Differential Effects of Short and Prolonged Exposure to Carvedilol on Voltage-Dependent Na\(^+\) Channels in Cultured Bovine Adrenal Medullary Cells

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ABSTRACT
We examined the effects of short and prolonged exposure to carvedilol, an antihypertensive and \(\beta\)-adrenoceptor blocking drug, on voltage-dependent Na\(^+\) channels in cultured bovine adrenal medullary cells. Carvedilol (1–100 \(\mu\)M) reduced 22Na\(^+\) influx induced by veratridine, an activator of voltage-dependent Na\(^+\) channels. Carvedilol also suppressed veratridine-induced 45Ca\(^{2+}\) influx and catecholamine secretion in a concentration-dependent manner similar to that of 22Na\(^+\) influx. Prolonged exposure of the cells to 10 \(\mu\)M carvedilol increased [\(\text{H}\)]saxitoxin ([\(\text{H}\)]STX) binding, which reached a plateau at 12 h and was still observed at 48 to 72 h. Scatchard analysis of [\(\text{H}\)]STX binding revealed that carvedilol increased the \(B_{\infty}\) value (control, 14.9 \(\pm\) 0.9 fmol/10\(^6\) cells; carvedilol, 23.8 \(\pm\) 1.2 fmol/10\(^6\) cells) \((n = 3, P < 0.05)\) without altering the \(K_d\) value, suggesting a rise in the number of cell surface Na\(^+\) channels. The increase in [\(\text{H}\)]STX binding by carvedilol was prevented by cycloheximide, an inhibitor of protein synthesis, whereas carvedilol changed neither \(\alpha\)-nor \(\beta\)-subunit mRNA levels of Na\(^+\) channels. The carvedilol-induced increase of [\(\text{H}\)]STX binding was abolished by brefeldin A and H-89, inhibitors of intracellular vesicular trafficking of proteins from the trans-Golgi network and of cyclic AMP-dependent protein kinase (protein kinase A), respectively. The present findings suggest that short-term treatment with carvedilol reduces the activity of Na\(^+\) channels, whereas prolonged exposure to carvedilol up-regulates cell surface Na\(^+\) channels. This may add new pharmacological effects of carvedilol to our understanding in the treatment of heart failure and hypertension.

Carvedilol, an antihypertensive agent, possesses the pharmacological properties of \(\beta\)-adrenoceptor blocking action and direct vasodilating action (Ruffolo et al., 1990). A series of investigations revealed that this agent possesses multiple actions, including those characteristic of a potent antioxidant, such as inhibition of superoxide release from human neutrophils and suppression of lipid peroxidation in rat brain homogenates (Yue et al., 1992), cardioprotective action (Feuerstein and Ruffolo, 1996), and inhibition of vascular smooth muscle cell proliferation (Ohlstein et al., 1993). Carvedilol is also reported to have neuroprotective activity via inhibition of glutamate release and the N-methyl-D-aspartate receptor (Lysko et al., 1992, 1994). However, it has not been reported whether/how carvedilol affects ion channels in cardiac, vascular, or neuronal cells.

Voltage-dependent Na\(^+\) channels are responsible for the rising phase of the action potential in the membranes of neurons and most electrically excitable cells (for review, see Catterall, 1992). Na\(^+\) channels consist of the principal \(\alpha\)-subunit, which may be associated with a noncovalently attached \(\beta\)-subunit, and a disulfide-linked \(\beta\)-subunit (Klugbauer et al., 1995; Catterall, 2000). The \(\alpha\)-subunits issued from a large multigene family contain the ion-pore and the toxin binding sites, i.e., site 1 for tetrodotoxin (TTX) and saxitoxin (STX), site 2 for veratridine, site 3 for \(\alpha\)-scorpion toxin, site 4 for \(\beta\)-scorpion toxin, and site 5 for Ptychodiscus brevis toxin-3 (PbTx-3) (Wada et al., 1992; Catterall, 2000). Structures of \(\beta\)-subunit are similar among various tissues, but \(\beta_2\)-subunit is cloned only in the brain (Isom et al., 1995).

