0026-895X/87/060792-06$00.00/0

MOLECULAR PHARMACOLOGY, 32:792-797

Characterization of the α+-like Na^+,K^+-ATPase which Mediates Ouabain Inhibition of Adrenergic Induction of *N*-Acetyltransferase (EC 2.3.1.87) Activity: Studies with Isolated Pinealocytes

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Received April 9, 1987; Accepted September 10, 1987

SUMMARY

Ouabain inhibits (IC_{50} ≃ 200 nM) the ≃ 100-fold adrenergic cyclic AMP stimulation of rat pineal arylalkylamine *N*-acetyltransferase (EC 2.3.1.87, serotonin *N*-acetyltransferase, NAT) activity in intact pineal glands. In the present study, ouabain binding sites in pineal membranes were characterized in detail and compared to sites in isolated pinealocytes, which mediate the inhibition of Na^+,K^+-ATPase, as indicated by ^{86}Rb uptake and norepinephrine (NE) stimulation of NAT activity. High affinity ouabain-binding sites were identified in crude preparations of pineal membranes (K_d ≃ 14 nM; B_{max} ≃ 4 pmol/mg of protein) and similar sites were also found in ovine and bovine pineal tissue. The ouabain K_d value for the rat pineal binding sites was similar to the estimated ouabain IC_{50} values for ^{86}Rb uptake and the NE stimulation of NAT activity in intact rat pinealocytes. In addition, the relative orders of potency of four cardiac glycosides in displacing [^{3}H]ouabain from high affinity binding sites and inhibiting both ^{86}Rb uptake and NE stimulation of NAT activity were the same (acetyldigitoxin > ouabain > digitoxin > strophanthidin). The similarities in the characteristics of the high affinity [^{3}H]ouabain-binding sites and the sites involved in the inhibition of ^{86}Rb uptake and stimulation of NAT activity indicate that an α+-like Na^+,K^+-ATPase mediates the inhibitory effects of ouabain on the adrenergic induction of pineal NAT activity.

The activity of rat pineal arylalkylamine *N*-acetyltransferase (NAT) (EC 2.3.1.87; serotonin *N*-acetyltransferase) is stimulated ≃100-fold at night in response to NE released from sympathetic nerve terminals (1–4). Release is regulated by a neural circuit, which includes a circadian clock located in the suprachiasmatic nucleus of the hypothalamus. The clock is entrained to the environmental lighting cycle by light acting through a retinohypothalamic projection terminating in the suprachiasmatic nucleus. NAT activity can also be stimulated by NE *in vitro*. This appears to involve α_1- and β-adrenergic receptor regulation of cyclic AMP, which acts through transcription, translation, and activation mechanisms to stimulate enzyme activity. The nocturnal increase in NAT activity elevates the production, release, and circulating levels of melatonin (1–4); accordingly, NAT plays an important role in neurochemical transduction in this tissue.

An interesting feature of the adrenergic cyclic AMP stimulation of pineal NAT activity in the adult is that it is completely blocked by K^+ and ouabain. The cardiac glycoside acts at a concentration (0.5 μM) that does not markedly affect protein synthesis or [^{3}H]tryptophan metabolism (5). This effect of ouabain and K^+ is of general interest because it indicates that induction of NAT and, perhaps, other neurally regulated enzymes requires a critical degree of membrane hyperpolarization. Similarly, it has been found that depolarizing treatments inhibit expression of the enkephalin gene in the rat adrenal gland (6, 7).

In an effort to better understand how ouabain acts in this system, we started to study pineal ouabain-binding sites. Our preliminary observations are in general agreement with the argument that an enzyme similar to α+ Na^+,K^+-ATPase, a high affinity ouabain-binding form of Na^+,K^+-ATPase found in rat brain cells and adipocytes (8, 9), may mediate these effects (10). These observations include limited evidence that high affinity ouabain-binding sites occur in pineal membranes, and that a close correlation exists between the developmental pattern of ouabain sensitivity of the NAT induction system and of α+-like Na^+,K^+-ATPase activity, as indicated by ^{86}Rb uptake by intact glands and catalytic activity of membrane preparations. In addition, α+ Na^+,K^+-ATPase has been identified in the pineal gland using immunoblot techniques.[2]

[1] Supported during the course of this work by Grant CCA-8411-029 from the United States-Spain Joint Committee for Scientific and Technological Cooperation.

[2] K. Sweadner, V. Ceña, C. González-García, and D. C. Klein, unpublished results.

ABBREVIATIONS: NAT, arylalkylamine *N*-acetyltransferase; NE, norepinephrine; EDTA, ethylenediaminetetraacetate.

