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Destabilization of $Na_v1.7$ Sodium Channel α-Subunit mRNA by Constitutive Phosphorylation of Extracellular Signal-Regulated Kinase: Negative Regulation of Steady-State Level of Cell Surface Functional Sodium Channels in Adrenal Chromaffin Cells

TOSHIHIKO YANAGITA, HIDEYUKI KOBAYASHI, YASUHITO UEZONO, HIROKI YOKOO, TAKASHI SUGANO, TOMOKAZU SAITOH, SHIN-ICHI MINAMI, SEIJI SHIRAISHI, and AKIHIKO WADA

Department of Pharmacology, Miyazaki Medical College, Miyazaki, Japan

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ABSTRACT

In cultured bovine adrenal chromaffin cells expressing $Na_v1.7$ isoform of voltage-dependent Na^+ channels, treatment (≥6 h) with serum deprivation, PD98059, or U0126 increased cell surface [^{3}H]saxitoxin ([^{3}H]STX) binding by ~58% ($t_{1/2}$ = 12.5 h), with no change in the K_d value. Immunoblot analysis showed that either treatment attenuated constitutive phosphorylation of extracellular signal-regulated kinase (ERK) 1 and ERK2 but not of p38 mitogen-activated protein kinase and c-Jun N-terminal kinase (JNK) 1 and JNK2. The increase of [^{3}H]STX binding and the attenuated phosphorylation of ERK1 and ERK2 returned to the control nontreated levels after the addition of serum or the washout of PD98059- or U0126-treated cells. Simultaneous treatment of serum deprivation with PD98059 or U0126 did not produce an additional increasing effect on [^{3}H]STX binding, compared with either treatment alone. In cells subjected to either treatment, veratridine-induced maximum $^{22}Na^+$ influx was augmented by ~47%, with no change in the EC_{50} value; *Ptychodiscus brevis* toxin-3 enhanced veratridine-induced $^{22}Na^+$ influx by 2-fold, as in nontreated cells. Serum deprivation, PD98059, or U0126 increased Na^+ channel α- but not β_1-subunit mRNA level by ~50% between 3 and 24 h; cycloheximide, an inhibitor of protein synthesis, increased α-subunit mRNA level and nullified additional increasing effect of either treatment on α-subunit mRNA level. Either treatment prolonged half-life of α-subunit mRNA from 17.5 to ~26.3 h without altering α-subunit gene transcription. Thus, constitutively phosphorylated/activated ERK destabilizes Na^+ channel α-subunit mRNA via translational event, which negatively regulates steady-state level of α-subunit mRNA and cell surface expression of functional Na^+ channels.

Density and activity of cell surface voltage-dependent Na^+ channels are finely regulated via as yet unknown mechanisms to meet development, differentiation, and survival of excitable cells (Linsdell and Moody, 1995). Aberrant properties of Na^+ currents are attributed to the dysregulated expression of Na^+ channel gene family in dorsal root ganglion (DRG) neurons (Waxman et al., 1994, 2000). Dysregulated expression of Na^+ channels is associated with abnormal excitability of cells in hypoxia/ischemia-induced injury (Urenjak and Obrenovitch, 1996), seizure (Xia et al., 2000; Isom, 2001), fatal cardiac arrhythmia (Goldin, 2001), and intolerable pain (Waxman et al., 2000; Isom, 2001). To understand the molecular basis for these physiological and pathological events, it is essential to explore the mechanisms whereby cell surface expression of Na^+ channels is regulated.

Na^+ channels consist of the principal α-subunit (~260 kDa), which may be associated with a noncovalently attached β_1-subunit (~36 kDa), and a disulfide-linked β_2-subunit

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ABBREVIATIONS: DRG, dorsal root ganglion; APP, amyloid precursor protein; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl ester; 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 α-subunit; JNK, c-Jun N-terminal kinase; kb, kilobase(s); KRP, Krebs-Ringer phosphate; MAPK, mitogen-activated protein kinases; MEK, MAPK/ERK kinase; nt, nucleotides; p38, p38 mitogen-activated protein kinase; pBII, plasmid Bluescript II; PbTx-3, *Ptychodiscus brevis* toxin-3; PKC, protein kinase C; RTK, receptor tyrosine kinase; SSC, saline-sodium citrate; STX, saxitoxin; TTX, tetrodotoxin; PD98059, 2′-amino-3′-methoxyflavone; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; SP600125, anthra[19-*cd*]pyrazol-6(2H)-one; U0126, 14-diamino-23-dicyano-14-*bis*(2-aminophenylthio)butadiene; A23187, calcimycin.

(~33 kDa) in some tissues and species (Goldin, 2001; Isom, 2001). The α-subunit is composed of four homologous domains (I–IV), each containing six transmembrane segments (S1–S6), and forms the ion-pore and the toxin binding sites [e.g., site 1 for tetrodotoxin (TTX)/saxitoxin (STX), site 2 for veratridine, and site 5 for *Ptychodiscus brevis* toxin-3 (PbTx-3)] (Cestèle and Catterall, 2000). The α-subunit arises from nine different genes and their alternative splicing (Goldin, 2001). The β_1- and β_2-subunits are type 1 transmembrane proteins containing a single transmembrane segment (Goldin, 2001; Isom, 2001). The β_1-subunit is encoded by a single gene, and the β_2-subunit is expressed only in brain.

In adrenal chromaffin cells (embryologically derived from the neural crest), α-subunit of Na^+ channels is the TTX/STX-sensitive human neuroendocrine type Na^+ channel α-subunit (hNE-Na) (Goldin, 2001). hNE-Na is the human homolog (~93% identity of amino acid sequence) of rat peripheral nerve type 1 Na^+ channel α-subunit and rabbit Schwann cell Na^+ channel α-subunit; they belong to the same α-subunit subfamily termed $Na_v1.7$, which is encoded by the gene SCN9A (Goldin, 2001). Our previous studies showed that cyclic AMP-dependent protein kinase (Yuhi et al., 1996), or insulin receptors, a member of receptor tyrosine kinase (RTK) family (Yamamoto et al., 1996), up-regulated cell surface expression of Na^+ channels without changing Na^+ channel α- and β_1-subunit mRNA levels. A slowly developing sustained moderate increase of cytoplasmic Ca^{2+} down-regulated Na^+ channels via promoting endocytic internalization of cell surface Na^+ channels; in addition, an immediate monophasic Ca^{2+} increase followed by the sustained plateau increase down-regulated Na^+ channels via lowering Na^+ channel α- and β_1-subunit mRNA levels (Shiraishi et al., 2001a). Calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase 2B, or the FK506 binding protein- and rapamycin-associated protein (FRAP), a serine/threonine protein kinase, up-regulated Na^+ channels via modulating cell surface externalization and internalization of Na^+ channels (Shiraishi et al., 2001b). Protein kinase C (PKC) down-regulated Na^+ channels via PKC isoform-specific mechanisms; conventional PKC-α promoted endocytic internalization of Na^+ channels, whereas novel PKC-ϵ accelerated degradation of α-subunit mRNA and decreased its level without altering α-subunit gene transcription (Yanagita et al., 1996, 1999, 2000). It is, however, unknown whether mitogen-activated protein kinases (MAPK), a family of serine/threonine protein kinases, modulate density and activity of Na^+ channels at any given tissue.

The mammalian MAPK, consisting of extracellular signal-regulated kinase (ERK), p38 MAPK (p38), and c-Jun N-terminal kinase (JNK), play crucial roles in various physiological and pathological states (Nozaki et al., 2001; Pearson et al., 2001). Each member of MAPK is activated by the phosphorylation of its tyrosine and serine/threonine residues, which is catalyzed by its own highly selective upstream MAPK kinase (MAPKK) family. ERK1 and ERK2 are activated by mitogenic stimuli, such as serum and growth factors, mainly via the cell surface RTK-Ras-MAPK/ERK kinase (MEK) pathway. In addition, ERK1 and ERK2 are activated by numerous neurotransmitters/hormones acting at G protein-coupled receptors and ligand-gated ion channels, and by cell adhesion molecules (Cox and Parsons, 1997; Bobrovskaya et al., 2001; Dudek and Fields, 2001; Pearson et al., 2001; Howe et al., 2002), Ca^{2+} (Agell et al., 2002), as well as by action potentials (Dudek and Fields, 2001). Our present study shows that chronic treatment of cultured bovine adrenal chromaffin cells with serum deprivation, PD98059 or U0126, an inhibitor of MEK (English and Cobb, 2002), decreased constitutive phosphorylation of ERK1/ERK2 (but not p38 and JNK1/JNK2), thereby increasing cell surface expression of functional Na^+ channels. It was associated with the increased level of Na^+ channel α- but not β_1-subunit mRNA, which was due to the increased stability of α-subunit mRNA, but not to the increased transcription of α-subunit gene.