In adrenal medullary cells (Yamamoto et al., 1996, 1997), the Na\(^+\) channel \(\alpha\)-subunits are homologous to the human neuroendocrine-type Na\(^+\) channel \(\alpha\)-subunit (hNE-Na) (Klugbauer et al., 1995), and the Na\(^+\) channel \(\beta\)-subunits are structurally similar to that of rat brain (Oh and Waxman, 1994). Bovine adrenal medullary Na\(^+\) channels share many physiological and pharmacological properties with those of the human cells (Wada et al., 1985, 1992). In the cells, veratridine caused catecholamine secretion, which was depen-

ABBREVIATIONS: TTX, tetrodotoxin; STX, saxitoxin; PbTx-3, Ptychodiscus brevis toxin-3; hNE-Na, human neuroendocrine-type Na\(^+\) channel \(\alpha\)-subunit; MEM, minimum essential medium; BFA, brefeldin A; DMSO, dimethyl sulfoxide; KRP, Krebs-Ringer phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SSC, saline-sodium citrate; S, segment; kb, kilobase.
dent on veratridine-induced $^{45}$Ca$^{2+}$ influx via voltage-dependent Ca$^{2+}$ channels as well as $^{22}$Na$^+$ influx via voltage-dependent Na$^+$ channels (Wada et al., 1985). In the regulation of Na$^+$ channel expression, cyclic AMP-dependent protein kinase (protein kinase A) (Yuhi et al., 1996) or activation of insulin receptors (Yamamoto et al., 1996) increased the cell surface density of Na$^+$ channels without elevating Na$^+$ channel mRNA levels. In contrast, chronic treatment with antiepileptic valproic acid increased both the density and mRNA of Na$^+$ channels (Yamamoto et al., 1997).

In a previous study (Morita et al., 1989), carvedilol inhibited the secretion of catecholamines induced by various secretagogues, by its stabilizing action on the plasma membranes rather than by its blocking action on Ca$^{2+}$ influx in bovine adrenal medullary cells. The aim of the present study was to investigate whether carvedilol directly interferes with Na$^+$ influx mediated through voltage-dependent Na$^+$ channels in cultured bovine adrenal medullary cells. In addition, the effects of prolonged exposure to carvedilol on the density of cell surface Na$^+$ channel proteins and levels of Na$^+$ channel mRNA were also evaluated in adrenal medullary cells. As a result, we found that carvedilol acutely reduces the functional activity of Na$^+$ channels, followed by an increase in cell surface Na$^+$ channels, without any change in levels of mRNA.

**Experimental Procedures**

**Materials.** Chemicals used were obtained from the following sources: Eagle’s minimum essential medium (MEM), Nissui Pharmaceutical, Tokyo, Japan; calf serum, Nacalai Tesque, Kyoto, Japan; brefeldin A (BFA), cycloheximide, TTX, carbachol, veratridine, a-scorpion venom (Leiurus quinquestratus quinquestratus), and b-scorpion venom (Centruroides sculpturatus), Sigma-Aldrich, St. Louis, MO; PhTx-3, Latoxan, Westbury, NY; and collagenase, Nitta Zerachin Inc., Osaka, Japan. Carvedilol was kindly donated from Daiichi Pharmaceutical Co. Ltd., Tokyo, Japan. Plasmids containing hNE-Na (Klugbauer et al., 1995) and rat brain Na$^+$ channel b1-subunit cDNA (Oh and Waxman, 1994) were generously donated by Dr. F. Hofmann (Technischen Universität München, Munich, Germany) and Dr. Y. Oh (University of Alabama, Birmingham, AL), respectively.

