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Up-Regulation of Cell Surface Sodium Channels by Cyclosporin A, FK506, and Rapamycin in Adrenal Chromaffin Cells

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ABSTRACT

Treatment of cultured bovine adrenal chromaffin cells with cyclosporin A (CsA) increased cell surface [^{3}H]saxitoxin ([^{3}H]STX) binding by 56% in a time ($t_{1/2}$ = 15.2 h)- and concentration (EC_{50} = 2.9 μM)-dependent manner but did not change the K_d value. In CsA-treated cells, veratridine-induced $^{22}Na^+$ influx was augmented with no change in the EC_{50} of veratridine; also, α- and β-scorpion venom and *Ptychodiscus brevis* toxin-3 enhanced veratridine-induced $^{22}Na^+$ influx in a more than additive manner, as in nontreated cells. CsA treatment for 1 to 24 h inhibited calcineurin activity, measured by the in vitro assay, with the IC_{50} of 0.6 μM but did not alter cellular level of calcineurin. FK506 or rapamycin elevated [^{3}H]STX binding by 36 or 25%, whereas GPI-1046, an immunophilin ligand incapable to inhibit calcineurin, or okadaic acid, an inhibitor of protein phosphatases 1 and 2A, had no increasing effect. The rise of [^{3}H]STX binding by CsA was attenuated by the coincident treatment with brefeldin A (BFA), an inhibitor of vesicular exit from the *trans*-Golgi network. The internalization rate of cell surface Na^+ channels, as determined in the presence of BFA, was decreased in CsA (but not rapamycin)-treated cells ($t_{1/2}$ = 20.3 h), compared with nontreated cells ($t_{1/2}$ = 13.7 h). CsA treatment, however, did not elevate cellular levels of Na^+ channel α-subunit and Na^+ channel α- and β_1-subunit mRNAs. In CsA-treated cells, veratridine-induced $^{45}Ca^{2+}$ influx via voltage-dependent Ca^{2+} channels and catecholamine secretion were enhanced, whereas high K^+-induced $^{45}Ca^+$ influx was not. Thus, the inhibition of calcineurin or rapamycin-binding protein causes up-regulation of cell surface functional Na^+ channels via modulating externalization and internalization of Na^+ channels, thus enhancing Ca^{2+} channel gating and catecholamine secretion.

Immunophilins, the intracellular receptors for immunosuppressive drugs, are markedly enriched in the nervous system, where they regulate an increasing number of target proteins (Snyder et al., 1998). Complexes of cyclosporin A (CsA)-cyclophilin and FK506-FK506 binding protein (FKBP) inhibit calcineurin, a Ca^{2+}- and calmodulin-dependent protein phosphatase 2B, thereby rapidly modulating secretion of glutamate, norepinephrine, and gating kinetics of glutamate receptors, voltage-dependent Na^+ and Ca^{2+} channels (Yakel, 1997). Rapamycin-FKBP complex has no effect on calcineurin but suppresses the activity of FKBP- and rapamycin-associated protein (FRAP), a serine and threonine protein kinase, thus inhibiting translation of mRNAs (Snyder et al., 1998). Also, cyclophilin and FKBP have the peptidyl prolyl *cis-trans* isomerase (PPIase) activity catalyzing the rate-limiting reaction in the folding of various proteins; the inhibition of immunophilin's PPIase activity by CsA, FK506, or rapamycin decreased cell surface expression of homo-oligomeric α_7-neuronal nicotinic receptors, type 3 serotonin receptors, Kir2.1 potassium channels, and insulin receptors (Shiraishi et al., 2000).

Chronic treatment with immunosuppressants is frequently associated with serious neurotoxicity, such as seizure (Gijtenbeek et al., 1999), whereas immunosuppressants also display neuroprotective and neuroregenerative effects via as yet fully defined calcineurin-dependent and -independent mechanisms (Gold, 1997). Treatment with CsA, FK506, and rapamycin promotes neurite outgrowth and axonal myelination (Lyons et al., 1994; Steiner et al., 1997) and protects

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ABBREVIATIONS: CsA, cyclosporin A; FKBP, FK506 binding protein; FRAP, FKBP- and rapamycin-associated protein; STX, saxitoxin; TTX, tetrodotoxin; PbTx-3, *Ptychodiscus brevis* toxin-3; hNE-Na, TTX/STX-sensitive human neuroendocrine type Na^+ channel α-subunit; TGN, *trans*-Golgi network; ARF, ADP-ribosylation factor; BFA, brefeldin A; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PAGE, polyacrylamide gel electrophoresis; KRP, Krebs-Ringer phosphate; SDS, sodium dodecyl sulfate; SSC, saline-sodium citrate; PPIase, peptidyl prolyl *cis-trans* isomerase.

brain striatal lesions in animal models of Parkinson's disease (Kitamura et al., 1994; Matsuura et al., 1997), coincident with the increased expression of FKBP during neuronal regeneration (Lyons et al., 1995; Araki et al., 1999). However, the molecules involved in the immunosuppressant-induced neuronal modulation remain largely unknown.

Density and activity of voltage-dependent Na^+ channels play crucial roles in synaptogenesis, neuronal differentiation, epileptic seizure (Yamamoto et al., 1996; Yanagita et al., 2000), and neuronal injury (Urenjak and Obrenovitch, 1996; Cummins and Waxman, 1997). 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 (~33 kDa) in some tissues and species (Yamamoto et al., 1996; Yanagita et al., 2000). The α-subunits form the ion-pore and the toxin binding sites [site 1 for tetrodotoxin (TTX) and saxitoxin (STX); site 2 for veratridine; site 3 for α-scorpion toxin; site 4 for β-scorpion toxin; and site 5 for *Ptychodiscus brevis* toxin] (Wada et al., 1985, 1992) and arise from multiple genes and their alternative splicing. Structures of β_1-subunits are similar among various tissues, and β_2-subunit is cloned so far only in brain.

In adrenal chromaffin cells (embryologically derived from the neural crest), the α-subunit of Na^+ channels is homologous to the TTX- and STX-sensitive human neuroendocrine type Na^+ channel α-subunit (hNE-Na) (Klugbauer et al., 1995). Previous studies showed that veratridine-induced Na^+ influx via Na^+ channels and the subsequent depolarization increases Ca^{2+} influx via voltage-dependent Ca^{2+} channels, thereby triggering catecholamine secretion (Wada et al., 1985, 1992; López et al., 1995). Either α- or β-scorpion venom or *Ptychodiscus brevis* toxin-3 (PbTx-3) enhanced veratridine-induced Na^+ influx, Ca^{2+} influx, and catecholamine secretion (Wada et al., 1992). In cultured bovine adrenal chromaffin cells, we showed that cyclic AMP-dependent protein kinase (PKA) (Yuhi et al., 1996) or activation of insulin receptors (Yamamoto et al., 1996), members of receptor tyrosine kinases, up-regulates density of Na^+ channels without elevating Na^+ channel α- and β_1-subunit mRNA levels. In contrast, translocative activation of protein kinase C (PKC) from cytosol to membranes down-regulates Na^+ channels via PKC isozyme-specific mechanisms: conventional PKC-α promotes internalization of cell surface Na^+ channels, whereas novel PKC-ϵ decreases α-subunit mRNA level by shortening the half-life of α-subunit mRNA with no effect on α-subunit gene transcription (Yanagita et al., 2000). In the present study, we examined whether and how chronic treatment with CsA, FK506, and rapamycin could modulate cell surface [^{3}H]STX binding, cellular levels of Na^+ channel α-subunit, as well as Na^+ channel α- and β_1-subunit mRNAs. To characterize Na^+ channel function, we also evaluated the effects of CsA treatment on $^{22}Na^+$ influx, $^{45}Ca^{2+}$ influx, and catecholamine secretion caused by veratridine, α- and β-scorpion venom, and PbTx-3.