Materials and Methods

Materials. Eagle's minimum essential medium was obtained from Nissui Seiyaku (Tokyo, Japan). Calf serum and nicotine were obtained from Nacalai Tesque (Kyoto, Japan). Actinomycin D, cytosine arabinoside, cycloheximide, TTX, ouabain, BAPTA-AM, and EGTA were obtained from Sigma-Aldrich (St. Louis, MO). PD98059, SB203580, and brain-derived neurotrophic factor were obtained from Calbiochem-Novabiochem (San Diego, CA). SP600125 was obtained from BIOMOL Reseach Laboratories (Plymouth Meeting, PA). Nerve growth factor 2.5S was obtained from Becton Dickinson Labware (San Jose, CA). TRIzol reagent was obtained from Invitrogen (Carlsbad, CA). Oligotex-dT30<Super>, and mini Quick Spin RNA columns were obtained from Roche Diagnostics (Tokyo, Japan). BcaBEST labeling kit and Noninterfering protein assay kit were obtained from Takara (Kyoto, Japan). Rabbit polyclonal antibodies raised against either ERK, p38, or JNK, and mouse monoclonal anti-phosphotyrosine ERK antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Rabbit polyclonal anti-phosphotyrosine/serine/threonine p38 antibody and anti-phosphotyrosine/serine/threonine JNK antibody, RQ1 RNase-free DNase, proteinase K, and U0126 were purchased from Promega (Madison, WI). [^{3}H]STX (20–40 Ci/mmol), ^{125}I-labeled donkey anti-rabbit IgG, ^{125}I-labeled sheep anti-mouse IgG, [α-^{32}P]dCTP (>3000 Ci/mmol), and [α-^{32}P]UTP (800 Ci/mmol) were obtained from PerkinElmer Life Sciences (Boston, MA). The Rapid-hyb buffer was purchased from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). cDNA for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from BD Biosciences Clontech (Palo Alto, CA). Plasmid Bluescript II (pBII) was purchased from Stratagene (La Jolla, CA). Plasmids containing hNE-Na cDNA, and rat brain Na^+ channel β_1-subunit cDNA were generously donated by Drs. F. Hofmann (Technischen Universität München) and Y. Oh (University of Alabama), respectively (Yamamoto et al., 1996; Yanagita et al., 1999, 2000; Shiraishi et al., 2001a,b).

Primary Culture of Adrenal Chromaffin Cells and Test Treatment. Isolated bovine adrenal chromaffin cells were cultured (4×10^6/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 (Yanagita et al., 1996, 1999, 2000). The cells were exposed to normal fresh medium or serum-free fresh medium (serum deprivation treatment) or treated without or with PD98059 or U0126 in normal fresh medium for up to 48 h, 3 days after plating. When effects of PD98059, U0126, SB203580, SP600125, cycloheximide, and actinomycin D were examined in normal medium or serum-free medium, these test compounds were dissolved in dimethyl sulfoxide (DMSO), the final concentration of DMSO in the test medium being ~0.25%. Treatment of chromaffin cells with 0.25% DMSO for 48 h did not alter [^{3}H]STX binding, immunoreactive ERK1 and ERK2 levels, as well as Na^+ channel α- and β_1-subunit mRNA levels, compared with nontreated cells. When chromaffin cells were purified by differential plating (Yamamoto et al., 1996), relative abundance of α- and β_1-subunit mRNAs/GAPDH

mRNA, as well as cellular levels of immunoreactive MAPK were similar between conventional and purified adrenal chromaffin cells.

[^{3}H]STX Binding. Cells were washed with ice-cold Krebs-Ringer phosphate (KRP) buffer (154 mM NaCl, 5.6 mM KCl, 1.1 mM $MgSO_4$, 2.2 mM $CaCl_2$, 0.85 mM NaH_2PO_4, 2.15 mM Na_2HPO_4, 5 mM glucose, and 0.5% bovine serum albumin, pH 7.4), and incubated with 1 to 25 nM [^{3}H]STX in 1 ml of KRP buffer at 4°C for 15 min in the absence (total binding) and presence (nonspecific binding) of 1 μM TTX (Yamamoto et al., 1996; Yanagita et al., 1996, 2000; Yuhi et al., 1996; Shiraishi et al., 2001a,b). 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.

$^{22}Na^+$ Influx. $^{22}Na^+$ influx was measured by incubating the cells with 2 μCi ^{22}NaCl at 37°C for 5 min in 1 ml of KRP buffer in the absence or presence of veratridine, ouabain, PbTx-3, and nicotine. The cells were washed with ice-cold KRP buffer, solubilized in 10% Triton X-100, and counted for radioactivity (Wada et al., 1986, 1992; Yamamoto et al., 1996, 1997; Yanagita et al., 1996; Yuhi et al., 1996; Shiraishi et al., 2001a,b).

Immunoblot. Cells were washed with ice-cold Ca^{2+}-free phosphate-buffered saline, and solubilized at 95°C for 3 min in 500 μl of 2× 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 15 h with antibodies raised against MAPK. After repeated washings, the immunoreactive bands were labeled with ^{125}I-anti-mouse lgG (1/1000) or ^{125}I-anti-rabbit lgG (1/1000), and analyzed by a bioimage analyzer BAS 2000 (Fuji Film, Tokyo, Japan).

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× saline-sodium citrate (SSC; 1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate) overnight, and cross-linked using a UV cross-linker (Funakoshi, Tokyo, Japan).

Northern Blot. Plasmids containing hNE-Na cDNA, and β_1-subunit cDNA were digested, respectively, with *Mun*I, and *Sac*II plus *Hin*dIII, to obtain nucleotide (nt) fragments for α-subunit (nt 1365–2948) and β_1-subunit (nt 457–790). These cDNA fragments and GAPDH cDNA (1.1 kilobase pairs) were labeled with [α-^{32}P]dCTP using BcaBEST labeling kit. The membrane was prehybridized and then hybridized with hNE-Na probe at 65°C for 4 h in the Rapid-hyb buffer. It was washed in 0.2 × SSC containing 0.1% SDS for 30 min twice and subjected to autoradiography. The same membrane was successively hybridized with probes for β_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.

Nuclear Run-On Assay. Cells were washed twice with ice-cold phosphate-buffered saline, dislodged, and centrifuged at 500*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*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 (1.2×10^7/100 μl) were incubated at 30°C for 30 min with 100 μl of buffer C (10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 200 mM KCl, 2 mM dithiothreitol, 0.5 mM ATP, CTP, and GTP, and 200 μCi [α-^{32}P]UTP), after which DNA was digested by exposing to 2 U of RQ1 RNase-free DNase for 10 min at 30°C. Proteins were digested in 200 μl of buffer D (20 mM Tris-HCl, pH 7.4, 10 mM EDTA, 20% SDS, and 200 μg/ml proteinase K) at 50°C for 1 h. 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 (5×10^6 cpm/ml) were hybridized overnight at 70°C in Rapid-hyb buffer with nylon membrane immobilizing 10 μg of 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 (Yanagita et al., 1999). The membrane was sequentially washed in 2× SSC containing 0.1% SDS at 65°C for 15 min, 2× SSC containing 10 μg/ml RNase A at 37°C for 10 min, 0.2× SSC containing 0.1% SDS at 65°C for 10 min, and then subjected to autoradiography.

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.

Results

Up-Regulation of Cell Surface [^{3}H]STX Binding by Serum Deprivation. Cells were treated without or with serum deprivation for up to 48 h, and [^{3}H]STX binding was assayed (Fig. 1A). Serum deprivation increased [^{3}H]STX binding by 10 and 25% at 6 and 12 h, causing the maximum plateau increase of ~58% between 24 and 48 h ($t_{1/2}$ = 12.5 h). When cells were treated with serum deprivation for the first 24 h, then exposed to serum (Fig. 1A, arrow), [^{3}H]STX binding gradually decreased toward the control level of nontreated cells between 30 and 48 h. Scatchard plot analysis (Fig. 1B) shows that 24-h treatment with serum deprivation significantly increased the B_{max} values from 58.3 ± 4.8 to 88.6 ± 5.2 fmol/4 × 10^6 cells without altering the K_d values (4.3 ± 0.5 nM, nontreated cells; 4.6 ± 0.5 nM, serum deprivation-treated cells; n = 3).

Immunoblot Analysis of ERK, p38, and JNK: Serum Deprivation-Induced Selective Decrease of Constitutive Phosphorylation of ERK1 and ERK2. Cells were

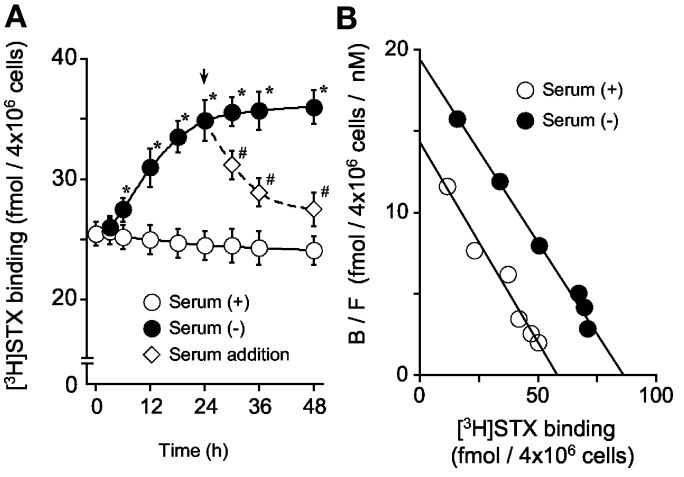

Fig. 1. Serum deprivation-induced time-dependent increase of cell surface [^{3}H]STX binding in cultured bovine adrenal chromaffin cells. A, cells were treated without or with serum deprivation for up to 48 h and subjected to [^{3}H]STX binding assay at the indicated times. In a parallel study, cells were initially treated with serum deprivation for 24 h, then exposed to serum (indicated by arrow) for up to 48 h, and subjected to [^{3}H]STX binding assay at 30, 36, and 48 h. Mean ± S.E.M. (n = 5). *, $P <$ 0.05, compared with serum-exposed cells; #, $P < 0.05$, compared with serum deprivation-treated cells. B, Scatchard plot analysis of a typical [^{3}H]STX binding assay data obtained in cells treated without or with serum deprivation for 24 h.

