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Expression of the Bovine Striatal D_2 Receptor, But Not the D_1 Receptor, in Bovine Adrenal Medulla

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SUMMARY

At 37°, the specific binding of [^{3}H]SCH23390 to purified adrenal medullary plasma membranes accounted for only 20% of total binding. At 4°, the binding did not saturate; therefore, equilibrium binding constants could not be estimated. Similar results were obtained with ^{125}I-SCH23982, a ligand that exhibits 25-fold higher specific activity, compared with [^{3}H]SCH23390. Of 11 dopamine receptor ligands used, only (+)-SCH23390 and (±)-SKF83566 inhibited the binding of [^{3}H]SCH23390, but with very low affinities (IC_{50} values of 446 and 635 nM, respectively). In striatal membranes, binding of [^{3}H]SCH23390 and of ^{125}I-SCH23982 followed saturation isotherms. [^{3}H]SCH23390 exhibited a K_d of 383 pM and a B_{max} of 479 fmol/mg of protein, and ^{125}I-SCH23982 exhibited a K_d of 664 pM and a B_{max} of 453 fmol/mg of protein. The radioligand was displaced by the D_1-selective compounds (+)-SCH23390 (IC_{50} of 3 nM), (±)-SKF83566 (IC_{50} of 5 nM), and (+)-SKF38393 (IC_{50} of 17 nM); spiperone and quinpirole were ineffective. [^{3}H]Spiperone binding to bovine striatal and adrenal medullary plasma membranes exhibited similar characteristics, compatible with a typical D_2 receptor. Northern blot analysis revealed the presence of D_1 receptor mRNA in poly(A)$^+$ RNA preparations from bovine brain striatum. When Northern blots containing poly(A)$^+$ from bovine adrenal medulla were probed, no specific hybridization band for D_1 receptors was observed; in contrast, a band of the expected size for D_2 receptors was obtained. Similar results were obtained with *in situ* hybridization techniques and with more sensitive reverse transcription-polymerase chain reaction methods. The data support the idea that the peripheral D_2 receptor present in bovine adrenal medulla is similar to striatal D_2 receptors; in contrast, striatal D_1 receptors do not seem to have a counterpart in bovine adrenal medullary tissues.

Multiple brain dopamine receptor subtypes have recently been cloned and expressed (1, 2). Receptor subpopulations have also been identified in peripheral sympathetic neurons (3, 4). However, in their close relatives, the adrenal medullary chromaffin cells, different experimental approaches have provided conflicting data on the presence of multiple types of dopamine receptors. For instance, using a fluorescent ligand (the rhodamine conjugate of the 4′-amino derivate of the D_1 antagonist SCH23390), Artalejo *et al.* (5) and Ariano *et al.* (6) detected D_1 sites in cultured chromaffin cells. In contrast, Bigornia *et al.* (7) could not detect significant specific binding of [^{3}H]-SCH23390 to crude microsomal membranes from bovine adrenal medulla. D_2 sites are identified much more effectively in bovine adrenal medullary microsomal membranes, using [^{3}H]-spiperone as selective ligand, as shown in three reports from three different laboratories (8–10). This may be due to a higher level of expression of D_2 versus D_1 receptors in bovine chromaffin cells.

Important regulatory functions have been suggested for D_1 and D_2 receptors in chromaffin cells. For instance, the selective D_1 receptor agonist SKF38393 activates a so-called "facilitation Ca^{2+} current"; on the basis of this observation, Artalejo *et al.* (5) suggested that a D_1 receptor coupled to an adenylate cyclase may form the basis for a positive feedback loop mechanism for catecholamine release. However, SKF38393 has recently been shown to have the opposite effect, inhibiting muscarinic receptor-mediated catecholamine release from perfused feline adrenal glands (11). On the other hand, D_2 receptor agonists also inhibit secretion in feline (12, 13) and bovine (8, 14–16) chromaffin cells, probably by blocking Ca^{2+} entry through voltage-dependent Ca^{2+} channels (7, 14, 15).

In light of these important regulatory functions claimed for peripheral D_1 and D_2 receptors, it is of utmost importance to unequivocally identify their presence in chromaffin cells, to estimate their relative densities, and to compare their characteristics with those of the well established striatal D_1 and D_2

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ABBREVIATIONS: RT, reverse transcription; PCR, polymerase chain reaction; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

receptors. Here we have tried to answer the question of whether bovine adrenal chromaffin cells express multiple types of dopamine receptors by using two methodological approaches. First, we have studied systematically and in parallel the characteristics of [^{3}H]SCH23390, ^{125}I-SCH23982, and [^{3}H]spiperone binding to membranes from the adrenal medulla, to intact chromaffin cells, and to brain striatal membranes of the same animal species, the cow. In relation to previous reports that used crude microsomal fractions, we have used here a purified plasma membrane preparation from bovine adrenal medulla and a radioligand, ^{125}I-SCH23982, with very high specific activity. This optimized the conditions to detect a low density of binding sites, especially for the D_1 receptor radioligands. Second, we have analyzed the D_1 and D_2 receptor gene expression in bovine caudate and adrenal medulla, using molecular biology techniques.

Materials and Methods

Biological material used. Bovine brain and adrenal glands (usually 20) were obtained from a local slaughterhouse within 30 min after death of the animals and were taken to the laboratory during the following 30 min, in an ice-cold solution of sucrose-Tris (0.3 M sucrose, 10 mM Tris·HCl, pH 7.4). All procedures to prepare subcellular fractions were performed at 4°.

Preparation of microsomal fractions from adrenal medullae and striata. Medullary tissue was dissected out from the adrenal cortex; striata were dissected out from the rest of the brain. Tissues were finely minced with scissors and homogenized in 10 volumes of sucrose-Tris, using a tight-fitting glass/glass Potter homogenizer at low speed (about 100 rpm). The homogenate was centrifuged at 800 × *g* for 12 min, and the supernatant was recentrifuged at 27,000 × *g* for 30 min. The resulting pellet was then resuspended and washed twice with 10 mM Tris·HCl by centrifugation at 100,000 × *g* for 30 min. The final pellet was resuspended in a small volume of the same medium, fractionated in 1-ml aliquots, and stored at −70° until used for radioligand binding experiments.

Purification of adrenal medullary plasma membranes. The homogenate was fractionated according to the method of Wilson and Kirshner (17), with some modifications (18). The final pellet was resuspended in 10 mM Tris·HCl, fractionated in aliquots (1 ml), and stored at −70°. Under these conditions, the plasma membrane fraction retained its acetylcholinesterase activity and binding capabilities for at least 1 month.

Acetylcholinesterase assay. The activity of this enzyme in different subcellular fractions of adrenal medulla was measured according to the procedure of Ellman *et al.* (19).

[^{3}H]SCH23390 and [^{3}H]spiperone binding assays. Binding assays with [^{3}H]SCH23390 and [^{3}H]spiperone were performed in suspensions of adrenal medullary purified plasma membranes and microsomal fractions and in striatal microsomal fractions. The samples (200 μg of protein/assay tube) were incubated at 4° for 120 min ([^{3}H]SCH23390 binding) or at 25° for 60 min ([^{3}H]spiperone binding), in a final volume of 2 ml of 50 mM Tris·HCl buffer, pH 7.4, containing 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$.

[^{3}H]SCH23390 equilibrium binding assays were performed by using increasing concentrations of ligand, ranging from 20 to 2000 pM in the adrenal medulla and from 20 to 1000 pM in the striatum; for the [^{3}H]-spiperone assays, concentrations of 20–1000 pM were used in the adrenal medulla and concentrations of 100–6000 pM were used in the striatum. The nonspecific binding was determined in the presence of 1 μM (+)-SCH23390 (for [^{3}H]SCH23390) or 10 μM unlabeled haloperidol (for [^{3}H]spiperone). Specific binding was estimated by subtracting nonspecific binding from total binding.

Displacement of ligand binding was done by simultaneous incubation of the purified adrenal medullary plasma membranes or microsomal fractions of striatum in the presence of [^{3}H]SCH23390 (1 nM) or [^{3}H]-spiperone (0.4 nM and 1 nM, respectively) and increasing concentrations of different drugs, at 4° for 120 min or at 25° for 60 min. Ascorbic acid (0.1%) was added when dopamine or apomorphine was used as the displacing drug. The binding reaction was stopped by filtration through Whatman GF/C glass fiber filters, under reduced pressure. Filters were washed four times with 5 ml of ice-cold 50 mM Tris·HCl buffer, and the radioactivity retained in the filters was counted in a liquid scintillation counter (Packard model L1500). IC_{50} values were calculated through logarithmic transformations of the displacing curves. The IC_{50} values were transformed to K_i values using the equation described by Cheng and Prusoff (20).

^{125}I-SCH23982 binding assays. Binding assays with ^{125}I-SCH23982 (2200 Ci/mmol) were performed in suspensions of adrenal medullary purified plasma membranes and striatal microsomal fractions. The samples (50 μg of protein/assay tube) were incubated at 4° for 120 min or at 25° for 60 min, in a final volume of 500 μl of 50 mM Tris·HCl buffer, pH 7.4, containing 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$.

