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A Sidhu, … , P A Jose, R A Felder

Research Article

The natriuretic effect of dopamine-1 (DA-1) agonists is reduced in spontaneously hypertensive rat (SHR), partly because of defective DA-1 receptor-adenylate cyclase (AC) coupling in renal proximal convoluted tubules. To investigate this defective coupling, DA-1 dopamine receptors from renal proximal tubules were solubilized and reconstituted into phospholipid vesicles. The binding of DA-1-selective ligand [125I]SCH 23982 was specific and saturable, with no differences in receptor density or Kd between SHR and normotensive rats (Wistar-Kyoto rats; WKY). Competition experiments of the reconstituted DA-1 dopamine receptors in WKY with a DA-1-selective agonist, SKF R-38393, revealed the presence of high- (Kh = 350 +/- 209 nM) and low-affinity (Kl = 70,500 +/- 39,500 nM) binding sites. 100 microM Gpp(NH)p abolished the agonist high-affinity sites, converting them to a low-affinity state (Ki = 33,650 +/- 10,850 nM). In SHR, one affinity site was noted (Ki = 13,800 +/- 500) and was not modulated by Gpp(NH)p (Ki = 11,505 +/- 2,295). The absence of guanine nucleotide-sensitive agonist high-affinity sites may explain the defective DA-1/AC coupling mechanism in the SHR.

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Persistent Defective Coupling of Dopamine-1 Receptors to G Proteins after Solubilization from Kidney Proximal Tubules of Hypertensive Rats

Anita Sidhu, Prayong Vachvanichsanong, Pedro A. Jose, and Robin A. Felder*

*Department of Pediatrics, Georgetown University, Washington, DC 20007; and *Department of Pathology, University of Virginia, Charlottesville, Virginia 22908*

Abstract

The natriuretic effect of dopamine-1 (DA-1) agonists is reduced in spontaneously hypertensive rat (SHR), partly because of defective DA-1 receptor–adenylate cyclase (AC) coupling in renal proximal convoluted tubules. To investigate this defective coupling, DA-1 dopamine receptors from renal proximal tubules were solubilized and reconstituted into phospholipid vesicles. The binding of DA-1-selective ligand [^{125}I]SCH 23982 was specific and saturable, with no differences in receptor density or K_d between SHR and normotensive rats (Wistar-Kyoto rats; WKY). Competition experiments of the reconstituted DA-1 dopamine receptors in WKY with a DA-1-selective agonist, SKF R-38393, revealed the presence of high- ($K_h = 350\pm209$ nM) and low-affinity ($K_l = 70,500\pm39,500$ nM) binding sites. 100 μM Gpp(NH)p abolished the agonist high-affinity sites, converting them to a low-affinity state ($K_i = 33,650\pm10,850$ nM). In SHR, one affinity site was noted ($K_i = 13,800\pm500$) and was not modulated by Gpp(NH)p ($K_i = 11,505\pm2,295$). The absence of guanine nucleotide–sensitive agonist high-affinity sites may explain the defective DA-1/AC coupling mechanism in the SHR. (*J. Clin. Invest.* 1992. 89:789–793.) Key words: radioligand binding • Wistar-Kyoto rats • dopamine-1 receptors • hypertension

Introduction

Dopamine exerts its biological effects through occupation of specific receptor subtypes. In the central nervous system and certain endocrine organs, the dopamine receptors are classified into the D-1 or D-2 subtypes on the basis of their ability to either stimulate or inhibit adenylate cyclase (AC),[1] respectively (1). These receptors have been cloned, including novel D-3, D-4, and D-5 dopamine receptors (2–10). In peripheral tissues, these receptors have been designated as DA-1 and DA-2 (11). The receptors in both the central nervous system and in peripheral tissues have marked similarities and some differences, the most notable of which is the lower affinity of the renal dopamine receptor than of the brain dopamine receptor for dopaminergic drugs (12, 13). Indeed, the RNA for the cloned brain dopamine receptors has not been detected in the kidney, except (maybe) for the D-3 dopamine receptor (8).