treated without or with serum deprivation for up to 24 h, and the cell lysates were subjected to immunoblot analysis for the measurement of phosphorylation levels and cellular levels of MAPK (Fig. 2A). In control cells incubated in serum-containing medium, ERK1 and ERK2 (top panel, upper part), p38 (middle panel, upper part), as well as JNK1 and JNK2 (bottom panel, upper part) were constitutively phosphorylated throughout the 24-h incubation period (lanes 1, 2, 4, 6, 8, 10, and 12). Serum deprivation caused a rapid (<15 min) and sustained (>24 h) decrease in the phosphorylation of ERK1 and ERK2 (top panel, upper part; lanes 3, 5, 7, 9, 11, and 13), with no change in the cellular levels of ERK1 and ERK2 (top panel, lower part; lanes 1–13). Quantification of these immunoreactive bands (Fig. 2B) shows that serum deprivation equipotently decreased phosphorylation of ERK1 and ERK2. The phosphorylation was rapidly decreased to 50% at 15 min, which was followed by the smaller, but sustained (>24 h) reduction. In contrast, serum deprivation slightly increased phosphorylation levels of p38, as well as JNK1 and JNK2 at ~3 h (Fig. 2A, middle and bottom panels, upper parts; and Fig. 2B), with no change in their cellular levels (Fig. 2A, middle and bottom panels, lower parts).

Serum deprivation selectively caused a rapid (<15 min) and sustained (>24 h) decrease in the phosphorylation levels of ERK1 and ERK2 (Fig. 2, A and B), which was followed by a gradual (~6 h) increase of [^{3}H]STX binding (Fig. 1A). In addition, serum deprivation-induced increase of [^{3}H]STX binding gradually returned to the control level between 6 and 24 h after the addition of serum (Fig. 1A, arrow). Thus, we examined whether addition of serum could also recover the phosphorylation of ERK1 and ERK2 to the control levels (Fig. 2C). Cells were treated with or without serum for the first 24 h, and then each group of cells was exposed to serum-containing medium or serum-free medium for 1 h; phosphor-

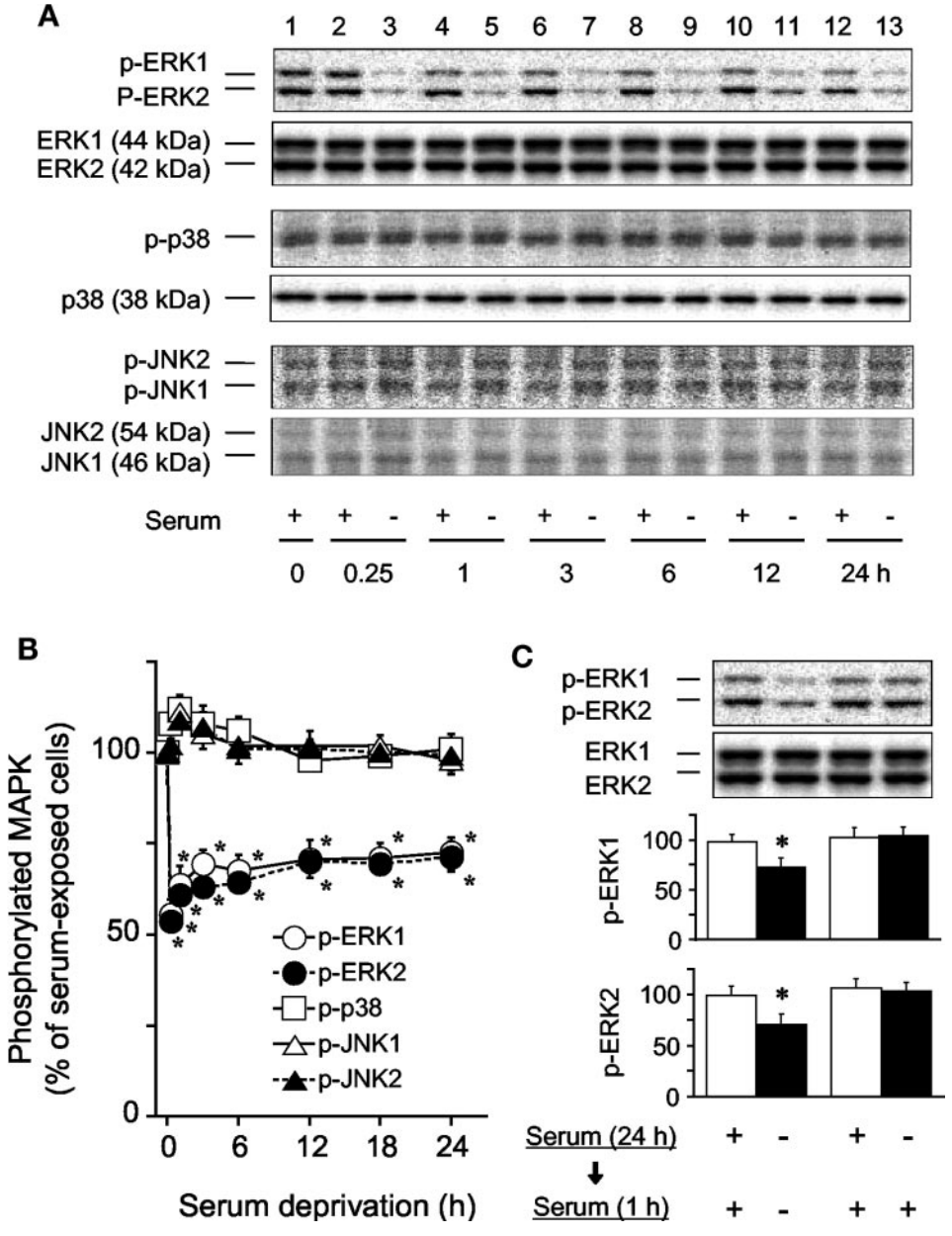

Fig. 2. Immunoblot analysis of constitutive phosphorylation of MAPK: serum deprivation-induced selective attenuation of ERK1 and ERK2 phosphorylation. A, cells were incubated with (+) or without (−) serum for up to 24 h; the cell lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred to membrane. The membrane was subjected to immunoblot analysis by using antibody raised against either phosphorylated ERK (p-ERK), ERK (top panels); p-p38, p38 (middle panels); or p-JNK, JNK (bottom panels). B, immunoreactivities of phosphorylated forms of MAPK in panel A were quantified by a bioimage analyzer. C, cells were treated with (+) or without (−) serum for 24 h, then exposed to serum-containing medium (+) or serum-free medium (−) for 1 h, and subjected to immunoblot analysis. Immunoblot data are typical from five independent experiments with similar results. A value of 100% represents phosphorylation level of MAPK in serum-exposed cells at each incubation time. Mean ± S.E.M. (n = 5). *, $P < 0.05$, compared with serum-exposed cells.

ylation levels of ERK1 and ERK2 were completely returned to the control levels within 1 h in serum-containing medium.

PD98059 and U0126: Concentration- and Time-Dependent Selective Blockade of Constitutive Phosphorylation of ERK1 and ERK2. Because serum deprivation-induced increase of [^{3}H]STX binding was associated with the selective reduction of constitutive phosphorylation of ERK1 and ERK2, we examined whether PD98059 or U0126 may selectively block constitutive phosphorylation of ERK1 and ERK2 in a concentration-dependent manner and whether its concentration-dependent inhibition may be accompanied by the concentration-dependent increase of [^{3}H]STX binding. PD98059 and U0126 have been used as inhibitors of ERK1 and ERK2 (English and Cobb, 2002). Previous in vivo and in vitro studies suggested that PD98059 or U0126 inhibited phosphorylation/activation of ERK1 and ERK2 by MEK presumably via the multiple mechanisms (Pereira et al., 2002), which include the inhibition of MEK activity and inhibition of MEK phosphorylation/activation by MEK kinase (English and Cobb, 2002). In bovine adrenal chromaffin cells, previous immunoblot analysis showed that either nicotinic receptor stimulation, depolarizing concentration of high K^+, Ca^{2+} ionophore A23187, or angiotensin II increased phosphorylation of ERK1 and ERK2 by ~9-fold; PD98059 (1–50 μM) or U0126 (~10 μM) almost completely blocked the enhanced phosphorylation of ERK1 and ERK2 but exhibited a much smaller inhibitory effect on basal phosphorylation of ERK1 and ERK2 (Cox and Parsons, 1997; Bobrovskaya et al., 2001). As shown in Fig. 3A, adrenal chromaffin cells were treated without or with 1 to 100 μM PD98059 or 1 to 100 μM U0126 for 15 min, and MAPK were subjected to immunoblot analysis. Quantification of these immunoreactive bands (Fig. 3, B and C) shows that PD98059 or U0126 blocked constitutive phosphorylation of ERK1 and ERK2 in a concentration-dependent manner with IC_{50} of 50 or 10 μM. In contrast, PD98059 or U0126 did not change constitutive phosphorylation of p38, as well as JNK1 and JNK2. Figure 3D shows that cells were treated without or with 50 μM PD98059 or 10 μM U0126 for up to 24 h and subjected to immunoblot analysis. PD98059 or U0126 attenuated constitutive phosphorylation of ERK1 and ERK2 by approximately 50% between 1 and 24 h. As shown in Fig. 3E, cells were treated without or with PD98059 or U0126 for 24 h, then washed, and incubated for 1 h in the absence or presence of either test compound; phosphorylation levels of ERK1 and ERK2 returned to the control nontreated levels within 1 h in the absence of either test compound.