^{125}I-SCH23982 equilibrium binding assays were performed using increasing concentrations of ligand, ranging from 40 to 2000 pM, in both tissues. Nonspecific binding was determined in the presence of 1 μM (+)-SCH23390. Specific binding was estimated by subtracting nonspecific binding from total binding.

Displacement of ligand binding was done by simultaneous incubation of the purified adrenal medullary plasma membranes or microsomal fractions of striatum in the presence of 1 nM ^{125}I-SCH23982 and increasing concentrations of (+)-SCH23390 (10^{-12} to 10^{-5} M), at 25° for 60 min. The binding reaction was stopped by filtration through Whatman GF/C glass fiber filters, under reduced pressure. Filters were washed four times with 5 ml of ice-cold 50 mM Tris·HCl buffer, and the radioactivity was counted in an LKB-Wallac 1282 CompuGamma CS counter. IC_{50} values were calculated through logarithmic transformation of the displacing curves. The IC_{50} values were transformed to K_i values using the equation described by Cheng and Prusoff (20).

Some experiments were also performed in intact chromaffin cells, which were prepared according to the method of Moro *et al.* (21) and plated in 24-well plastic culture plates (1×10^6 cells/well). Displacement of ligand binding was studied by using 50 mM Tris·HCl buffer, pH 7.4, containing 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$. The cells were washed two times with 1 ml of buffer before the binding reaction was started. ^{125}I-SCH23982 (1 nM) was incubated with increasing concentrations of (+)-SCH23390 (10^{-12} to 10^{-4} M) at 25° for 60 min or at 4° for 120 min, in a final volume of 500 μl. After washing of the cells three times with 1 ml of buffer, 0.5 ml of buffer was added to each well, cells were scraped and collected, and their radiactivity was counted as described above.

Protein content. Protein contents were measured by the method of Lowry *et al.* (22), using bovine serum albumin as the standard.

RNA analysis and RT-PCR. Extraction of total RNA, Northern blotting, and hybridization were carried out as described (23). cDNAs for rat dopamine D_1 and D_2 receptors (kindly provided by Dr. R. T. Fremeau) and human and bovine dopamine D_1 receptors were labeled with [^{32}P]dCTP, using the random oligonucleotide primer method (24), to specific activities of ≥7×10^8 cpm/μg.

For RT of the dopamine D_1 receptor mRNA, 0.1 μg of poly(A)$^+$-enriched RNA from bovine caudate or bovine adrenal medulla was first transcribed by incubation for 45 min at 42° with a random mixture of hexadeoxynucleotides in the presence of Moloney murine leukemia virus reverse transcriptase (200 units/reaction; BRL). The cDNAs generated were then amplified by PCR (25), using two degenerate oligonucleotides previously described [5′ primer, GAGTCGACCTGTG-(C/T)G(C/T)(C/G)AT(C/T)(A/G)CIIT(G/T)GAC(C/A)G(C/G)TAC; 3′ primer, CAGAATTCAG(T/A)AGGGCAICCAGCAGAI(G/C)(G/A)-(T/C)GAA] (26). Thirty cycles of PCR with 1 min of denaturation at 95°, 2 min of annealing at 45°, and 3 min of extension at 72° were performed. The PCR products were double-digested with *Eco*RI and

*Sal*I; after agarose gel separation, the 300–700-base pair portion was extracted (GeneClean) and subcloned. Colony screening was performed as described (27), and the filters were hybridized with a probe encompassing the third to sixth transmembrane domains of the rat dopamine D_1 receptor. Six positive clones from caudate were sequenced, and in addition a total of 24 randomly chosen clones from adrenal medulla and caudate were sequenced by the Sanger dideoxy termination method, using Sequenase (United States Biochemicals).

For RT-PCR amplification of the dopamine D_2 receptor, we used two 30-mers, 5′-ATGATGATCCAGAGAGCCGGAACTGGAGCC and 5′-GTCCTGTCCTTCACCATCTCCTGCCCGAG, corresponding or complementary, respectively, to positions +29 and +510 in bovine dopamine D_2 receptor cDNA (28). Forty cycles of PCR with 1 min of denaturation at 95° and 1 min of annealing-extension at 72° were performed.

Preparation of chromaffin cells and catecholamine release studies. Bovine adrenal medullary chromaffin cells were isolated following standard methods, with some modifications (21). Cells were suspended in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. For secretion experiments, 5×10^6 cells were plated on plastic 3-cm-diameter Petri dishes containing 10 ml of Dulbecco's modified Eagle's medium. For ^{125}I-SCH23982 binding studies, cells were plated at a density of 5×10^5 cells/well in 24-multiwell Costar plates.

Chemicals. [^{3}H]SCH23390 (87 Ci/mmol), ^{125}I-SCH23982 (2200 Ci/mmol), and [^{3}H]spiperone (25 Ci/mmol) were obtained from New England Nuclear. (+)-SCH23390 HCl (±)-SKF83566 HCl, (+)-SKF38393 HCl, (+)-butaclamol HCl, *cis*-(*Z*)-flupentixol dihydrochloride, quinpirole HCl, spiperone HCl, dopamine HCl, and (+)-bromocryptine methanesulfonate were from Research Biochemicals. Flunarizine and domperidone were obtained from Janssen (Beerse, Belgium), apomorphine HCl, acetyl-β-methylcholine, adenosine *N*-oxide-3′,5′-cyclic monophosphate sodium salt, and haloperidol from Sigma (Madrid, Spain), and clozapine from Sandoz (Madrid, Spain).

Results

Purity of the Adrenal Medullary Plasma Membrane Preparation

To estimate the enrichment of subcellular fractions in plasma membranes, acetylcholinesterase activity was measured. According to Rosenheck (29), this enzyme is the best marker for chromaffin cell plasma membranes. The final fraction was enriched 10–12-fold in plasma membranes, a value similar to those reported by Rosenheck (29).

Binding Characteristics of [^{3}H]SCH23390

In the purified plasma membrane preparation used here, the density of binding sites for any receptor is enhanced with respect to crude microsomal fractions previously used in studies to identify dopamine receptors in adrenal medullary chromaffin cells (8). However, data obtained in initial experiments performed at 37° were disappointing. With 1 nM [^{3}H]SCH23390, after a 30-min incubation period at 37° in the presence of 1 μM unlabeled (+)-SCH23390 the specific binding accounted for only 23% of the total binding. In light of these poor results, binding experiments were performed at lower temperatures. After a 160-min incubation period at 4° in the presence of 1 μM unlabeled (+)-SCH23390, the specific binding corresponded to 70% of the total binding. Under these experimental conditions, the specific binding of [^{3}H]SCH23390 to bovine adrenal medulla was greatly improved.

In the striatum, the signal to noise ratio for the binding of [^{3}H]SCH23390 to microsomal membranes was much more favorable. The specific binding amounted to 96% of total binding. At 4° these results were quite similar.

The rate of association of [^{3}H]SCH23390 to striatal membranes was strongly dependent on temperature. For instance, at 37° equilibrium was attained in only 10–15 min (Fig. 1B). At 4° the equilibrium binding was reached only after 80 min of incubation. The maximal binding was similar at both temperatures after 80 min. In contrast, little specific binding was found in purified adrenal medullary plasma membranes at 37° (Fig. 1A). The specific binding improved at 25° but was maximal at 4°. After a 2-hr incubation, however, the maximal binding of [^{3}H]SCH23390 was 8–10-fold lower than in striatum. This difference is more pronounced when it is noted that comparisons are being made between purified plasma membranes in the case of the adrenal medulla and a substantially cruder microsomal fraction for the striatum.