**Isolation and Primary Culture of Adrenal Medullary Cells and Drug Treatment.** Bovine adrenal medullary cells were isolated by collagenase digestion and maintained in Eagle’s MEM, containing 10% calf serum and antibiotics (4 $\times$ 10$^5$ cells/dish; Falcon 35-mm dish), as reported previously (Yanagihara et al., 1994). To remove nonchromaffin cells, such as fibroblasts or epithelial cells, the differential plating procedure was used, and the final cell preparation contained at least 80 to 90% chromaffin cells. The cells were cultured under 5% CO$_2$/95% air in a CO$_2$ incubator and used for experiments within 3 to 5 days of culture. For prolonged exposure to carvedilol, cells were incubated with or without 10 $\mu$M carvedilol in Eagle’s MEM containing 10% calf serum for the various periods indicated. After treatment, the cells were washed three times with 1 ml of Krebs-Ringer phosphate buffer (see below) and used for the experiments. There was not a significant change in cell viability between control cells and cells treated with carvedilol. Carvedilol was dissolved in dimethyl sulfoxide (DMSO). To avoid the possible influence of DMSO on cells, all reaction media, including the control media, were standardized at a final concentration of 0.25% DMSO.

$^{22}$Na$^+$ and $^{45}$Ca$^{2+}$ Influx. Oxygenated Krebs-Ringer phosphate (KRP) buffer was used throughout. It had the following composition: 154 mM NaCl, 5.6 mM KCl, 1.1 mM MgSO$_4$, 2.2 mM CaCl$_2$, 0.85 mM Na$_2$HPO$_4$, 2.15 mM NaH$_2$PO$_4$, and 10 mM glucose, adjusted to pH 7.4. Cells were incubated with 1.5 $\mu$Ci of $^{22}$NaCl (6–17 Ci/mmol; PerkinElmer Life Sciences, Boston, MA) or 1.5 $\mu$Ci of $^{45}$CaCl$_2$ (0.5–2.0 Ci/mmol; Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) at 37°C for 5 min with or without various secretagogues, a- and b-scorpion venoms, and/or PhTx-3 in KRP buffer. After reaction, the cells were quickly washed four times with 1 ml of ice-cold KRP buffer. Then, the cells were solubilized with 1 ml of 10% Triton X-100. Radioactivity of $^{22}$Na$^+$ and $^{45}$Ca$^{2+}$ in the cells was counted by an Aloka ARC-2005 gamma counter (Aloka Co., Ltd., Tokyo, Japan) and a Beckman LS-7000 liquid scintillation counter (Beckman Coulter Inc., Fullerton, CA), respectively. The influx of $^{22}$Na$^+$ and $^{45}$Ca$^{2+}$ was calculated from the initial specific radioactivity of these ions in the incubation medium.

**Cholecoclinamine Secretion.** Cells were incubated at 37°C for 5 min with or without carvedilol in the presence or absence of 100 $\mu$M veratridine (final volume 1.0 ml). After the reaction, the incubation medium was immediately transferred to a test tube containing 4 ml of ice-cold 0.5 M perchloric acid. Cholecoclinamines (norepinephrine and epinephrine) secreted into the incubation medium were adsorbed to aluminum hydroxide and estimated by the ethylenediamine condensation method with a fluorescence spectrophotometer (Hitachi 650-10S; Hitachi Ltd., Tokyo, Japan) with an excitation wavelength of 420 nm and an emission of 540 nm.

$^{[3]H}JSTX$ Binding. Cells were washed with ice-cold KRP buffer and incubated with 1 to 25 nM $^{[3]H}JSTX$ (20–40 Ci/mmol; Amersham Biosciences) in 1 ml of KRP buffer at 4°C for 15 min in the absence (total binding) or presence (nonspecific binding) of 1 $\mu$M TTX. The cells were immediately washed, solubilized in 10% Triton X-100, and counted for radioactivity to determine specific binding, which was calculated as the total binding minus nonspecific binding. A mere addition of carvedilol to the binding assay medium per se did not alter $^{[3]H}JSTX$ binding.

