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REGULATION BY CHRONIC DRUG ADMINISTRATION OF NEURONAL AND CARDIAC CALCIUM CHANNEL, BETA-ADRENOCEPTOR AND MUSCARINIC RECEPTOR LEVELS

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Abstract—Chronic administration of atropine (40–100 mg/kg, 23 days) produced a 29–33% increase in muscarinic receptors, measured by [^{3}H]quinuclidinyl benzilate binding, in rat brain. Diisopropyl phosphorofluoridate (0.9 mg/kg, 14 days) produced a 35% decrease in muscarinic receptors. Propranolol administration (800 μg/kg/hr, 10 days) increased beta-adrenoceptors, measured by [^{3}H]dihydroalprenolol binding, by 69 and 50% in brain and heart respectively. Isoproterenol administration (800 μg/kg/hr, 10 days) produced a 50% reduction in cardiac beta-adrenoceptors but did not alter brain receptors. These drug treatments were without effect on binding of the Ca^{2+} channel ligands, [^{3}H]nimodipine and [^{3}H]nitrendipine, to brain or heart respectively. However, chronic administration of nifedipine for 20 days (36 and 360 μg/kg/hr) did produce down-regulation of both cardiac and neuronal Ca^{2+} channels and a similar down-regulation of beta-adrenoceptors. Co-regulation of Ca^{2+} channels and neurotransmitter receptors may occur but may not be an automatic consequence of either receptor or channel regulation.

An elevation in the concentration of free intracellular Ca^{2+} in response to cell stimulation is central to many cellular events. One major pathway by which intracellular Ca^{2+} levels are elevated is by activation of voltage-dependent Ca^{2+} channels. The Ca^{2+} channel antagonists including the clinically available verapamil, diltiazem and nifedipine are potent blockers of Ca^{2+} channel function in smooth and cardiac muscle and are being used clinically to treat several cardiovascular disorders [1–3]. The voltage-dependent Ca^{2+} channels sensitive to these organic antagonists represent one subset of channels [4–6] and may be linked directly or indirectly to membrane receptors for neurotransmitters and hormones by electrical or biochemical processes, the latter including the guanine nucleotide binding proteins [7–9].

Because of the extensive use of these antagonists in cardiovascular medicine, it is important to know whether chronic use produces tolerance and whether adverse effects occur upon withdrawal. There are currently few reports concerning such phenomena for the Ca^{2+} channel blockers and clear evidence for the development of either tolerance or withdrawal is not available [6, 10–12], but adverse symptoms have been associated with withdrawal of the beta-receptor antagonist propranolol [13, 14].

Chronic administration of receptor-specific agonist or antagonist ligands is usually associated with decreases and increases, respectively, in ligand binding densities [15–17], and these changes may be associated with the development of tolerance and withdrawal. Receptor occupancy is linked to activation of Ca^{2+} channels in excitable tissues, including cardiac muscle and neurons. Accordingly, it was of interest to determine whether receptor regulation in these tissues is associated with co-regulation of Ca^{2+} channels and whether chronic administration of a Ca^{2+} channel antagonist is accompanied by regulation of both channels and receptors.

It is known that neuronal Ca^{2+} channels can be regulated homologously by chronic administration of antagonists in food [18] and heterologously by several influences including alcohol [19], membrane potential [20], and thyroid hormone [21]. Additionally, receptor and channel numbers are altered simultaneously under some conditions including treatment with 6-hydroxydopamine [22] and thyroid hormone [21].

MATERIALS AND METHODS

Chronic drug treatment. Male Holtzman laboratory rats (Charles Rivers, Wilmington, MA) were anesthetized with ether. A cannula consisting of a 21-gauge hypodermic needle joining a section of PE 60 tubing to a section of silastic tubing was introduced into the external jugular vein. The cannula was anchored to the vessel by a suture of 4.0 surgical silk and subcutaneously positioned to the anterior dorsal scapular region. An osmotic pump (model 2ML1, Alza Corp., Palo Alto, CA), subcutaneously placed between the second and thirteenth thoracic vertebrae, was connected to the jugular cannula. Animals received a continuous intravenous drug infusion for 10 or 20 days with free access to food

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and water. When intraperitoneal pump implantation was used, a 2-cm abdominal incision was made in the left suprainguinal region, the pump was inserted intraperitoneally, and the incision was closed with sutures of 2.0 silk.