Experimental Procedures

Materials. Eagle's minimum essential medium was from Nissui Seiyaku (Tokyo, Japan). Calf serum was from Nacalai Tesque (Kyoto, Japan). CsA, cytosine arabinoside, veratridine, α-scorpion venom (*Leiurus quinquestriatus quinquestriatus*), β-scorpion venom (*Centruroides sculpturatus*), TTX, ouabain, and brefeldin A (BFA) were from Sigma (St. Louis, MO). PbTx-3 was from Latoxan (Westbury, NY). Rapamycin and okadaic acid were from Calbiochem-Novabiochem Corp. (San Diego, CA). FK506 and GPI-1046 [3-(3-pyridyl)-1-propyl (2*S*)-1-(3,3-dimethyl-1,2-dioxopentyl)-2-pyrrolidinecarboxylate] were kindly donated from Fujisawa Pharmaceutical Co. (Osaka, Japan) and Amgen Inc. (Thousand Oaks, CA), respectively. The serine and threonine phosphatase assay system was from Promega (Madison, WI). Mouse monoclonal antibody against calcineurin was from Transduction Laboratories (Lexington, KY). Rabbit polyclonal antibody against Na^+ channel α-subunit was from Upstate Biotechnology (Lake Placid, NY). TRIzol reagent was from Life Technologies (Rockville, MD). Oligotex-dT30<Super> was from Nippon Roche Co. (Tokyo, Japan). BcaBEST labeling kit and Noninterfering Protein Assay kit were from Takara (Kyoto, Japan). ^{125}I-Labeled sheep anti-mouse IgG, ^{125}I-labeled donkey anti-rabbit IgG, [^{3}H]STX (20–40 Ci/mol), ^{22}NaCl (6–17 Ci/mmol), $^{45}CaCl_2$ (0.5–2 Ci/mmol), and [α-^{32}P]dCTP (>4000 Ci/mmol) were from Amersham (Buckinghamshire, UK). cDNA for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was from Clontech Laboratories (Palo Alto, CA). Plasmids containing hNE-Na cDNA (Klugbauer et al., 1995) and rat brain Na^+ channel β_1-subunit cDNA (Oh and Waxman, 1994) were generously donated by Drs. F. Hofmann (Technischen Universität München, München, Germany) and Y. Oh (University of Alabama, Tuscaloosa, AL), respectively.

Primary Culture of Adrenal Chromaffin Cells and Drug Treatment. Isolated bovine adrenal chromaffin cells were cultured (4×10^6 per dish, Falcon; 35 mm in diameter) in Eagle's minimum essential medium containing 10% calf serum under 5% CO_2 and 95% air in a CO_2 incubator (Wada et al., 1985). Three days (60–62 h) after plating, the cells were treated in the fresh medium with or without 0.01 to 100 μM CsA, 100 nM FK506, 1 μM rapamycin, 100 nM GPI-1046, and 50 nM okadaic acid for up to 48 h in the absence and presence of BFA. The culture medium contained 3 μM cytosine arabinoside to suppress the proliferation of nonchromaffin cells; when chromaffin cells were further purified by differential plating (Yamamoto et al., 1996), the concentration-response curve of CsA for [^{3}H]STX binding increase was similar between the conventional and purified chromaffin cells.

Chronic treatment with CsA did not impair the cell viability because veratridine-induced $^{22}Na^+$ influx occurred to a greater extent in CsA (10 μM for 48 h)-treated cells than in nontreated cells, and catecholamine contents were comparable between nontreated and CsA (10 μM for 48 h)-treated cells.

[^{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 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; Yuhi et al., 1996). The cells were immediately washed, solubilized in 10% Triton X-100, and counted for radioactivity. Specific binding was calculated as the total binding minus nonspecific binding. A mere addition of 10 μM CsA, FK506, and rapamycin to the binding assay medium per se did not alter [^{3}H]STX binding.

Calcineurin Assay. Calcineurin activity was measured by a serine and threonine phosphatase assay system, according to the instruction from Promega, the manufacturer. Briefly, cells were incubated with or without various concentrations of CsA for up to 24 h in the culture medium, washed with 50 mM Tris-HCl buffer (pH 7.2), and homogenized by Polytron at 4°C for 30 s in the storage buffer [50 mM Tris-HCl (pH 7.2), 0.1 mM EGTA, 0.1% 2-mercaptoethanol, and 50% glycerol]. The homogenate was centrifuged at 100,000*g* for 1 h; the resultant supernatant was subjected to a Sephadex G-25 spin column to remove free phosphate, and the subsequent eluate was used as the enzyme preparation. Dephosphorylation of synthetic phosphopeptide was determined at 30°C for 20 min in the reaction buffer [250 mM imidazole (pH 7.2), 1 mM EGTA, 50 mM $MgCl_2$, 50

nM okadaic acid, and 0.1% 2-mercaptoethanol] supplemented with or without 5 mM $NiCl_2$ and 250 μg/ml calmodulin. Protein concentration was measured with the Noninterfering Protein Assay kit.

^{22}Na Influx, ^{45}Ca Influx, and Catecholamine Secretion. Cells were washed with KRP buffer and incubated with 2 μCi of $^{22}NaCl$ in 1 ml of KRP buffer at 37°C for 5 min with or without veratridine, α- and β-scorpion venom, PbTx-3, and ouabain; then they were washed, solubilized, and counted for radioactivity. Previous electrophysiological and $^{22}Na^+$ influx studies showed that whole venom from *Leiurus quinquestriatus quinquestriatus* (Catterall, 1976) and that from *Centruroides sculpturatus* (Meves et al., 1982) exert effects similar to those of their major α- and β-scorpion toxin, respectively. Addition of 10 μM CsA to the KRP buffer did not change by itself veratridine (100 μM)-induced $^{22}Na^+$ influx in the absence and presence of ouabain.

To measure $^{45}Ca^{2+}$ influx and catecholamine secretion, cells were incubated with 2 μCi of $^{45}CaCl_2$ for 5 min in 1 ml of KRP buffer with or without veratridine or for 1 min in high K^+ solution, in which NaCl was reduced to maintain the isotonicity of KRP buffer. Incubation medium was saved into a test tube for catecholamine (epinephrine plus norepinephrine) assay by high performance liquid chromatography (Yamamoto et al., 1996; Yuhi et al., 1996), and the cells were washed, solubilized, and counted for radioactivity (Wada et al., 1985, 1992).