Equilibrium Binding of [^{3}H]SCH23390 to Adrenal Medullary and Striatal Membranes

Adrenal medulla. In light of the better signal obtained at 4°, attempts were made to obtain equilibrium saturation binding of [^{3}H]SCH23390 to purified adrenal medullary plasma membranes. Increasing concentrations of [^{3}H]SCH23390 (20–2,000 pM) were incubated at 4° for 2 hr, as described in Materials and Methods. The nonspecific binding was estimated in parallel in tubes containing 1 μM (+)-SCH23390. The Scat-

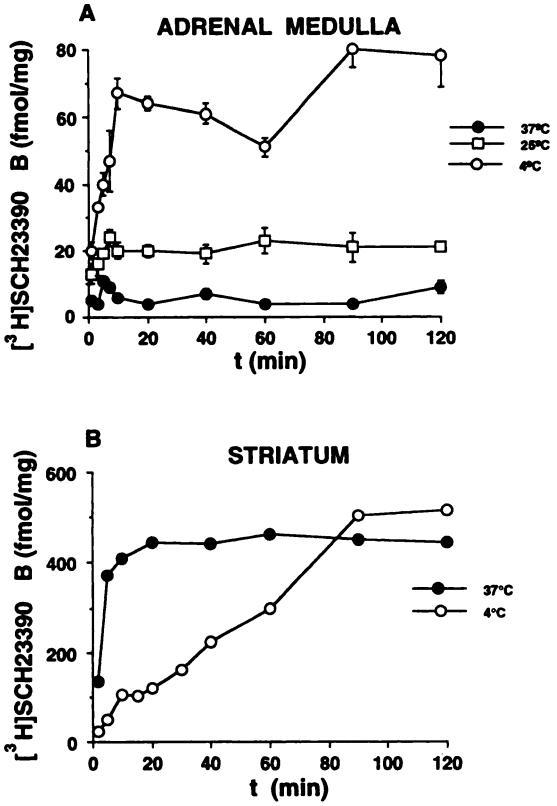

Fig. 1. Effects of temperature on the time course for the binding of [^{3}H]-SCH23390 to purified bovine adrenal medullary plasma membranes (A) or to striatal microsomal membranes (B). The incubation mixture was identical to that described for Table 1. *Abscissae*, incubation times; *ordinates*, specifically bound ligand (*B*). For adrenal medulla, the data are means ± standard errors of three experiments assayed in duplicate, performed with separate batches of membranes. For striatum, the data are from a single experiment assayed in triplicate.

chard plot of the results (Fig. 2A) produced a poor correlation coefficient, which precluded the calculation of any meaningful binding constants.

In spite of this, attempts were made to test whether displacement experiments showed a more consistent picture. The results on the ability of various selective and nonseletive dopamine receptor agonists and antagonists to displace the binding of 1 nM [^{3}H]SCH23390 to adrenal medullary plasma membranes are shown in Table 1. The most effective displacing agent was (+)-SCH23390, followed by the D_1-selective antagonist (±)-SKF83566. The D_1-selective agonist (+)-SKF38393 was a very poor displacing agent. The remaining nonselective

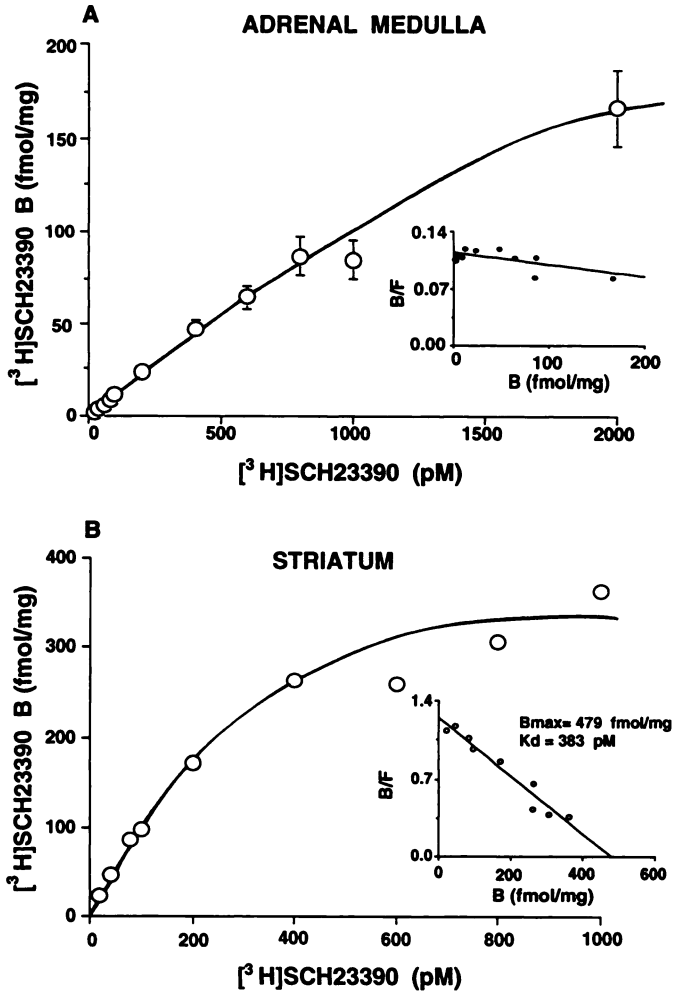

Fig. 2. A, Equilibrium binding of [^{3}H]SCH23390 to purified bovine adrenal medullary plasma membranes. Membranes (200 μg/ml) were incubated at 4° for 2 hr in the reaction mixture described in Materials and Methods, in the presence of increasing concentrations of the ligand (*abscissa*). Nonspecific binding was determined in the presence of 1 μM (+)-SCH23390. *Ordinate*, [^{3}H]SCH23390 specifically bound (fmol/mg of plasma membrane protein). *Inset*, Scatchard plot of the data. *B*, bound ligand (fmol/mg of protein); *F*, free ligand concentration (pM). Data are means ± standard errors of three duplicate experiments performed using different batches of membranes. B, Saturation equilibrium isotherm for the binding of [^{3}H]SCH23390 to bovine striatal microsomal membranes. Membranes (200 μg/ml) were incubated for 2 hr at 4° with increasing concentrations of the ligand (*abscissa*) in the reaction mixture described in Materials and Methods. Nonspecific binding was determined in the presence of 1 μM (+)-SCH23390. *Ordinate*, radioactivity bound to membranes (fmol/mg of membrane protein). *Inset*, Scatchard plot of the saturation data.

TABLE 1

Comparative IC_{50} values to displace [^{3}H]SCH23390 binding to purified bovine adrenal medullary plasma membranes and striatal microsomal membranes

Membranes were incubated at 4° for 2 hr in the presence of 1 nM [^{3}H]SCH23390 and increasing concentrations of each displacing agent. Each value is the mean ± standard error of three experiments assayed in duplicate, with membranes from different batches.

Displacing drug	IC_{50}	
	Adrenal medulla	Striatum
	nM	
(+)-SCH23390	446 ± 44	3 ± 0.4
(±)-SKF83566	635 ± 51	5 ± 0.4
(+)-SKF38393	33,700 ± 3,000	171 ± 29
Flunarizine	ND[a]	284 ± 11
Haloperidol	ND	515 ± 24
Apomorphine	ND	702 ± 63
Dopamine	ND	5,890 ± 212
Spiperone	ND	12,510 ± 1,925
Quinpirole	ND	ND
cAMP	ND	ND
Ketanserine	ND	ND

[a] ND, nondetectable (at 10^{-4} M).

ligands (haloperidol, apomorphine, and dopamine) and D_2-selective ligands (spiperone and quinpirole) did not affect the binding of [^{3}H]SCH23390.

Striatum. At equilibrium, the binding of [^{3}H]SCH23390 to bovine striatal microsomal membranes at 4° followed a saturation isotherm (Fig. 2B). The points in the Scatchard plot fit a straight line with a Hill coefficient of 0.95, suggesting the presence of a homogeneous population of binding sites for [^{3}H]SCH23390. The K_d for the binding was 383 ± 14 pM and the B_{max} amounted to 479 ± 26 fmol/mg of membrane protein.

The results of competition experiments appear in Table 1. The D_1-selective antagonists (+)-SCH23390 and (±)-SKF83566 were the most potent displacing agents. It is interesting to note that both IC_{50} values were 100-fold lower than those estimated in adrenal medullary membranes. Dopamine, spiperone, and quinpirole were very poor displacing agents. Neither cAMP nor the 5-hydroxytryptamine type 2 receptor antagonist ketanserine affected the binding of [^{3}H]SCH23390.

Characteristics of ^{125}I-SCH23982 Binding

A low densitiy of D_1 receptors in chromaffin cells could explain the lack of consistency of the results obtained with [^{3}H]SCH23390. Therefore, it was desirable to use ^{125}I-SCH23982, a D_1-selective ligand with a 25-fold higher specific activity.

Displacement of ^{125}I-SCH23982 binding by (+)-SCH23390 was studied in isolated adrenal medullary plasma membranes and in intact bovine adrenal medullary chromaffin cells maintained in primary culture for 2 days. (+)-SCH23390 displaced the binding of ^{125}I-SCH23982 to striatal membranes with an IC_{50} of 3.75 nM, compared with an IC_{50} of 730 nM for adrenal medullary plasma membranes (about 200-fold higher) (Fig. 3A). The binding of ^{125}I-SCH23982 to intact cells was performed at 25 and 4°; the IC_{50} values for SCH23390 were 3,760 nM at 25° and 21,700 nM at 4° (Fig. 3B). The IC_{50} values for striatal and adrenal medullary membranes were close to those obtained when [^{3}H]SCH23390 was used as the ligand.