Concentration- and Time-Dependent Up-Regulation of [^{3}H]STX Binding by PD98059 and U0126 but Not by SB203580 and SP600125: No Additional Increasing Effect of Serum Deprivation. Because PD98059 or U0126 caused a selective and sustained (>24 h) blockade of constitutive phosphorylation of ERK1 and ERK2 in a concentration-dependent manner, we then examined whether PD98059 or U0126 could increase [^{3}H]STX binding capacity. Figure 4A shows that treatment with PD98059 or U0126 for 24 h raised [^{3}H]STX binding by ~50 to ~58% in a concentration-dependent manner with EC_{50} of 2.2 or 6.4 μM. These EC_{50} values of PD98059 and U0126 to increase [^{3}H]STX binding were slightly different from the IC_{50} values of PD98059 (50 μM) and U0126 (10 μM) to attenuate tyrosine phosphorylation of ERK1 and ERK2. Tyrosine phosphorylation of ERK1 and ERK2, however, may not be precisely correlated to the enzyme activity of ERK1 and ERK2. It has been shown that phosphorylation of both tyrosine and threonine residues of ERK2 is essential and sufficient to the activation of ERK2; dephosphorylation of either residue inactivates ERK2 (Ferrell and Bhatt, 1997; Pearson et al., 2001). In addition, Ferrell and Bhatt (1997) demonstrated that ERK2 is first phosphorylated usually, but not invariably, at the tyrosine residue; the monophosphorylated ERK2 then dissociates from MEK and reassociates with MEK to undergo the secondary phosphorylation.

Figure 4B shows that 50 μM PD98059 or 10 μM U0126 elevated [^{3}H]STX binding by 11 and 25% at 6 and 12 h, causing the maximum plateau rise of 48% between 24 and 48 h ($t_{1/2}$ = 10.6 h). Cells were initially treated with PD98059 or U0126 for 24 h, then washed (Fig. 4B, arrow) and incubated in the absence of PD98059 or U0126; [^{3}H]STX binding lowered toward control level of nontreated cells between 30 and 48 h, consistent with the restoration of ERK1 and ERK2 phosphorylation levels to the control nontreated levels after the washout of PD98059- or U0126-treated cells (Fig. 3E). Scatchard plot analysis (Fig. 4C) shows that PD98059 (50 μM for 24 h) or U0126 (10 μM for 24 h) increased the B_{max} values from 58.9 ± 5.2 to 87.2 ± 4.8 or 89.5 ± 4.1 fmol/4 × 10^6 cells, without altering the K_d values (4.4 ± 0.4 nM, nontreated cells; 4.6 ± 0.5 nM, PD98059-treated cells; 4.5 ± 0.3 nM, U0126-treated cells; n = 3).

With respect to the attenuation of constitutive phosphorylation of ERK1 and ERK2 (Figs. 2 and 3), and the increase of [^{3}H]STX binding (Figs. 1 and 4), the abilities of serum deprivation were comparable with those of 50 μM PD98059 and 10 μM U0126. Thus, we examined whether serum deprivation-induced increase of [^{3}H]STX binding may be attributed exclusively to the attenuation of constitutive phosphorylation of ERK1 and ERK2 or may involve other intracellular signaling pathway(s) (Fig. 4D). Treatment for 24 h with serum deprivation, 50 μM PD98059 or 10 μM U0126 increased [^{3}H]STX binding, whereas concurrent treatment of serum deprivation with either PD98059 or U0126 did not produce additional increasing effect on [^{3}H]STX binding, compared with either treatment alone. SB203580, an inhibitor of p38, or SP600125, an inhibitor of JNK, did not increase [^{3}H]STX binding and did not alter serum deprivation-induced rise of [^{3}H]STX binding. Thus, these results implicate that serum deprivation, PD98059 or U0126 increases [^{3}H]STX binding via a similar mechanism, and the attenuation of constitutive phosphorylation of ERK1 and ERK2 by either treatment is causally related to the up-regulation of [^{3}H]STX binding. Taken together, our present study suggests that in normal extracellular milieu, constitutive phosphorylation of ERK1 and ERK2 negatively regulates cell surface expression of Na^+ channels, thereby maintaining the steady-state density of Na^+ channels at the physiological level.

It is known that ERK1 and ERK2 are phosphorylated by various growth factors contained in serum (Pearson et al., 2001). In our present study, the increase of [^{3}H]STX binding caused by serum deprivation was reversible after the addition of serum (Fig. 1A). Little is known, however, about the concentrations of growth factors in serum. Therefore, we added back conventional experimental concentrations of various growth factors to serum-free medium and examined which growth factor(s) causes phosphorylation of ERK1 and ERK2, thus lowering serum deprivation-induced increase of [^{3}H]STX binding to the level obtained in serum-containing

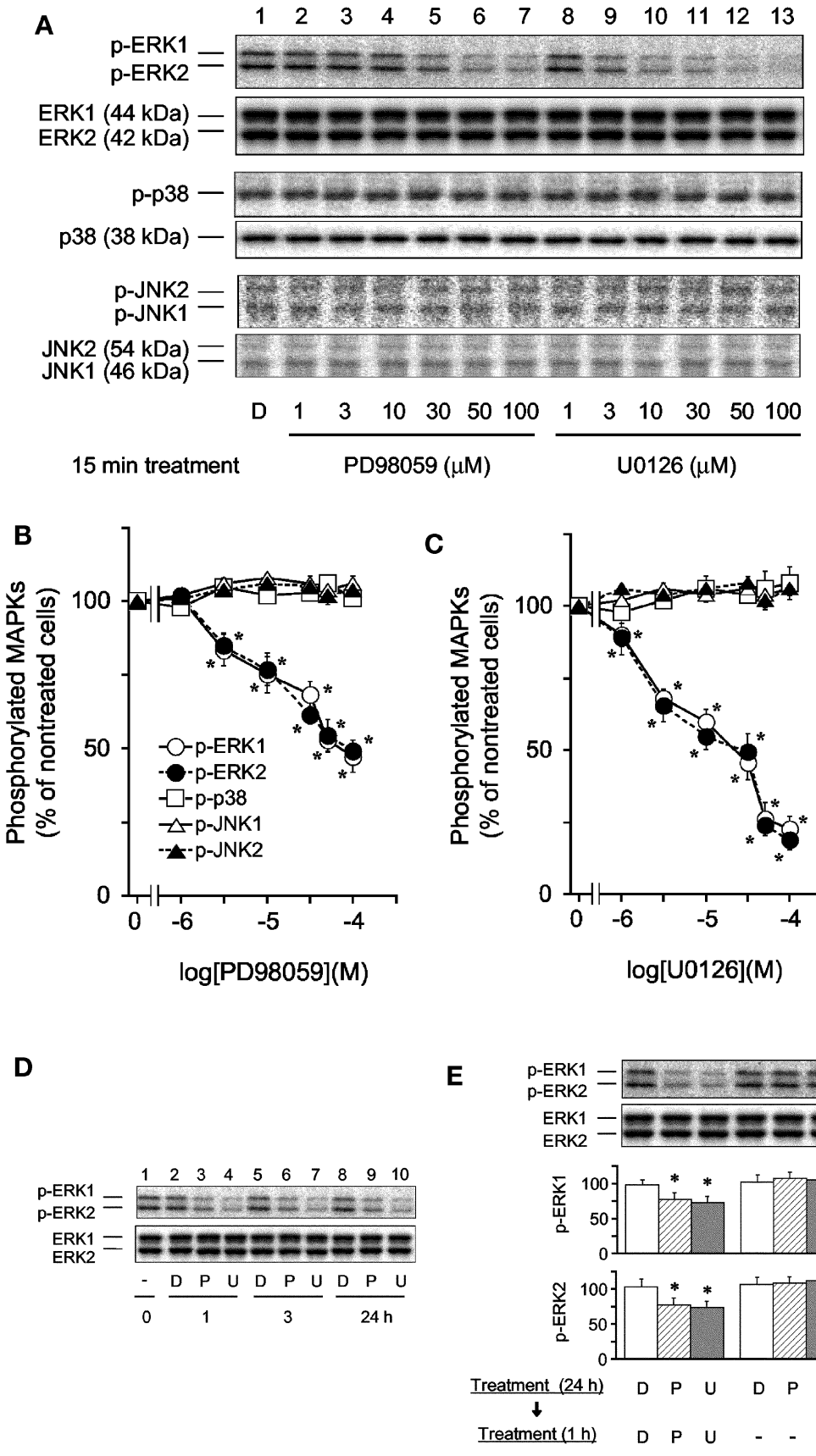

Fig. 3. Selective blockade of ERK1 and ERK2 phosphorylation by PD98059 and U0126: concentration-dependent sustained blockade. A, cells were treated without (DMSO, D) or with 1 to 100 μM PD98059 or 1 to 100 μM U0126 for 15 min; cell lysates were subjected to immunoblot analysis of MAPK (Fig. 2, legend). B and C, immunoreactivities of phosphorylated forms of MAPK in panel A were quantified by a bioimage analyzer. D, cells were treated without (−) or with DMSO (D), 50 μM PD98059 (P), or 10 μM U0126 (U) for up to 24 h and subjected to immunoblot analysis. E, cells were treated with DMSO (D), 50 μM PD98059 (P), or 10 μM U0126 (U) for 24 h, then washed with culture medium three times, and incubated for 1 h in the presence (D, P, and U) or absence (−) of either treatment before immunoblot analysis. Immunoblot data are typical from five independent experiments with similar results. Mean ± S.E.M. (n = 5). *, $P < 0.05$, compared with nontreated cells.

medium. In serum-free medium, a 1-h treatment with nerve growth factor (50 ng/ml) or brain-derived neurotrophic factor (50 ng/ml) increased phosphorylation of ERK1 and ERK2; in addition, phosphorylation level was ~25% greater when compared with the constitutive phosphorylation of ERK1 and ERK2 in serum-containing medium. Unexpectedly, 24 h of treatment with either growth factor in serum-free medium rather increased per se [^{3}H]STX binding; its extent was ~25% greater, compared with serum deprivation-induced increase of [^{3}H]STX binding.