Nifedipine, dissolved in polyethylene glycol (PEG 400), was delivered at rates of 36 and 360 μg/kg/hr for 10 or 20 days. Whole blood was collected from the ventral caudal artery during this treatment, centrifuged at 300 *g*, and the unhemolyzed serum used to determine nifedipine levels by radioreceptor assay [23, 24]. Control animals received PEG 400 and pump implantation alone. Similarly, propranolol, dissolved in saline, was administered at a rate of 800 μg/kg/hr for 10 days [25]. Isoproterenol was administered by the intraperitoneal route at a rate of 800 μg/kg/hr for 10 days [25]. In some experiments, nifedipine was administered by the intraperitoneal route using a delivery of 360 μg/kg/hr for 20 days. For studies of muscarinic receptor regulation, atropine and diisopropyl phosphorofluoridate (DFP) were administered by injection. Atropine sulfate was administered daily at doses of 40 and 100 mg/kg, i.p., for 23 days. Control animals received 0.9% saline. DFP was administered in peanut oil by giving first a loading dose of 1.25 mg/kg, s.c., and thereafter seven doses of 0.9 mg/kg, s.c., every other day. Control animals received peanut oil alone. Acetylcholinesterase activity was assayed by the method of Ellman *et al.* [26] after the loading dose and when the animals were killed. Animals were killed 24 hr after the pumps had been removed or 24 hr after the last injection of drug or vehicle, and tissues were prepared for radioligand binding.

Radioligand binding. Animals were killed by decapitation, and the intact heart and brain were rapidly removed and placed in ice-cold 50 mM tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.4. The cerebral cortices and cardiac ventricles were dissected, minced with scissors, and homogenized in Tris buffer with a glass Teflon homogenizer. The homogenate was centrifuged at 1,100 *g* for 20 min at 4°, the supernatant fraction was recentrifuged at 10,000 *g* for 10 min, and this supernatant was centrifuged at 45,000 *g* for 45 min. The resultant membrane pellet was resuspended for radioligand binding, but in the case of nifedipine-treated animals and their controls, it was resuspended and recentrifuged twice prior to radioligand binding. Protein concentrations were determined by the method of Bradford [27]. Radioligand binding assays were performed by established protocols and filtration employed a Brandel cell harvester (model M-24R, Gaithersberg, MD) and Whatman GF/B filters, save for the [^{3}H]dihydroalprenolol assay where GF/C filters presoaked in 0.1% polyethyleneimine were used.

For [^{3}H]nitrendipine binding, our previously described protocols were employed [28, 29] with concentrations of [^{3}H]nitrendipine from 3×10^{-11} M to 10^{-9} M, an assay volume of 5 ml Tris at pH 7.4 with 50–150 μg protein per assay volume, and an incubation time of 60 min at 25°. Specific binding was defined by 10^{-6} M nitrendipine, and after filtration the filters were washed twice with ice-cold buffer. For muscarinic receptors (brain only), [^{3}H]quinuclidinyl benzilate, 10^{-11} to 10^{-9} M, was incubated with protein, 50–150 μg per 5 ml assay volume in Tris buffer, pH 7.4, at 25° for 30 min using 10^{-7} M atropine to define specific binding and washing the filters twice with ice-cold buffer [30]. [^{3}H]Dihydroalprenolol, from 5×10^{-11} to 3×10^{-9} M, was incubated with membrane protein, 150–250 μg per assay volume, for 30 min in 2.5 ml Tris buffer, pH 7.4, with 10^{-6} M propranolol to define specific binding [31]. The filters were washed three times with ice-cold buffer. In some experiments beta-adrenoceptors were measured with [^{125}I]iodocyanopindolol. Membrane protein was resuspended in medium M-199 (Gibco Laboratories, Grand Island, NY) containing 1.8 mM $CaCl_2$, 1 mM KCl, 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes) and 1 mg/ml serum albumin, pH 7.4, at a protein concentration of 80 μg/ml. Incubations were carried out in polypropylene tubes with various concentrations of [^{125}I]iodocyanopindolol, 2×10^{-12} M to 10^{-10} M) in a volume of 0.25 ml for 60 min. The filters were washed four times with 3-ml portions of Tris buffer, 50 mM at pH 7.4 containing 154 mM NaCl. Specific binding was determined by the presence of 10^{-6} M propranolol [32]. ^{3}H-labeled materials were counted by scintillation spectrometry at efficiencies of 40–45% and ^{125}I-labeled materials by gamma counting at an efficiency of 78%.

Radioligand binding data were analyzed by a nonlinear Ligand-based program implemented on an IBM personal computer.

