-subunit mRNA by constitutive activity of ERK pathway, or by PKC-epsilon activation was dependent on synthesis of short-lived protein(s) that destabilizes alpha-subunit mRNA. In addition, we may not exclude the possibility that constitutive activity of ERK pathway, or PKC-epsilon activation destabilized alpha-subunit mRNA by a mechanism similar to that of cycloheximide; thus, inhibition of synthesis of stabilizing-protein(s) inevitably led to the destabilization of alpha-subunit mRNA. However, $beta_1$-subunit mRNA level was decreased by cycloheximide, while being increased by PKC-epsilon activation, with no change by constitutive activity of ERK pathway. These differential effects on $beta_1$-subunit mRNA level may raise the question of whether constitutive activity of ERK pathway, or PKC-epsilon activation specifically inhibited synthesis of stabilizing-protein(s) for alpha-subunit mRNA, in contrast to cycloheximide that inhibited translation of various mRNAs in a nonselective manner. Multiple lines of evidence have shown that the situation may be more complicated. In addition to the translation-dependent autoregulation of mRNA degradation, translation of mRNA is intimately linked to the stability of mRNA via as yet, not fully-defined mechanisms, which may be common and/or specific to individual mRNAs (reviewed in 36). Protein synthesis inhibitors, even if they inhibit translation of mRNA by different mechanisms, stabilize most mRNAs, proposing several plausible explanations for the link between translation and mRNA stability (reviewed in 36). Conversely, several cases have been documented, in which mRNA-stabilizing/destabilizing *trans*-acting nucleotide-binding proteins also regulate translational rate of mRNAs via interacting with their cognate *cis*-elments (reviewed in 42).

5.1.2. Internalization and externalization of Na^+ channels

Activation of conventional PKC-alpha or rise of $[Ca^{2+}]_i$ accelerated endocytic internalization of Na^+ channels, causing down-regulation of cell surface Na^+ channels. $[Ca^{2+}]_i$-induced down-regulation of Na^+ channels was prevented by Gö6976 or calpain inhibitor. In contrast, inhibition of calcineurin activity by cyclosporin A retarded endocytic internalization of Na^+ channels, causing

up-regulation of cell surface Na^+ channels. It has been shown that internalization of cell surface receptors/ion channels (e.g. renal epithelial Na^+ channels) via clathrin-coated vesicles is cooperatively regulated by calpain, calcineurin, and PKC (reviewed in 43; 44). Calpain binds to clathrin-coated vesicles in a Ca^{2+}-dependent manner, and catalyzes proteolytic removal of spectrin, a cytoskeletal protein, thus triggering budding of clathrin-coated vesicles (reviewed in 43; 45). Calpain is associated with ankyrin, which accelerates calpain-induced degradation of spectrin (46). Association of Na^+ channels with ankyrin and spectrin has been documented in brain and skeletal muscle (5, 47, 48). Among calcineurin's substrates, dynamin I, a monomeric GTPase, exhibits higher affinity for calcineurin, and calcineurin-catalyzed dephosphorylation of dynamin I initiates the fission of invaginated clathrin-coated vesicles (reviewed in 43). In addition, dynamin I internalized into cytoplasm with clathrin-coated vesicles is phosphorylated by PKC for an another round of endocytosis, and the phosphorylation/dephosphorylation cycles of dynamin I are assumed to be indispensable to the continuation of internalization of clathrin-coated vesicles (reviewed in 43).

By using cyclosporin A, FK506, or rapamycin, we observed that inhibition of either calcineurin or FRAP caused up-regulation of cell surface Na^+ channels via accelerating cell surface externalization of newly-synthesized proteins from the *trans*-Golgi network. Activation of insulin receptor tyrosine kinase or PKA caused up-regulation of cell surface Na^+ channels via a transcription/translation-dependent mechanism(s). Na^+ channel alpha- and $beta_1$-subunits are heavily glycosylated proteins, thus N-linked glycosylation of both subunits presumably playing indispensable roles in the conformational maturation/subunit assembly of Na^+ channels (reviewed in 49-52), as previously shown in the case of insulin receptors by our laboratory (53). Our previous study showed that treatment of adrenal chromaffin cells with tunicamycin (0.05 μg/ml for 24h), an inhibitor of N-linked glycosylation, lowered cell surface density of Na^+ channels by ~20% (19). With respect to ion channels/receptors, including Na^+ channels, specific evidence is still lacking about their quality control system in the endoplasmic reticulum and Golgi complex (reviewed in 49-52; 53), as well as cell surface vesicular externalization from the *trans*-Golgi network (reviewed in 54, 55).

5.2. Biological significance of regulated and dysregulated expression of Na^+ channels

Expression of various Na^+ channel isoform genes is finely regulated presumably by the isoform-specific, as yet defined, mechanisms to meet physiological demands (reviewed in 6, 9). In immature nonmyelinated neurons, $Na_v1.2$ Na^+ channel isoform is expressed evenly in the entire axolemma; during conformational maturation into the myelinated neurons, $Na_v1.2$ is replaced by $Na_v1.6$ Na^+ channel isoform, which localizes at the node of Ranvier, and conducts saltatory action potentials (56). In contrast, dysregulated de-repression of otherwise silent $Na_v1.8$ Na^+ channel isoform gene was documented in cerebellar Purkinje cells from experimental mouse allergic encephalomyelitis, and from human patients with neurodegenerative multiple sclerosis, presumably accounting for the pathognomonic cerebellar ataxia (reviewed in 6; 57). In dorsal root ganglion neurons, nerve growth factor, in cooperation with glial cell-derived neurotrophic factor, constitutively abrogated inappropriate expression of $Na_v1.3$ Na^+ channel gene in physiological state; its dysregulated de-repression contributed to chronic pain associated with injury of sensory neurons (58). Thus, ERK pathway's activity-dependent constitutive down-regulation of $Na_v1.7$ Na^+ channel gene expression is a novel regulatory mechanism of cell excitability, which may play crucial roles in various physiological and pathological states.

