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Competitive binding assays simply done– with a native marker and mass spectrometric quantification

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Chemicals. SCH 23390, pimozide, (*S*)-sulpiride and trifluoperazine were purchased from RBI/Biotrend, Köln, Germany. Chlorpromazine, (+)-butaclamol, haloperidol and SKF 83566 were purchased from RBI/Sigma, Taufkirchen, Germany. [^{3}H]SCH 23390 (3182 GBq mmol^{-1}) was purchased from Perkin Elmer Life Sciences, Freiburg, Germany.

Porcine striatal membrane fraction. Striatum from pig brains (from the local slaughterhouse) was homogenised in 10 volumes of 0.32 M sucrose with a potter (PotterS, Braun, Melsungen, Germany, 1200 rpm, 10 up-and-down strokes), and centrifuged at 1000 *g* for 10 min at 4 °C. The supernatant was centrifuged at 20000 *g* for 10 min at 4 °C again. The resulting pellets (P_2) were resuspended in 50 mM Tris-HCl pH 7.4 and centrifuged at 30000 *g* for 20 min at 4 °C. The last centrifugation was repeated. Resuspension of the pellet in 50 mM Tris-HCl pH 7.4 yielded a membrane fraction, which was frozen at – 80 °C.

General procedure of competitive binding assays using SCH 23390 as marker. A portion of the membrane fraction was thawed, centrifuged at 48000 g for 30 min at 4 °C and resuspended in 50 mM ammonium formiate pH 7.4 (the pH was adjusted by addition of aqueous ammonia). Aliquots of about 250 µg protein (estimated according to Bradford using

bovine serum albumin as standard)[1] were incubated in the presence of 1.25 nM SCH 23390, test compounds in varying concentrations and 50 mM ammoniumformiate pH 7.4 in a total volume of 250 µl in 1.5 ml polypropylene tubes in a shaking water bath for 40 min at 25 °C. The samples were repeatedly vortexed to avoid sedimentation of the membrane particles. The incubation was stopped by centrifugation at 50000 g for 20 min at 4 °C (Biofuge Stratos, Kendro, Osterode, Germany). From the supernatant 220 µl were centrifuged again as before. From the second supernatant 200 µl were transferred to autosampler vials. Control samples without test compounds were used to define total binding. Samples with 10 µM (+)-butaclamol were used to define non specific binding. Samples without SCH 23390 and without test compounds were treated in the same manner as described above to obtain the matrix for external standards. To 180 µl of the second supernatant of those matrix samples 20 µl of 2 nM, 4 nM, 7 nM, 10 nM and 12.5 nM of SCH 23390 or 20 µl 50 mM ammoniumformiate pH 7.4 were added to obtain external standards and blanks. All samples including the controls, external standards and blanks were performed as quadruplicates.

Procedure of LC-ESI-MS-MS Analysis. The quantitation of SCH 23390 was carried out on an Agilent 1100 HPLC instrument (vacuum degasser, quaternary pump, autosampler and oven, Agilent, Waldbronn, Germany) coupled to an API 2000 triple quadrupole mass spectrometer with an electrospray ionisation source (Applied Biosystems, Darmstadt, Germany). A Merck (Darmstadt, Germany) Superspher 60 RP-select B column (2 x 125 mm, 4 µm) in connection with a Phenomenex (Torrance, CA, USA)

RP8 guard column (4 x 2 mm) was utilised for separation. The column temperature was set at 20 °C. The mobile phase consisted of 50 % of 0.1 % formic acid in water and 50 % of acetonitril. The total run time was 4 min at a flow rate of 300 µl min^{-1}. A 50 µl aliquot of the resulting second supernatant was injected without further purification on to the column. For routine quantification the effluent up to 1.5 min and effluent from 3.5 min to 4.0 min was diverted to waste by a Valco valve in order to protect the mass spectrometer. The operating parameters of the MS detector in the MRM mode[2] were set as follows: source temperature 485 °C, ion spray voltage +1500 V, collision energy 37 V, nitrogen was used as the curtain (25 psi), as the nebulizing (70 psi), as the auxiliary (30 psi) and as the collision gas (12 psi). The transition from the precursur-ion 288.1 (*m*/*z*) to the product-ion 91.2 (*m*/*z*) was monitored operating Q1 and Q3 under low mass resolution conditions and dwell times of 500 ms. Data were collected and quantitated (without further manipulation like smoothing etc.) using Analyst 1.2 (Applied Biosystems, Darmstadt, Germany). Under these conditions the signal to noise ratio of a 70 pM external standard was > 15.