**mRNA Isolation and Electrophoresis.** Total cellular RNA was isolated from cells treated with or without carvedilol by acid guanidinium thiocyanate-phenol-chloroform extraction, using TRIzol reagent (Invitrogen, Carlsbad, CA). Poly(A)$^+$ RNA was purified using Oligotex-dT30 <$\superscript{>}$Super$<$> (Nippon Roche Co., Ltd., Tokyo, Japan), electrophoresed on a 1% agarose gel containing 6.3% formaldehyde in the running buffer (40 mM 3-(N-morpholino)propanesulfonic acid, pH 7.2, 0.5 mM EDTA, and 5 mM sodium citrate), then transferred to a nylon membrane (Hybond-N; Amersham Biosciences) in 20x saline-sodium citrate (SSC; 1 x SSC = 0.15 M NaCl and 0.015 M sodium citrate) overnight, and cross-linked using a UV cross-linker (Funakoshi, Tokyo, Japan).

**Northern Blot.** cDNA fragments of hNE-Na (435–2666) and b1-subunit (457–790), as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1.1 kb pair) were labeled with [$\alpha$-32P]deoxyctidine 5'-triphosphate (>$\sim$4000 Ci/mmol; Amersham Biosciences) using the BeaBEST labeling kit (Takara, Kyoto, Japan). The membrane was prehybridized and hybridized with hNE-Na probe at 42°C for 18 h in 6 x SSC, 10 x Denhardt’s solution (2% bovine serum albumin fraction V, 2% polyvinylpyrrolidone, and 2% Ficoll 400), 500 formamide, 0.5% SDS, and 50 $\mu$g/ml salmon sperm DNA; it was washed at 65°C in 2 x, 1 x, and 0.2 x SSC containing 0.1% SDS, each for 30 min, and subjected to autoradiography. The same membrane was sequentially hybridized to probes for b1-subunit and GAPDH (BD Biosciences Clontech, Palo Alto, CA) after it was thoroughly washed in 0.1% SDS at 100°C to remove the former probe. Autoradiogram was quantified by a BAS 2000 bioimage analyzer (FujiFilm, Tokyo, Japan).

**Statistical Analysis.** Data are expressed as mean ± S.D. The statistical evaluation of the data was performed by analysis of variance, followed by post hoc test. Values were considered statistically different when $P$ was less than 0.05. Student’s t test was used when two means of group were compared.
Results

Effect of Carvedilol on Veratridine-Induced $^{22}$Na$^+$ Influx in Cultured Bovine Adrenal Medullary Cells.

Veratridine (100 µM), an activator of voltage-dependent Na$^+$ channels, caused the influx of $^{22}$Na$^+$ (42.5 ± 3.3 nmol/10$^6$ cells) (Fig. 1A). Carvedilol reduced veratridine-evoked $^{22}$Na$^+$ influx in a concentration-dependent manner (IC$_{50}$ = 1.8 µM). A significant reduction by carvedilol was observed at 1 µM, and a maximal reduction was observed at 100 µM. The basal $^{22}$Na$^+$ influx was not changed by carvedilol at any concentration used.

When cells were stimulated with various concentrations of veratridine, veratridine increased $^{22}$Na$^+$ influx in a concentration-dependent manner. The suppressive effect of carvedilol (10 µM) was not overcome even when the concentration of veratridine was increased to 560 µM (Fig. 1B).

Effect of Carvedilol on Veratridine-Induced $^{45}$Ca$^{2+}$ Influx and Catecholamine Secretion. As shown in Fig. 2, veratridine produced $^{45}$Ca$^{2+}$ influx (1.25 ± 0.05 nmol/10$^6$ cells) and catecholamine secretion (1.05 ± 0.04 µg/10$^6$ cells), respectively. Carvedilol (1–100 µM) also attenuated veratridine-induced $^{45}$Ca$^{2+}$ influx (IC$_{50}$ = 1.9 µM) and catecholamine secretion (IC$_{50}$ = 2.3 µM) in a concentration-dependent manner similar to that of $^{22}$Na$^+$ influx. Carvedilol did not affect any basal responses.