Immunoblot. Adrenal chromaffin cells were washed with ice-cold Ca^{2+}-free phosphate-buffered saline and solubilized in 500 μl of 2× sodium dodecyl sulfate (SDS) electrophoresis sample buffer [125 mM Tris-HCl (pH 6.8), 20% glycerol, 10% 2-mercaptoethanol, and 4% SDS]. Rat brain was obtained by decapitation of Sprague-Dawley rats (8 weeks old; Charles River, Shizuoka, Japan), mixed with 10-fold volume of 2× SDS electrophoresis sample buffer, and homogenized. These samples were boiled for 3 min and used for the immunoblot analysis. Total quantities of cellular proteins, as estimated with the Noninterfering Protein Assay kit, were not changed between nontreated and CsA-treated cells; the same amounts of proteins (10–20 μg/lane) were separated by SDS-7.5% polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane. The membrane was preincubated at room temperature with 5% dry milk in phosphate-buffered saline and then reacted for 6 h with mouse monoclonal antibody raised against calcineurin or rabbit polyclonal antibody against Na^+ channel α-subunit. 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, Japan).

Northern Blot. Total cellular RNA was isolated from cells by acid guanidine thiocyanate-phenol-chloroform extraction using TRIzol reagent. Poly(A)$^+$ RNA was purified by Oligotex-dT30<Super>, electrophoresed on a 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). cDNA fragments of hNE-Na [nucleotides 435-2666] and of the β_1-subunit (nucleotides 457–790) were obtained according to Yanagita et al. (2000); they and GAPDH cDNA (1.1 kilobase pairs) were labeled with [α-^{32}P]dCTP using the BcaBEST labeling kit. The membrane was prehybridized at 42°C in 6× SSC, 10× Denhardt's (2% bovine serum albumin fraction V, 2% polyvinylpyrrolidone, and 2% Ficoll 400), 50% formamide, 0.5% SDS, and 50 μg/ml salmon sperm DNA, and then hybridized with the hNE-Na probe under the same condition for 18 h. It was washed at 55°C successively in 2×, 1×, and 0.2× SSC containing 0.1% SDS, each being performed for 30 min twice, and subjected to autoradiography. The same membrane was sequentially rehybridized with β_1-subunit and GAPDH probes after being thoroughly washed in 0.1% SDS at 100°C to remove the former probe. The autoradiogram was quantified by a Bioimage analyzer BAS 2000 (Fuji Film).

Statistical Methods. [^{3}H]STX binding was performed in triplicate, and all experiments were repeated at least three times (mean ± S.E.). 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

[^{3}H]STX Binding to Adrenal Chromaffin Cells Treated with CsA, FK506, Rapamycin, GPI-1046, and Okadaic Acid. When adrenal chromaffin cells were treated with 10 μM CsA for up to 48 h, cell surface [^{3}H]STX binding did not significantly increase for the first 6 h but elevated by 22% at 12 h, developing to the maximum plateau rise of 50 to 56% between 24 and 48 h (Fig. 1A). Treatment for 24 h with 1 to 100 μM CsA increased [^{3}H]STX binding in a concentration-dependent manner with the EC_{50} value of 2.9 μM (Fig. 1B). Scatchard plot analysis (Fig. 1C) revealed that CsA treatment (10 μM for 24 h) raised the B_{max} from 57.0 ± 3.2 to 82.5 ± 4.9 fmol/4 × 10^6 cells without altering the K_d values (3.7 ± 0.2 nM for nontreated cells, 4.0 ± 0.2 nM for CsA-treated cells; $n = 5$).

As shown in Fig. 1D, treatment with either 100 nM FK506

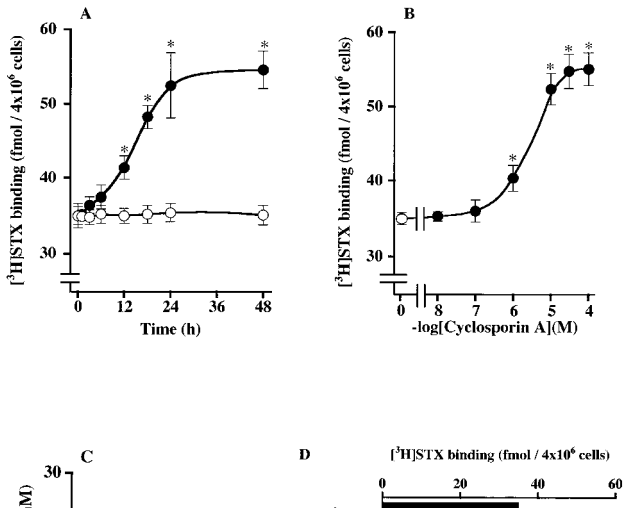

Fig. 1. Treatment of adrenal chromaffin cells with CsA, FK506, rapamycin, GPI-1046, and okadaic acid: the effects on cell surface [^{3}H]STX binding. Cultured cells (4×10^6) were treated without (○) or with (●) 10 μM CsA for the indicated periods (A), or 0.01 to 100 μM CsA for 24 h (B), then washed, and subjected to [^{3}H]STX binding assay. Mean ± S.E. ($n = 5$). C, Scatchard plot of [^{3}H]STX binding to the cells treated without (○) or with (●) 10 μM CsA for 24 h. Data are typical of one from five separate experiments with similar results. D, cells were treated without (Control) or with 100 nM FK506, 1 μM rapamycin, 100 nM GPI-1046, or 50 nM okadaic acid for 24 h and subjected to [^{3}H]STX binding assay. Mean ± S.E. ($n = 5$). *$P < 0.05$, compared with nontreated cells.

or 1 μM rapamycin for 24 h increased [^{3}H]STX binding by 36 or 25%. Because CsA, FK506, and rapamycin are known to inhibit PPIase activities of their cognate immunophilins (Snyder et al., 1998), we then evaluated whether the inhibition of PPIase activity may be involved in the immunosuppressant-induced increase of [^{3}H]STX binding. GPI-1046 is a synthetic immunophilin ligand that inhibits PPIase activity of FKBP with an IC_{50} of 7.2 nM but cannot bind to calcineurin, thus being incapable to suppress calcineurin activity (Snyder et al., 1998). Figure 1D shows that treatment with 100 nM GPI-1046 for 24 h failed to increase [^{3}H]STX binding. In contrast, treatment for 24 h with 50 nM okadaic acid, an inhibitor of protein phosphatases 1 and 2A, decreased [^{3}H]STX binding by 30%.