The molecular mechanisms of dopamine action on DA-1 dopamine receptors in the kidney and the series of events that leads to physiological responses are beginning to be elucidated. In the kidney, there is evidence that dopamine, which is produced in the proximal tubule, increases sodium excretion by inhibition of Na$^+$/H$^+$ antiport activity via cAMP (14). Dopamine also inhibits Na$^+$/K$^+$ ATPase activity (15) and this effect is apparently mediated by protein kinase C (16). In renal tissue, DA-1 agonists stimulate phospholipase C (17, 18).

We recently reported the existence of abnormalities in the renal dopaminergic system in the genetically hypertensive rat (spontaneously hypertensive rat; SHR) of the Okamoto Aoki strain (19). We also demonstrated that the natriuretic and the antinatriuretic effects of DA-1 agonists and antagonists, respectively, were reduced in the SHR but not in normotensive rat controls (Wistar-Kyoto rat; WKY) (20). In the SHR, both the potency of DA-1-selective agonists to compete for binding sites on the receptor and the ability of these agonists to stimulate AC were vastly diminished. The decreased ability of DA-1 agonists to stimulate AC activity was not due to a defective AC enzyme per se but rather to a defective DA-1 receptor–AC coupling mechanism (19). To investigate the mechanism for the defective DA-1 dopamine receptor in the SHR and to develop a cell-free system (21, 22) in which these mechanisms can be studied, we decided to analyze the guanine nucleotide–sensitive agonist high-affinity state of the receptor, which represents the activated receptor. We report that coupling of solubilized and reconstituted DA-1 dopamine receptors to G proteins is deficient in SHR, but not in WKY, as evidenced by a complete lack of agonist high-affinity sites.

Methods

Materials. The DA-1-selective agonist SKF R-38393 and the DA-1-selective antagonist SCH 23390 were from Research Biochemicals Inc. (Natick, MA), Gpp(NH)p was from Boehringer Mannheim Corp. (Indianapolis, IN), and [^{125}I]SCH 23982 was from New England Nuclear (Boston, MA). Sodium cholate, collagenase (type IV), crude phospholipids (type VII), and polyethyleneimine were purchased from Sigma Chemical Co. (St. Louis, MO), whereas SM-2 Bio-Beads were from Bio-Rad Laboratories (Richmond, CA). Dulbecco's PBS (DPBS) was purchased from Gibco Laboratories (Grand Island, NY). All other reagents were of the highest purity commercially available. 20-wk-old WKY and SHR (Okamoto-Aoki strain) were purchased from Taconic Farms, Inc., (Germantown, NY).

Membrane preparation. Renal cortical tubules enriched with proximal tubules were prepared as previously described, with modifications

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1. *Abbreviations used in this paper:* AC, adenylate cyclase; DA-1, dopamine receptor subtype 1; DPBS, Dulbecco's PBS; PCT, proximal convoluted tubules; SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto rat (normotensive controls).

(23). In brief, the rats were anesthetized with pentobarbital (50 mg/kg i.p.) and the kidneys exposed via a midline abdominal incision. A polyethylene catheter was inserted into the abdominal aorta just above the bifurcation into the iliac arteries. A direct blood pressure recording was obtained to verify the elevated systolic arterial pressure in the SHR (185–210 mmHg) and normal pressure in the WKY (135–145 mmHg). The kidneys were perfused with 25 ml of Ca^{2+}-free PBS, pH 7.4, with collagenase (30 mg, Sigma type IV). The kidneys were then excised and the cortex isolated from the medulla. The cortical tissue, which was minced to a fine paste, was passed successively through a series of stainless steel sieves (Newark Wire Cloth Co., Newark, NJ) (in order: 212, 85, and 75 μm). The tubules (from the 85-μm sieve) were resuspended in ice-cold DPBS and allowed to settle by gravity for 30 min. The resulting pellet contained mainly proximal tubular cells, as ascertained by AC activation by DA-1 agonists and parathyroid hormone (19), alkaline and acid phosphatase determinations, and light microscopic examination (23). The tubules were free of glomeruli, as determined by acid phosphatase determinations and by light microscopic examination (23). In addition, most of the tubules took up fluorescein, which is characteristic of proximal but not distal tubular cells (24). Moreover, these tissues behaved in a fashion consistent with proximal convoluted tubules (PCT) obtained from WKY and SHR (19). The only other cortical tubule with AC-linked DA-1 dopamine receptor is present in the cortical collecting duct (12). However, the coupling of DA-1 dopamine receptors in the cortical collecting duct to AC is similar in WKY and SHR (25). Viability was determined by the failure of cells to take up 1% trypan blue (23).