Evidence has accumulated that phosphorylation and dephosphorylation of ERK1 and ERK2 are regulated by intracellular Ca^{2+} signaling pathways (Dudek and Fields, 2001; Agell et al., 2002). Therefore, we employed EGTA or BAPTA-AM, a cell membrane-impermeable or -permeable Ca^{2+} chelator and examined whether intracellular Ca^{2+} may contribute to the down-regulation of Na^+ channels caused by the constitutive phosphorylation of ERK1 and ERK2. Treatment of adrenal chromaffin cells with 5 mM EGTA or 50 μM BAPTA-AM for 24 h did not alter [^{3}H]STX binding (fmol/4 $\times$ 10^6 cells; 25.4 $\pm$ 1.5, nontreated cells; 24.8 $\pm$ 1.7 EGTA-treated cells; 25.2 $\pm$ 1.6, BAPTA-AM-treated cells; n = 3), whereas the same treatment with EGTA or BAPTA-AM completely prevented reduction of [^{3}H]STX binding caused by the concurrent 24-h treatment with A23187 or with thapsigargin, an inhibitor of sarco(endo)plasmic reticulum Ca^{2+}-ATPase (Shiraishi et al., 2001a).

Up-Regulation of $^{22}Na^+$ Influx via Na^+ Channels in Cells Treated with Serum Deprivation, PD98059, and U0126: No Effect of Either Treatment on $^{22}Na^+$ Influx via Nicotinic Receptor-Associated Cation Channels. In adrenal chromaffin cells, our previous studies showed that veratridine-induced Na^+ influx via Na^+ channels is indispensable to the gating of voltage-dependent Ca^{2+} channels, a

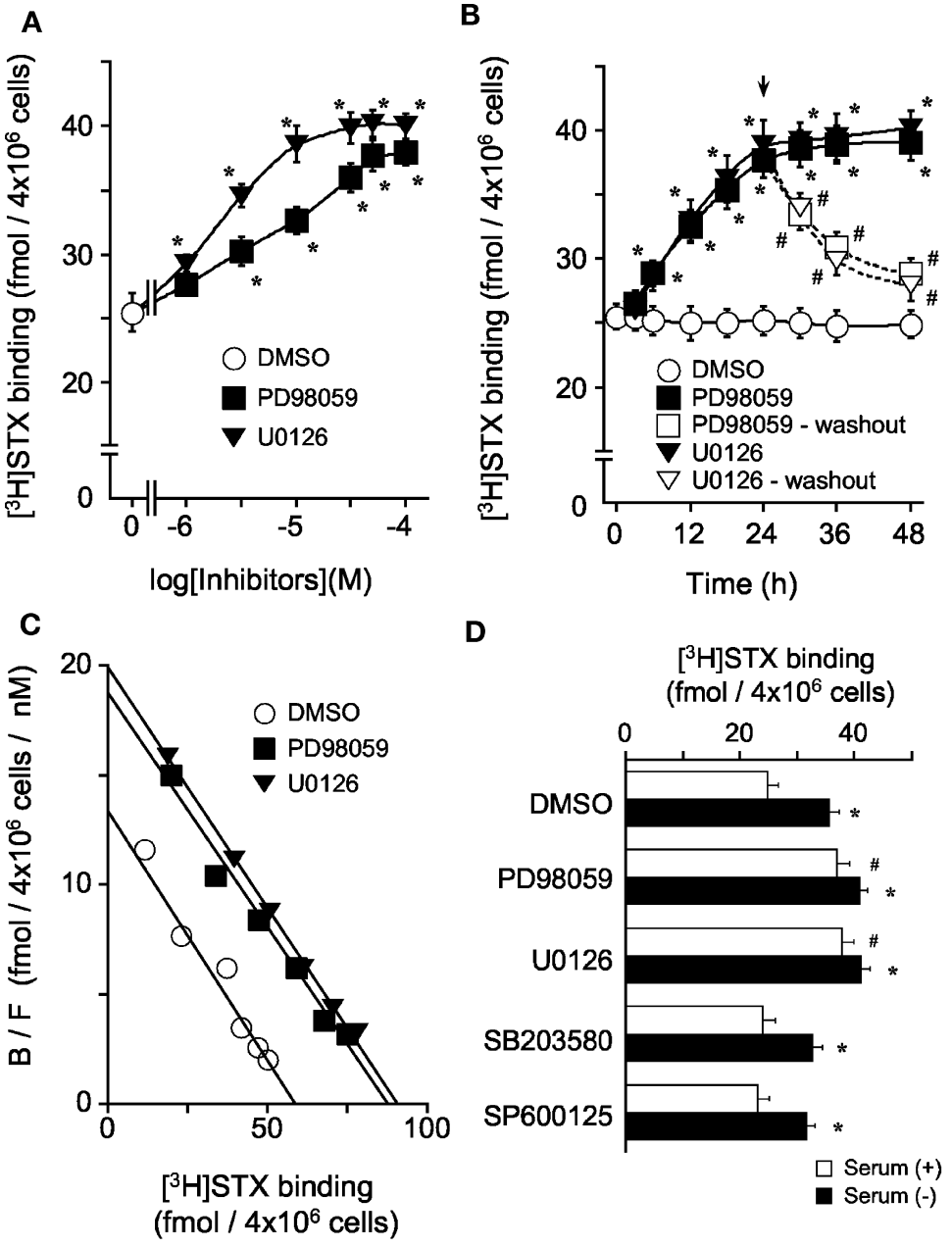

Fig. 4. Concentration- and time-dependent increase of cell surface [^{3}H]STX binding by PD98059 and U0126 but not by SB203580 and SP600125: no additional increasing effect between serum deprivation, PD98059, and U0126. A, cells were treated without or with indicated concentrations of PD98059 or U0126 for 24 h and subjected to [^{3}H]STX binding. B, cells were treated without or with 50 μM PD98059 or 10 μM U0126 for up to 48 h, and subjected to [^{3}H]STX binding assay at the indicated times. In parallel study, cells were initially treated with PD98059 or U0126 for 24 h; the cells were washed with culture medium three times (indicated by arrow), then incubated without PD98059 or U0126 for up to 48 h, and subjected to [^{3}H]STX binding assay at 30, 36, and 48 h. Mean $\pm$ S.E.M. (n = 5). *, $P < 0.05$, compared with nontreated cells; #, $P < 0.05$, compared with test drug-treated cells. C, Scatchard plot analysis of typical [^{3}H]STX binding assay data obtained in cells treated without or with 50 μM PD98059 or 10 μM U0126 for 24 h. D, in the presence (open columns) or absence (closed columns) of serum, cells were treated without (DMSO) or with 50 μM PD98059, 10 μM U0126, 50 μM SB203580, or 10 μM SP600125 for 24 h, and subjected to [^{3}H]STX binding assay. Mean $\pm$ S.E.M. (n = 3). *, $P < 0.05$, compared with cells not subjected to test treatment; #, no significant difference within each cell group.

prerequisite for exocytic secretion of catecholamines (Wada et al., 1992). Also, up-regulation of Na^+ channels caused by cyclic AMP-dependent protein kinase (Yuhi et al., 1996), insulin (Yamamoto et al., 1996), calcineurin inhibitor (Shiraishi et al., 2001b), and FRAP inhibitor (Shiraishi et al., 2001b) enhanced veratridine-induced $^{22}Na^+$ influx, $^{45}Ca^{2+}$ influx and catecholamine secretion. Figure 5A shows that cells were treated without or with serum deprivation, 50 μM PD98059, or 10 μM U0126 for 24 h, and $^{22}Na^+$ influx was assayed in the absence or presence of veratridine, a toxin acting at site 2 in segment 6 of domain I (DIS6) of Na^+ channel α-subunit (Cestèle and Catterall, 2000). In adrenal chromaffin cells, veratridine causes a persistent influx of $^{22}Na^+$ for at least 5 min that passes through TTX/STX-sensitive Na^+ channels (Wada et al., 1992). In cells treated with serum deprivation, PD98059 or U0126, veratridine ($\geqq$30 μM)-induced $^{22}Na^+$ influx was augmented by ~47%, with no change in EC_{50} values of veratridine (85 μM, nontreated cells; 91 μM, serum deprivation-treated cells; 84 μM, PD98059-treated cells; 79 μM, U0126-treated cells). Our previous study showed that Na^+ influx increases the activity of Na^+,K^+-ATPase, whereby Na^+, once it enters chromaffin cells, is continuously pumped out (Wada et al., 1986). Figure 5B shows that ouabain at 100 μM, a concentration at which ouabain totally inhibits the activity of Na^+,K^+-ATPase (Wada et al., 1986), increased accumulation of $^{22}Na^+$, and it was not changed by serum deprivation, PD98059, or U0126. In the presence of ouabain, however, veratridine (100 μM)-induced $^{22}Na^+$ influx occurred to a greater extent in cells treated with serum deprivation, PD98059, or U0126, compared with nontreated cells.