Effects of CsA Treatment on Calcineurin Activity and Cellular Level of Calcineurin. Because GPI-1046 failed to elevate [^{3}H]STX binding, we examined whether CsA treatment may inhibit calcineurin activity in adrenal chromaffin cells. As shown in Fig. 2, chromaffin cells were treated with or without the indicated concentrations of CsA for 24 h, and the partially purified cell lysates were subjected to the in vitro assay of calcineurin activity, using the synthetic phosphopeptide substrate. CsA treatment ($\geq$1 μM) significantly inhibited calcineurin activity in a concentration-dependent manner with an IC_{50} of 0.6 μM, and the maximum inhibition of 65% was obtained with CsA ($\geq$10 μM). When chromaffin cells were treated with or without 10 μM CsA for up to 12 h, calcineurin activity was suppressed by 84.2 $\pm$ 4.2, 84.0 $\pm$ 5.0, 81.2 $\pm$ 6.2, and 78.4 $\pm$ 4.8% at 1, 3, 6, and 12 h (n = 3), respectively, in CsA-treated cells, compared with nontreated cells. By using Western blot analysis, we also tested whether cellular level of calcineurin may be changed during the chronic treatment of chromaffin cells with 10 μM CsA. Figure 3 shows that anti-calcineurin antibody recognized one major (~61 kDa) band, consistent with the molecular size of calcineurin recognized with this antibody (Artalejo et al., 1996; the manufacturer's instruction). When the levels of calcineurin were quantified by a Bioimage analyzer, CsA treatment did not significantly alter cellular levels of calcineurin (99.1 $\pm$ 6.3, 105.2 $\pm$ 5.8, 103.7 $\pm$ 6.4, 98.2 $\pm$ 4.7, and 100.1 $\pm$ 3.2% of levels in nontreated cells, n = 3) at 3, 6, 12, 24, and 48 h, respectively.

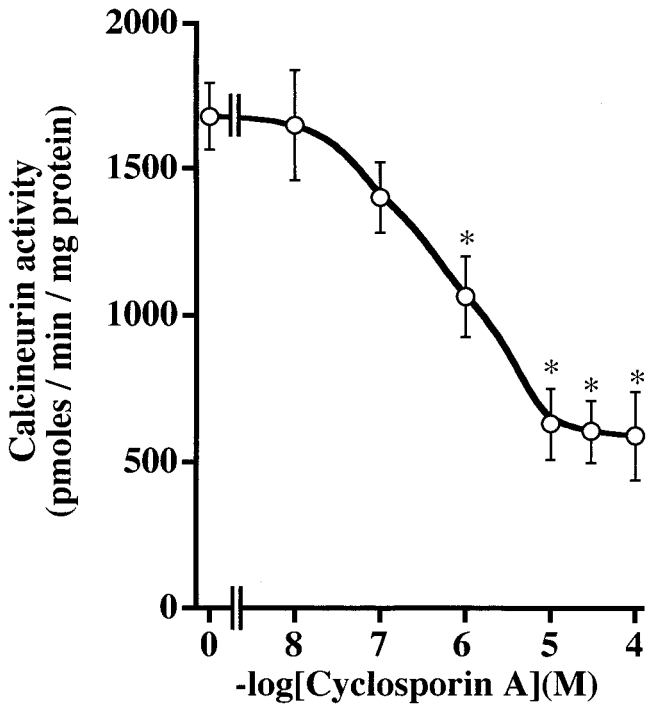

Fig. 2. Concentration-dependent inhibitory effect of CsA on calcineurin activity. Cells were incubated without or with the indicated concentrations of CsA for 24 h in the culture medium, washed with Tris-HCl buffer, and homogenized. The homogenate was centrifuged at 100,000g for 1 h, and the resultant supernatant was subjected to a Sephadex G-25 spin column to obtain the enzyme preparation. Calcineurin activity was determined by the difference in the amount of phosphate released from the synthetic phosphopeptide substrate in the absence and presence of $NiCl_2$ and calmodulin. Mean $\pm$ S.E. (n = 5). *$P < 0.05$, compared with nontreated cells.

Effect of CsA Treatment on Na^+ Channel Activity. We characterized the pharmacological properties of up-regulated Na^+ channels in CsA-treated cells by using veratridine, α- and β-scorpion venom, and PbTx-3 because cooperative activation caused by these distinct classes of toxins and venoms occurs in a Na^+ channel isoform-specific manner (Wada et al., 1992; Yamamoto et al., 1996). In adrenal chromaffin cells, veratridine, a toxin acting at site 2 in segment 6 of domain I (S6I) of Na^+ channel α-subunit (Trainer et al., 1996), causes a persistent influx of $^{22}Na^+$ for at least 5 min that passes through TTX- and STX-sensitive Na^+ channels (Wada et al., 1985, 1992). As shown in Table 1, CsA treatment (10 μM for 24 h) did not alter the basal $^{22}Na^+$ influx (Table 1, legend) but augmented veratridine-induced maximum influx of $^{22}Na^+$ by 30% without changing the EC_{50} value of veratridine. In adrenal chromaffin cells, Na^+ influx stimulates the activity of Na^+,K^+-ATPase, whereby Na^+, once it has entered chromaffin cells, is continuously pumped out (Wada et al., 1986). Table 1 shows that even in the presence of ouabain at 100 μM, a concentration that totally inhibits the activity of Na^+,K^+-ATPase (Wada et al., 1986), CsA treatment potentiated veratridine-induced maximum $^{22}Na^+$ influx by 21% but did not alter the EC_{50} value of veratridine.

As shown in Table 2, either α-scorpion venom, which binds to site 3 between S3IV and S4IV (Rogers et al., 1996), or β-scorpion venom, which interacts with site 4, or PbTx-3, which binds site 5 between S5IV and S6I (Trainer et al., 1994), had little effect per se but enhanced veratridine (30 μM)-induced $^{22}Na^+$ influx in a more than additive manner in CsA-treated cells, as in nontreated cells (Wada et al., 1992); also, the magnitudes of potentiation by α-scorpion venom (2.2-fold), β-scorpion venom (2.4-fold), and PbTx-3 (3.6-fold) were comparable between nontreated and CsA-treated cells. PbTx-3 in combination with α- or β-scorpion venom further

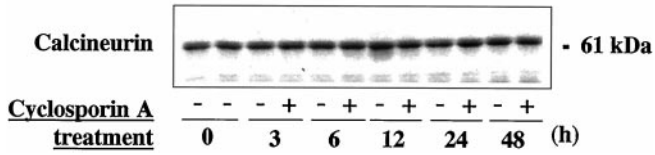

Fig. 3. Lack of effect of CsA treatment on cellular level of calcineurin. Adrenal chromaffin cells were treated without (−) or with (+) 10 μM CsA for up to 48 h and solubilized. The proteins were separated by SDS-7.5% PAGE, transferred to a membrane, and subjected to immunoblot analysis of calcineurin. Data are typical of one from three independent experiments with similar results.