Activities of PKC isoforms fluctuate in various physiological and pathological states (reviewed in 59, 60); thus, PKC isoform-selective inhibitors/activators are expected to be effective for the treatment of a diversity of diseased states (e.g. diabetes mellitus, neurodegeneration, and hypoxia/ischemia) (reviewed in 61). One example is shown in insulin-deficient diabetes mellitus, in which membrane translocation and enzyme activity of PKC-alpha and –epsilon were increased (62), and the density of Na^+ channels at the node of Ranvier was decreased with a defective axonal conduction (63). In this context, our present study may be informative, because PKC-alpha and –epsilon down-regulated Na^+ channels via distinct mechanisms, whereas insulin up-regulated Na^+ channels. In efferent and afferent myelinated neurons, insulin receptors are localized at the node of Ranvier, where Na^+ channels are concentrated (64). In experimental animal and human insulin-dependent diabetes mellitus, insulin deficiency culminated in the disruption of paranodal myelination, independent of hyperglycemia, and dislocated Na^+ channels from nodal to paranodal axolemma, causing nodal Na^+ channel down-regulation and defective nerve conduction (63); the defective conduction velocity of motor and sensory neurons was prevented by local injection of low dose of insulin (65).

It has been shown that noninactivating Na^+ currents (reviewed in 66) via TTX/STX-sensitive Na^+ channels initiates hypoxia/ischemia-induced neuronal injury primarily by causing Ca^{2+} overload via reversed operation of Na^+-Ca^{2+} exchanger (reviewed in 3). Hypoxia/ischemia-induced ATP depletion perturbs Ca^{2+} sequestration into the endoplasmic reticulum catalyzed by sarco(endo)plasmic Ca^{2+}-ATPase, and also compromises extracellular Ca^{2+} extrusion by plasma membrane Ca^{2+}-ATPase, thus aggravating Ca^{2+} overload (reviewed in 3). Our present study showed that 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. $[Ca^{2+}]_i$-induced down-regulation of Na^+ channels may be a compensatory defensive event against Ca^{2+} overload, because down-regulation of Na^+ channels has been assumed to prevent hypoxia/ischemia-induced neuronal injury (reviewed in 3), and failure of normal down-regulation of Na^+ channels in response to hypoxia may generate seizure (4).

5.3. Clinical implications of Na^+ channel up-regulation by therapeutic drugs

In our present study, valproic acid, NS-7, carvedilol, bupivacaine enantiomers, and ropivacaine

caused up-regulation of Na^+ channels via multiple mechnanisms, whereas riluzole and lidocaine had no effect. During hypoxia, steady-state inactivation of Na^+ currents was shifted to a more hyperpolarizing potential in human cortical neurons (67), presumably as the compensatory defensive response against hypoxia-induced neuronal injury. Also, in response to hypoxia, density of cell surface Na^+ channels in brain was down-regulated to prevent neuronal injury (reviewed in 3; 4). Compared with adult rat brain, neonatal rat brain is more resistant to hypoxia, which is postulated to be attributed to the lower density of brain Na^+ channels in the neonate than in adult rat (reviewed in 3). In addition, Na^+ influx via Na^+ channels caused down-regulation of Na^+ channels by promoting internalization of Na^+ channels in fetal and neonatal (but not adult) rat brain (68). These previous results led us to pose the question of whether Na^+ channel up-regulation caused by these drugs may compromise the therapeutic effectiveness of these drugs.

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Abbreviations: BFA, brefeldin A, $[Ca^{2+}]_i$, cytoplasmic Ca^{2+}, DMSO, dimethyl sulfoxide, ERK, extracellular signal-regulated kinase, FRAP, FK506 binding protein- and rapamycin-associated protein, GAPDH, glyceraldehyde-3-phosphate dehydrogenase, hNE-Na, human neuroendocrine type Na^+ channel alpha-subunit, JNK, *c-Jun* N-terminal kinase, KRP, Krebs-Ringer phosphate, MAPK, mitogen-activated protein kinase, mTOR, mammalian target of rapamycin, pBII, plasmid Bluescript II, PBS, phosphate-buffered saline, PbTx-3, *Ptychodiscus brevis* toxin-3, PKA, protein kinase A, PKC, protein kinase C, SDS, sodium dodecyl sulfate, SSC, saline-sodium citrate, STX, saxitoxin, TMX, thymeleatoxin, TPA, 12-O-tetradecanoylphorbol 13-acetate, TTX, tetrodotoxin

Key Words: ERK, Sodium channel, mRNA stability, Internalization, Externalization, Phosphorylaiton, kinase, Protein kinase C, Ca^{2+}, Neuroprotective drug, Adrenal chromaffin cell

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