In contrast to adrenal medullary plasma membranes, good saturation equilibrium isotherms were obtained with striatal membranes. Fig. 4 shows that increasing concentrations of ^{125}I-

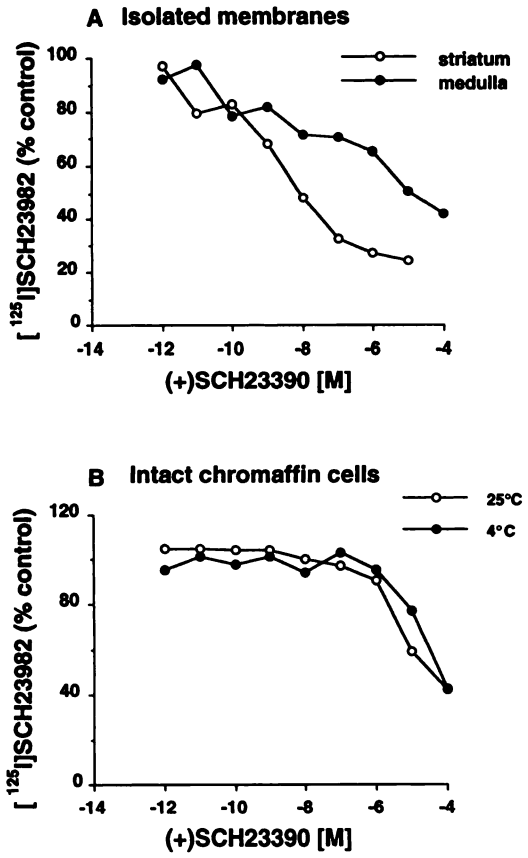

Fig. 3. Displacement by (+)-SCH23390 (10^{-12} to 10^{-4} M) of ^{125}I-SCH23982 (1 nM) binding to purified bovine adrenal medullary plasma membranes and bovine striatal microsomal membranes (A) or to intact chromaffin cells (B). Membranes (50 μg/0.5 ml) were incubated at 25° for 1 hr and chromaffin cells were incubated at two temperatures (25° for 1 hr and 4° for 2 hr), in the reaction mixture described in Materials and Methods. Data are from one experiment assayed in duplicate.

SCH23982 bound to striatal membranes following a saturation isotherm with a Hill coefficient close to 1. The Scatchard plot gave a B_{max} of 453 fmol/mg of protein and a K_d of 664 pM. These are values close to those obtained with [^{3}H]SCH23390 (compare with Fig. 2B). Attempts were also made to obtain saturation equilibrium isotherms using adrenal medullary plasma membranes. At 25°, the data points obtained were very dispersed, which precluded the construction of a saturation isotherm or the estimation of binding constants (Fig. 5A). Once more, the data seemed more reliable at 4° but, again, saturation was not achieved; the Scatchard plots of these data did not allow the calculation of meaningful equilibrium binding constants. In summary, in spite of a much stronger signal, the data obtained with ^{125}I-SCH23982 could be superimposed on those obtained with [^{3}H]SCH23390.

General Characteristics of [^{3}H]Spiperone Binding

The binding of [^{3}H]spiperone (0.4 nM) to plasma membranes was studied at 25°, as described in Materials and Methods. Under these conditions, the specific binding of [^{3}H]spiperone generally corresponded to as much as 85–90% of the total binding.

Equilibrium Binding of [^{3}H]Spiperone to Purified Bovine Adrenal Medullary Plasma Membranes and to Striatal Microsomal Membranes

Adrenal medullary plasma membranes. Increasing concentrations of [^{3}H]spiperone (20–1000 pM) produced saturation equilibrium isotherms (Fig. 6A). Scatchard plots were constructed from these curves, giving a K_d of 0.19 ± 0.02 nM and a B_{max} of 359 ± 21 fmol/mg of protein (means ± standard errors of three experiments performed in duplicate with different batches of plasma membranes). The Hill coefficient was 0.98, a result suggesting the presence of a single homogeneous population of binding sites for [^{3}H]spiperone.

A saturation equilibrium binding experiment using a microsomal membrane preparation from adrenal medullary tissue instead of purified plasma membranes was also performed. The K_d obtained was 0.19 nM, a value identical to the K_d value found in purified plasma membranes. However, the number of sites in microsomal membranes (87 fmol/mg of protein) was 4-fold lower than that in purified plasma membranes.

Increasing concentrations of various D_1 and D_2 receptor agonists and antagonists displaced [^{3}H]spiperone (0.4 nM) in a concentration-dependent manner. The well known D_2 antagonists spiperone and (+)-butaclamol most potently displaced the binding of [^{3}H]spiperone, with similar K_i values (Table 2). The

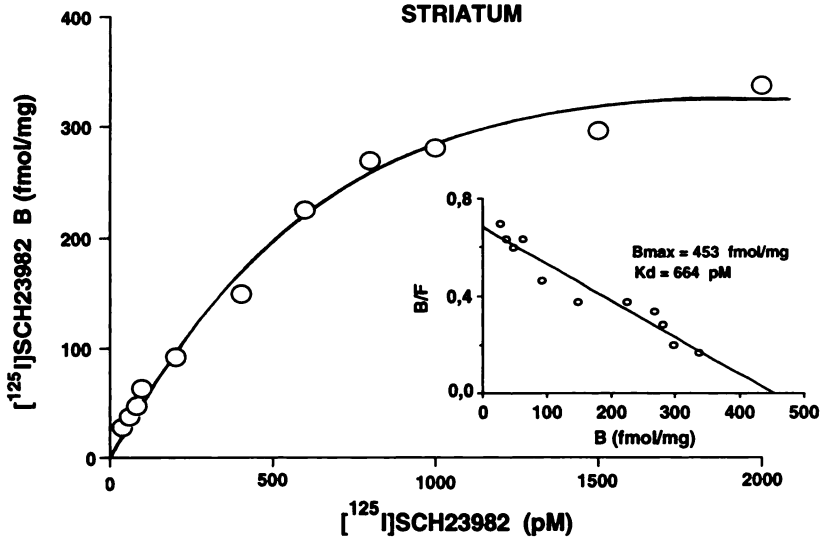

Fig. 4. Saturation equilibrium isotherm for the binding of ^{125}I-SCH23982 to bovine striatal microsomal membranes. Membranes (50 μg/0.5 ml) were incubated for 1 hr at 25° with increasing concentrations of the ligand (*abscissa*) in the reaction mixture described in Materials and Methods. Nonspecific binding was determined in the presence of 1 μM (+)-SCH23390. *Inset*, Scatchard plot of the data. *B*, bound ligand (fmol/mg of protein); *F*, free ligand concentration (pM). Data are from one experiment assayed in duplicate.

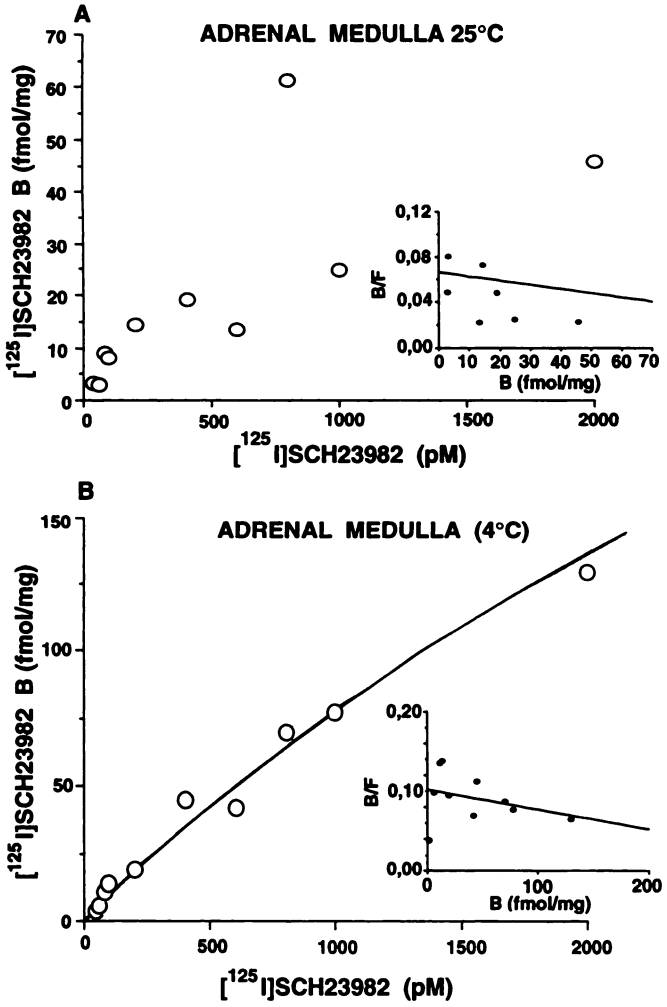

Fig. 5. A, Equilibrium binding of ^{125}I-SCH23982 to purified bovine adrenal medullary plasma membranes. Membranes (50 μg/0.5 ml) were incubated at 25° for 1 hr in the reaction mixture described in Materials and Methods, in the presence of increasing concentrations of the ligand (*abscissa*). Nonspecific binding was determined in the presence of 1 μM (+)-SCH23390. *Inset*, Scatchard plot of the data. *B*, bound ligand (fmol/mg of protein); *F*, free ligand concentration (pM). Data are from one experiment assayed in duplicate. B, Equilibrium binding of ^{125}I-SCH23982 to purified bovine adrenal medullary plasma membranes. Membranes (50 μg/0.5 ml) were incubated at 4° for 2 hr in the reaction mixture described in Materials and Methods, in the presence of increasing concentrations of the ligand (*abscissa*). Nonspecific binding was determined in the presence of 1 μM (+)-SCH23390. *Inset*, Scatchard plot of the data from one experiment assayed in duplicate.