TABLE 1

Effects of CsA treatment on $^{22}Na^+$ influx caused by veratridine in the absence and presence of ouabain

Cells were treated with or without 10 μM CsA for 24 h, then washed, and incubated with 2 μCi of $^{22}NaCl$ at 37°C for 5 min in the absence or presence of 1 to 560 μM veratridine and 100 μM ouabain. $^{22}Na^+$ influx values (nmol/4 $\times$ 10^6 cells/5 min; n = 5) at 37°C and ouabain alone were comparable between nontreated (18.9 ± 1.7; 73.8 ± 2.9) and CsA-treated cells (19.8 ± 1.6; 71.7 ± 2.7), and they were subtracted from the data. Mean ± S.E. (n = 5).

	$^{22}Na^+$ Influx	
	Nontreated Cells	Cyclosporin A-Treated Cells
Veratridine		
EC_{50} (μM)	110.0 ± 4.5	98.0 ± 6.6
Maximum (nmol/4 $\times$ 10^6 cells/5 min)	390.6 ± 8.8	509.5 ± 10.2[a]
Veratridine + ouabain (100 μM)		
EC_{50} (μM)	65.4 ± 2.0	63.0 ± 3.8
Maximum (nmol/4 $\times$ 10^6 cells/5 min)	712.5 ± 7.8	860.4 ± 12.4[a]

[a] $P < 0.05$, compared with nontreated cells.

TABLE 2

Cooperative enhancement of $^{22}Na^+$ influx by veratridine, α- and β-scorpion venom, and PbTx-3 in nontreated and CsA-treated cells

Cells were treated with or without 10 μM CsA for 24 h, washed, and incubated with 2 μCi of $^{22}NaCl$ at 37°C for 5 min in the absence or presence of 30 μM veratridine, 5 μg/ml α-scorpion venom, 0.5 μg/ml β-scorpion venom, and 1 μM PbTx-3. Mean ± S.E. (n = 5).

	$^{22}Na^+$ Influx	
	Nontreated Cells	Cyclosporin A-Treated Cells
	nmol/4 $\times$ 10^6 cells/5 min	
α-Scorpion venom (5 μg/ml)	5.8 ± 1.0	7.5 ± 2.0
β-Scorpion venom (0.5 μg/ml)	4.2 ± 0.7	5.9 ± 0.9
PbTx-3 (1 μM)	9.6 ± 4.8	12.6 ± 3.6
Veratridine (30 μM)	68.0 ± 4.1	115.0 ± 2.3[a]
α-Scorpion venom + veratridine	149.6 ± 6.5[b]	250.0 ± 5.5[a,b]
β-Scorpion venom + veratridine	161.0 ± 4.8[b]	274.0 ± 8.5[a,b]
PbTx-3 + veratridine	244.0 ± 10.3[b]	410.0 ± 6.1[a,b]
α-Scorpion venom + PbTx-3 + veratridine	424.0 ± 16.2[c]	710.0 ± 28.2[a,c]
β-Scorpion venom + PbTx-3 + veratridine	405.0 ± 12.4[c]	686.0 ± 10.9[a,c]

[a] $P < 0.05$, compared with CsA-nontreated cells.
[b] $P < 0.05$, compared with veratridine alone within each CsA-nontreated and -treated cell group.
[c] $P < 0.05$, significant enhancement by scorpion venom compared with cells exposed to veratridine and PbTx-3.

TABLE 3

Effects of CsA treatment on veratridine-induced $^{45}Ca^{2+}$ influx and catecholamine secretion

Cells were treated without or with 10 μM CsA for 24 h, then washed, and incubated with 2 μCi of $^{45}CaCl_2$ at 37°C for 5 min in the absence or presence of 1 to 560 μM veratridine. Basal $^{45}Ca^{2+}$ influx and catecholamine secretion per 4 $\times$ 10^6 cells per 5 min were similar between nontreated (0.48 ± 0.03 nmol; 0.14 ± 0.03 μg) and CsA-treated cells (0.47 ± 0.05 nmol; 0.17 ± 0.02 μg), and they were subtracted from the data. Cellular amounts of catecholamines (μg/4 $\times$ 10^6 cells) was similar between nontreated (65.6 ± 1.8) and CsA-treated cells (65.2 ± 0.7). Mean ± S.E. (n = 5).

	$^{45}Ca^{2+}$ Influx	
	Nontreated Cells	Cyclosporin A-Treated Cells
Veratridine		
EC_{50} (μM)	110.8 ± 8.6	105.6 ± 10.0
Maximum (nmol/4 $\times$ 10^6 cells/5 min)	8.7 ± 0.4	10.5 ± 0.4[a]
	Catecholamine Secretion	
	Nontreated Cells	Cyclosporin A-Treated Cells
Veratridine		
EC_{50} (μM)	102.8 ± 6.8	97.6 ± 9.2
Maximum (μg/4 $\times$ 10^6 cells/5 min)	8.5 ± 0.4	10.8 ± 0.5[a]

[a] $P < 0.05$, compared with CsA-nontreated cells.

enhanced veratridine-induced $^{22}Na^+$ influx by 6.2- or 6.0-fold in both nontreated and CsA-treated cells.

Effects of CsA Treatment on Veratridine-Induced $^{45}Ca^{2+}$ Influx via Voltage-Dependent Ca^{2+} Channels and Catecholamine Secretion. In adrenal chromaffin cells, veratridine causes a sustained influx of Ca^{2+} via Ca^{2+} channels and catecholamine secretion for at least 5 min, and they were blocked by Mg^{2+} (20 mM) or Ni^{2+} (1 mM), an inhibitor of voltage-dependent Ca^{2+} channels (Wada et al., 1985; López et al., 1995). Table 3 shows that CsA treatment (10 μM for 24 h) did not change the basal $^{45}Ca^{2+}$ influx and catecholamine secretion (Table 3, legend) but enhanced veratridine-induced maximum $^{45}Ca^{2+}$ influx and catecholamine secretion by 21 and 27% without altering the EC_{50} values of veratridine.

Effect of CsA Treatment on High K^+-Induced $^{45}Ca^{2+}$ Influx via Voltage-Dependent Ca^{2+} Channels. We examined whether the enhancement of veratridine-induced $^{45}Ca^{2+}$ influx in CsA-treated cells may be due to the CsA-induced up-regulation of functional Na^+ channels and the consequent augmentation of Ca^{2+} channel gating or whether CsA treatment may produce the direct stimulatory effect on

