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Regeneration of [^{3}H]Ouabain Binding to (Na^+ + K^+)-ATPase in Chemically Sympathectomized Cat Peripheral Organs

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SUMMARY

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Regeneration of specific binding of [^{3}H]ouabain in particulate fractions and Na^+-dependent [^{3}H]norepinephrine uptake in tissue slices from salivary gland, heart, spleen and nictitating membrane were measured in control and chemically sympathectomized cats. Regeneration of ouabain binding was relatively rapid in salivary gland and relatively slow in heart and nictitating membrane particulate fractions. Similarly, recovery of norepinephrine uptake was relatively rapid in salivary gland and relatively slow in heart and spleen slices. Thus rates of reappearance of ouabain binding and norepinephrine uptake appear to be similar in separate organs. Kinetic analyses of ouabain binding show that the return of ouabain binding sites in regenerating sympathetic nerve endings is due to augmentation of density of binding sites. The norepinephrine uptake and ouabain binding in salivary glands degenerated to a similar extent 24 hours after administration of 6-hydroxydopamine. These results support the idea that a major fraction of ouabain binding sites in several sympathetically innervated organs is localized at the sympathetic nerve terminals.

INTRODUCTION

Chemical sympathectomy following administration of 6-hydroxydopamine (6-OHDA)[2] to cats or rabbits leads to a marked decrease in the densities of ouabain-binding sites in microsomal preparation from salivary glands, heart, nictitating membrane, vas deferens, and slow- and fast-contracting skeletal muscles (1–4). There are three possible reasons for a remarkable decrease in the densities of ouabain-binding sites in microsomal preparations derived from several peripheral organs of cats or rabbits following chemical sympathectomy. First, a reduction of the total number of ouabain-binding sites may be due to predominant localization of (Na^+ + K^+)-ATPase in the sympathetic nerve terminals which degenerate during chemical sympathectomy. Second, 6-OHDA may have nonspecific effects on the cell plasma membrane so that it either decreases the densities of ouabain-binding sites in the membrane suspensions or alters the sedimentation coefficient of the microsomes. Third, sympathetic nerves may be involved, in part, in the regulation of number of oua-

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[2] The abbreviations used are: 6-OHDA, 6-hydroxydopamine; NE, norepinephrine.

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bain-binding sites on the surface of some peripheral tissues so that ablation of sympathetic input leads to a decrease in the number of ouabain-binding sites.

In order to obtain further evidence in support of the hypothesis that a large proportion of ouabain-binding sites are localized at the sympathetic nerve terminals (1), we have examined the reappearance of ouabain-binding sites in regenerating sympathetic nerve terminals. We have taken advantage of the fact that the rate of regeneration of adrenergic nerve terminals innervating salivary glands is many times faster than the rate of recovery of sympathetic nerve endings to heart or nictitating membrane in a chemically sympathectomized animal (5–11) to show that rates of reappearance of ouabain-binding sites in microsomes of different peripheral organs are similar to the rates of regeneration of sympathetic nerve endings in these organs. These results provide further evidence that toxic effects of 6-OHDA on the end organs do not appear to be involved in the reduction of densities of ouabain-binding sites in microsomal preparations of several tissues following chemical sympathectomy.

MATERIALS AND METHODS

Adult male cats were used in all experiments and were divided into two groups. In the first group, catecholaminergic nerve endings were effectively destroyed with intravenous injections of 6-OHDA hydrobromide, two doses of 20 mg/kg on the first day and two doses of 50 mg/kg one week later, as described by Thoenen and Tranzer (6). The second group, which served as control, received equal volumes of normal saline instead of the drug solution. The animals were killed 2, 4, and 12 weeks after the first dose of 6-OHDA or saline. Cats were anesthetized with sodium pentobarbital (30 mg/kg; Nembutal, Abbott). Organs of interest—salivary glands, heart, spleen, and nictitating membrane—were removed immediately and placed on ice. A part of the tissue was minced and homogenized with a Brinkman Polytron PT-20 in 20 volumes of ice-cold 50 mM Tris buffer (pH 7.4 at 37°), and the homogenate was passed first through four and then through eight layers of cheesecloth to remove connective tissue and lipids and then was centrifuged for 15 min at 49,000 × *g*. The pellet was rehomogenized in the same buffer and centrifuged as before. The pellet was finally resuspended in 20 volumes of 50 mM Tris buffer and used for the binding assay.