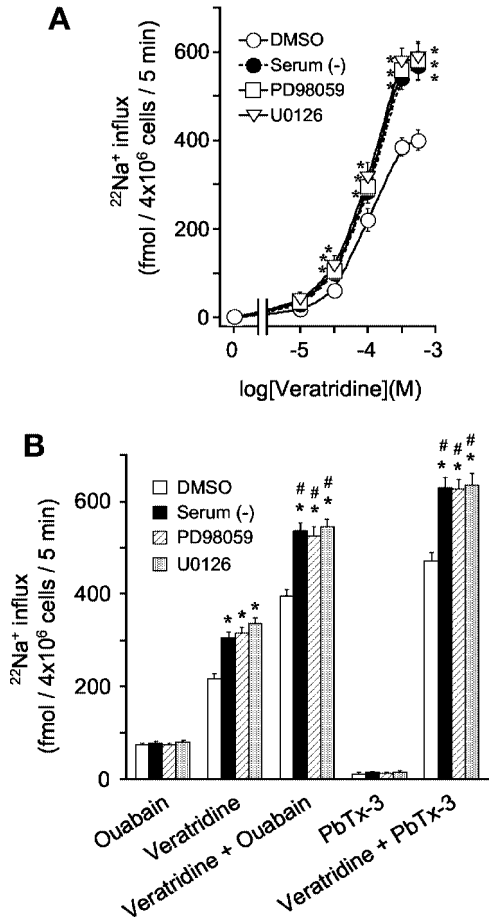

Fig. 5. $^{22}Na^+$ influx measured in the absence and presence of veratridine, ouabain, and PbTx-3: up-regulation in cells treated with serum deprivation, PD98059, and U0126. Cells were treated without (DMSO) or with 50 μM PD98059 or 10 μM U0126 in serum-containing medium or with DMSO plus serum deprivation for 24 h. $^{22}Na^+$ influx was assayed by incubating the cells with 2 μCi $^{22}NaCl$ for 5 min in the absence or presence of 1 to 500 μM veratridine (A), or 100 μM ouabain, 100 μM veratridine and/or 1 μM PbTx-3 (B). Basal $^{22}Na^+$ influx value at 37°C (nmol/4 $\times$ 10^6 cells/5 min) was not changed in cells treated with serum deprivation (18.7 $\pm$ 1.6), PD98059 (18.5 $\pm$ 2.1), or U0126 (18.9 $\pm$ 1.8), compared with nontreated cells (18.8 $\pm$ 1.6). These basal values are subtracted from the data. Mean $\pm$ S.E.M. (n = 5). *, $P < 0.05$, compared with nontreated cells; #, $P < 0.05$, compared with veratridine alone.

In adrenal chromaffin cells, our previous studies showed that nicotinic receptor agonists rapidly increase $^{22}Na^+$ influx within 1 min, which passes through nicotinic receptor-associated cation channels (but not through voltage-dependent Na^+ channels) and gates voltage-dependent Ca^{2+} channels (Wada et al., 1986; Yamamoto et al., 1997). In the present study, we examined whether treatment of adrenal chromaffin cells with serum deprivation, 50 μM PD98059 or 10 μM U0126 for 24 h could alter the activity of nicotinic receptor-associated cation channels. Nicotine (100 μM)-induced $^{22}Na^+$ influx (fmol/4 $\times$ 10^6 cells/1 min) was not changed by either treatment (225.8 $\pm$ 14.2, nontreated cells; 218.9 $\pm$ 15.6, serum deprivation-treated cells; 228.4 $\pm$ 14.6, PD98059-treated cells; 230.5 $\pm$ 19.4, U0126-treated cells; n = 3).

Up-Regulation of Na^+ Channel α- but Not β_1-Subunit mRNA Level in Cells Treated with Serum Deprivation, PD98059, and U0126: Effect of Cycloheximide. Cells were treated without or with serum deprivation, 50 μM PD98059, or 10 μM U0126 for up to 24 h, and the steady-state levels of Na^+ channel α- and β_1-subunit mRNAs were measured by Northern blot analysis (Fig. 6A). Evidence has accumulated that Na^+ channel β-subunit family regulates gating and cell surface expression of Na^+ channels; in particular, Na^+ channel β-subunit family is structurally similar to the immunoglobulin superfamily of cell adhesion molecules, and functions as a cell adhesion molecule to interact with adhesion molecules (e.g., neurofascin) and extracellular matrix proteins (e.g., tenascin), as well as intracellular scaffold proteins (e.g., ankyrin) (Goldin, 2001; Isom, 2001). It is noted that cell-to-cell adhesion enhances activation of ERK pathway initiated by RTK, and ERK pathway is impaired in cells held in suspension, compared with cells anchored to cell adhesion molecules (Howe et al., 2002); however, it is unclear whether this adhesion-dependent mechanism also operates in the constitutive phosphorylation of ERK1 and ERK2 in quiescent cells. In our present study, hNE-Na probe hybridized to one major (~9.4 kb) and two minor (~11.0 and ~7.0 kb) transcripts; β_1-subunit probe hybridized to a single (~1.5 kb) transcript, as reported previously (Yamamoto et al., 1996, 1997; Yanagita et al., 1999, 2000; Shiraishi et al.,

2001a,b). Levels of α- (~9.4 kb) and β_1-subunit mRNAs were normalized against that of GAPDH mRNA (Fig. 6, B and C). Serum deprivation, PD98059 or U0126 increased α- but not β_1-subunit mRNA level by ~15% as early as 3 h, causing the maximum plateau increase of ~53% between 12 and 24 h ($t_{1/2}$ = 6.1 h).

Steady-state level of mRNA is dependent on gene transcription, processing of heterogeneous nuclear RNA to mRNA and mRNA degradation. These transcriptional and post-transcriptional events are regulated by constitutively expressed or stimuli-inducible *trans*-acting nucleotide binding proteins that shuttle between nucleus and cytoplasm (Shyu and Wilkinson, 2000; Guhaniyogi and Brewer, 2001). Thus, we examined whether ERK pathway-induced up-regulation of α-subunit mRNA level 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 (Yanagita et al., 1999). Figure 7 shows that treatment with cycloheximide alone for 12 h increased α-subunit mRNA (~9.4 kb) level by 2.4-fold, in agreement with our previous study that cycloheximide increased α-subunit mRNA level by ~2.4-fold while decreasing Na^+ channel β_1-subunit mRNA level by ~41% between 3 and 24 h (Yanagita et al., 1999). In the presence of cycloheximide, however, serum deprivation, 50 μM PD98059, or 10 μM U0126 failed to exert additional increasing effect on α-subunit mRNA level.

Increased Stability of Na^+ Channel α-Subunit mRNA in Cells Treated with Serum Deprivation, PD98059, and U0126: No Effect on α-Subunit Gene Transcription. Cells were treated without or with serum deprivation, 50 μM PD98059, or 10 μM U0126 for 6 h, and the transcription rate of α-subunit gene was measured by nuclear run-on assay (Yanagita et al., 1999). Figure 8A shows that either treatment did not alter the transcription rate of α-subunit gene.

We then measured the degradation rate of α-subunit mRNA by using actinomycin D, an inhibitor of RNA synthesis. Figure 8B shows that cells were treated for the first 6 h without or with serum deprivation, 50 μM PD98059 or 10 μM U0126, then exposed to actinomycin D in the continuous absence or presence of either test treatment, and subjected to Northern blot analysis at the indicated times. Serum deprivation, PD98059 or U0126 elongated half-life ($t_{1/2}$) of α-subunit mRNA (~ 9.4 kb) from 17.5 to ~26.3 h.