Chemicals. [^{3}H]Quinuclidinyl benzilate ([^{3}H]QNB, sp. act. 30.2 Ci/mmol), [^{3}H]nitrendipine (sp. act. 79 Ci/mmol), [^{3}H]dihydroalprenolol (sp. act. 96.1 Ci/mmol) and [^{125}I]iodocyanopindolol (sp. act. 2200 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) were purchased from Du Pont–New England Nuclear, Boston, MA. Nitrendipine was a gift from Dr. A. Scriabine, Miles Institute for Preclinical Pharmacology, New Haven, CT. Other chemicals were commercially available and were of the highest purity available.

Statistics. The results are reported as means ± SEM. Student's *t*-test was performed to evaluate mean values at the 5% level of significance.

RESULTS

The chronic administration of atropine resulted in a significant increase in the number of [^{3}H]QNB binding sites in rat cerebral cortex (Fig. 1). B_{max} for the control animals was 3.69 ± 0.33 pmol/mg protein with a K_D value of $7.7 \pm 0.06 \times 10^{-11}$ M. Chronic atropine treatment at 40 and 100 mg/kg caused significant increases in B_{max} from control to 4.75 ± 0.24 and 4.91 ± 0.32 pmol/mg protein, respectively, but these increases were not significantly different from each other. There were also significant increases in K_D from the control value accompanying atropine treatment. [^{3}H]Nimodipine binding was unaltered by the atropine treatment (Table 1).

The initial loading dose of DFP caused a reduction of acetylcholinesterase activity to 35% of control, and the activity was reduced further to 10% of control after the subsequent treatment with DFP. There

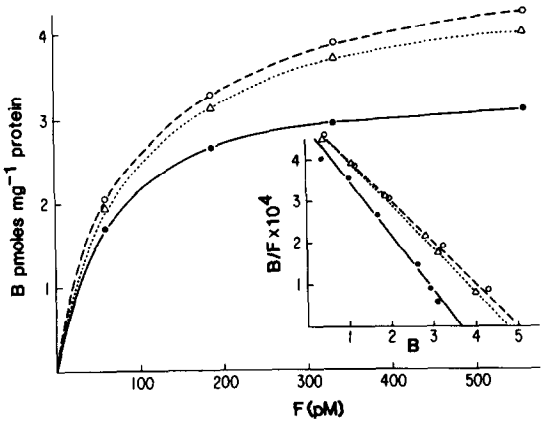

Fig. 1. Specific binding of [^{3}H]QNB to cerebrocortical membranes from rats chronically treated with atropine. Key: control (——), atropine at 40 mg/kg (. . . .) and atropine at 100 mg/kg (– – – –). Inset: Scatchard analysis of the specific binding data. One representative experiment is depicted; the experiment was repeated eight times yielding similar results (Table 1).

was a marked reduction in the number of [^{3}H]QNB binding sites (Fig. 2) from 4.20 ± 0.22 pmol/mg protein to 2.75 ± 0.18 pmol/mg protein without significant change in K_D. [^{3}H]Nimodipine binding was unaltered by the DFP treatment (Table 1).

Propranolol administration resulted in a significant increase in [^{3}H]DHA binding sites in both brain and heart (Table 1). In the brain B_{max} increased from 234 ± 11 fmol/mg protein to 389 ± 13 fmol/mg protein, and this was also accompanied by a significant increase in K_D which increased from 0.96 ± 0.1 × 10^{-9} M to 1.68 ± 0.3 × 10^{-9} M. In the heart B_{max} increased from 140 ± 11 fmol/mg protein to 200 ± 20 fmol/mg protein with an accompanying change in K_D (Table 1). Isproterenol was without effect on brain [^{3}H]DHA binding but caused a 50% reduction in binding sites in the heart (Table 1).

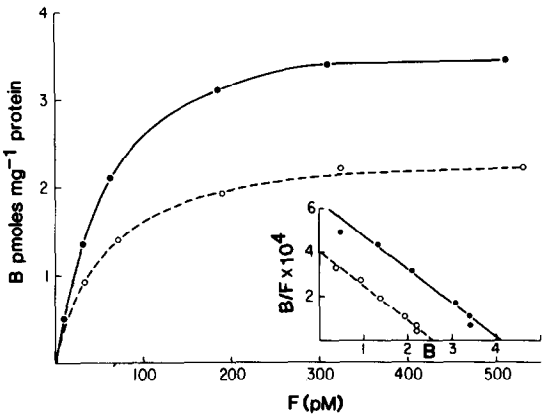

Fig. 2. Specific binding of [^{3}H]QNB to cerebrocortical membranes from rats treated with DFP. Key: control (——) and DFP-treated (– – – –). Inset: Scatchard analysis of the specific binding data. One representative experiment is depicted; the experiment was repeated eight times yielding similar results (Table 1).