However, the results from studies with cultured pineal glands are somewhat troublesome because the K_d value for ouabain binding is about 1/10 the IC_{50} value for inhibition of NAT induction. We have now reexamined this issue in more detail, using isolated pinealocytes rather than intact pineal glands; the use of pinealocytes eliminates the problem of diffusion barriers inherent to organ cultures. In addition, we have used four cardiac glycosides to characterize and compare these binding sites and the sites mediating the inhibition of ^{86}Rb uptake and NE induction of NAT activity.

Materials and Methods

Animals. Male Sprague-Dawley rats (150–200 g) were used for the binding experiments and organ culture studies. The animals were obtained from Zivic-Miller Co. (Allison Park, PA), who also removed or decentralized the superior cervical ganglia. Female Sprague-Dawley rats were used for cell culture experiments. These animals were also used for some binding experiments for which data are not presented. Animals were housed in our facilities for 2 weeks in an automatically regulated lighting (light/dark 14:10) cycle or in continual lighting (light/dark 24:0), stunned by a blow to the head, and decapitated. Pineal glands were rapidly removed within 1 min of death and were either used immediately for organ culture experiments or frozen on solid CO_2 for future analytical use.

Bovine pineal glands were obtained from a local slaughterhouse within 30 min of death and transported to the laboratory frozen on solid CO_2. Sheep (6–12 months old, Dorset × Rambouillet crosses weighing 30–40 kg) were obtained from a breeding herd of the Division of Intramural Research, National Heart, Lung and Blood Institute. Pineal glands were removed within 3 min of death and frozen on solid CO_2.

Tissue preparation. Rat pineal glands were individually homogenized by sonication (Biosonik IV, Bronwill Scientific Company, Rochester, NY; dial setting at 45 W, 10 times for 5 sec, 4°) in 50 μl of buffer I (150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, and 50 mM Tris-HCl, pH 7.4). Homogenates were mixed, brought to 1 ml with buffer I (approximately 10 glands/ml), and then centrifuged (10,000 × *g*, 1 min, 4°); the supernatant was used to perform all binding assays, unless otherwise indicated.

Ovine or bovine tissue was homogenized in a motor-driven (Greiner Scientific, New York, NY) glass-Teflon homogenizer (30 sec, dial 10) in 10 volumes of buffer I. The homogenate was centrifuged (10,000 × *g*, 1 min, 4°), and the supernatant was used as a source of membranes for most experiments. In addition, a microsomal fraction was prepared by centrifuging the homogenate at 10,000 × *g* (20 min, at 4°) and then centrifuging the supernatant at 100,000 × *g* (1 hr, 4°); the pellet was resuspended in buffer I and used as the microsomal fraction.

Protein was determined as described by Lowry *et al.* (11), using bovine serum albumin as internal standard.

Glycoside-binding site analysis. Membranes (50 μg of protein/tube unless otherwise indicated) were incubated (90 min, 37°) with [^{3}H]ouabain (1–200 nM; specific activity = 20 Ci/mmol) in buffer I containing ATP (1.25 mM; final volume = 1 ml). The incubation was terminated by immersing the tubes in a 4° bath for 10 min. No appreciable loss of bound radioactivity was observed during this period of time. In displacement studies, glycosides were added 10 min before addition of [^{3}H]ouabain (20 nM). This concentration was chosen because it approximated the K_d value estimated by Scatchard analysis. Nonspecific [^{3}H]ouabain binding measured in the presence of 100 μM unlabeled ouabain was less than 10% of the total binding.

Membrane-bound [^{3}H]ouabain was collected with a Cell Harvester M-24 (Brandel, Gaithersburg, MD) using Whatman glass-fiber filters (GF/C). The filters were washed three times with 2 ml of buffer I (4°), dried (30 min, room temperature), and then placed in a vial containing 10 ml of scintillation liquid (HP Ready-Solv, Beckman, Fullerton, CA). Radioactivity was measured by conventional techniques in a Beckman LS5801 scintillation counter.

Saturation and displacement studies were analyzed using the nonlinear curve-fitting program LIGAND (12). Kinetic dissociation experiments were determined with EXPFIT.[3] Other kinetic parameters (K_{+1}, K_{-1}, K_d, K_{obs}) were calculated using published formulas (13).

Cell culture. Pinealocytes were prepared from glands by trypsinization and were incubated for 24 hr in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (14). Cells were aliquoted into 1.5-ml microtubes (10^5 cells/0.5 ml/tube) and then incubated with the desired cardiac glycoside concentration for 0.5 hr before addition of NE (1 μM). The incubation was continued for 4 hr more in the presence of both NE and the cardiac glycoside. The treatment was ended by centrifuging the sample (10,000 × *g*, 30 sec), removing the culture medium, and placing the tube containing the cell pellet on solid CO_2.