Discussion

Treatment (≥6 h) of adrenal chromaffin cells with serum deprivation, 50 μM PD98059, or 10 μM U0126 increased

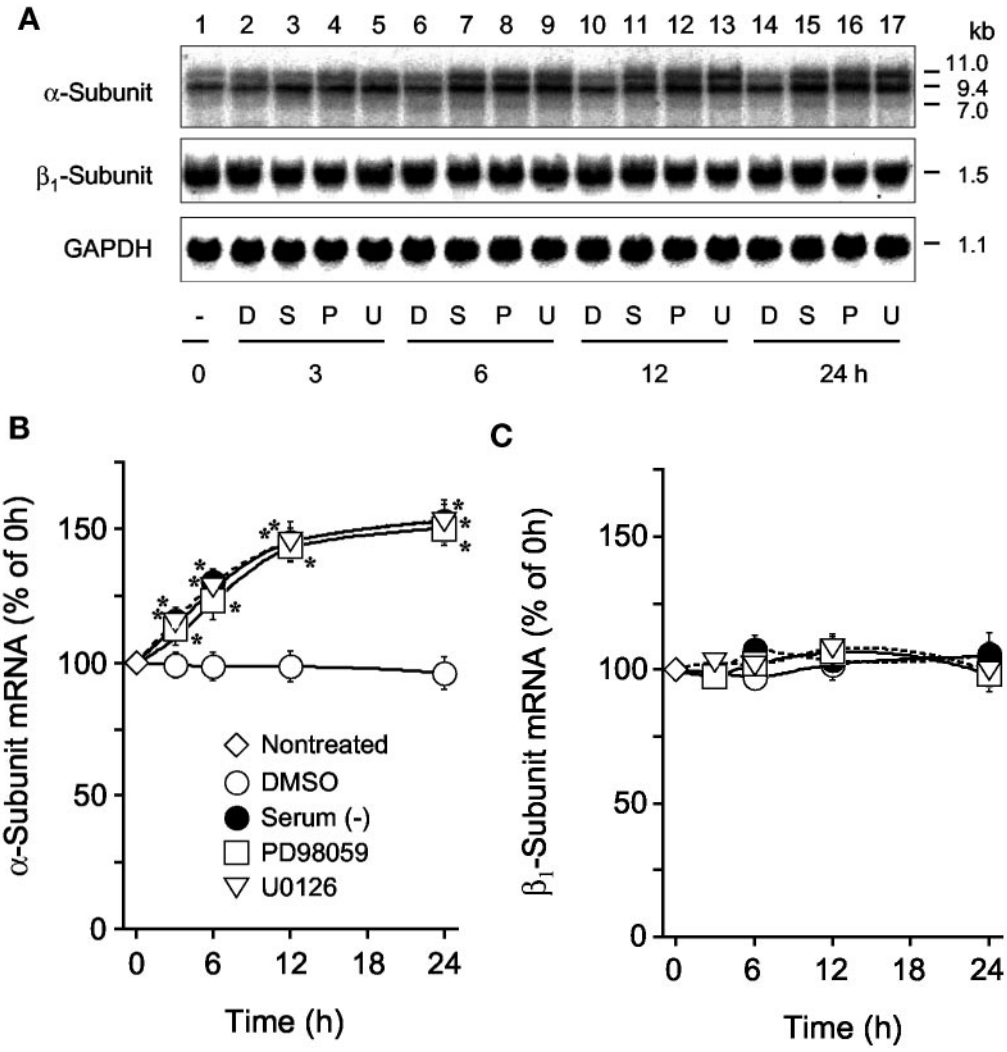

Fig. 6. Northern blot analysis: up-regulation of Na^+ channel α- but not β_1-subunit mRNA level in cells treated with serum deprivation, PD98059, and U0126. A, cells were treated without (−) or with DMSO (D), DMSO plus serum deprivation (S), 50 μM PD98059 (P), or 10 μM U0126 (U) for up to 24 h; poly(A)$^+$ RNA was extracted, electrophoresed, and transferred to membrane. The membrane was hybridized with each ^{32}P-labeled cDNA probe for hNE-Na (top), Na^+ channel β_1-subunit (middle), or GAPDH (bottom), after removing the former probe. B and C, levels of α-subunit mRNA (~9.4 kb), β_1-subunit mRNA, and GAPDH mRNA were quantified by a bioimage analyzer; relative level of α- or β_1-subunit mRNA/GAPDH mRNA is shown. The relative level in nontreated cells at 0 h is assigned a value of 100%. Mean ± S.E.M. (n = 3). *, $P < 0.05$, compared with nontreated cells.

B_{max} value of [^{3}H]STX binding by ~58% with no change in the K_d value. Serum deprivation, PD98059, or U0126 increased [^{3}H]STX binding in a time-dependent manner ($t_{1/2}$ = ~12.5 h); in either treatment, [^{3}H]STX binding developed into the almost maximum plateau increase between 24 and 48 h. In cells treated with serum deprivation, PD98059, or U0126, veratridine-induced maximum influx of $^{22}Na^+$ was augmented by ~47% with no change in the EC_{50} of veratridine. PbTx-3 potentiated veratridine-induced $^{22}Na^+$ influx by 2-fold in cells subjected to either treatment, as in nontreated cells. Thus, serum deprivation, PD98059, or U0126 causes up-regulation of functional Na^+ channels, their pharmacological properties, characterized by STX, veratridine, and PbTx-3 (Cestèle and Catterall, 2000), being similar to those of native Na^+ channels.

Serum deprivation, PD98059 or U0126 attenuated constitutive phosphorylation of ERK1 and ERK2 (but not p38, as well as JNK1 and JNK2). In addition, PD98059 or U0126 decreased constitutive phosphorylation of ERK1 and ERK2 in a concentration-dependent manner between 1 and 100 μM, a concentration range at which PD98059 or U0126 caused a concentration-dependent increase of [^{3}H]STX binding. Concurrent treatment of serum deprivation with either PD98059 or U0126 did not produce additional increasing effect on [^{3}H]STX binding, compared with either treatment alone. These correlative results implicate that up-regulation of cell surface Na^+ channels by serum deprivation, PD98059, or U0126 occurs via a similar mechanism and proceeds coincident with the attenuation of constitutive phosphorylation of ERK1 and ERK2.

In cells treated with serum deprivation, PD98059, or U0126, steady-state level of Na^+ channel α- but not β_1-subunit mRNA was increased by ~15% as early as 3 h, whereas [^{3}H]STX binding became increased by ~11% at 6 h. In cells subjected to either treatment, the α-subunit mRNA level further developed into the maximum plateau ~53% increase between 12 and 24 h, whereas [^{3}H]STX binding developed into the maximum plateau ~58% increase between 24 and 48 h. Serum deprivation, PD98059, or U0126 attenuated constitutive phosphorylation of ERK1 and ERK2

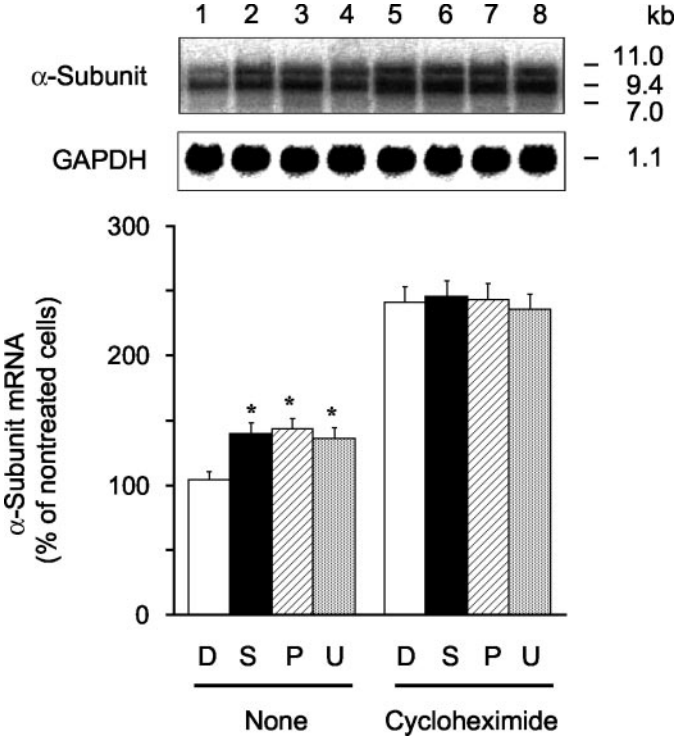

Fig. 7. Up-regulation by cycloheximide of Na^+ channel α-subunit mRNA level: no additional increasing effect of serum deprivation, PD98059, and U0126. In the absence (none) or presence of 10 μg/ml cycloheximide, cells were treated without or with DMSO (D), DMSO plus serum deprivation (S), 50 μM PD98059 (P), or 10 μM U0126 (U) for 12 h, and subjected to Northern blot analysis. Data show relative level of α-subunit mRNA (~9.4 kb)/GAPDH mRNA; a value of 100% represents the level obtained in nontreated cells. Mean ± S.E.M. (n = 3). *, $P < 0.05$, compared with DMSO-treated cells.