The assay for specific binding of [^{3}H]ouabain to particulate fractions has been described before (1, 12, 13). Total binding was estimated in the presence of 1 mM Mg^{2+}, 1 mM Na_2ATP, 0.1 M Na^+, and 0.2 ml of tissue particulate fraction in a total volume of 2 ml at 37° for 30 min. The concentration of [^{3}H]ouabain ranged between 0.04 and 0.32 μM. Corrections were made for nonspecific accumulation of radioactivity by assaying parallel incubations in which ATP and Mg^{2+} were omitted from the reaction mixtures. In the presence of Na^+ and the absence of nucleotide, such as ATP, [^{3}H]ouabain does not bind to the (Na^+ + K^+)-ATPase enzyme system (14). The nonspecific accumulation of radioactivity was generally less than 15% of the total binding.

The Na^+-dependent [^{3}H]NE uptake in tissue slices was measured as described previously (15). Ventricle, spleen, and salivary gland slices were prepared with a Stadie-Riggs tissue microtome. The slices were weighed and immersed in 10 ml beakers containing 3.8 ml of modified Krebs solution. Modified Krebs solution had the following composition (mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 0.54; $NaHCO_3$, 25; NaH_2PO_4, 1; and glucose, 11 (pH 7.4). Ethylenediaminetetraacetic acid, 20 μg/ml, ascorbic acid, 200 μg/ml, and nialamide (12.5 μM) were added to retard the spontaneous oxidation of [^{3}H]NE (16, 17). The concentration of [^{3}H]NE was 3.2 nM. The beakers containing the slices were shaken in a Dubnoff metabolic incubator at 37° under an atmosphere of 95% oxygen and 5% carbon dioxide for 30 min. Preliminary experiments indicated that in all tissues examined, the rate of [^{3}H]NE uptake remained linear for at least 30 min. Nonspecific accumulation of radioactivity or blank was routinely determined by parallel incubations at 37° in the absence of external Na^+ for 30 min. After incubation, slices were rinsed three times with ice-cold modified Krebs solu-

tion, blotted on filter paper, and placed in 1 ml of ice-cold 0.4 N perchloric acid. Tissues were extracted in acid for 21 h at 4°, and this was followed by centrifugation at 3000 × *g* for 10 min in a Sorvall centrifuge (Model GLC-2). An aliquot of 0.5 ml of each sample was transferred to a vial containing 9.5 ml of Scintiverse (Fisher Co.) and counted in a Packard Tri-Carb liquid scintillation spectrometer (Model 3380) at 30% efficiency as determined with an internal standard. Tissue/medium ratios for [^{3}H]NE uptake were calculated as (counts/min/g wet weight tissue)/(counts/min/ml of medium). Alumina chromatography by the method described by Snyder *et al.* (18) of all tissues used in the present study confirmed that more than 90% of tissue radioactivity was unmetabolized norepinephrine.

[^{3}H]Ouabain (10 Ci/mmole) and [^{3}H]NE (10 Ci/mmole) were obtained from New England Nuclear Corporation, Boston, Massachusetts. Comparisons of the difference between means of separate experimental groups was determined using Student's *t*-test, and linear regression analyses were calculated by the least-squares method.