Organ culture. Pineal glands were incubated (37°, 95% O_2, 5% CO_2) in chemically defined medium (BGJ^b, 1 gland/well, 200 μl/well) in a 24-multiwell plate for 48 hr before treatment (5, 15). Presynaptic elements degenerate during this period, resulting in a relatively pure preparation of pinealocytes. The medium was changed after 24 hr to remove any NE released by degenerating nerve terminals. After 48 hr, each gland was placed into fresh medium for 1 hr and then NE (10 μM) was added; the gland was removed 4 hr later. Ouabain, when present, was added 0–60 min before NE and maintained through the stimulation period. At the end of the treatment period, each gland was placed in a 1.2-ml microtest tube in solid CO_2 and stored at −75° for up to 3 days before being analyzed for NAT activity.

NAT activity assay. NAT activity was assayed by our modification (5) of the Deguchi and Axelrod method (16). Cell pellets or glands were sonicated (Biosonik IV, Bronwill Scientific Company; dial setting 40 "lo," 10 sec, 4°) in 100 μl of 0.1 M sodium phosphate buffer, pH 6.8, which contained ^{14}C-acetyl-coenzyme A (0.5 mM, specific activity 1 μCi/mmol) and tryptamine (10 mM). Tubes were incubated at 37° for 20 min. The product [^{14}C]acetyltryptamine was extracted into chloroform, which was evaporated to dryness in a scintillation vial. Radioactivity was then determined by conventional techniques.

^{86}Rb uptake. ^{86}Rb uptake was measured by the method of Vara *et al.* (17). Pinealocytes (10^5 cells/tube; 0.5 ml/tube) were incubated (1 hr at 37°) with a range of concentrations of cardiac glycosides; ^{86}Rb (0.1 mM; specific activity 66.7 Ci/mol) was then added. After a 2-min incubation period, a 0.5-ml volume of a solution (4°) containing 0.1 M $MgCl_2$ and 10 mM RbCl was added. The cells were then placed on top of 0.5 ml of a dinonyl phthalate and silicone oil mixture (18) 1:1, v/v), and the tube was centrifuged (10,000 × *g*, 2 min) to pellet the cells. The fluid layers were carefully removed, and the pellet containing the cells was resuspended in 100 μl of deionized H_2O and sonicated (Biosonik IV, Brownwill Scientific Company, dial setting "lo," 10 sec, room temperature). A 50-μl sample was used to determine ^{86}Rb.

Chemicals. NE, digitoxin, acetyldigitoxin, ouabain, strophanthidin, RbCl, and disodium ATP were obtained from Sigma Chemical Company (St. Louis, MO); dinonyl phthalate was from ICN Biomedicals Inc. (Plainview, NY); silicone oil was from Aldrich Chemical Company (Milwaukee, WI); and [^{3}H]ouabain (specific activity 20 Ci/mmol), ^{86}RbCl (specific activity 3 Ci/g), and ^{14}C-acetyl-coenzyme A (specific activity 45 mCi/mol) were from New England Nuclear (Boston, MA). All other reagents were obtained from commercial sources and were of the highest quality available.

Results

Characterization of Pineal [^{3}H]Ouabain-Binding Sites

Specific [^{3}H]ouabain binding was linear between 10 and 200 μg of membrane protein for the three species studied (Fig. 1). Binding was ATP dependent (in unpublished experiments, binding was reduced to 2% in the absence of ATP). Scatchard analysis (12) of the saturation isotherm of [^{3}H]ouabain binding (1–100 nM) revealed a single class of high affinity binding sites

[3] V. Guardabaso, P. Munson, and D. Rodbard, submitted for publication.

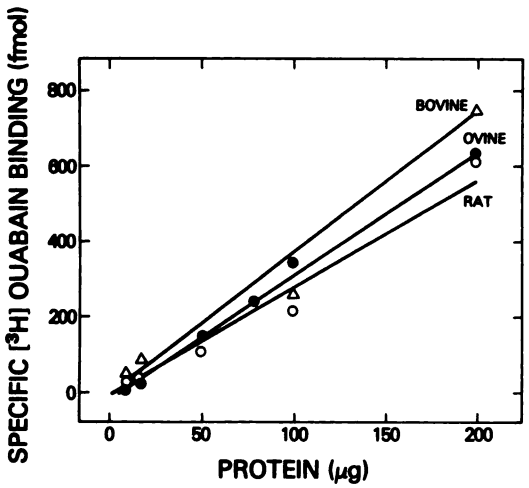

Fig. 1. Specific [^{3}H]ouabain binding as a function of pineal membrane protein. Membranes were prepared from three mammalian species. [^{3}H] Ouabain (20 nM) binding was done as indicated under Materials and Methods. Data represent a typical experiment. Each point was done in triplicate. For further details see Materials and Methods.

with an apparent affinity constant (K_d) of ≃14 nM and a maximal binding capacity (B_{max}) of 4.2 pmol/mg of protein (n = 20) (Table 1, Fig. 2). A similar binding site was also present in ovine and bovine pineal membranes (Table 1). The B_{max} value for [^{3}H]ouabain-binding sites was nearly 10-fold greater in the microsomal fraction of the bovine pineal gland, which is consistent with their location in plasma membranes (Table 1, Fig. 2). This indicates that these binding sites are a constant feature of the mammalian pineal gland.