The proximal tubules were homogenized at a protein concentration of 1 mg/ml in 10 mM Tris-HCl, pH 7.4, containing protease inhibitors (1 mM PMSF, 1 mM EDTA, and 5 μg/ml each of leupeptin and pepstatin). Cellular debris and nuclei were pelleted at 270 g for 5 min, and the resulting supernatant was centrifuged at 18,000 g for 20 min to pellet membranes. The crude membranes were washed once and were either used immediately or stored frozen in small aliquots at −80°C in 10 mM Tris HCl, pH 7.4, 5 mM $MgCl_2$, and 250 mM sucrose (26).

Solubilization and reconstitution. Proximal tubular membranes were solubilized by 1% sodium cholate, after pretreatment with 10 μM SKF R-38393, essentially as described before for rat striatal D-1 dopamine receptors (21, 22). Briefly, membranes were suspended at 1–2 mg/ml in buffer A (50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$) in the presence of 10 μM SKF R-38393, a D-1 selective agonist. After 20 min at 37°C, the membranes were centrifuged and suspended in solubilizing buffer (50 mM Tris-HCl, pH 7.4, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 250 mM sucrose, 1 mM EDTA, 1.5 mM PMSF, and 1 mM DTT). In some studies, as noted in the legends to figures, 1 M NaCl was included in the solubilizing buffer. Sonicated phospholipids were added to a final concentration of 1.2 mg/ml and sodium cholate was added to a final concentration of 1%. After a 15–20-min incubation on ice, the mixture was centrifuged at 31,300 g for 45 min. The clear supernatant obtained after high-speed centrifugation was stored frozen in small aliquots at −80°C (22, 27).

For reconstitution of soluble DA-1 dopamine receptors into phospholipid vesicles, sonicated phospholipids (20 mg/ml) were readded to the soluble extract to a final concentration of 1.2 mg/ml. Protease inhibitors (1 mM PMSF and 5 μg/ml each of leupeptin and pepstatin) were added directly to the soluble extracts before addition of phospholipids (26). Cholate was removed by the addition of moist SM-2 Bio-Beads (1.2 g/ml of extract), and the mixture was shaken gently for 1 h at 4°C. After the beads were allowed to settle, the turbid supernatant containing the proteoliposomes was aspirated and used directly in binding studies.

Radioligand binding assays. For saturation studies, it was essential to first reduce the specific activity of [125I]SCH 23982 to 220 Ci/mmol (dried before use) by diluting the stock radioligand 1:10 with its unlabeled isomer, SKF 103108-A. This permitted us to use concentrations of the ligand up to 50 nM (final) in our incubation mixture. For competition studies, 1 nM of the undiluted radioligand [125I]SCH 23982 (sp act 2,200 Ci/mmol) was routinely used. The [125I]SCH 23982 binding

to reconstituted receptors was assayed by filtration onto glass fiber filters (28, 29) pretreated with 200 μl of ice-cold 0.3% polyethyleneimine solution. The binding assay was performed by using 25–50 μl of protein extract, 1 nM [125I]SCH 23982, and varying concentrations of D-1 dopamine-specific compounds. Nonspecific binding was determined with 10 μM SCH 23390; all dilutions were done using buffer B (50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM PMSF, and 5 μg/ml each of leupeptin and pepstatin) (26). After incubation at room temperature for 90 min, binding was terminated by filtering the assay system onto glass fiber filters.