RESULTS

The Na^+-dependent [^{3}H]NE uptake into slices of salivary gland, heart, and spleen obtained from control and 6-OHDA treated cats is shown in Table 1. The initial velocity of Na^+-dependent [^{3}H]NE uptake in the slices of salivary gland is about three times greater than that observed with ventricular slices. Of the three tissues examined the uptake velocity for [^{3}H]NE was lowest in spleen slices and was about 60% of that found in heart slices. Administration of 6-OHDA in two divided doses at an interval of seven days decreased the Na^+-dependent [^{3}H]NE uptake by about 83% in salivary gland, 86% in heart, and 90% in spleen slices, suggesting that degeneration of sympathetic nerve endings leads to a marked decrease in Na^+-dependent [^{3}H]NE transport. The rate of recovery of Na^+-dependent [^{3}H]NE uptake in various tissues was found to be different. The initial velocity of [^{3}H]NE uptake nearly doubled in salivary gland and heart slices at four weeks after the initial administration of 6-OHDA compared with the rates at two weeks; on the other hand, in spleen slices the uptake velocity increased by about 60% at the same time period, again compared with the rates at two weeks (Table 1). Twelve weeks after the initial administration of 6-OHDA, the rates of Na^+-dependent [^{3}H]NE uptake in salivary gland, heart, and spleen slices were found to be 87%, 26%, and 16% of control values, respectively.

Specific binding of [^{3}H]ouabain in particulate fractions of salivary gland, heart, and nictitating membrane is shown in Table 2. Specific binding of [^{3}H]ouabain in particulate fractions of salivary gland was approximately two and one-half times the binding seen in the cardiac preparation. Ouabain binding in nictitating membrane was little more than half of that observed in heart particulate fractions. These results are

TABLE 1

Na^+-dependent [^{3}H]norepinephrine uptake in tissue slices

The results shown are the mean of six determinations ± standard error of the mean obtained from six cats in each group. The concentration of [^{3}H]norepinephrine was 3.2 nM. The Na^+-dependent [^{3}H]norepinephrine uptake was measured as described in the text. Tissue/medium ratios for control salivary gland, heart, and spleen were 37.84, 13.44, and 8.41, respectively.

Time after first injection of 6-OHDA	Na^+-dependent [^{3}H]norepinephrine uptake		
	Salivary gland	Heart	Spleen
		pmole/g/min	
Control	14.10 ± 1.25	5.00 ± 0.26	3.10 ± 0.19
2 weeks	2.50 ± 0.16*	0.70 ± 0.03*	0.30 ± 0.02*
4 weeks	4.70 ± 0.29*	1.20 ± 0.08*	0.50 ± 0.03*
12 weeks	12.30 ± 0.89	1.30 ± 0.08*	0.50 ± 0.02*

* Significantly different from control, $p < 0.001$.

TABLE 2

Specific binding of [^{3}H]ouabain in particulate fractions obtained from various organs of cats

The concentration of [^{3}H]ouabain was 80 nM and values are means ± standard errors of six determinations obtained from six cats in each group. Specific binding of [^{3}H]ouabain was measured as described in the text.

Time after first injection of 6-OHDA	Specific binding of [^{3}H]ouabain		
	Salivary gland	Nictitating membrane	Heart
Control	17.40 ± 1.29	4.00 ± 0.21	7.10 ± 0.51
2 weeks	4.10 ± 0.30*	0.80 ± 0.06*	2.30 ± 0.12*
4 weeks	7.50 ± 0.44*	0.80 ± 0.05*	2.80 ± 0.11*
12 weeks	18.10 ± 1.26	1.20 ± 0.08*	3.40 ± 0.12*

* Significantly different from control, $p < 0.001$.

qualitatively similar to, but quantitatively different from, the values reported earlier (1). Quantitative differences may be accounted for by the fact that in the previous report purified microsomal fractions were used in binding studies whereas particulate fractions were used in the present study. Since one explanation for the decrease in ouabain binding following chemical sympathectomy may be the toxic effect of 6-OHDA on peripheral end organs so that the sedimentation coefficient of the microsomes is altered, in this investigation all binding studies have been performed in particulate fractions.