[^{3}H]Ouabain (50 nM) binding to rat membranes reached equilibrium in approximately 60 min and was stable for at least 2 hr. The association rate constant (K_{+1}) was 0.93×10^6 liters/mol·min (Fig. 3); this represents 1/10 the value reported for rat striatum (19). Specifically bound [^{3}H]ouabain was rapidly displaced by ouabain (100 μM). The dissociation curve revealed a monophasic dissociation rate with a half-life of 35 min (data not shown). The calculated dissociation rate constant (K_{-1}) was 0.0150 min^{-1},[4] which is similar to the values described for rat striatum. The K_d value for ouabain calculated from kinetic parameters (13) was ≃16 nM, similar to that obtained from Scatchard analysis.

[^{3}H]Ouabain (20 nM) displacement from rat binding sites by four cardiac glycosides was determined (Fig. 4). The order of potency was acetyldigitoxin (K_d ≃ 15 nM) > ouabain (K_d ≃20 nM) > digitoxin (K_d ≃ 220 nM) > strophanthidin (K_d ≃ 1000 nM; Table 2). The displacement curves appeared to be generally parallel. Unexplained variation (±10%) in displacement at low concentrations was detected (Fig. 4), but was not consistently observed in all experiments. In an effort to reveal a lower affinity binding site, a higher concentration of [^{3}H]ouabain was used in displacement studies (200 nM). The results were similar to those presented here (data not shown).

To determine whether high affinity [^{3}H]ouabain-binding sites were located primarily on postsynaptic structures, binding sites were measured in denervated rat pineal glands. Denervation was produced by removal of the superior cervical ganglia 14 days before the glands were obtained from animals. This did not alter the [^{3}H]ouabain K_d value and only slightly decreased

[4] V. Guardabaso, P. Munson, and D. Rodbard, submitted for publication.

TABLE 1

K_d and B_{max} values for [^{3}H]ouabain binding in pineal membranes from three mammalian species

Data are presented as the mean ± standard error of the K_d values obtained in the indicated number of experiments (n). [^{3}H]Ouabain binding was performed at 37° for 90 min. In each experiment seven or nine concentrations of [^{3}H]ouabain (1, 2, 4, 10, 20, 40, 50, 75, and 100 nM) were used. Binding at each concentration was assayed in triplicate. For more details see Materials and Methods.

Species	Membrane preparation	n	K_d	B_{max}
			nM	pmol/mg protein
Rat	crude	20	13.8 ± 1.8	4.2 ± 0.7
Bovine	crude	6	17.8 ± 0.3	3.5 ± 1
Bovine	microsomal	1	35.5	36.9
Ovine	crude	3	14.6 ± 4.4	7.8 ± 1

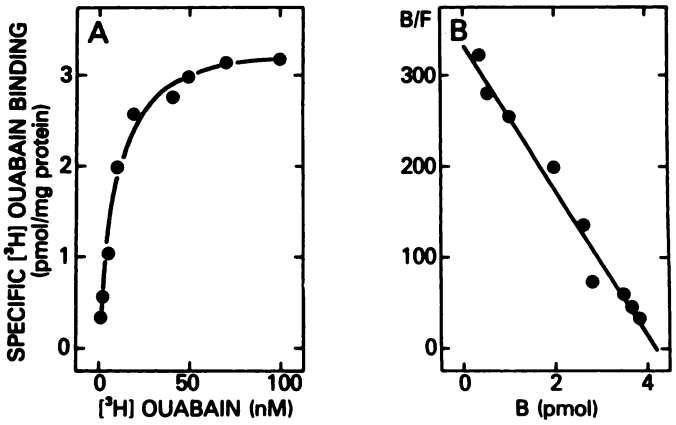

Fig. 2. Saturation isotherm (A) and Scatchard plot (B) for [^{3}H]ouabain (1–100 nM) binding to rat pineal membranes. Unlabeled ouabain (100 μM) was used to determine nonspecific binding, which was always less than 10% of the total binding. Scatchard analysis revealed an average K_d value of 13.8 ± 1.8 nM and a B_{max} value of 4.2 ± 0.7 pmol/mg of protein (12). Data represent a typical experiment in which each point is the average of three determinations. For further details see Materials and Methods.