Other procedures. Protein was determined by the method of Lowry et al. (30). The computer-fitted program LIGAND (31) was used to analyze the binding data. In each case, a two-site model was considered to be a better fit according to the F test at $P < 0.05$. All values are summarized as means±SEM from two to six separate experiments.

Results

[125I]SCH 23982 binding to solubilized and reconstituted receptor preparations. We had earlier demonstrated that, on solubilization and reconstitution of brain D-1 dopamine receptors into phospholipid vesicles, the agonist high-affinity sites were potentiated and comprised 40–50% of the total receptor population (21, 22). These potentiated sites were due to increased coupling of receptor to G protein and were abolished by guanine nucleotide analogues. We decided to use a similar approach to study the guanine nucleotide–sensitive agonist high-affinity DA-1 sites in proximal tubules of normotensive WKY and hypertensive SHR rats.

DA-1 dopamine receptors from proximal tubules of WKY rats were extracted in the presence of 1 M NaCl and reconstituted into phospholipid vesicles, as described under Methods. The binding of the DA-1 dopamine-selective ligand [125I]SCH 23982 to the reconstituted DA-1 dopamine receptors was specific and saturable (Fig. 1). Specific binding represented the bulk of total binding; nonspecific binding, defined as binding in the presence of 10 μM of the DA-1 receptor–selective antagonist SCH 23390, was between 25 and 40% of total binding. Scatchard analysis (Fig. 1, *inset*) of the saturation data revealed that the ligand bound to a single site with an apparent K_d of 15.3±8.5 nM and a maximum specific binding (B_{max}) of 663.0±142.3 fmol/mg of protein, which represents a slight enrichment over the PCT membranes of WKY rats (479.1±22.2 fmol/mg protein) (19). This increase in specific activity suggests a partial purification of the DA-1 dopamine receptors on solubilization, possibly because of loss of cellular proteins. The results also suggest that this method of solubilization and reconstitution is well suited for extracting DA-1 dopamine receptors from PCT.

Similar results were also obtained with DA-1 dopamine receptors extracted from proximal tubular membranes of the SHR (Fig. 2). Accordingly, after solubilization and reconstitution of SHR receptors, the Scatchard analysis (Fig. 2, *inset*) of the saturation data revealed a K_d of binding of 22.0±6.7 nM and B_{max} of 730±95 fmol/mg protein, representing a slight increase in specific activity over the membrane-bound receptor (601.0±91.3 fmol/mg protein) (19). The K_d values of [125I]SCH 23982 binding to reconstituted DA-1 dopamine receptors was only 0.4–2-fold increased compared with the membrane-bound receptors in both WKY and SHR, where values of 10.9±1.3 and 10.7±0.9 nM, respectively, were reported (19). These decreases in [125I]SCH 23982 binding affinity may be reflective of specific environmental requirements that have been disrupted on solubilization (21, 22).
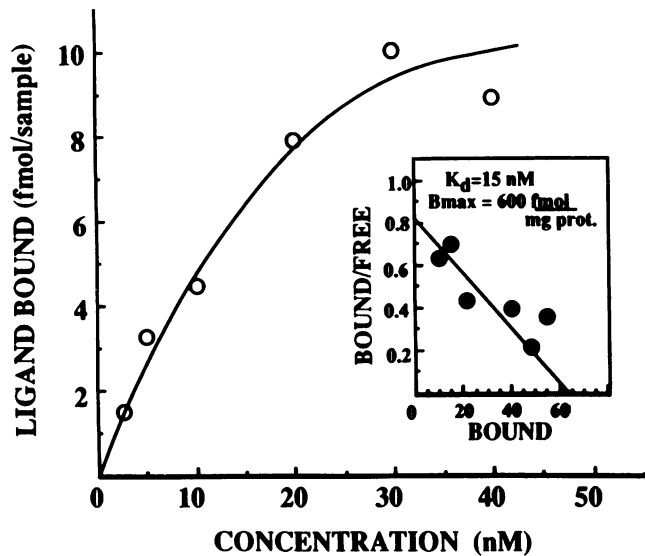
Figure 1. Concentration dependence of [^{125}I]SCH 23982 binding to reconstituted DA-1 dopamine receptors extracted from the proximal tubules of WKY rats, in the presence of 1 M NaCl. Proteoliposomes containing the reconstituted DA-1 receptors (25 μg of protein/assay) were incubated in triplicate with increasing concentrations of the radioligand (sp act 220 Ci/mmol) for 90 min at room temperature. Specific binding was obtained by subtracting nonspecific binding from the total binding, and the data were analyzed by a Scatchard plot (*inset*). The amount of bound and free ligand was calculated in femtomoles and nanomolar, respectively. The data are from a representative experiment.