Two weeks after the first injection of 6-OHDA, specific binding of [^{3}H]ouabain in heart, salivary gland, and nictitating membrane particulate fractions decreased by about 67%, 76%, and 80%, respectively (Table 2). In chemically sympathectomized cats, the recovery of specific binding of [^{3}H]ouabain was relatively fast in salivary gland and relatively slow in heart and nictitating membrane. For example, 12 weeks after the first administration of 6-OHDA, ouabain binding in cardiac and nictitating membrane particulate fractions was 48% and 30% of control values, respectively, in contrast to almost complete recovery in salivary glands (Table 2). Thus the results shown in Tables 1 and 2 indicate that rates of recovery of ouabain binding and regeneration of sympathetic nerve terminals in different peripheral organs of chemically sympathectomized cats appear to be similar. A more careful examination of the data in Tables 1 and 2 indicates that the return of ouabain binding precedes the recovery of Na^+-dependent [^{3}H]NE uptake. For example, 12 weeks after the first treatment with 6-OHDA, ouabain binding in cardiac particulate fractions was 48% of the control in contrast to 26% of the control of [^{3}H]NE uptake in heart slices.

Previously, we have demonstrated that a profound decrease in ouabain binding in cardiac microsomes following chemical sympathectomy is due to changes in number of ouabain-binding sites (1). It is possible that partial recovery seen in cardiac and nictitating membrane particular fraction 12 weeks after initial administration of 6-OHDA may be due to changes in apparent affinity of ouabain-binding sites. To rule out this possibility, equilibrium dissociation constants and densities of ouabain-binding sites in salivary gland, heart, and nictitating membrane particulate fractions were estimated by Scatchard analysis (Fig. 1). The kinetic constants obtained from Fig. 1 are shown in Table 3. These results indicate that restoration of ouabain binding in various peripheral tissue particulate fractions following chemical sympathectomy was not due to an increase in apparent affinity of ouabain-binding sites for cardiac glycosides. The observed increase in ouabain binding appears to be due to enhancement of densities of these binding sites in particulate fractions. The equilibrium dissociation constant for ouabain in microsomal preparation decreased in heart and increased in salivary gland following chemical sympathectomy (1). In the particulate fraction, however, administration of 6-OHDA did not produce significant change in dissociation constants in these two tissues (Table 3).

The data presented so far provide strong

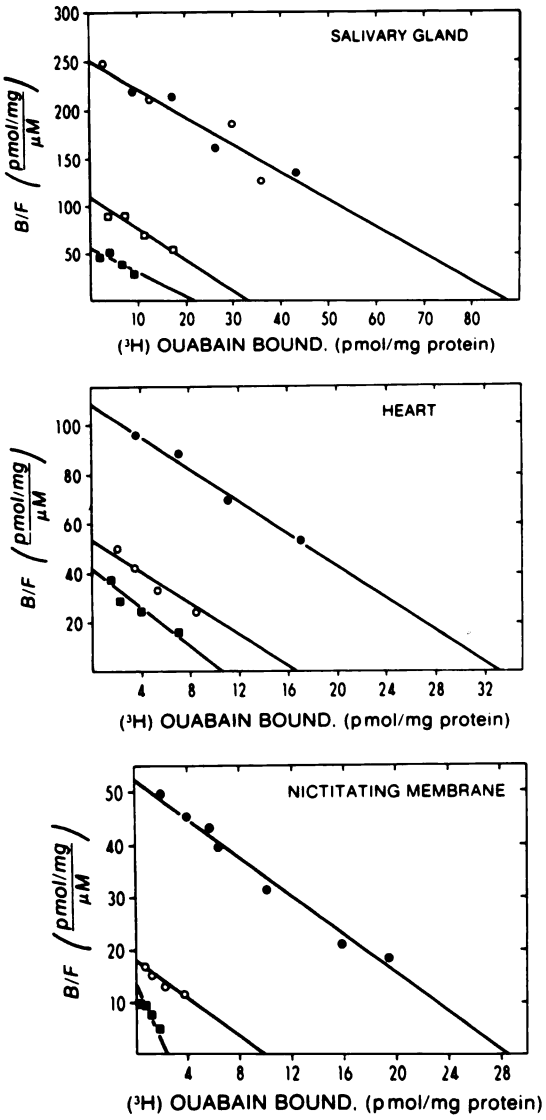

FIG. 1. *Scatchard plots of specific binding of [³H]ouabain in particulate fractions obtained from salivary glands, heart, and nictitating membrane*