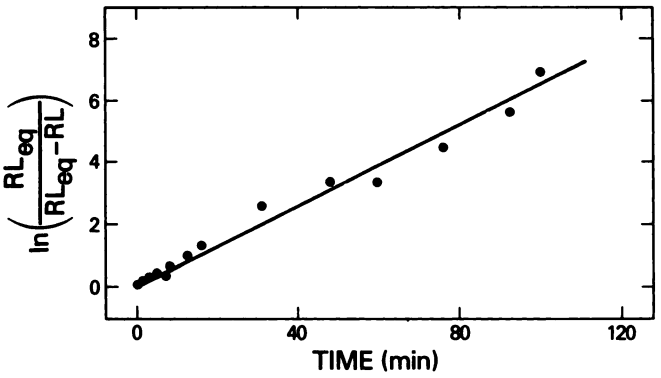

Fig. 3. Semilogarithmic plot of the association curve for [^{3}H]ouabain binding to rat pineal membranes. Tissue was incubated at 37° with 50 nM [^{3}H]ouabain for various time intervals ranging from 1 to 120 min. The slope, determined by linear regression analysis, is equal to k_{obs}, the pseudo-first order rate constant (13). Data are from a single experiment; essentially the same results were obtained when the experiment was repeated. Each point is the average of four determinations. For further details see Materials and Methods.

the B_{max} value (Table 3). In addition, these binding sites were not altered by other treatments which block neural stimulation of the pineal gland via the superior cervical ganglia (4), i.e., decentralization of the superior cervical ganglia or exposure to

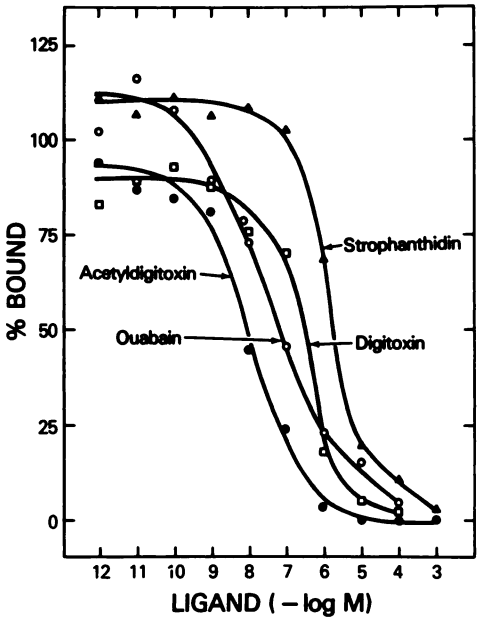

Fig. 4. Displacement of [^{3}H]ouabain (20 nM) bound to rat pineal membranes by cardiac glycosides. Acetyldigitoxin (●), ouabain (○), digitoxin (□), and strophanthidin (▲) were used. Tissue was incubated with [^{3}H] ouabain at 37° for 90 min. Each cardiac glycoside was present for 10 min before the addition of [^{3}H]ouabain. Data represent a typical experiment, in which each point was done in triplicate. K_d values given in Table 1 were calculated with the LIGAND program (12) from the data used to generate these curves. The experiment was repeated twice for digitoxin and acetyldigitoxin, four times for strophanthidin, and six times for ouabain with similar results. For further details see Materials and Methods.

TABLE 2

Summary of cardiac glycoside K_d and IC_{50} values (nM) for inhibition of NE (1 μM)-induced NAT activity and ^{86}Rb uptake in isolated pinealocytes

K_d values were calculated from [^{3}H]ouabain displacement curves using rat pineal membranes (Fig. 4). In the case of strophanthidin and ouabain the standard error is given; the number of binding experiments appears in parentheses. The K_d values for acetyldigitoxin and digitoxin are from two experiments. The IC_{50} values were estimated graphically from data in Fig. 5 and from a second experiment.

Drug	Binding (K_d)	Inhibition of NE stimulation of NAT activity (IC_{50})	Inhibition of ^{86}Rb uptake (IC_{50})
Acetyldigitoxin	14.8, 18.7	4, 6	22, 17
Ouabain	21.8 ± 4.1 (6)	40, 50	34, 25
Digitoxin	254, 210	220, 250	280, 220
Strophanthidin	1038 ± 218 (4)	1310, 1120	880, 1100

TABLE 3

K_d and B_{max} values for specific [^{3}H]ouabain binding to rat pineal membranes

Glands were obtained from control animals, animals with bilateral superior cervical ganglia removal (ganglionectomized) or decentralization (decentralized), or animals that had been in constant lighting. Constant light treatment and post surgery periods were 14 days. See Materials and Methods and the legend to Table 1 for details on the procedures used to measure [^{3}H]ouabain binding. Data are presented as the mean ± standard error of the values generated in 3–20 experiments.