R-38393, the DA-1 receptor selective agonist. NaCl was omitted from the solubilizing buffers in these studies, since the presence of the salt may adversely affect the agonist-binding properties of the receptor (unpublished observations). As seen in Fig. 3, the curves were shallow and fit best to a two-site model with a high-affinity site (K_h) of 350±209 nM and a low-affinity site (K_l) of 70,500±39,500 nM. Approximately 40±5.6% of the receptors were in the high-affinity state. These high-affinity sites of the receptors were due to coupling to G proteins. Thus, in the presence of 100 μM Gpp(NH)p, the high-affinity sites were abolished and a single site corresponding to the low-affinity state of the receptor was obtained with a K_i of 33,650±10,850 nM (Fig. 3 and Table I). Competition curves with the DA-1 dopamine-selective antagonist SCH 23390 were monophasic, with a K_i value of 731±130 nM (Table I).

Absence of guanine nucleotide–sensitive agonist high-affinity sites in reconstituted DA-1 dopamine receptors from SHR. DA-1 dopamine receptors from proximal tubules of SHR were solubilized and reconstituted into phospholipid vesicles in the absence of sodium chloride. Agonist competition curves of these reconstituted receptors from SHR were monophasic, and the K_i of binding of SKF R-38393 was 13,800±500 nM, corresponding to the low-affinity state of the receptor (Fig. 4). Further, this affinity state was not due to coupling to G proteins, since in the presence of 100 μM Gpp(NH)p, the K_i was virtually unaffected (Table I). These data suggest that the SHR DA-1 dopamine receptor is unable to couple to G proteins under the present experimental conditions. This aberrant coupling between receptor and G protein occurs in conditions that pro-

Agonist competition of reconstituted DA-1 dopamine receptors of WKY. DA-1 dopamine receptors of WKY were extracted with sodium cholate and reconstituted into phospholipid vesicles; competition curves were obtained with SKF
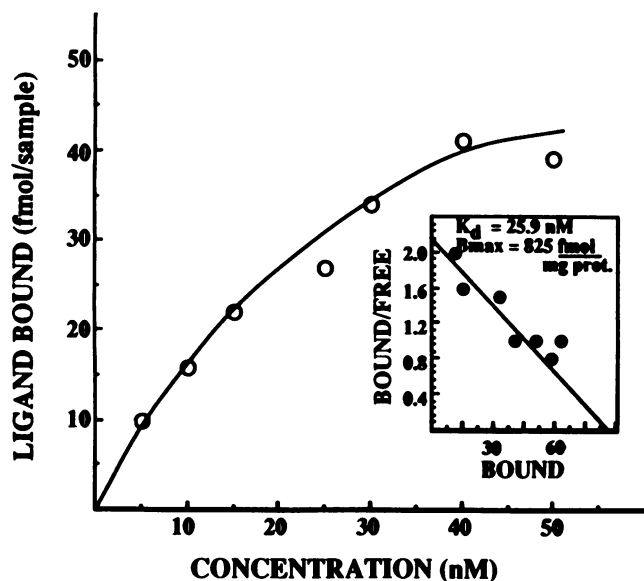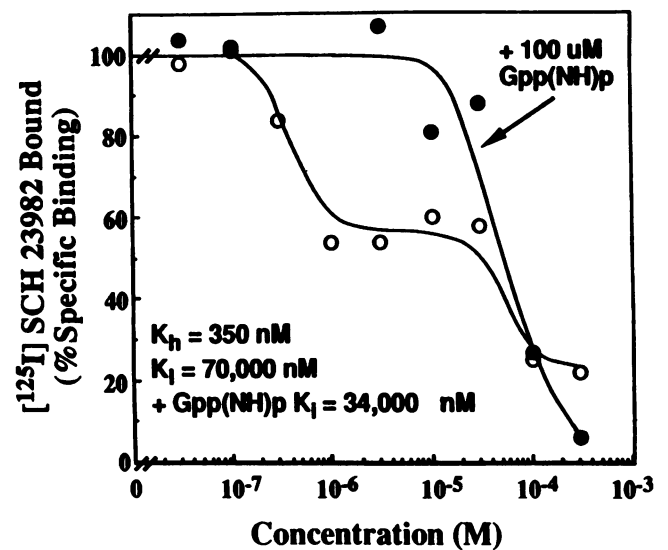