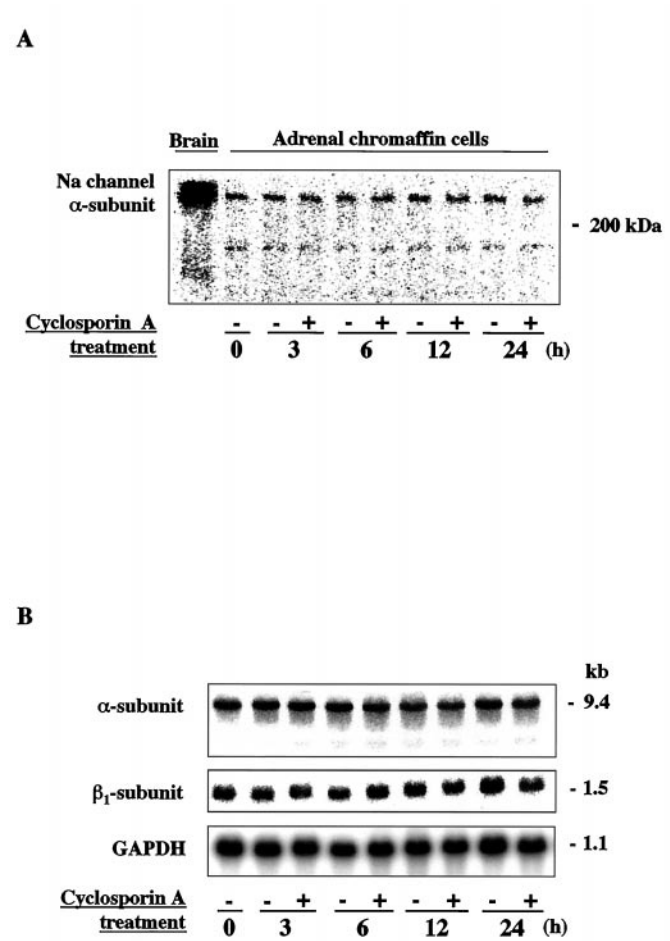

Fig. 4. Lack of effect of CsA treatment on cellular levels of Na^+ channel α-subunit as well as Na^+ channel α- and β_1-subunit mRNAs. Adrenal chromaffin cells were treated without (−) or with (+) 10 μM CsA for the indicated periods. A, the whole-cell lysates of adrenal chromaffin cells and rat brain homogenate were solubilized, size-fractionated by SDS-7.5% PAGE, and transferred to a membrane for the immunoblot analysis of Na^+ channel α-subunit. B, poly(A)$^+$ RNA was extracted from adrenal chromaffin cells, electrophoresed on a 1% agarose gel, and transferred to a membrane. The membrane was sequentially hybridized with ^{32}P-labeled cDNA probes for hNE-Na (upper panel), β_1-subunit (middle panel), and GAPDH (lower panel) after removing the former probe. Data are typical of one from three independent experiments with similar results.

Ca^{2+} channel gating. In adrenal chromaffin cells, we previously showed that in contrast to veratridine, high K^+ directly increases $^{45}Ca^{2+}$ influx via voltage-dependent Ca^{2+} channels independent of $^{22}Na^+$ influx, and it attains to its maximum at 1 min (Wada et al., 1985). In our present study, high K^+ increased $^{45}Ca^{2+}$ influx (nmol/4 $\times$ 10^6 cells/min; $n = 3$) over the basal value (0.6 $\pm$ 0.1) by 1.6 $\pm$ 0.4, 5.1 $\pm$ 0.6, 11.2 $\pm$ 0.8, 11.6 $\pm$ 0.6 and 11.0 $\pm$ 0.7 at 28, 56, 84, 98, and 140 mM, respectively. CsA treatment (10 μM for 24 h) did not change the basal (0.7 $\pm$ 0.2) nor high K^+-induced $^{45}Ca^{2+}$ influx (1.4 $\pm$ 0.4, 5.0 $\pm$ 0.2, 11.0 $\pm$ 0.6, 11.4 $\pm$ 0.8 and 11.2 $\pm$ 0.5 at 28, 56, 84, 98, and 140 mM, respectively) ($n = 3$).

Effects of CsA Treatment on Cellular Levels of Na^+ Channel α-Subunit, as Well as Na^+ Channel α- and β_1-Subunit mRNAs. Because the gradual development ($t_{1/2}$ = 15.2 h) of CsA-induced increase of [^{3}H]STX binding may implicate the involvement of transcriptional and translational events in the CsA-induced up-regulation of Na^+ channels, we measured the cellular levels of Na^+ channel α-subunit as well as Na^+ channel α- and β_1-subunit mRNAs in adrenal chromaffin cells that had been treated with or without 10 μM CsA for up to 24 h. For Western blot analysis, we used the antibody raised against the highly conserved segment in the intracellular loop between S6III and S1IV of the Na^+ channel α-subunit, which is identical among all known vertebrate Na^+ channels (the manufacturer's instruction; Klugbauer et al., 1995). As shown in Fig. 4A, the antibody recognized one major (~220 kDa) band in rat brain and bovine adrenal chromaffin cells, consistent with the molecular size of Na^+ channel α-subunit recognized with this antibody (the manufacturer's instruction). When the levels of Na^+ channel α-subunit were quantified by a Bioimage analyzer, CsA treatment did not elevate cellular levels of Na^+ channel α-subunit (104.0 $\pm$ 2.0, 101.0 $\pm$ 5.0, 97.0 $\pm$ 0.6, and 94.0 $\pm$ 2.0% of levels in nontreated cells, $n = 3$) at 3, 6, 12, and 24 h, respectively. Northern blot analysis (Fig. 4B) showed that the hNE-Na probe and the β_1-subunit probe hybridized to α (~9.4 kb)- and β_1 (~1.5 kb)-subunit mRNAs, respectively, as reported previously (Oh and Waxman, 1994; Klugbauer et al., 1995; Yamamoto et al., 1996; Yanagita et al., 2000). When the levels of α- and β_1-subunit mRNAs were normalized against those of GAPDH mRNA, CsA treatment did not elevate α-subunit mRNA levels (105.0 $\pm$ 5.2, 93.4 $\pm$ 4.4, 93.7 $\pm$ 3.1, and 93.3 $\pm$ 4.2% of levels in nontreated cells) and β_1-subunit mRNA levels (98.7 $\pm$ 7.7, 104.0 $\pm$ 7.9, 99.3 $\pm$ 3.3, and 94.6 $\pm$ 2.6% of levels in nontreated cells) at 3, 6, 12, and 24 h, respectively ($n = 3$).

Effect of Treatment with BFA on CsA-Induced Rise of [^{3}H]STX Binding. Because CsA-induced increase of [^{3}H]STX binding was not associated with the increased cellular levels of Na^+ channel α-subunit, as well as Na^+ channel α- and β_1-subunit mRNAs, it is suggested that CsA treatment may stimulate the post-translational intracellular vesicular trafficking of Na^+ channels from the *trans*-Golgi network (TGN). BFA is an inhibitor of the guanine nucleotide-exchange protein of ADP-ribosylation factor 1 (ARF1), a monomeric GTPase. BFA blocks cell surface externalization of newly synthesized ion channels (Shimkets et al., 1997; Staub et al., 1997) and receptors (Schonhorn and Wessling-Resnick, 1994; Hirasawa et al., 1998) from the TGN, whereas BFA has no effect on ARF6-catalyzed endocytic internalization of cell surface proteins (Schonhorn and Wessling-Resnick, 1994; Hirasawa et al., 1998). In adrenal chromaffin cells, previous fluorescence study showed that treatment with BFA (0.28–2.8 μg/ml for 2 h) was sufficient to cause the disassembly of Golgi membrane in most chromaffin cells (>90%) (Xu and Tse, 1999). As shown in Fig. 5A, treatment with 10 μg/ml BFA for 24 h lowered by itself [^{3}H]STX binding by 55%; the concurrent treatment with CsA significantly elevated [^{3}H]STX binding even in the presence of BFA, but its magnitude became remarkably smaller, compared with CsA alone.