Treatment	n	K_d	B_{max}
		nM	pmol/mg protein
Control	20	13.8 ± 1.8	4.2 ± 0.7
Ganglionectomized	12	19.6 ± 4.3	3.3 ± 0.6
Decentralized	3	27.6 ± 5.4	3.8 ± 0.5
Constant light	10	24.6 ± 6.8	3.4 ± 0.4

constant light (Table 3). The pineal gland is stimulated on a daily basis by the suprachiasmatic nucleus → pineal circuit (4). This stimulation regulates some pineal proteins (1, 3, 20, 21). However, according to these results it does not seem to control the number of [^{3}H]ouabain-binding sites because treatments that produce stimulus deprivation do not modify [^{3}H]ouabain binding. This suggests that the number of pineal α+-like Na^+,K^+-ATPase molecules is not under neural control.

Functional Studies

Inhibition of adrenergic induction of NAT activity. NE induction of NAT activity in isolated pinealocytes was inhibited by ouabain (IC_{50} ≃50 nM; Fig. 5A). The order of potency of the four cardiac glycosides was acetyldigitoxin (IC_{50} ≃5 nM)> ouabain (IC_{50} ≃50 nM)> digitoxin (IC_{50} ≃250 nM)> strophanthidin (IC_{50} ≃1200 nM; Table 2). The inhibition curves were monophasic and nearly parallel (Fig. 5A).

As indicated in the introduction, ouabain inhibits NE induction of NAT activity in the intact rat pineal gland with an IC_{50}

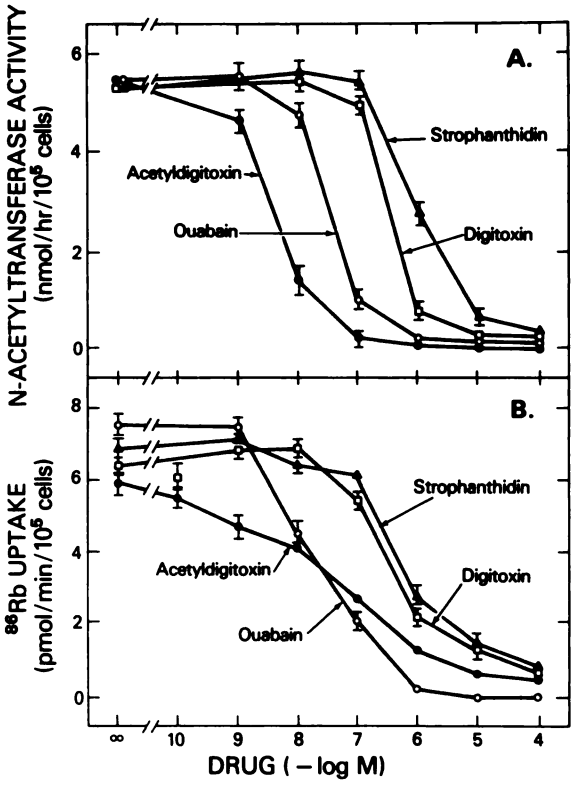

Fig. 5. Effect of cardiac glycosides on NE stimulation of NAT activity (A) and ^{86}Rb uptake (B) in isolated pinealocytes. For NAT studies, cells (10^5/0.5 ml) were incubated in Dulbecco's modified Eagle's medium (1 hr, 37°) with the indicated concentrations of acetyldigitoxin (●), ouabain (○), digitoxin, (□), and strophanthidin (▲). NE (1 μM) was then added and the incubation continued for an additional 4 hr. Incubation was terminated by centrifugation (10,000 × g, 30 sec), removing the supernatant and placing the tube on solid CO_2. For ^{86}Rb uptake the protocol was similar, but ^{86}Rb (1 μCi, specific activity 66.7 Ci/mol) was added for 2 min after a 60-min treatment with the cardiac glycoside of interest. The cell suspension was diluted with 0.5 ml of 0.1 M $MgCl_2$ containing 10 mM RbCl (4°), placed on top of 0.5 ml of a mixture of dinonyl phthalate and silicone oil (1:1, v/v), and centrifuged (12,000 × g, 2 min). The fluid layers were carefully aspirated, the pellet was sonicated in 100 μl of H_2O, and radioactivity in the homogenate was determined. Data represent the mean ± standard error of three experiments in which each value for each point was based on three samples of cells. For further details see Materials and Methods.

of about 200 nM (5). This is about 5-fold higher than the IC_{50} in isolated pinealocytes (Fig. 5A). A possible explanation of this discrepancy is that the mass of the gland presented a diffusion barrier to ouabain and that insufficient time (15 min) was allowed for ouabain to act before the start of the 4-hr NE treatment period. We tested the effects of longer ouabain exposure periods on glands and found that the maximal effect was produced by a 45-min treatment before addition of NE (Table 4). Treatment for 1 hr before NE was added decreased the apparent IC_{50} from 200 nM to about 40 nM (Fig. 6), indicating that the apparent potency of ouabain in isolated cells and in intact glands is similar when sufficient time is allowed for ouabain to penetrate the gland and act, prior to the initiation of NE treatment.