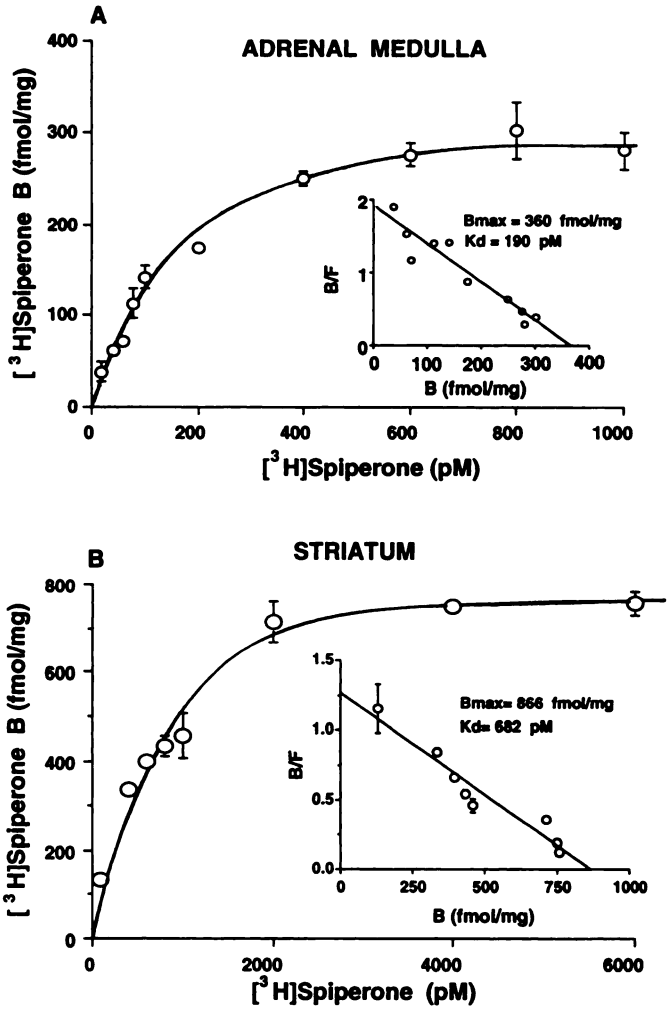

Fig. 6. A, Equilibrium binding of [^{3}H]spiperone to purified bovine adrenal medullary plasma membranes. Plasma membranes were incubated with increasing concentrations of [^{3}H]spiperone for 60 min at 25°; nonspecific binding was determined in the presence of 10 μM haloperidol. *Inset*, Scatchard plot of the saturation curve, with the K_d and B_{max} values. *B*, bound ligand (fmol/mg of protein); *F*, free ligand concentration (pM). The data are the mean ± standard error of duplicate experiments performed with three different batches of bovine adrenal medullary plasma membranes. B, Equilibrium binding of [^{3}H]spiperone to bovine striatal microsomal membranes. The membranes were incubated with [^{3}H]spiperone for 60 min at 25°; nonspecific binding was measured in the presence of 10 μM haloperidol. *Inset*, Scatchard plot of the saturation curve, with the K_d and B_{max} values. The data are the mean ± standard error of duplicate experiments performed with three different batches of microsomal membranes of bovine striatum.

dopamine receptor antagonist *cis*-(*Z*)-flupentixol was the third most potent drug. Domperidone and haloperidol were fairly potent displacing agents, but (+)-SCH23390 was a poor displacing ligand.

In general, the dopamine receptor agonists were poor displacing agents. Bromocryptine was the most potent, followed by apomorphine, dopamine, and the D_1-selective agonist (+)-SKF38393. It is worth noting the poor affinity of quinpirole for [^{3}H]spiperone binding sites.

Flunarizine displaced [^{3}H]spiperone binding with an intermediate potency. cAMP and the muscarinic receptor agonist methacholine did not compete with [^{3}H]spiperone for its binding sites, at least up to the highest concentration used (100 μM).

Striatal microsomal membranes. In bovine striatal microsomal membranes, increasing concentrations of [^{3}H]spiperone (100–6000 pM) produced binding curves that saturated at 2 nM (Fig. 6B) The specific binding, in the presence of haloperidol (10 μM), was 85–90% of the total binding. Scatchard plots of these saturation isotherms yielded a K_d of 0.68 ± 0.02 nM and a B_{max} of 866 ± 25 fmol/mg of protein (mean ± standard error of three experiments performed in duplicate with different batches of microsomal membranes). The Hill coefficient was 1.03, a result compatible with the presence of a single homogeneous population of binding sites for [^{3}H]spiperone. The numbers of binding sites found in these membranes are 2.5-fold higher than those found in purified adrenal medullary

TABLE 2

Comparative K_i values for various agents to displace [^{3}H]spiperone binding to bovine adrenal medullary plasma membranes and striatal microsomal membranes

Each result is the mean ± standard error of three experiments assayed in duplicate. The assay conditions are described in Materials and Methods.

Displacing drug	K_i	
	Adrenal medulla	Striatum
	nM	
Spiperone	0.4 ± 0.1	0.82 ± 0.2
(+)-Butaclamol	0.4 ± 0.1	2 ± 0.3
cis-(*Z*)-Flupentixol	2 ± 1	5 ± 1
Bromocryptine	4 ± 0.1	8 ± 1
Domperidone	5 ± 0.4	15 ± 2
Haloperidol	5 ± 1	24 ± 3
Flunarizine	248 ± 28	108 ± 25
Apomorphine	62 ± 9	335 ± 12
Clozapine	419 ± 33	481 ± 40
(+)-SCH23390	1154 ± 86	1760 ± 55
Dopamine	2205 ± 207	2410 ± 180
(±)-SKF83566	2327 ± 110	2390 ± 296
Quinpirole	3290 ± 85	4360 ± 663
(+)-SKF38393	8257 ± 582	7337 ± 735
cAMP	ND[a]	ND
Methacholine	ND	ND

[a] ND, nondetectable (at 10^{-4} M).

plasma membranes and about 10-fold higher than the number of sites found in a microsomal fraction from adrenal medullary tissue (8).

Increasing concentrations of various D_1 and D_2 receptor ligands competed with [^{3}H]spiperone (at 1 nM) with different affinities (Table 2). As in the case of adrenal medullary plasma membranes, spiperone and (+)-butaclamol most potently displaced the binding of the ligand. The K_i for (+)-butaclamol was 5-fold higher in the striatum than in the adrenal medulla. The antagonist *cis*-(*Z*)-flupentixol had similar K_i values in striatum and adrenal medulla.

A good correlation was also found with respect to the best displacing agonists. Dopamine, quinpirole, (+)-SKF38393, (+)-SCH23390, and (±)-SKF83566 were poor displacing agents.

Concerning nondopaminergic drugs, it is worth noting the differences observerd in K_i values for flunarizine in the two tissues. Neither cAMP nor methacholine (even at 100 μM) displaced [^{3}H]spiperone binding.

Analysis of D_1 and D_2 Dopamine Receptor Gene Expression in Bovine Caudate and Adrenal Medulla

To strengthen the results obtained in radioligand binding studies, we investigated the presence of specific transcripts of D_1 and D_2 dopamine receptor genes in the bovine caudate and adrenal medulla. In a preliminary analysis using Northern blots and cDNA probes for rat dopamine D_1 and D_2 receptors, we observed the presence of these mRNAs in poly(A)$^+$ RNA samples from bovine caudate but only the D_2 dopamine receptor mRNA appeared to be expressed in bovine adrenal medulla (data not shown). The hybridization signal for the D_1 dopamine receptor in bovine caudate was about 4 kilobases, a size that approximately corresponds to the size reported for this messenger in human tissues (26, 30, 31). To unambiguously demonstrate the presence or absence of specific transcripts of the dopamine D_1 receptor in bovine adrenal medullary tissues, we attempted the cloning of this receptor from bovine tissue using RT-PCR and the two degenerate oligonucleotides used in the cloning of the human dopamine D_1 receptor (26). Poly(A)$^+$ mRNA from bovine adrenal medulla served as the template for RT, and as a positive control we included poly(A)$^+$ mRNA from bovine caudate. After subcloning of the RT-PCR products and colony screening with a probe derived from the human dopamine D_1 receptor, several positive clones were obtained from the caudate-derived filters, whereas no positive signals were observed on the filters with adrenal medulla-derived colonies. Similar negative results were obtained in three independent trials using adrenal medullary material, even when less stringent conditions were used during the hybridization.