Specific binding of [³H]ouabain was measured in the particulate fractions from different organs of cats which were either untreated control (●) or treated with 6-OHDA for 2 weeks (■), 4 weeks (□), or 12 weeks (○) before sacrifice. Each experimental point is the average of six measurements of two separate experiments obtained from two cats, which varied less than 15%.

evidence that [³H]ouabain binding is primarily associated with the sympathetic nerve endings. However, it is not certain if regeneration of ouabain-binding sites following chemical sympathectomy is due to the reappearance of ouabain-binding sites in the end organs or sympathetic nerve terminals. In order to differentiate between these two possible mechanisms, we measured Na^+-dependent norepinephrine uptake and specific binding of [³H]ouabain in cat salivary glands 24 hr after 6-OHDA or saline treatment. Under these conditions, administration of 6-OHDA decreased Na^+-dependent norepinephrine uptake and specific binding of [³H]ouabain in salivary glands by about 60% and 50%, respectively (Table 4). If ouabain binding is associated with the end organ and regulated by sympathetic nerves, then [³H]ouabain binding should change minimally, since the loss of the modulator (sympathetic impulses) should not alter the number of binding sites in so short a time as 24 hours. Therefore, decrease in ouabain binding following chemical sympathectomy appears to be due to degeneration of ouabain-binding sites located in the sympathetic nerve terminals.

DISCUSSION

Chemical sympathectomy decreases ouabain binding in microsomal preparations of various peripheral organs of cats and rabbits (1–4). This decrease in ouabain binding may be due to toxic effects of 6-OHDA on the end organs or loss of regulation by adrenergic nerve of ouabain-binding sites on organ cell surface or destruction of ouabain-binding sites located at the sympathetic nerve endings. Since in adult animals adrenergic cell bodies remain unaffected following administration of 6-OHDA, the regeneration of sympathetic nerve terminals occurs rapidly (7–11). The rates of regeneration of sympathetic nerve endings in different organs are not the same (9–11).

In order to eliminate the possibility that the decrease in ouabain binding in 6-OHDA treated cats is due to toxic effects of the drug, the recovery of ouabain binding in the particulate fraction of salivary glands, heart and nictitating membrane of chemically sympathectomized cats was determined. Although by the end of 12 weeks after the first administration of 6-OHDA ouabain binding in the particulate fractions of salivary glands had returned to control

TABLE 3

Kinetic constants of specific binding of [^{3}H]ouabain in particulate fractions of different organs of cats

The specific binding of various concentrations of [^{3}H]ouabain in particulate fractions from different organs of the cat was assayed as described in the text. The equilibrium dissociation constants (K_D) and maximal number of binding sites (B_{max}) were estimated from Scatchard plots. Values are means (± SE) of six measurements obtained from two cats.

Time after first injection of 6-OHDA	Salivary glands		Heart		Nictitating membrane	
	K_D	B_{max}	K_D	B_{max}	K_D	B_{max}
	μM	*pmole/mg*	*μM*	*pmole/mg*	*μM*	*pmole/mg*
Control	0.35 ± 0.03	87.5 ± 3.4	0.30 ± 0.02	33.2 ± 2.1	0.56 ± 0.04	28.5 ± 1.2
2 weeks	0.39 ± 0.04	21.5 ± 1.2	0.23 ± 0.02	10.5 ± 0.8	0.17 ± 0.01	2.4 ± 0.5
4 weeks	0.30 ± 0.02	33.0 ± 1.3	—	—	—	—
12 weeks	0.35 ± 0.04	87.5 ± 4.6	0.31 ± 0.03	16.6 ± 0.8	0.49 ± 0.04	8.8 ± 0.3

TABLE 4

Na^+-dependent [^{3}H]norepinephrine uptake and specific binding of [^{3}H]ouabain in cat salivary glands

Three cats received 40 mg of 6-OHDA hydrobromide per kg intravenously. A second group of three cats, which served as control, were given equal volumes of normal saline instead of drug. The animals were sacrificed 24 hours after treatment. The Na^+-dependent [^{3}H]norepinephrine uptake and specific [^{3}H]ouabain binding in salivary glands were measured as described in the text. The concentrations of [^{3}H]-norepinephrine and [^{3}H]ouabain were 3.2 nM and 80 nM, respectively. Values are mean ± SE for three determinations.