Inhibition of ^{86}Rb uptake. Ouabain inhibits ^{86}Rb uptake by isolated pinealocytes (Fig. 5B), as in the case in the intact pineal gland. We attempted to obtain a better characterization of the receptor involved by comparing the effects of the cardiac glycosides used above. All inhibited ^{86}Rb uptake; the relative potency was acetyldigitoxin (IC_{50} ≃20 nM > ouabain (IC_{50} ≃30 nM) > digitoxin (IC_{50} ≃250 nM) > strophanthidin (IC_{50} ≃1000 nM) (Table 2, Fig. 5B). The inhibition curves appeared to be monophasic and parallel except for that of acetyldigitoxin, which was distinctly less steep.

Discussion

The data presented here provide further evidence that rat pineal membranes possess a high affinity binding site for [^{3}H] ouabain similar to that on rat brain and adipocytes α+ Na^+,K^+-ATPase (8, 9). The evidence includes the finding that the approximate K_d values of four cardiac glycosides for binding are similar to their estimated IC_{50} values for inhibition of ^{86}Rb uptake, and that maximal inhibition by ouabain occurs at concentrations below 1 μM (8, 9). In addition, immunoblot analysis has revealed the presence of α+ Na^+,K^+-ATPase in homogenates of denervated rat pineal glands and isolated rat pinealocytes.[5] It would appear that the published discrepancy (5) between binding data and inhibition by ouabain of adrenergic-induced NAT activity in intact glands reflects an experimental artifact more than real differences, because the discrepancy was not observed when isolated pinealocytes were used. It appears that this discrepancy is due to diffusion barriers inherent to organ culture systems.

Our studies also indicate that this enzyme mediates the inhibition of NE induction of NAT, because the order of potency of the four cardiac glycosides studied is the same for inhibition of both ^{86}Rb uptake and NE-induced NAT activity in isolated pinealocytes and displacement of bound [^{3}H]ouabain from rat pineal membranes. Also, the IC_{50} and K_d values are generally similar for each cardiac glycoside. To our knowledge this is the first demonstration in any neural system that the relative potency of a series of cardiac glycosides in displacing [^{3}H]ouabain is the same as that in functional tests, reflecting both a direct and indirect action of cardiac glycosides.

The [^{3}H]ouabain-binding site described here generally resembles the high affinity site present in brain cortex, which is probably α+ Na^+,K^+-ATPase (8, 19). The K_d found in that study (≃10 nM) agrees very well with the K_d described here (≃14 nM). Moreover, displacement studies show that ouabain is more potent than strophanthidin in displacing [^{3}H]ouabain from high affinity ouabain-binding sites in rat pineal and brain (Fig. 4; Ref. 19). Similarly, strophanthidin is less potent than ouabain in inhibiting α+-like Na^+,K^+-ATPase activity of both rat brain and pineal preparations (8, 19, 22). Although the relative affinity of the binding site for ouabain and strophanthidin is similar in both the rat brain and pineal, there is a discrepancy in the absolute values reported. The IC_{50} value for ouabain calculated from displacement studies reported by Hauger *et al.* (19) (750 nM) is different from that reported here (≃60 nM).

This discrepancy may reflect experimental differences. In the displacement studies in this paper we incubated the membranes for 10 min with the unlabeled cardiac glycosides before we added [^{3}H]ouabain; Hauger *et al.* (19) added labeled and cold drug at the same time. In addition, we used a 90-min ouabain incubation period rather than the 60-min period used in the brain study; longer incubation periods are required to achieve equilibrium at low concentrations of ligand. Another possible cause for a difference in the apparent binding characteristics is the amount of protein used in the present studies (50 μg/ml) and the studies by Hauger *et al.* (19) (600 μg/ml).

To test the validity of the IC_{50} values reported here and in the Hauger *et al.* (19) report, we have used the Cheng-Prussof

TABLE 4

Relationship of the length of ouabain treatment preceding initiation of NE treatment to ouabain inhibition of NE-induced NAT activity in cultured rat pineal glands

Ouabain (1 μM) was incubated with pineal glands for the indicated periods of time before the addition of NE (10 μM) and was present during NE treatment (4 hr). Data represent the mean ± standard error of four determinations. Basal NAT activity in nonstimulated pineal glands was 0.03 ± 0.02 nmol/min/mg of protein (n = 4).