Effect of Carvedilol on Carbachol-Evoked $^{22}$Na$^+$ Influx and High K$^+$-Evoked $^{45}$Ca$^{2+}$ Influx. Carbachol (0.1 mM), an activator of nicotinic acetylcholine receptor-ion channels, caused $^{22}$Na$^+$ influx (43.3 ± 1.3 nmol/10$^6$ cells). Carvedilol at 3 to 100 µM significantly reduced carbachol-evoked $^{22}$Na$^+$ influx in a concentration-dependent manner (IC$_{50}$ = 4.3 µM) (Fig. 3A). On the other hand, 56 mM K$^+$, an activator of voltage-dependent Ca$^{2+}$ channels, stimulated the influx of $^{45}$Ca$^{2+}$ (2.12 ± 0.13 nmol/10$^6$ cells) (Fig. 3B). Carvedilol at concentrations of 0.1 to 10 µM had no effect on 56 mM K$^+$-induced $^{45}$Ca$^{2+}$ influx. Only at 100 µM did carvedilol slightly suppress $^{45}$Ca$^{2+}$ influx caused by 56 mM K$^+$. The basal $^{22}$Na$^+$ influx and $^{45}$Ca$^{2+}$ influx were not changed by carvedilol at any concentrations used.

Effect of Prolonged Exposure to Carvedilol on Veratridine-Induced $^{22}$Na$^+$ Influx in Combination with Either α- or β-Scorpion Venom and/or PbTx-3. We examined whether/how prolonged exposure to carvedilol may alter $^{22}$Na$^+$ influx induced by veratridine, using several distinct classes of toxins of Na$^+$ channels (Wada et al., 1992). Cells were treated for 12 h with or without 10 µM carvedilol, washed three times with 1 ml of KRP buffer, and used for $^{22}$Na$^+$ influx assay. α-Scorpion venom, which binds to site 3 between segment (S) 3 to 4 of domain IV (Rogers et al., 1996), enhanced $^{22}$Na$^+$ influx evoked by veratridine, a toxin acting at site 2 of S6I (Trainer et al., 1996) (Fig. 4). Similarly, β-scorpion venom, which interacts with site 4 (Catterall, 1992), and PbTx-3, which binds to site 5 (Trainer et al., 1994) between S5IV and S6I, augmented veratridine-induced $^{22}$Na$^+$ influx. Furthermore, PbTx-3 in the presence of either α- or β-scorpion venom enhanced $^{22}$Na$^+$ influx induced by veratridine to a greater extent than did either toxin/venom alone. Pretreatment with carvedilol, however, rather suppressed veratridine-induced $^{22}$Na$^+$ influx. Furthermore, veratridine-induced $^{22}$Na$^+$ influx was also decreased in the presence of α- or β-scorpion venom and/or PbTx-3 in cells pretreated with carvedilol compared with nontreated cells. Nevertheless, these four toxins still enhanced veratridine-
induced $^{22}$Na$^+$ influx even in the cells treated with carvedilol.

**Effect of Prolonged Exposure to Carvedilol on Cell Surface $[^3$H]$STX$ Binding.** As shown in Fig. 4, prolonged exposure to carvedilol reduced the functional activity of Na$^+$ channels that showed pharmacological properties similar to that of nontreated cells. Next, we examined the cell surface expression of Na$^+$ channels by using $[^3$H]$STX$ binding. Surprisingly, when cells were treated with 10 $\mu$M carvedilol for 3 to 72 h, increases in $[^3$H]$STX$ binding were found between 3 and 72 h and reached a plateau at 12 h with the maximal increase of 61.2% (Fig. 5A). Carvedilol also increased $[^3$H]$STX$ binding in a concentration-dependent manner (Fig. 5B).

Scatchard plot showed that carvedilol treatment (10 $\mu$M, 12 h) significantly increased the $B_{\text{max}}$ (14.9 ± 0.9 to 23.8 ± 1.2 fmol/10$^6$ cells) without altering the $K_d$ values (control cells, 4.2 ± 0.3 nM; carvedilol-treated cells, 4.5 ± 0.4 nM) (Fig. 6).