Sequencing of several bovine caudate positive clones revealed a common sequence (Fig. 7A) highly homologous to the human and rat dopamine D_1 receptor sequences. This cDNA, probably corresponding to a part of the bovine dopamine D_1 receptor gene, hybridized with a band of approximately 4 kilobases in Northern blots of bovine caudate but did not show any hybridization signal in adrenal medullary samples (data not shown).

As a quality control for the RT-PCR cloning, we randomly chose several colonies from the caudate and adrenal medulla cDNA collections and characterized the inserts by sequencing. In this way, a cDNA strongly represented in both cDNA collections was revealed. This cDNA corresponds to a previously cloned orphan receptor (32). Several other cDNAs, some of them coding for G protein-coupled receptors (e.g., the adenosine A_1 receptor and the α subunit of the vitronectin receptor), were obtained from either the caudate or adrenal medulla collections.

Finally, to analyze the expression of the dopamine D_2 receptor gene in adrenal medulla, we used RT-PCR and primers derived from the sequence of the bovine dopamine D_2 receptor cDNA (28). As shown in Fig. 7B, a band of the expected size of 481 base pairs was obtained in bovine (Fig. 7B, *lanes 2* and *3*) and rat (Fig. 7B, *lanes 4* and *5*) caudate and adrenal medulla tissues.

Effects of SKF38393 on Catecholamine Release from Superfused Bovine Chromaffin Cells

Cells were superfused with Krebs-HEPES solution and allowed to equilibrate for 15 min. After stabilization of the resting catecholamine release, the cells were stimulated at 7-min intervals with brief depolarizing pulses (10 sec), using a solution containing 70 mM K^+ (with equimolar reduction of NaCl). The initial electrochemical measurements of secretory responses equaled approximately 500 nA (Fig. 8A). Such responses tended to decline slightly with time. At the 10th K^+ stimulus, the responses were 25–30% lower than the initial secretion peaks.

In three separate experiments, SKF38393 (1 μM) was introduced into the superfusion system and four depolarizing pulses were applied in its presence. Secretion tended to decrease slightly but in a manner similar to that of the controls (Fig. 8, compare A and B). Washout of SKF38393 did not change this slow decline of the secretory responses seen in the absence of the compound.

Discussion

The results of this investigation show the existence of a D_2 dopamine receptor in the bovine adrenal medulla, with a pharmacological profile equivalent to that exhibited by the D_2 receptor of the bovine brain striatum. In contrast, under the present experimental conditions, it is doubtful that the striatal