Ouabain (1 μM)	Ouabain treatment	NAT activity
	min before NE	*nmol/hr/mg protein*
−	0	91.2 ± 1.8
+	0	29.4 ± 6.6
+	10	16.8 ± 3
+	20	14.4 ± 3
+	30	9.0 ± 2.4
+	45	4.8 ± 1.2
+	60	4.2 ± 0.6

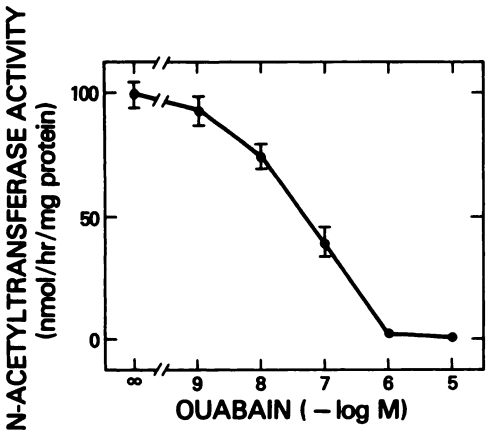

Fig. 6. Inhibitory action of ouabain on NE (10 μM)-induced NAT activity in cultured pineal glands. In this study ouabain was present for 1 hr before addition of NE. The experimental procedure is similar to that described in Fig. 5. Data represent the mean ± standard error of the NAT activity in four pineal glands. For further details see Materials and Methods.

[5] K. Sweadner, V. Ceña, C. González-García, and D. C. Klein, unpublished results.

equation (23) to calculate K_d values from data obtained from displacement studies. Using this approach, we have found that the K_d for ouabain in rat pineal tissue is about 24 nM, which is similar to that obtained using LIGAND analysis of saturation isotherms (14 nM). However, application of this equation to the data reported by Hauger *et al.* (19) generates a K_d value of 375 nM, which is >40-fold larger than that obtained from LIGAND analysis of rat brain saturation isotherms. Accordingly, it appears that the absolute IC_{50} value for ouabain reported for rat brain is not accurate, possibly reflecting the differences in methodology identified above. In addition, it appears likely that the absolute IC_{50} values reported for the other agents in that study are inaccurate.

Our studies not only indicate that an α+-like Na^+,K^+-ATPase activity is present in the pineal gland, but also indicate that this form of Na^+,K^+-ATPase is the dominant mechanism through which K^+ is pumped into this cell, because low concentrations of cardiac glycosides nearly completely inhibit ^{86}Rb uptake within the 2-min test period. This indicates that isolated pinealocytes can be used to study the function and regulation of this form of the enzyme in an intact neural cell, which has not been done. The similarities of the pineal and brain enzymes suggest that studies on the pinealocyte may lead to a better understanding of how this enzyme is regulated in intact brain cells. Studies in isolated adipocytes suggest that insulin may regulate α+ Na^+,K^+-ATPase (9). It will be of interest to determine whether insulin or other agents can influence α+-like Na^+,K^+-ATPase in intact pinealocytes. In preliminary unpublished studies along these lines, we have already found that NE does not cause a significant change in ^{86}Rb uptake.

Although the available evidence indicates that α+ Na^+,K^+-ATPase is present in pinealocytes and is primarily responsible for K^+ uptake, as indicated by ^{86}Rb uptake studies, it also appears that both α+ and α Na^+,K^+-ATPase are present in pinealocytes.[5] Our inability to obtain evidence that the α-like low affinity ouabain-binding form of the enzyme is functional, as indicated by ^{86}Rb uptake, is puzzling. Perhaps the contribution of α Na^+,K^+-ATPase provides to ^{86}Rb uptake is too small to be detectable by the techniques used. Similarly, technical limitations might also preclude the detection of the low affinity cardiac glycoside-binding site using [^{3}H]ouabain because this site appears to have a very rapid dissociation rate (24). In addition, the number of binding sites present in the preparation could be too low to detect with the methodology employed. Perhaps future studies will reveal the functional importance of this apparently silent form of Na^+,K^+-ATPase.

Finally, the rat had been considered to be a ouabain-resistant species (25, 26). However, it is clear from studies on rat cortex, adipocytes, and pinealocytes (9) that this is not true for all rat tissues; ouabain binds to high affinity sites in these tissues, where low concentrations of ouabain have also been found to inhibit α+ Na^+,K^+-ATPase as indicated by ^{86}Rb uptake or other functions. In the pinealocyte this interaction also leads to inhibition of adrenergic or cyclic AMP stimulation of NAT activity. Perhaps ouabain blocks gene expression in other rat tissues through inhibition of α+ Na^+,K^+-ATPase and subsequent depolarization.

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