![Fig. 4. Effects of prolonged exposure to carvedilol on $^{22}$Na$^+$ influx caused by veratridine, $\alpha$- or $\beta$-scorpion venom, and PbTx-3. Cells were treated with (closed column) or without (open column) 10 $\mu$M carvedilol for 12 h. After treatment, the cells were washed three times with 1 ml of KRP buffer and then incubated with $^{22}$Na$^+$ (1.5 $\mu$Ci) at 37°C for 5 min in the presence (+) or absence (−) of 30 $\mu$M veratridine, 100 $\mu$g/ml $\alpha$-scorpion venom, 100 $\mu$g/ml $\beta$-scorpion venom, and 1 $\mu$M PbTx-3. Basal values of $^{22}$Na$^+$ influx without any toxin or venom were subtracted from the data. Data are mean ± S.D. of three separate experiments carried out in triplicate.](image)

![Fig. 5. Effect of prolonged exposure to carvedilol on $[^3$H]$STX$-specific binding. After treatment of cells for the indicated periods with (●) or without (○) 10 $\mu$M carvedilol (A), or for 12 h with increasing concentrations of carvedilol (B), cells were washed three times with 1 ml of KRP buffer and incubated at 4°C for 15 min with 25 nM $[^3$H]$STX$ in the presence or absence of 1 $\mu$M unlabeled TTX. Data are mean ± S.D. of three separate experiments. * P < 0.05; ** P < 0.01; *** P < 0.001 versus nontreated cells.](image)

![Fig. 6. Scatchard plot of $[^3$H]$STX$-specific binding. Binding was assayed with 1 to 25 nM $[^3$H]$STX$ in the presence or absence of 1 $\mu$M unlabeled TTX after treatment of the cells with (●) or without (○) 10 $\mu$M carvedilol for 12 h. Scatchard plot of $[^3$H]$STX$ binding to the cells. Data are typical of three independent experiments, with similar results. B/F, bound/free.](image)
Effect of Carvedilol Treatment on Na\(^+\) Channel \(\alpha\)- and \(\beta_1\)-Subunit mRNA Levels. From the results of Scatchard analysis (Fig. 6), carvedilol-induced increase of \[^{3}H\]STX binding may implicate an up-regulation of Na\(^+\) channel proteins through its transcriptional events. Therefore, we estimated the steady-state levels of Na\(^+\) channel \(\alpha\)- and \(\beta_1\)-subunit mRNAs in cells that had or had not been treated with 10 \(\mu\)M carvedilol for 0, 1, 3, 6, 12, and 24 h. cDNA probes for hNE-Na and \(\beta_1\)-subunit hybridized, respectively, to one major \(\alpha\)-subunit mRNA (9.4 kb) and to a single \(\beta_1\)-subunit mRNA (1.5 kb), as reported previously (Oh and Waxman, 1994; Klugbauer et al., 1995; Yamamoto et al., 1997). When levels of \(\alpha\)- and \(\beta_1\)-subunit mRNAs were normalized against those of GAPDH mRNA, densitometric analysis revealed that treatment by carvedilol did not significantly change the levels of \(\alpha\)-subunit mRNA (102 \(\pm\) 4, 100 \(\pm\) 4, 99 \(\pm\) 4, 100 \(\pm\) 5, and 106 \(\pm\) 7% of control) \((n = 3)\) and \(\beta_1\)-subunit mRNA (98 \(\pm\) 5, 100 \(\pm\) 4, 102 \(\pm\) 7, 102 \(\pm\) 3, and 105 \(\pm\) 3% of control) \((n = 3)\) at 1, 3, 6, 12, and 24 h, respectively (data not presented).

Effects of Cycloheximide, BFA, H-7, and H-89 Treatment on Carvedilol-Induced Increase in \[^{3}H\]STX Binding. We used various inhibitors of translational or post-translational events of membrane proteins to know which step(s) on carvedilol-induced increase in \[^{3}H\]STX binding is affected by carvedilol. Treatment with 10 \(\mu\)g/ml cycloheximide, an inhibitor of protein synthesis, abolished per se \[^{3}H\]STX binding stimulated by carvedilol (Fig. 7). BFA is an inhibitor of the Golgi network (Moss and Vaughan, 1995; Morinaga et al., 1997). Exposure to 10 \(\mu\)g/ml BFA lowered basal \[^{3}H\]STX binding by 50.4% but nullified the rise of \[^{3}H\]STX binding caused by carvedilol. Furthermore, to test an involvement of protein kinases in the carvedilol effect, we used H-7 and H-89 as inhibitors of protein kinase C and cyclic AMP-dependent protein kinase, respectively. H-7 (100 \(\mu\)M) did not affect the stimulatory effect of carvedilol, whereas H-89 (30 \(\mu\)M) completely suppressed it.