A

```
bov      1 TACACCAGGA TCTACAGGAC CGCCCAGAAA CAAATACGGC GTATCTCAGC
hum    915                     T T  T                   C  TG G
rat   1054          T          T T        G      C      C

bov     51 CTTGGAGAGG GCAGCAGTCC ATGCCAAGAA CTGCCAGACC ACTACAGGTA
hum    965                       C         T            C
rat   1104                                 T            CG

bov    101 ATGGAAACCC CATGGAGTGT TCTCAACCAG AAAGCTCCTT TAAGATGTCC
hum   1015        G   TG C  A            G      T  T
rat   1154  C  G       G C  A  C G C  GT T      T

bov    151 TTCAAAAGAG AGACTAAAGT TCTGAAGACT CTGTCAGTGA TCATGGGGGT
hum   1065             A         C                 G            T
rat   1204      G  G      G          A     G      T

bov    201 GTTCGTGTGC TGCTGGCTCC CTTTCTTCAT C
hum   1115    T         T     A
rat   1254    T
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B

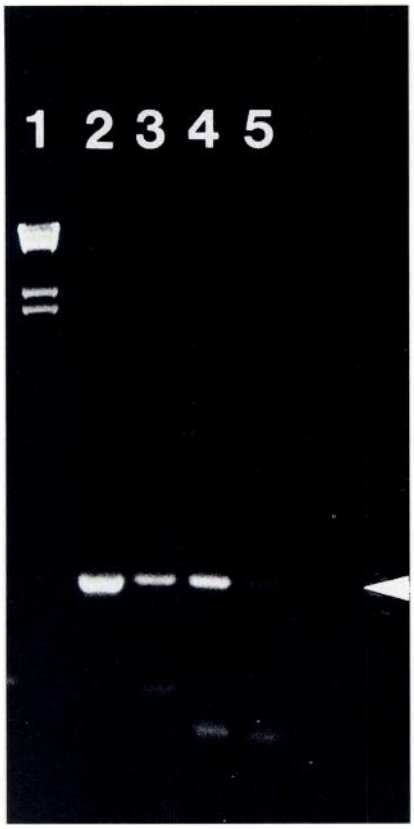

Fig. 7. A, Nucleotide sequence of a partial clone for the putative bovine (*bov*) dopamine D_1 receptor cDNA. Comparison with human (*hum*) and rat cDNAs is also shown. B, RT-PCR analysis with specific primers for the bovine dopamine D_2 receptor, showing its expression in bovine (*lanes 2* and *3*) and rat (*lanes 4* and *5*) caudate (*lanes 2* and *4*) and adrenal medulla (*lanes 3* and *5*). *Lane 1*, size markers. The agarose gel was stained with ethidium bromide.

D_1 receptor has a counterpart in bovine adrenal medullary tissues.

D_1 receptors. (+)-[^{3}H]SCH23390 and ^{125}I-SCH23982 are reputed to be highly selective D_1 ligands in brain tissues (1, 2, 33). Their binding to bovine striatal membranes is compatible with a D_1 receptor. However, there could also be a D_5 receptor subtype exhibiting a pharmacological profile similar to that of D_1 receptors (34). D_5 receptors exhibit 10-fold higher affinity for dopamine, compared with D_1 receptors, but lower affinity for antagonists such as (+)-butaclamol, haloperidol, and spiperone. The IC_{50} values for various displacing drugs against [^{3}H]SCH23390 binding to striatal membranes (Table 1) better fit the profile of a D_1 receptor. However, we could not demonstrate selective binding of [^{3}H]SCH23390 to purified plasma membranes from bovine adrenal medulla. Bigornia *et al.* (7) also failed to show specific binding of [^{3}H]SCH23390 to adrenal medullary microsomal membranes. The membrane purification led to a 4-fold increase in the density of D_2 receptors, suggesting that the conditions to detect specific binding sites for [^{3}H]-SCH23390 improved at least 4-fold. These conditions were further optimized by using the D_1-selective radioligand ^{125}I-SCH23982 (33), which exhibits a 25-fold higher specific activity than the tritiated parent compound. However, the results with the two ligands, in striatal and adrenal medullary membranes, could be superimposed. In intact chromaffin cells, at different

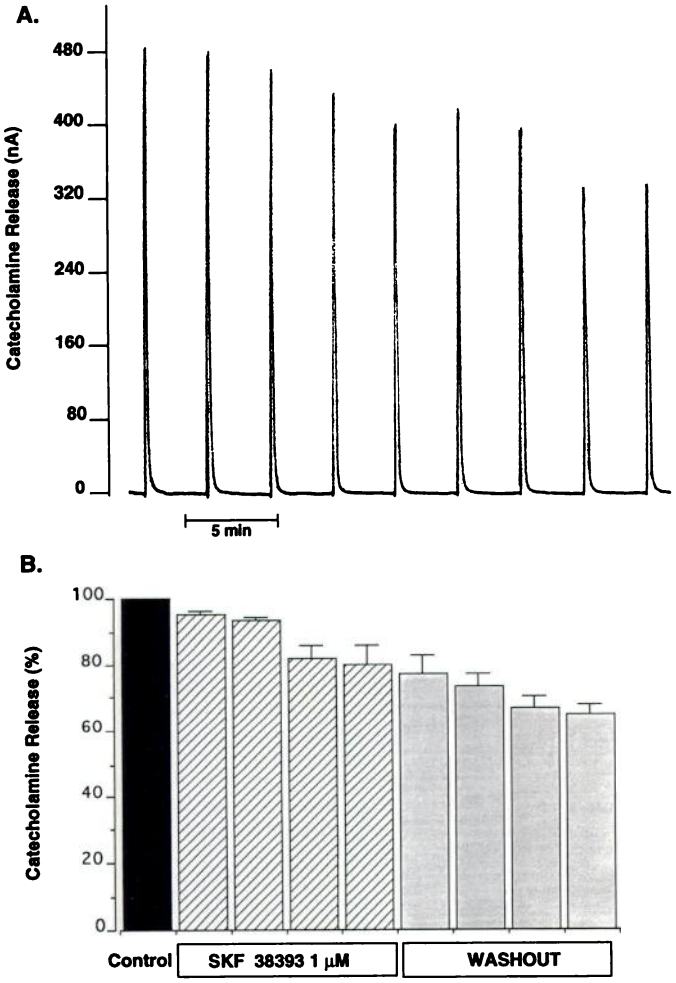

Fig. 8. On-line measurement of catecholamine release from superfused chromaffin cells. Cells were repeatedly stimulated to secrete catecholamines, at 7-min intervals. Stimuli consisted of a 10-sec superfusion with a solution containing 70 mM K^+ (iso-osmotically adjusted by reducing the NaCl concentration). A, An experiment is shown to indicate that the peak secretory responses tend to decrease slightly with time. B, Secretion was normalized as a percentage of the initial secretory response. SKF38393 was introduced as shown (*horizontal bars at the bottom*). Data are means ± standard errors of three experiments performed with cells from different batches.

temperatures, specific binding could not be detected using ^{125}I-SCH23390 as ligand.

Another interesting difference between striatal and adrenal medullary binding sites for [^{3}H]SCH23390 rests in their behavior at low and high temperatures. For instance, a temperature of 4° was necessary to detect minimal specific binding of [^{3}H]-SCH23390 to adrenal medullary plasma membranes. At 4° the total binding was the same as that at 37° (808 versus 873 cpm); however, the nonspecific binding decreased upon lowering of the temperature (from 620 cpm to 260 cpm). In contrast, the total binding and specific binding were similar in the striatum (8268 versus 6998 cpm for total binding and 317 versus 534 cpm for nonspecific binding). Thus, qualitative as well as quantitative differences were observed between the binding sites for [^{3}H]SCH23390 in adrenal medulla and striatum.

The lack of high affinity binding sites for [^{3}H]SCH23390 and ^{125}I-SCH23982 and the lack of results compatible with the presence of D_1 receptors agree with the molecular biology studies in adrenal medullary tissue. We have also characterized a cDNA from bovine caudate that shows very strong homology to a segment between the third and sixth transmembrane domains of the human and rat dopamine D_1 receptor genes. The failure to obtain a similar cDNA from bovine adrenal medulla and the finding that specific hybridization signals were not observed on Northern blots of rat (26) and bovine adrenal medulla suggest that the dopamine D_1 receptor gene is not expressed in this tissue. Furthermore, *in situ* hybridization experiments using a rat riboprobe failed to detect the expression of the D_1 dopamine receptor in bovine or rat adrenal medullary tissues.

D_2 receptors. The expression of the dopamine D_2 receptor gene in caudate and adrenal medulla was indisputably observed in both bovine and rat samples. The binding of [^{3}H]spiperone to membranes from bovine adrenal medulla and from bovine brain striatum was in line with these findings. In adrenal medulla, the increase in B_{max} (4-fold) with respect to values obtained in microsomal fractions reflects the advantages of using this purified preparation in binding studies. The question is whether this D_2 receptor belongs to a D_2 receptor subtype (so-called D_3 or D_4 receptors). Dopamine D_4 receptors exhibit a pharmacological profile similar to that of D_2 and D_3 receptors; they are distinguished by the fact that clozapine exhibits 1 order of magnitude higher affinity for D_4 receptors (dissociation constant of 9 nM) (35). In striatal microsomal membranes and in adrenal medullary plasma membranes the K_i values for clozapine to displace [^{3}H]spiperone binding were 0.48 and 0.42 μM, respectively. This low affinity of clozapine suggests the lack of D_4 receptors labeled by [^{3}H]spiperone in bovine striatal and adrenal bovine medullary tissues.

The displacement experiments strengthen the expected conclusion that those binding sites correspond to D_2 dopamine receptors. Spiperone and (+)-butaclamol displaced with the highest potency the binding of [^{3}H]spiperone in both tissues. In contrast, (+)-SCH23390, (±)-SKF83566, and (+)-SKF38393, considered D_1-selective ligands, were extremely poor displacing agents. It is interesting to note that the same order of the best displacing drugs was observed for striatum and adrenal medulla. Thus, the D_2 receptor recognized by [^{3}H]spiperone seems to exhibit similar pharmacological characteristics for striatal and adrenal medullary membranes.

An interesting observation relates to the finding that the dopaminergic agonist bromocryptine is a potent displacer in both the adrenal medulla and the striatum. It could be interesting to use this molecule as a tool to explore functional correlates of these binding experiments in bovine chromaffin cells. Apomorphine, which shows pronounced functional effects in regulating catecholamine release in feline (12, 13) and bovine (8) adrenal glands, occupies an intermediate position among the displacing agents (K_i values of 62 nM for the adrenal medulla and 335 nM for the striatum). However, dopamine and quinpirole (considered to be a selective D_2 agonist) exhibited poor affinity for adrenal medullary D_2 receptors.

By comparing the B_{max} of [^{3}H]ouabain in purified plasma membranes and knowing the density of binding sites for this ligand on each single chromaffin cell, a fairly good approximation of the number of [^{3}H]spiperone binding sites/single chromaffin cell can be obtained. The B_{max} for [^{3}H]ouabain is 9750 fmol/mg of protein in purified plasma membranes and 244 fmol/10^6 bovine chromaffin cells (18). The B_{max} for

[^{3}H]spiperone binding to purified plasma membranes was 359 fmol/mg of protein. Thus, these membranes contain 27 [^{3}H]-ouabain binding sites/[^{3}H]spiperone binding site. Assuming that the same ratio is maintained in living chromaffin cells in culture, we can extrapolate the number of binding sites for [^{3}H]spiperone in living cells. The resulting value is 11 fmol/10^6 cells. This means that a single chromaffin cell binds 6908 molecules of [^{3}H]spiperone. If a chromaffin cell is considered as a sphere of 20-μm diameter, the surface area is 1256 μm^2. Thus, the density of [^{3}H]spiperone binding sites, and of D_2 receptors, on the bovine chromaffin cell is 5.5/μm^2 of surface area.

The displacement by flunarizine of [^{3}H]spiperone binding deserves a comment. De Vries and Beart (36) observed the ability of the calcium channel antagonists verapamil, D600, and nicardipine to inhibit the binding of [^{3}H]spiperone to rat brain membranes (IC_{50} values of 2 μM, 2 μM, and 6 μM, respectively) through interactions between these molecules and D_2 sites. This finding is not due to nonspecific membrane interactions but might depend on a phenylalkylamine (dopamine-like) moiety present in those molecules. The displacement potency of flunarizine in the bovine adrenal medulla (K_i of 248 nM) and bovine striatum (K_i of 108 nM) is in the range of potencies of the aforementioned drugs for the rat striatum. It is possible that the flunarizine-displacing effects of [^{3}H]spiperone binding provide additional evidence for a functional association between calcium channels and D_2 receptors in modulating catecholamine release evoked by nicotinic stimulation of chromaffin cells. In this context, González *et al.* (8) observed that the dihydropyridine calcium antagonist nitrendipine did not inhibit [^{3}H]spiperone binding to a microsomal fraction of bovine adrenal medulla. Therefore, it seems that the inhibitory effect of flunarizine on [^{3}H]spiperone binding could be specific for the piperazine-type calcium channel antagonists. The interaction between flunarizine and D_2 dopamine receptors could help explain the Parkinson-like syndrome produced by flunarizine-type drugs (37), on the basis of the observed inhibitory effects on [^{3}H]spiperone binding.