Neither propranolol nor isoproterenol treatment affected [^{3}H]nimodipine or [^{3}H]nitrendipine binding in the brain or heart respectively (Table 1).

Nifedipine administration resulted in decreases in the number of [^{3}H]nitrendipine binding sites according to the amount administered and the duration of the chronic treatment. Administration at the highest dose levels for 20 days which resulted in a plasma free concentration of 7 ng/ml (2 × 10^{-8} M) produced a 23% reduction of binding sites in brain and a 49% reduction in heart (Table 2; Fig. 3). At the lower intravenously infused dose of nifedipine, 36 μg/kg/hr for 20 days, which resulted in a significantly lower serum concentration of 0.21 ng/ml (6 × 10^{-10} M), a significant reduction in [^{3}H]nitrendipine binding site density was seen only in cardiac tissue (Table 2; Fig. 3). Administration of nifedipine by the intraperitoneal route for 20 days at the rate of 360 μg/kg/hr resulted in a plasma level of 0.35 ng/ml (1 × 10^{-9} M) and reduced [^{3}H]nitrendipine binding site density by 10 and 34% in the brain and heart respectively (Table 2; Fig. 3); only the latter change was significant. Accompanying studies of [^{125}I]iodocyanopindolol binding showed that beta-adrenoceptor binding site density was reduced significantly in both brain and heart to 65 and 62%, respectively, of control following chronic nifedipine administration at 360 μg/kg/hr for 20 days. No significant changes in either [^{3}H]nitrendipine or [^{125}I]iodocyanopindolol binding were observed following 10 days of chronic nifedipine treatment (data not shown).

DISCUSSION

The major focus of this work was to determine whether regulation of one receptor type by a specific ligand was also accompanied by changes in other receptors or excitable membrane components that may be linked to, or activated by, the regulated receptor. In particular, it was of interest to determine whether voltage-dependent Ca^{2+} channels and neurotransmitter receptors are co-regulated. Cardiac beta-adrenoceptor stimulation is accompanied by voltage-dependent Ca^{2+} channel activation [7], and it appeared of interest to compare this system with others, including cardiac and neuronal muscarinic receptors and neuronal beta-adrenoceptors which either do not activate Ca^{2+} channels or where channel association is undefined.

Chronic administration of atropine and other muscarinic antagonists has been shown by several groups to produce up-regulation of muscarinic receptors [33–35]. In a similar fashion, propranolol causes up-regulation of beta-adrenoceptors [36, 37]. DFP, a potent acetylcholinesterase inhibitor, causes abnormally high acetylcholine concentrations leading to down-regulation of muscarinic receptors [34, 38], and chronic administration of isoproterenol similarly causes down-regulation of beta-adrenoceptors [25]. The present study confirms these findings. The increases in muscarinic and beta-adrenoceptor binding sites following atropine and propranolol treatment were accompanied by significant changes in K_D values. These changes in K_D values likely represent the presence of residual drug in the system, although

Table 1. Effects of chronic muscarinic and β-adrenoceptor drug treatment on specific [^{3}H]QNB, [^{3}H]DHA, [^{3}H]nimodipine and [^{3}H]nitrendipine binding to rat brain and heart membranes