Effect of CsA Treatment on Endocytic Internalization of Cell Surface Na^+ Channels. In *Xenopus* oocytes expressing renal epithelial Na^+ channels, two laboratories have measured the internalization rate of renal Na^+ channels from plasma membrane, in which condition cell surface sorting of newly synthesized renal Na^+ channels from the TGN was blocked by BFA treatment (10 μg/ml for 8 h), and the reduction of Na^+ currents during BFA treatment was estimated by the two-electrode voltage-clamp technique

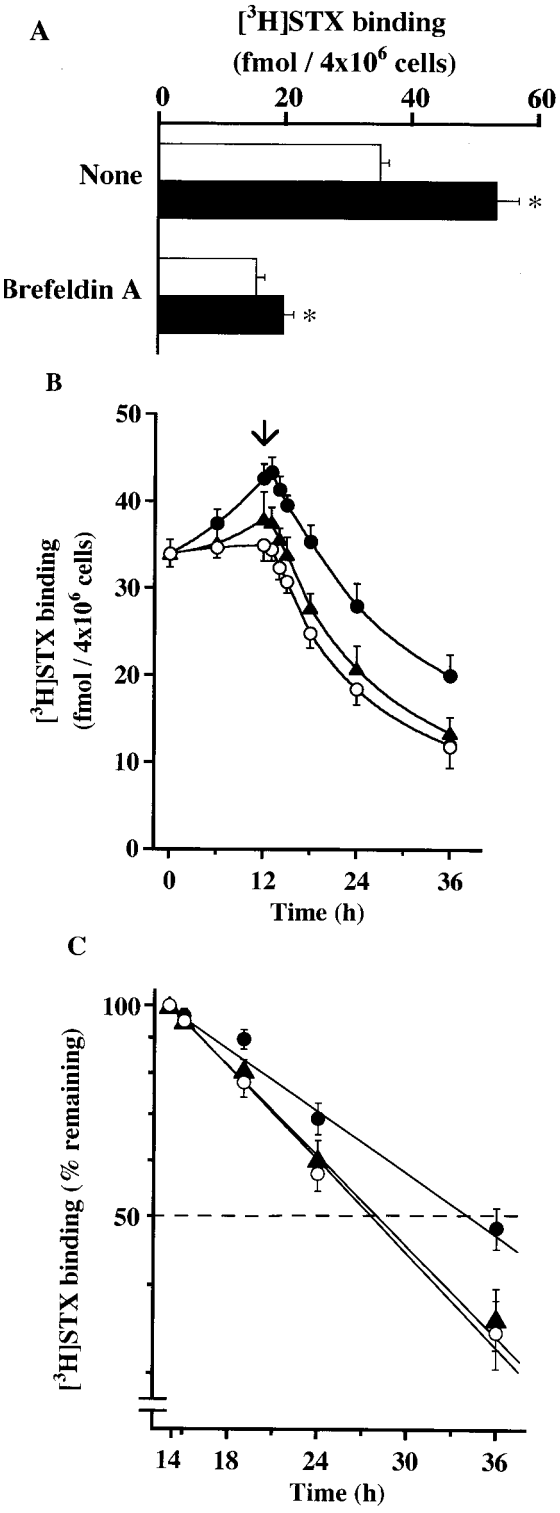

Fig. 5. Effects of CsA treatment on externalization and internalization of Na^+ channels. A, cells were treated for 24 h without (open columns) or with (closed columns) 10 μM CsA in the absence (None) or presence of 10 μg/ml BFA and subjected to [^{3}H]STX binding assay. Mean ± S.E. (n = 5). *$P < 0.05$, compared with CsA-nontreated cells within each cell group. B, cells were treated without (○) or with 10 μM CsA (●) or 1 μM rapamycin (▲) for 12 h, then exposed to 10 μg/ml BFA (indicated by arrow), and incubated for up to 36 h in the continued absence or presence of CsA or rapamycin. At each time point indicated, cells were washed and subjected to [^{3}H]STX binding assay. C, values of [^{3}H]STX binding obtained in B were plotted against the time after the initial 14-h incubation period. A value of 100% represents [^{3}H]STX binding obtained at 14 h in nontreated, CsA-, or rapamycin-treated cells. Mean ± S.E. (n = 3).

(Shimkets et al., 1997; Staub et al., 1997). As shown in Fig. 5B, cells were treated for the first 12 h without or with 10 μM CsA or 1 μM rapamycin, then incubated with 10 μg/ml BFA in the continued absence or presence of CsA or rapamycin for an additional 24 h, and subjected to [^{3}H]STX binding assay at the indicated times. The addition of BFA at 12 h caused a time-dependent reduction of [^{3}H]STX binding in nontreated and rapamycin-treated cells; in CsA-treated cells, however, [^{3}H]STX binding increased slightly at 13 h even after the addition of BFA but decreased after 14 h in a time-dependent manner. When the level of [^{3}H]STX binding at 14 h was assigned a value of 100% in each cell group (Fig. 5C), [^{3}H]STX binding decreased with a similar rate between nontreated ($t_{1/2}$ = 13.7 h) and rapamycin ($t_{1/2}$ = 14.0 h)-treated cells. In contrast, [^{3}H]STX binding decayed with a $t_{1/2}$ of 20.3 h in CsA-treated cells; thus, CsA treatment prolonged the $t_{1/2}$ of cell surface Na^+ channels by 48%.

Discussion

In our present study, long-term (≥12 h) treatment of adrenal chromaffin cells with 10 μM CsA elevated cell surface [^{3}H]STX binding with the $t_{1/2}$ of 15.2 h, reaching the almost maximum rise of 50% at 24 h. The increasing effect of CsA was concentration-dependent between 1 and 100 μM with the EC_{50} of 2.9 μM, a value close to the plasma concentrations (~1.5 μM) of CsA in patients receiving oral administration of CsA (4.5 mg/kg) (Fahr, 1993). Scatchard analysis showed that CsA treatment (10 μM for 24 h) raised the B_{max} of [^{3}H]STX binding by 45% without changing the K_d value. CsA treatment (10 μM for 24 h) enhanced veratridine-induced maximum influx of $^{22}Na^+$ but did not alter the EC_{50} value of veratridine, when assayed in the absence or presence of ouabain. Either α- or β-scorpion venom or PbTx-3 potentiated veratridine-induced $^{22}Na^+$ influx to the comparable fold between nontreated and CsA-treated cells. Veratridine-induced $^{45}Ca^{2+}$ influx and catecholamine secretion were enhanced in CsA-treated cells, whereas high K^+-induced $^{45}Ca^{2+}$ influx was not augmented in CsA-treated cells. These results suggest that chronic treatment with 1 to 100 μM CsA up-regulates density of cell surface functional Na^+ channels with similar pharmacological properties to the native Na^+ channels, and it enhances Ca^{2+} channel gating and catecholamine secretion.