In conclusion, the combination of molecular biology and radioligand binding techniques shows that dopamine D_2 receptors of similar characteristics are expressed centrally in bovine brain striatum and peripherally in bovine adrenal medullary chromaffin cells. With these techniques, we have also partially cloned and characterized a dopamine D_1 receptor expressed in the bovine striatum; this receptor is not expressed in the bovine adrenal medulla. These results are relevant in light of previous reports suggesting an important functional role for D_1 receptors in facilitating the Ca^{2+} current flowing through voltage-dependent Ca^{2+} channels, by a cAMP-mediated mechanism (5). Therefore, the reported effects of D_1 probes [i.e., the agonist SKF38393 and the antagonist (+)-SCH23390] on such currents (5) cannot be associated with D_1 receptors; rather, they might reflect pharmacological effects of those drugs unrelated to D_1 receptors. It is also likely that the rhodamine conjugate of the 4′-amino derivate of SCH23390 used in previous reports (5, 6) does not label true D_1 receptors in bovine chromaffin cells. Thus, inferences regarding the facilitation of catecholamine release by D_1 receptors in bovine chromaffin cells or the intact adrenal gland seem to be physiologically irrelevant. In fact, we showed here that catecholamine release, induced by brief depolarizing pulses, from superfused bovine chromaffin cells was unaffected by the D_1-selective agonist SKF38393 (Fig. 8). Doubts also emerge concerning the functional role for D_2 receptors in bovine chromaffin cells. Catecholamine release triggered by nicotinic stimulation of these cells was inhibited by apomorphine but not by dopamine or quinpirole. Thus, Huettle *et al.* (16) suggested that the inhibitory actions of apomorphine were unrelated to D_2 receptors. Species differences, differences in cell culture conditions or cell culture times, or simply differences in protocols (long versus short stimulation times, temperature, parameters measured, etc.) could explain such differences. Therefore, only additional thorough studies in intact glands and isolated chromaffin cells from various animal species will clearly define the role of dopamine receptor subtypes in controlling adrenal catecholamine secretory responses to stress.

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References

1. Andersen, P. H., M. D. B. Gingrich, A. Dearry, P. Falardeau, S. E. Senogles, and M. Garon. Dopamine receptor subtypes: beyond the D_1/D_2 classification. *Trends Pharmacol. Sci.* **11**:231–235 (1990).
2. Sibley, D. R., and F. J. Monsma, Jr. Molecular biology of dopamine receptors. *Trends Pharmacol. Sci.* **13**:61–69 (1992).
3. Alkahadi, S. A., M. J. Sabouni, A. F. Ansar, and M. F. Likhandwala. Activation of DA_1 receptors by dopamine or fenoldapam increases cyclic AMP levels in the renal artery but not in the superior cervical ganglion of the rat. *J. Pharmacol. Exp. Ther.* **238**:547–553 (1986).
4. Ariano, M. A. Comparison of dopamine binding sites in the rat superior cervical ganglion and caudate nucleus. *Brain Res.* **421**:245–254 (1991).
5. Artalejo, C. R., M. A. Ariano, R. L. Perlman, and A. P. Fox. Activation of facilitation calcium channels in chiomaffin cells by D_1 dopamine receptors through a cAMP protein kinase A dependent mechanism. *Nature (Lond.)* **348**:239–242 (1990).
6. Ariano, M. A., H. C. Kang, R. P. Haugland, and D. R. Sibley. Multiple fluorescent ligands for dopamine receptors. II. Visualization in neural tissues. *Brain Res.* **547**:208–222 (1991).
7. Bigornia, L., C. N. Allen, C.-R. Jan, R. Lyon, M. Titeler, and A. Schneider. D_2 dopamine receptors modulate calcium channel current and catecholamine secretion in bovine adrenal chromaffin cells. *J. Pharmacol. Exp. Ther.* **252**:586–592 (1990).
8. González, M. C., A. R. Artalejo, C. Montiel, P. P. Hervás, and A. G. García. Characterization of a dopaminergic receptor that modulates adrenomedullary catecholamine release. *J. Neurochem.* **47**:382–387 (1986).
9. Quik, M., L. Bergeron, H. Mount, and J. Philie. Dopamine D_2 receptor binding in adrenal medulla: characterization using [^{3}H]spiperone. *Biochem. Pharmacol.* **36**:3707–3713 (1987).
10. Lyon, R. A., M. Titeler, L. Bigornia, and A. S. Schneider. D_2 dopamine receptor on bovine chromaffin cell membranes: identification and characterization by [^{3}H]*N*-methylspiperone binding. *J. Neurochem.* **48**:631–635 (1987).
11. Albillos, A., F. Abad, and A. G. García. Cross-talk between M_2 muscarinic and D_1 dopamine receptors in the cat adrenal medulla. *Biochem. Biophys. Res. Commun.* **183**:1019–1024 (1992).
12. Artalejo, A. R., A. G. García, C. Montiel, and P. Sánchez-García. A dopaminergic receptor modulates catecholamine release from the cat adrenal gland. *J. Physiol. (Lond.)* **362**:359–368 (1985).
13. Montiel, C., A. R. Artalejo, P. M. Bermejo, and P. Sánchez-García. A dopaminergic receptor in adrenal medulla as a possible site of action for the droperidol-evoked hypertensive response. *Anesthesiology* **65**:65–71 (1987).
14. Bigornia, L., M. Suozzo, K. A. Ryan, D. Napp, and A. Schneider. Dopamine receptors on adrenal chromaffin cells modulate calcium uptake and catecholamine release. *J. Neurochem.* **51**:999–1006 (1988).
15. Sontag, J.-M., P. Sanderson, M. Klepper, D. Aunis, K. Takeda, and M.-F. Bader. Modulation of secretion by dopamine involves decreases in calcium and nicotinic currents in bovine chromaffin cells. *J. Physiol. (Lond.)* **427**:495–517 (1990).
16. Huettle, G. A. G., M. D. Browning, and J. M. Masserano. Effects of dopamine receptor agonists and antagonists on catecholamine release in bovine chromaffin cells. *J. Pharmacol. Exp. Ther.* **257**:567–574 (1991).
17. Wilson, S. P., and N. Kirshner. Isolation and characterization of plasma membranes from the adrenal medulla. *J. Neurochem.* **27**:1289–1298 (1976).
18. Castillo, C. J. F., R. J. Fonteriz, M. G. López, K. Rosenheck, and A. G. García. (+)-PN200–110 and ouabain binding sites in purified bovine adrenomedullary plasma membranes and chromaffin cells. *J. Neurochem.* **53**:1342–1349 (1989).

19. Ellman, G. L., K. D. Courtney, B. Andres, Jr., and R. M. Featherstone. A new rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7**:88–95 (1961).
20. Cheng, Y. L., and W. H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
21. Moro, M. A., M. G. López, L. Gandía, P. Michelena, and A. G. García. Separation and culture of living adrenaline- and noradrenaline-containing cells from bovine adrenal medulla. *Anal. Biochem.* **185**:243–248 (1990).
22. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
23. Naranjo, J. R., B. Mellström, M. Achaval, and P. Sassone-Corsi. Molecular pathways of pain: Fos/Jun-mediated activation of the predynorphin gene through a non-canonical AP-1 site. *Neuron* **6**:607–617 (1991).
24. Feinberg, A. P., and B. Vogelstein. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**:6–13 (1983).
25. Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science (Washington D. C.)* **239**:487–494 (1988).
26. Zhow, Q.-Y., D. K. Grandy, L. Thambi, J. A. Kushner, H. H. M. Van Tol, R. Cone, D. Pribnow, J. Salon, J. R. Bunzow, and O. Civelli. Cloning and expression of human and rat D_1 dopamine receptors. *Nature (Lond.)* **347**:76–80 (1990).
27. Buluwela, L., A. Forster, T. Boehm, and T. H. Rabbitts. A rapid procedure for colony screening using nylon filters. *Nucleic Acids Res.* **17**:452 (1989).
28. Chio, C. L., G. F. Hess, R. S. Graham, and R. M. Huff. A second molecular form of D_2 dopamine receptor in rat and bovine caudate nucleus. *Nature (Lond.)* **343**:266–269 (1990).
29. Rosenheck, K. The chromaffin cell plasma membrane, in *Stimulus-Secretion Coupling in Chromaffin Cells* (K. Rosenheck and P. I. Lelkes, eds.), Vol. 2. CRC Press, Boca Raton, FL, 37–50 (1986).
30. Dearry, A., J. A. Gingrich, P. Falerdeau, R. T. Fremaeu, M. D. Bates, and M. G. Caron. Molecular cloning and expression of the gene for a human D_1 dopamine receptor. *Nature (Lond.)* **347**:72–76 (1990).
31. Sunahara, R. W., H. B. Niznik, D. M. Weiner, T. M. Stormann, M. R. Brann, J. L. Kennedy, J. E. Gelernter, R. Rozmahel, Y. Yang, Y. Israel, P. Seeman, and B. F. O'Dowd. Human dopamine D_1 receptor encoded by an intronless gene on chromosome 5. *Nature (Lond.)* **347**:80–83 (1990).
32. Eidne, K. A., J. Zabavnik, T. Peters, S. Yoshida, L. Anderson, and P. L. Taylor. Cloning, sequencing and tissue distribution of a candidate G-protein coupled receptor from rat pituitary gland. *FEBS Lett.* **292**:243–248 (1991).
33. Lovenberg, T. W., R. H. Roth, D. E. Nichols, and R. B. Mailman. D_1 dopamine receptors of NS204 neuroblastoma cells are functionally similar to rat striatal D_1 receptors. *J. Neurochem.* **57**:1563–1569 (1991).
34. Sunahara, R. K., H.-G. Guan, B. F. O'Dowd, P. Sfeman, L. G. Laurier, G. Ng, S. R. Gponge, M. J. Torchia, H. H. M. Vantol, and H. B. Niznik. Cloning of the gene for a human dopamine D_5 receptor with higher affinity for dopamine than D_1. *Nature (Lond.)* **350**:614–619 (1991).
35. Van Tol, H. H. M., J. R. Bunzow, H.-C. Guan, R. K. Sunhara, P. Seeman, H. B. Niznik, and O. Civelli. Cloning of the gene for a human dopamine D_4 receptor with high affinity for the antipsychotic clozapine. *Nature (Lond.)* **350**:610–614 (1991).
36. De Vries, D. J., and P. M. Beart. Competitive inhibition of [^{3}H]spiperone binding to D-2 dopamine receptors in striatal homogenates by organic calcium channel antagonists and polyvalent cations. *Eur. J. Pharmacol.* **106**:133–139 (1985).
37. Chouza, C., A. Scaramelli, J. L. Caamaño, O. De Medina, R. Aljanati, and S. S. Romer. Parkinsonism, tardive dyskinesia, akathisia and depression induced by flunarizine. *Lancet* **1**:1303–1304 (1986).

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