Treatment	[^{3}H]Norepinephrine uptake	Specific binding [^{3}H]ouabain
	pmole/g/min	*pmole/mg protein*
Saline	13.4 ± 1.4	18.1 ± 1.6
6-OHDA	5.2 ± 0.8*	9.3 ± 0.5*

* Significantly different from control, $p < 0.001$.

values, it remained inhibited by more than 50% in cardiac and nictitating membrane preparations (Table 2). Similarly, the Na^+-dependent [^{3}H]NE uptake was restored to control values in salivary gland slices despite less than 30% return of sympathetic nerve endings in the cardiac tissue 12 weeks after the first treatment with 6-OHDA (Table 1). These observations indicate that there is parallel recovery of ouabain binding and Na^+-dependent NE uptake in different organs following chemical sympathectomy. Thus, it appears highly unlikely that the decrease in ouabain binding in 6-OHDA-treated cats is due to toxic effects of the drug on the peripheral organs.

Chemical sympathectomy may decrease ouabain binding in particulate fractions by abolishing regulation by sympathetic nerves of ouabain-binding sites in organ plasma membranes. This possibility appears unlikely because 6-OHDA treatment did not decrease ouabain binding in spleen microsomal preparations (1). It may be argued that the proposed mechanism will be operative in tissues where postjunctional supersensitivity may be observed in sympathectomized animals. Postjunctional supersensitivity after chronic surgical denervation or chronic reserpine treatment could not be demonstrated in the spleen (19). Thus, a negative result on ouabain binding in spleen microsomes does not invalidate the above possibility.

Perec *et al.* (11) have reported that in 6-OHDA-treated rats, the postjunctional type of supersensitivity to NE or isoproterenol-stimulated secretory responses in salivary glands could be seen 67 days after the first injection of 6-OHDA despite the return of the endogenous content of NE to 84% of control values. Again, whereas there is good correlation between the reappearance of nerve terminals and the recovery of NE content (6, 9, 10), a complete response to sympathetic nerve stimulation returns when the regeneration of the nerve terminals is far from being complete (20, 21). This premature recovery of function is believed to be partially due to the existence of postjunctional supersensitivity (7, 8). Thus, there is good evidence that the rate of reappearance of sympathetic nerve terminals or function is faster than the rate of disappearance of postjunctional supersen-

sitivity in the peripheral organs of chemically sympathectomized animals. Since the recovery of ouabain binding is parallel to, or faster than, the return of sympathetic nerve terminals in 6-OHDA-treated cats (Tables 1 and 2), it is unlikely that the decrease in the density of ouabain-binding sites in salivary gland, heart, and nictitating membrane preparations in chemically sympathectomized cats (Table 3, Fig. 1) is due to abolition of regulation by adrenergic nerves of ouabain binding at the postjunctional sites. Furthermore, 24 hours after a single dose of 6-OHDA, there was parallel decrease in ouabain binding and [^{3}H]norepinephrine uptake in salivary glands (Table 4). Since it is unlikely that postjunctional changes will take place in so short a time as 24 hours, sympathetic nerves may not be involved in the regulation of ouabain-binding sites in the end organ. Finally, 6-OHDA treatment does not modify ouabain binding in cardiac sarcolemmal membrane.[3]

In conclusion, a decrease in ouabain binding in particulate fractions of various peripheral organs of chemically sympathectomized cats does not appear to be due to toxic effects of 6-OHDA on the peripheral end organs or loss of adrenergic regulation of postjunctional binding sites. These conclusions would suggest that in several sympathetically-innervated organs of cats, a large proportion of the ouabain-binding sites are localized at the sympathetic nerve endings.

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