Discussion

Suppression of Na\(^+\) Channel Activity by Carvedilol. In the present study, we demonstrated that carvedilol (1–100 \(\mu\)M) reduced \(^{22}\)Na\(^+\) influx evoked by veratridine in cultured bovine adrenal medullary cells (Fig. 1A). Our study also provides evidence that the reduction of veratridine-induced \(^{22}\)Na\(^+\) influx by carvedilol was not reversed by increasing concentrations of veratridine (Fig. 1B), suggesting that carvedilol does not share common binding sites (site 2) with veratridine. Carvedilol decreased the veratridine-induced \(^{45}\)Ca\(^{2+}\) influx and catecholamine secretion similar to that for the \(^{22}\)Na\(^+\) influx (Fig. 2). In our previous study, veratridine-induced Na\(^+\) influx mediated through Na\(^+\) channels increased Ca\(^{2+}\) influx via activation of voltage-dependent Ca\(^{2+}\) channels and produced the exocytotic secretion of catecholamines (Wada et al., 1985). Thus, it appears that carvedilol reduces veratridine-induced Na\(^+\) influx which, in turn, suppresses Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels and finally produces the reduction of catecholamine secretion.

In addition to voltage-dependent Na\(^+\) channels, in bovine adrenal medullary cells two ion channels are involved in catecholamine secretion: nicotinic acetylcholine receptor-ion channels (activated by carbachol) and voltage-dependent Ca\(^{2+}\) channels (activated by 56 mM K\(^+\)) (Wada et al., 1985). In the present study, we observed that carvedilol reduced the carbachol-evoked influx of \(^{22}\)Na\(^+\) at 3 to 100 \(\mu\)M and suppressed the 56 mM K\(^+\) -stimulated influx of \(^{45}\)Ca\(^{2+}\), but only at a high concentration (100 \(\mu\)M) (Fig. 3). These findings suggest that carvedilol mainly interferes with voltage-dependent Na\(^+\) channels and nicotinic acetylcholine receptor-ion channels. The order of potency of the suppressive effect by carvedilol was voltage-dependent Na\(^+\) channels \(\gg\) nicotinic acetylcholine receptor-ion channels \(\gg\) voltage-dependent Ca\(^{2+}\) channels. Clinical plasma concentrations of carvedilol in humans reached 173 \(\mu\)g/l (425 nM) after intravenous infusion of 12.5 mg, and 66 \(\mu\)g/ml (162 nM) after oral administration of 50 mg (Neugbauer et al., 1987). Although these concentrations are about one order of magnitude lower than the concentration of 1 \(\mu\)M that significantly reduced the activity of voltage-dependent Na\(^+\) channels (Fig. 1), the concentrations used in the present study are comparable with those previously reported in other systems such as cultured human pulmonary artery vascular smooth muscle cells (Ohlstein et al., 1993) and cultured rat cerebellar granule cells (Lysko et al., 1994).

Up-Regulation of Na\(^+\) Channels by Carvedilol Treatment. Prolonged exposure of adrenal medullary cells to carvedilol enhanced \[^{3}H\]STX binding, which reached a plateau at 12 h with the increase of 61% (Fig. 5). Scatchard analysis revealed that carvedilol raised the number of \[^{3}H\]STX binding sites without altering the \(K_d\) value (Fig. 6).