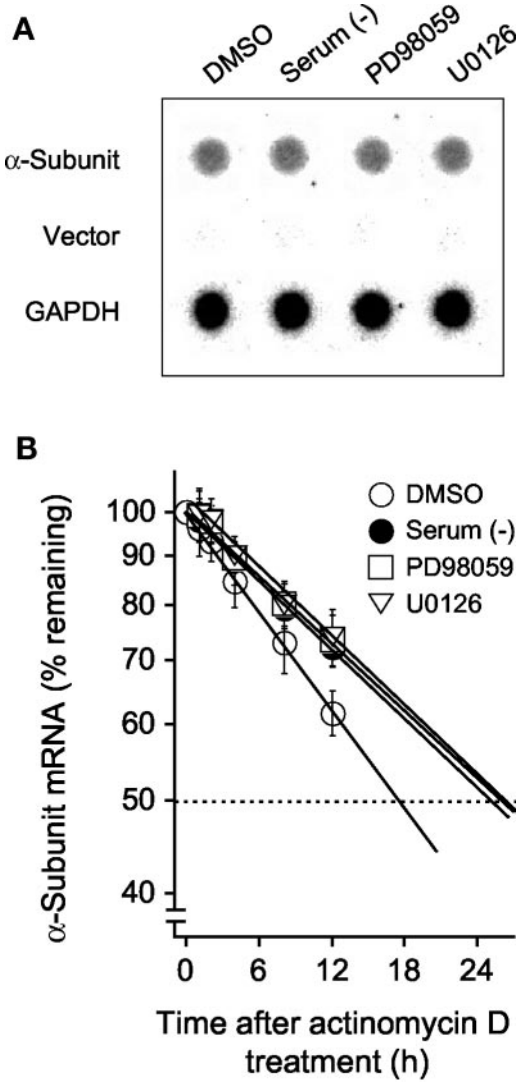

Fig. 8. Increased stability of Na^+ channel α-subunit mRNA in cells treated with serum deprivation, PD98059, and U0126: no effect on α-subunit gene transcription. A, cells were treated with DMSO, DMSO plus serum deprivation, 50 μM PD98059, or 10 μM U0126 for 12 h; nuclei were isolated and used for in vitro nuclear run-on assay using [α-^{32}P]UTP. ^{32}P-Labeled transcripts were purified and hybridized to 10 μg of pBII alone (vector), pBII containing hNE-Na cDNA (α-subunit), or GAPDH cDNA immobilized on membrane. Data are typical from three independent experiments with similar results. B, cells were pretreated with DMSO, DMSO plus serum deprivation, 50 μM PD98059, or 10 μM U0126 for 6 h (see Fig. 6B) and incubated with 10 μg/ml actinomycin D in the continuous absence or presence of either test treatment. At the indicated times, poly(A)$^+$ RNA was isolated and subjected to Northern blot analysis. The level of α-subunit mRNA (~9.4 kb) was quantified by a bioimage analyzer. Mean ± S.E.M. (n = 3).

as early as 15 min, when α-subunit mRNA level was not yet elevated by either treatment. These temporal and quantitative correlations among ERK phosphorylation, α-subunit mRNA level, and [^{3}H]STX binding suggest that attenuation of constitutive phosphorylation of ERK1 and ERK2 is intimately involved in the increase of α-subunit mRNA level, which contributes to the up-regulation of Na^+ channels. In cells treated with serum deprivation, PD98059, or U0126, $t_{1/2}$ of α-subunit mRNA was prolonged from 17.5 to ~26.3 h, whereas the transcription rate of α-subunit gene was not changed. Because mRNA stability is a major determinant in the control of gene expression (Guhaniyogi and Brewer, 2001), our results suggest that serum deprivation, PD98059, or U0126 retards degradation rate of α-subunit mRNA, thus leading to the increased steady-state level of α-subunit mRNA and the increased cell surface expression of Na^+ channels. Concentration-response curves of PD98059 and U0126 show that the attenuated extent of constitutive phosphorylation of ERK1 and ERK2 was inversely related to the increased extent of [^{3}H]STX binding. This inverse relation between ERK phosphorylation and [^{3}H]STX binding may support the notion that the phosphorylation level of ERK1 and ERK2 is tightly linked to the stability of α-subunit mRNA in a quantitative manner, thereby accommodating cell surface expression of Na^+ channels. In addition, the increase of [^{3}H]STX binding and the attenuated phosphorylation of ERK1 and ERK2 caused by serum deprivation, PD98059, or U0126 were rapidly reversible after the removal of either treatment. This observation raises the possibility that constitutive phosphorylation of ERK1 and ERK2, as well as stability of α-subunit mRNA may be regulated in a moment-to-moment manner.

Constitutively expressed and external stimuli-inducible *trans*-acting nucleotide-binding proteins in cytoplasm and nucleus bind to specific nucleotide *cis*-elements at the 3′- and 5′-untranslated regions, as well as coding region, thereby causing stabilization or destabilization of mRNA (Shyu and Wilkinson, 2000; Guhaniyogi and Brewer, 2001). In some mRNA (e.g., β-tubulin mRNA), nucleotide-binding proteins are encoded in their target mRNA, thus exerting translation-dependent autoregulation of mRNA levels (Guhaniyogi and Brewer, 2001). In our previous study, sustained gradual increase of α-subunit mRNA level by cycloheximide led us to consider that constitutively expressed nucleotide-binding protein(s) with a short half-life destabilizes α-subunit mRNA, thereby negatively regulating the steady-state level of α-subunit mRNA (Yanagita et al., 1999). Our present study showed that in the presence of cycloheximide, serum deprivation, PD98059, or U0126 failed to produce additional increasing effect on α-subunit mRNA level, compared with cycloheximide alone. The most straightforward interpretation of these results may be that reduction of constitutive phosphorylation of ERK1 and ERK2 by serum deprivation, PD98059, or U0126 accelerates the synthesis of protein(s) that stabilizes α-subunit mRNA, thus increasing the steady-state level of α-subunit mRNA. In addition, we could not exclude another possibility that serum deprivation, PD98059, or U0126 increased α-subunit mRNA level by a mechanism similar to that of cycloheximide; reduction of constitutive phosphorylation of ERK1 and ERK2 inhibits synthesis of short-lived protein(s) that destabilizes α-subunit mRNA. However, the situation may be more complicated. In addition to mRNA autoregulation, translation of mRNA is intimately linked to the stability of mRNA via complex and, as yet, not fully defined mechanisms, and mRNA turnover may not be precisely measured by using translation inhibitor; protein synthesis inhibitors, even if they inhibit translation of mRNA by different mechanisms, stabilize most mRNA via unknown mechanism(s) (Ross, 1997; Guhaniyogi and Brewer, 2001).

In PC12 cells, Lee and Malek (1998) showed that chronic treatment (~15 days) with nerve growth factor or basic fibroblast growth factor elongated $t_{1/2}$ of m_4 muscarinic receptor mRNA from 1.4 to ~5.6 h, and it was associated with ~4-fold increase in the number of cell surface binding sites of [^{3}H]quinuclidinyl benzilate, an antagonist of muscarinic receptors. Also, they observed that nerve growth factor-induced stabilization of m_4 mRNA was prevented by 50 μM PD98059 or cycloheximide. In peripheral blood mononuclear cells, Westmark and Malter (2001) documented that treatment with phorbol 12-myristate 13-acetate for ~4 h increased nucleolin mRNA level by ~2.5-fold, and it was prevented approximately 50% by ~20 μM U0126. Phorbol ester treatment elongated $t_{1/2}$ of nucleolin mRNA from 1.8 to 3.2 h and increased nucleolin protein level. Nucleolin bound to the instability *cis*-element in the 3′-untranslated region of Alzheimer's amyloid precursor protein (APP) mRNA, thus decreasing APP mRNA stability and APP protein synthesis. Our present results are in striking contrast to the previous one, because constitutive activity of the ERK pathway destabilizes α-subunit mRNA in quiescent cells, and negatively regulates steady-state levels of α-subunit mRNA and cell surface Na^+ channels in normal extracellular milieu.

Finally, we should raise possible biological significance of our present findings. In addition to generating action potentials, Na^+ influx via Na^+ channels regulated phosphorylation and dephosphorylation of ERK, thus directing genotypic and phenotypic events of excitable cells, such as DRG and hippocampal neurons (Dudek and Fields, 2001). Inappropriate up-regulation of Na^+ channels, and the failure of Na^+ channel down-regulation are responsible for hypoxia/ischemia-induced cell injury (Urenjak and Obrenovitch, 1996), seizure (Xia et al., 2000), intolerable pain (Waxman et al., 1994, 2000), and defective development of embryonic skeletal myocytes (Linsdell and Moody, 1995). Neonatal rat brain is more tolerable to hypoxia, compared with adult rat brain, and its hypoxia tolerance is supposed to be due to the lower density of brain Na^+ channels in the neonate than in adult rat (Urenjak and Obrenovitch, 1996). In DRG neurons, Leffler et al. (2002) documented that nerve growth factor, in cooperation with glial cell-derived neurotrophic factor, constitutively abrogated inappropriate expression of $Na_v1.3$ Na^+ channel gene in physiological condition; its dysregulated expression contributed to chronic pain associated with injury of sensory neurons. In addition, dysregulated expression of otherwise silent $Na_v1.8$ Na^+ channel gene was documented in cerebellar Purkinje cells from experimental mouse allergic encephalomyelitis and humans with multiple sclerosis, a neurodegenerative disease (Black et al., 2000). Thus, ERK pathway-induced 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.

Previous in vivo and in vitro studies have increasingly

shown that ERK pathway plays neuroprotective (Hu et al., 2000; Irving et al., 2000) and neurotoxic effects (Murray et al., 1998; Namura et al., 2001; Mori et al., 2002), depending on the types of cells and the kinds of insults employed (Nozaki et al., 2001). Thus, in vivo and in vitro studies documented that PD98059 and U0126 prevented neuronal injury due to excitotoxicity (Murray et al., 1998) and mechanical trauma (Mori et al., 2002), as well as ischemia-induced brain infarction (Namura et al., 2001). Although ERK pathway has been intensively studied in neuronal apoptosis and cerebral ischemia, the target molecules of ERK pathway are not yet defined (Nozaki et al., 2001). Our present study provides the first evidence that ERK pathway is constitutively involved in the surveillance of Na^+ channel α-subunit mRNA level, and negatively regulates cell surface expression of functional Na^+ channels, thereby determining the steady-state level of Na^+ channels.

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Address correspondence to: Akihiko Wada, Department of Pharmacology, Miyazaki Medical College, Kiyotake, Miyazaki 889-1692, Japan. E-mail: akihiko@fc.miyazaki-med.ac.jp