	[^{3}H]QNB		[^{3}H]DHA		[^{3}H]Nimodipine		[^{3}]Nitrendipine	
Treatment	B_{max} (pmole/mg)	K_D ($\times 10^{-12}$ M)	B_{max} (fmoles/mg)	K_D ($\times 10^{-9}$ M)	B_{max} (fmoles/mg)	K_D ($\times 10^{-12}$ M)	B_{max} (fmoles/mg)	K_D ($\times 10^{-12}$ M)
Rat brain								
Control saline	3.69 ± 0.33	77 ± 6 (8)	234 ± 11	0.96 ± 0.1 (3)	702 ± 37	263 ± 63 (8)		
Atropine 40 mg/kg, i.p.	4.75 ± 0.24	95 ± 4* (8)			711 ± 21	266 ± 31 (8)		
Atropine, 100 mg/kg, i.p.	4.91 ± 0.32*	102 ± 6* (8)			688 ± 55	225 ± 18 (8)		
Propranolol, 800 μg/kg/hr, i.p.			389 ± 13*	1.68 ± 0.3* (3)	701 ± 78	397 ± 92 (3)		
Isoproterenol, 800 μg/kg/hr, i.p.			230 ± 12	1.19 ± 0.1 (3)	684 ± 90	349 ± 54 (3)		
Control peanut oil, s.c.	4.20 ± 0.22	76 ± 10 (8)			715 ± 36	275 ± 21 (8)		
DFP, 0.9 mg/kg, s.c.	2.75 ± 0.18*	81 ± 12 (8)			722 ± 13	281 ± 16 (8)		
Rat heart								
Control			140 ± 11	0.37 ± 0.04 (2)			620 ± 35	150 ± 17 (2)
Vehicle, 0.1% ascorbic acid saline			140 ± 10	0.30 ± 0.04 (2)			605 ± 31	126 ± 13 (2)
Propranolol, 800 μg/kg/hr, i.p.			200 ± 20*	0.46 ± 0.04* (2)			595 ± 32	136 ± 10 (2)
Isoproterenol, 800 μg/kg/hr, i.p.			70 ± 8*	0.38 ± 0.03 (2)			603 ± 26	153 ± 7 (2)

Values are means ± SE except where the number in parentheses is less than 3; for values where the number is 2, the mean ± average is shown.
* Significantly different from control, $P < 0.05$.

Table 2. Effect of chronic nifedipine treatment on specific [^{3}H]nitrendipine and [^{125}I]iodocyanopindolol binding in rat brain and heart membranes

Treatment	[^{3}H]Nitrendipine			[^{125}I]Iodocyanopindolol	
	B_{max} (fmoles/mg)	K_D ($\times 10^{-12}$ M)	Serum level (ng/ml)	B_{max} (fmoles/mg)	K_D ($\times 10^{-12}$ M)
Control, Vehicle PEG 400, i.v.					
Heart	600 ± 20	169 ± 33 (5)		160 ± 11.3	8.65 ± 0.8 (2)
Brain	220 ± 10	138 ± 33 (5)		235 ± 7.1	12.7 ± 0.7 (2)
Nifedipine, 360 μg/kg/hr, i.v., 20 days			7.0		
Heart	320 ± 20*	130 ± 10 (10)		99 ± 10*	9.7 ± 1.0 (6)
Brain	170 ± 10*	136 ± 18 (10)		152 ± 27*	11.2 ± 0.6 (6)
Nifedipine 36 μg/kg/hr, i.v., 20 days			0.21		
Heart	480 ± 50*	173 ± 19 (5)			
Brain	210 ± 20	147 ± 18 (5)			
Nifedipine, 360 μg/kg/hr, i.p., 20 days			0.35		
Heart	410 ± 10*	185 ± 13 (2)			
Brain	200 ± 10	106 ± 15 (2)			

Values are means ± SE except where the number in parentheses is less than 3; for values where the number is 2, the mean ± average is shown.

* Significantly different from control, $P < 0.05$.

the animals were not administered drugs for 24 hr prior to killing.

Cardiac beta-adrenoceptor regulation by either propranolol or isoproterenol was not accompanied by changes in Ca^{2+} channel density although it is known that these systems are functionally linked. However, a similar absence of channel regulation was seen in the other systems, including cardiac and neuronal muscarinic receptors and neuronal beta-adrenoceptors, where linkages to these voltage-dependent Ca^{2+} channels are either absent or not defined. Despite the absence of co-regulation of cardiac beta-adrenoceptors and Ca^{2+} channels reported here, other protocols have indicated co-regulation of these species. Thus, 6-hydroxydopamine treatment increases equally cardiac beta-adrenoceptors and Ca^{2+} channels [22], and reserpine treatment increases both Ca^{2+} channels and alpha-adrenoceptors in smooth muscle [39]. Furthermore, isoproterenol and propranolol cause