Treatment for 24 h with 100 nM FK506 raised [^{3}H]STX binding by 36%, whereas either okadaic acid treatment (50 nM for 24 h) or GPI-1046 treatment (100 nM for 24 h) failed to increase [^{3}H]STX binding. The inability of okadaic acid suggests that the increase of [^{3}H]STX binding is not attributed to the inhibition of protein phosphatases 1 and 2A. In contrast to CsA and FK506 that inhibit calcineurin activity, GPI-1046, an inhibitor of PPIase activity of FKBP, is unable to suppress calcineurin activity (Snyder et al., 1998). Our Western blot analysis of calcineurin and in vitro assay of calcineurin activity showed that CsA (10 μM) treatment did not alter cellular level of calcineurin for up to 48 h; however, CsA (≥1 μM) inhibited calcineurin activity for at least 24 h with the IC_{50} of 0.6 μM, and the maximum inhibition was obtained between 10 and 100 μM CsA. In adrenal chromaffin cells, previous in vitro assay showed that cell extracts prepared from CsA (1 μM for 30 min)- and FK506 (100 nM for 30 min)-treated cells suppressed Ca^{2+}- and calmodulin-induced

dephosphorylation of ^{32}P-labeled calcineurin substrate, i.e., regulatory subunit of PKA, by 92 and 88%, respectively (Artalejo et al., 1996). Similar study in insulinoma MIN6 cells showed that CsA treatment (0.5–5 μM for 60 min) inhibited the activity of calcineurin by up to 76% with the IC_{50} value of 0.9 μM (Ebihara et al., 1996). Although the in vitro assay condition of calcineurin activity does not precisely reflect the in vivo activity of calcineurin in the native intracellular milieu, our present and their previous results raise the possibility that CsA- or FK506-induced rise of cell surface Na^+ channels is due to the inhibition of calcineurin activity (but not to the suppression of PPIase activity of cyclophilin or FKBP). We also observed that treatment for 24 h with 1 μM rapamycin elevated [^{3}H]STX binding by 25%. Rapamycin-FKBP complex inhibits FRAP but has no effect on calcineurin (Snyder et al., 1998), implicating that inhibition of FRAP-dependent as yet fully defined signaling pathways up-regulates cell surface Na^+ channels.

In our present study, CsA treatment did not elevate cellular levels of Na^+ channel α-subunit and Na^+ channel α- and β_1-subunit mRNAs from 3 to 24 h, when [^{3}H]STX binding was progressively raised by CsA. These results suggest that CsA-induced up-regulation of cell surface Na^+ channels is not attributed to the increased synthesis of Na^+ channels. In contrast, CsA-induced increment of [^{3}H]STX binding was significantly prevented by the concurrent 24 h treatment with 10 μg/ml BFA. In various intact cells, it has been shown that BFA treatment (2.5–10 μg/ml for 2–36 h) blocks cell surface vesicular trafficking from the TGN of renal epithelial Na^+ channels (Shimkets et al., 1997; Staub et al., 1997), α_{1B}-adrenoceptors (Hirasawa et al., 1998), and transferrin receptors (Schonhorn and Wessling-Resnick, 1994), whereas the same BFA treatment does not change the internalization of receptors and ion channels (Schonhorn and Wessling-Resnick, 1994; Shimkets et al., 1997; Staub et al., 1997; Hirasawa et al., 1998). Thus, our present and their previous findings support the notion that inhibition of calcineurin-dependent signaling pathways due to CsA treatment promotes vesicular transport of Na^+ channels from the TGN to plasma membrane, thereby causing up-regulation of cell surface Na^+ channels in adrenal chromaffin cells. Multiple lines of evidence have documented that phosphorylation events catalyzed by PKA and PKC accelerate constitutive cell surface trafficking of the post-Golgi vesicles from the TGN (Muñiz et al., 1997), but the molecular machinery regulating the externalization of as yet fully defined post-Golgi vesicles remains largely unknown (Traub and Kornfeld, 1997).

Our present study also showed that internalization rate of cell surface [^{3}H]STX binding was reduced by 48% in CsA-treated cells ($t_{1/2}$ = 20.3 h), compared with nontreated ($t_{1/2}$ = 13.7 h) and rapamycin ($t_{1/2}$ = 14.0 h)-treated cells. Thus, our present results may imply that inhibition of calcineurin-dependent signaling pathways delays internalization rate of Na^+ channels, thereby contributing to the up-regulation of cell surface Na^+ channels. Evidence has emerged that internalization of cell surface receptors and ion channels, including renal epithelial Na^+ channels (Shimkets et al., 1997), occurs via clathrin-coated vesicles, in which process calcineurin may play crucial role. Among calcineurin's substrates, dynamin I, a monomeric GTPase, exhibits higher affinity for calcineurin; calcineurin-catalyzed dephosphorylation of dynamin I triggers the fission of invaginated clathrin-coated vesicles (Liu and Robinson, 1995), and the dephosphorylation of dynamin I was blocked by 1 μM CsA but not by 100 nM okadaic acid (Nichols et al., 1994). Also, dynamin I is phosphorylated by PKC, and the phosphorylation/dephosphorylation cycles of dynamin I are assumed to be important for the continuation of internalization of clathrin-coated vesicles (Liu and Robinson, 1995). In adrenal chromaffin cells, our previous study showed that translocative activation of PKC-α from cytosol to membranes accelerated internalization of Na^+ channels, thus causing down-regulation of cell surface Na^+ channels (Yanagita et al., 2000). Although mechanisms regulating internalization of Na^+ channels remain largely unknown at any given tissue, our previous and present findings may implicate that PKC-α and calcineurin accelerate Na^+ channel internalization, at least, in adrenal chromaffin cells.

CsA, FK506, and rapamycin accelerated neurite outgrowth in PC12 cells and dorsal root ganglion, as well as promoted myelination of crushed sciatic nerve (Lyons et al., 1994; Steiner et al., 1997). In animal models of Parkinson's disease, CsA and FK506 protected depletion of nigrostriatal dopaminergic neurons caused by 6-hydroxydopamine or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Kitamura et al., 1994; Matsuura et al., 1997). These neurotrophic effects of immunophilin ligands may require the increased synthesis and tightly regulated cell surface expression of Na^+ channels (Vabnick and Shrager, 1998). In contrast, aberrant up-regulation and hyperactivity of Na^+ channels may culminate in epileptic seizure (Yanagita et al., 2000), spinal cord injury (Cummins and Waxman, 1997), and ischemic brain damage (Urenjak and Obrenovitch, 1996). Therapeutic concentrations of CsA and FK506 frequently invoke an array of neurotoxic effects (Gijtenbeek et al., 1999). Thus, our present study may provide the molecular basis for the better understanding of the neurotrophic and neurotoxic aspects of immunophilins and immunophilin ligands.

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