Figure 2. Concentration dependence of [^{125}I]SCH 23982 binding to reconstituted DA-1 dopamine receptors extracted from proximal tubules of SHR, in the presence of 1 M NaCl. DA-1 dopamine receptors were extracted from proximal tubules of SHR and were assayed as described in the legend to Fig. 1. The data are from a representative experiment.

Figure 3. Effect of Gpp(NH)p on agonist competition for [^{125}I]SCH 23982 binding to reconstituted DA-1 dopamine receptors from proximal tubules of WKY rats. DA-1 dopamine receptors from proximal tubules of WKY rats were solubilized in the absence of any NaCl. After reconstitution into phospholipid vesicles, the receptors were incubated at room temperature for 90 min with 1 nM [^{125}I]SCH 23982 (sp act 2,200 Ci/mmol) and increasing concentrations of the agonist SKF R-38393, in the absence (o——o) or presence (●——●) of 100 μM Gpp(NH)p. Total binding was determined in the absence of any drug, whereas nonspecific binding was in the presence of 10 μM SCH 23390. After accounting for nonspecific binding, the specific counts at each drug concentration were expressed as percentage of total specific binding. The data are from a representative experiment.

Table I. Agonist and Antagonist K_is for Reconstituted DA-1 Dopamine Receptors from Proximal Tubules of WKY and SHR

Source of receptors	Competing drug	Without Gpp(NH)p	With Gpp(NH)p
		nM	
WKY	SKF R-38393	350±209 (K_h)	
		70,500±39,500 (K_l)	33,650±10,850
WKY	SCH 23390	731±130	ND
SHR	SKF R-38393	13,800±500	11,505±2,295
SHR	SCH 23390	620±142.0	ND

DA-1 dopamine receptors from proximal tubules of either WKY or SHR were solubilized in the absence of NaCl, reconstituted into phospholipid vesicles, and incubated with 1 nM [125I]SCH 23982 and increasing concentrations of the drugs. After 90 min at room temperature, the incubation was terminated by filtering onto glass fiber filters. Values (nanomolar) represent the mean±SEM of two to four independent experiments.

K_h, inhibition constant for the high-affinity site; K_l, inhibition constant for the low-affinity site; ND, not done.

mote such couplings in WKY (Fig. 3). The antagonist competition curves of SCH 23390 binding to reconstituted DA-1 receptors from SHR were monophasic, with K_i values of 620±142.0 nM (Table I).