Cycloheximide, an inhibitor of the ribosomal synthesis of proteins, halted the stimulatory effect of carvedilol on \[^{3}H\]STX binding (Fig. 7), suggesting that carvedilol treat-
ment induces the up-regulation of $\text{Na}^+$ channels through a de novo synthesis of protein(s). The mRNA levels of the $\alpha$- and $\beta$-subunit $\text{Na}^+$ channels, however, were not changed by carvedilol treatment. A simple interpretation of these results is that carvedilol may stimulate the translational or post-translational step(s) of $\text{Na}^+$ channel synthesis or may inhibit the internalization/degradation of $\text{Na}^+$ channels. Yamamoto et al. (1996) also observed that treatment of cultured adrenal medullary cells with insulin up-regulates the functional $\text{Na}^+$ channels without causing any change in the level of mRNA encoding the $\text{Na}^+$ channel $\alpha$-subunit. In the present study, brefeldin A, an inhibitor of vesicle-mediated externalization of newly synthesized proteins from the trans-Golgi network, abolished the up-regulation of $\text{Na}^+$ channels by carvedilol (Fig. 7). Several lines of evidence have indicated a regulatory role of protein kinase A in the production of constitutive transport vesicles from the trans-Golgi network of endocrine cells (Muniz et al., 1997). Indeed, in the present study, H-89, an inhibitor of protein kinase A, abolished the stimulatory effect of carvedilol. These findings led us to hypothesize that carvedilol up-regulates $\text{Na}^+$ channels via increasing vesicular trafficking from the trans-Golgi network, in which protein kinase A may be involved. Yoshimura et al. (1998), however, reported that long-term treatment with the anticonvulsant carbamazepine up-regulates $\text{Na}^+$ channels through a cycloheximide-sensitive but cyclic AMP-independent pathway in cultured adrenal medullary cells. Thus, the up-regulation of $\text{Na}^+$ channels induced by $\text{Na}^+$ channel blockers might be more complex than is generally acknowledged. Indeed, $\text{Na}^+$ channels have been reported to be associated with various cytoskeletal proteins (Flucher and Daniels, 1989) at plasma membranes (Kordeli et al., 1990). Little, however, is known as to the molecular machinery that regulates intracellular vesicular transport (Novick and Brenwald, 1993) of ion channels/receptors, including $\text{Na}^+$ channels, toward plasma membranes or the internalization of cell surface $\text{Na}^+$ channels (Dargent et al., 1994). Therefore, it is interesting to characterize precisely the processes of externalization or internalization of $\text{Na}^+$ channels induced by these $\text{Na}^+$ channel blockers.

The clinical implication of the up-regulation of $\text{Na}^+$ channel blockers induced by carvedilol is not clear at present. When carvedilol increased cell surface number of $[^3\text{H}]$STX binding sites (Figs. 5 and 6), the functional activity of $\text{Na}^+$ channels, such as $\text{Na}^+$ influx, was rather suppressed in cells treated with carvedilol for 12 h (Fig. 4). However, $\alpha$ (site 3 toxin)- or $\beta$ (site 4 toxin)-scorpion venom, or PfTx-3 (site 5 toxin) enhanced the $^{22}\text{Na}^+$ influx induced by veratridine in the presence or absence of carvedilol. Therefore, it appears that carvedilol does not interact with these distinct functional segments of the $\text{Na}^+$ channel $\alpha$-subunit (Trainer et al., 1994; Rogers et al., 1996), and prolonged exposure to carvedilol does not alter the pharmacological features of $\text{Na}^+$ channels. It may be that carvedilol accumulates in the plasma membranes during prolonged exposure and is not readily removed by photography because this drug is highly lipophilic (Neugebauer et al., 1987), thus producing the continued suppression of $\text{Na}^+$ channel gating (Fig. 4). Therefore, carvedilol might produce a persistent suppressive effect on $\text{Na}^+$ channels and exert long-lasting blocking actions. Further in vivo studies would clarify whether administration of carvedilol influences the $\text{Na}^+$ channel activity in experimental animals.

In conclusion, carvedilol reduced the functional activity of voltage-dependent $\text{Na}^+$ channels and subsequently up-regulated cell surface $\text{Na}^+$ channels in cultured adrenal medullary cells. The present findings may add new pharmacological actions of carvedilol to our understanding in the treatment of heart failure and hypertension.

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