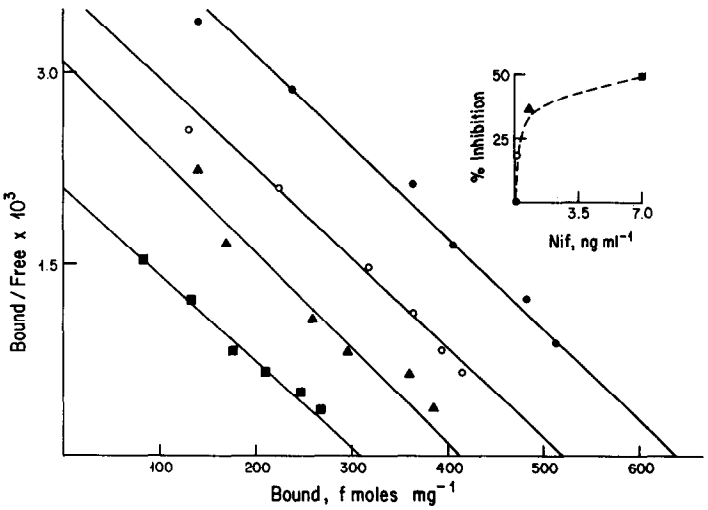

Fig. 3. Scatchard representation of [^{3}H]nitrendipine binding to rat cardiac membranes. Key: (●) control binding; (○) binding to animals chronically treated with nifedipine, 36 μg/kg/hr, i.v., for 20 days; (▲) binding to animals chronically treated with nifedipine, 360 μg/kg/hr, i.p., for 20 days; and (■) binding to animals chronically treated with nifedipine, 360 μg/kg/hr, i.v., for 20 days. Inset: Percent inhibition of specific [^{3}H]nitrendipine binding as a function of plasma nifedipine levels. Shown are representative plots; data are summarized in Table 2.

increases and decreases, respectively, in the numbers of [^{3}H]nitrendipine binding sites in chick skeletal muscle cells [40], and cAMP regulates the appearance of voltage-dependent Ca^{2+} channels in neurons [41]. It is likely that the extent to which co-regulation of a channel and an associated receptor occurs is dependent upon whether synaptogenesis or other developmental change is occurring in the regulated tissue.

Few reports have appeared of the effects of chronic Ca^{2+} channel occupancy on Ca^{2+} channel density. The subject is of both basic and clinical interest, the latter because of the possibility of adverse "rebound effects" following withdrawal from Ca^{2+} channel blockers; however, few documented reports of this phenomenon exist [6, 11]. Our data showing down-regulation of [^{3}H]nitrendipine binding sites following chronic intravenous and intraperitoneal administration of nifedipine confirm previous reports of down-regulation in brain by chronic administration of nifedipine or verapamil [18]. However, chronic nifedipine treatment for 14 days has been reported not to regulate [^{3}H]nitrendipine binding sites in the rat heart [42]. The duration of the chronic treatment and the blood levels of antagonist achieved are likely to be important factors determining down-regulation since 10 days of intravenous administration was ineffective and the lower levels of circulating nifedipine were clearly less effective in producing down-regulation (data not shown).

The possibility exists that the down-regulation of [^{3}H]1,4-dihydropyridine binding sites observed after chronic nifedipine effects is an indirect effect mediated by increased sympathetic discharge caused by the vasodilatation produced by nifedipine. This is unlikely since neither isoproterenol nor propranolol treatment produced regulation of 1,4-dihydropyridine sites, and chronic treatment with minoxidil (64 μg/kg/hr, 20 days: M. Hawthorn and D. J. Triggle, unpublished data), a non Ca^{2+} channel blocking vasodilator, was also without effect on cardiac 1,4-dihydropyridine binding sites.

The down-regulation of [^{3}H]nitrendipine binding sites observed with chronic nifedipine treatment was accompanied by corresponding changes in the density of beta-adrenoceptors as measured by [^{125}I]iodocyanopindolol binding (Table 2). This contrasts with the lack of effect of chronic beta-adrenoceptor ligand administration on Ca^{2+} channel density reported here and by Nishiyama *et al.* [42]. However, studies by other workers have suggested that mutual regulation of Ca^{2+} channels and beta-adrenoceptors does occur in cardiac tissue. In human cardiac tissue from patients treated with beta-adrenoceptor antagonists or Ca^{2+} channel blockers, the density of beta-adrenoceptors was some 50% above control levels [43]. Additionally, in a group of patients with hypertrophic cardiomyopathy a positive correlation was found between the densities of beta-adrenoceptors and Ca^{2+} channels [44]. In contrast, hyper- and hypothyroid animals show apparently compensating changes whereby beta-adrenoceptor and Ca^{2+} channel densities change in opposing direction according to thyroid state [21]. Clearly, co-regulation of receptors and ion channels exhibits complex features. We are currently studying regulation in cell cultures in an attempt to avoid some of the complexities of regulation that occur *in vivo*.

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