Discussion

We have earlier shown that DA-1 dopaminergic receptors from PCT of SHR are unable to stimulate AC (19). Furthermore, these receptors appear to have a lowered affinity for agonists compared with receptors from WKY rats. Photoaffinity labeling of DA-1 receptors from WKY and SHR, using the photoaffinity ligand [125I]MAB, did not reveal any differences in the
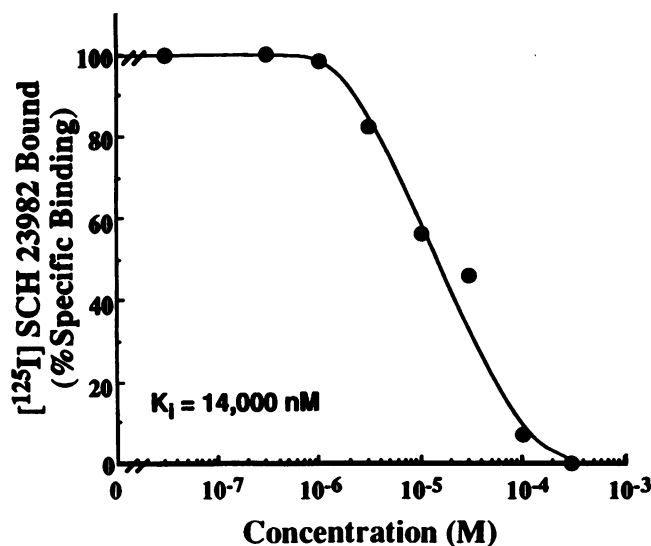

Figure 4. Ability of SKF R-38393 to compete for [125I]SCH 23982 binding to reconstituted DA-1 dopamine receptors from proximal tubules of SHR. DA-1 dopamine receptors from proximal tubules of SHR were solubilized in the absence of any NaCl and reconstituted into phospholipid vesicles. The binding assay was conducted as described in the legend to Fig. 3. The data are from a representative experiment.

molecular weights or subunit composition of the labeled receptors (19). To further investigate the molecular mechanisms underlying the properties of DA-1 receptors in SHR rats, we decided to undertake the current study, using a model system that promotes receptor–G protein couplings (21, 22). We found that renal receptors from the proximal tubules of WKY rats in a cell-free system existed in both high- and low-affinity states. The high-affinity sites were fully modulated by guanine nucleotides, suggesting that under the experimental conditions these sites were due to coupling between receptors and G proteins. Under similar experimental conditions, however, the DA-1 dopamine receptors from proximal tubules of SHR were unable to couple to G proteins; instead, there was only a single low-affinity, guanine nucleotide–insensitive site.

This inability of the receptor to couple to G proteins in SHR may be due to defective receptor, defective G protein, or both. Since we found earlier that the potency of DA-1 dopamine selective agonists to compete for binding sites on the receptor is diminished in SHR relative to WKY (19), it is possible that alterations in the molecular structure also exist at the agonist-binding site of the receptor. Further, the inability of receptor to couple to G proteins in SHR suggests that alterations may exist in the third cytoplasmic loop of the receptor, the domain through which coupling to G proteins occurs. While our data suggest that the defect may be at the receptor level, the possibility that the G proteins themselves are defective cannot be ruled out, since receptor and cyclase coupling sites on the G proteins are represented by distinct domains (33).

The inability of SHR DA-1 receptors to couple to G proteins may be of significance in understanding the mechanisms underlying certain types of hypertension. Interestingly, from radioligand binding studies, defects in D-1 dopamine receptors of the central nervous system have been associated with other pathophysiological conditions (34). It has been shown in postmortem Huntington's diseased putamen and amygdala that D-1 receptors are unable to couple to G proteins, as evidenced by a complete lack of agonist high-affinity binding sites. From indirect evidence, others have also suggested that similar coupling defects may exist in schizophrenia and Huntington's disease, but not in Parkinson's or Alzheimer's disease (35). Although these studies warrant further investigation, it is tempting to speculate that defective D-1/DA-1 receptor–G protein couplings exist in certain D-1 dopaminergic-linked diseases. On the basis of the presence of the DA-1 dopamine receptor defect in specific organs, and, indeed, in a specific nephron segment in spontaneous hypertension, it is possible that defects in discrete areas of certain organs may lead to different clinical manifestations.

We have recently demonstrated (36) that solubilized and reconstituted brain D-1 dopamine receptors were able to couple to exogenously added G proteins after inactivation of endogenous G proteins. A similar approach with DA-1 receptors from SHR rats may be useful to elucidate whether defective couplings are due to defective receptor, defective G proteins, or both.

Acknowledgments

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