General procedure of radioligand binding assays. A portion of the membrane fraction was thawed, centrifuged at 48000 g for 30 min at 4 °C and resuspended in 50 mM ammonium formiate pH 7.4. In competition experiments aliquots of about 50 µg protein (estimated according to Bradford using bovine serum albumin as standard)[1] were incubated in the presence of 1 nM [^{3}H] SCH 23390, test compounds in varying concentrations, 50 mM ammoniumformiate pH 7.4 in a total volume of 250 µl in 3.5 ml polystyrene tubes in a shaking water bath for 40 min at 25

°C. Incubation was terminated by filtration through Whatman GF/C filters pre-soaked for 1 h in 0.5 % polyethylenimine using a Brandel M-24R harvester (Gaithersburg, MD, USA). The filters were rapidly rinsed with cold buffer (4 x 2 ml) and [^{3}H] SCH 23390 bound on the filters was counted in 3 ml Rotiszint Eco Plus (Roth, Karlsruhe, Germany) using a Packard TriCarb 1600 (Perkin Elmer Life Sciences, Freiburg, Germany) liquid scintillation counter. Control samples without test compounds were used to define total binding. Samples with 10 µM (+)-butaclamol were used to define non specific binding. Saturation experiments were performed analogously with 8 concentrations [^{3}H] SCH 23390 in the range from 0.2 nM to 10 nM. All experiments were carried out with samples in triplicates. In order to document that binding to D_1-receptors in 50 mM ammonium formiate shows a rank order of potency which is in agreement with results deduced from experiments in conventional buffers used in radioligand binding assays, we carried out the same binding experiments as described above in 50 mM Tris-HCl pH 7.4 and in 1 mM EDTA, 120 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$ 50 mM Tris-HCl pH 7.4 (Tris HCl + salts) as well (table 1).

Analysis of binding experiments. In all experiments specific binding defined as difference between total and non specific binding was analysed. The concentration of a competing drug that inhibits 50 % of specific binding (IC_{50}) was calculated with equation (1) using Prism 2.01 (GraphPad Software, San Diego, CA, USA) for sigmoidal dose-response curves. K_i-values were calculated according to Cheng and Prussoff when [^{3}H] SCH 23390 was used as marker and depletion of the radioligand was

negligible.[3,4] K_i-values were calculated with equation (2) when SCH 23390 was used as marker and depletion of the marker was substantial.[4]Values of the dissociation constant (K_d) and of the density of binding sites (B_{max}) were calculated from saturation isotherms of specific binding by means of the non-linear curve-fitting program Prism 2.01 (GraphPad Software, San Diego, CA, USA). If not stated otherwise all data are expressed as mean ± standard error of the mean of at least three separate experiments.

$$Y = A + \frac{(B - A)}{1 + 10^{\log IC50 - X}} \qquad (1)$$

A: bottom (concentration of SCH 233390 in samples without inhibitor); B: top (concentration of SCH 233390 in samples with 10 µM (+)-butaclamol)

$$K_i = \frac{IC_{50}}{2\frac{(L^* - L_0^*)}{L_0^*} + 1 + \frac{L^*}{K_d}} \qquad (2)$$

L*: concentration of SCH 233390 at the IC_{50}; L_0*: concentration of free SCH 23390 in the absence of a competing ligand

Characteristics of [^{3}H] SCH 23390 binding to the porcine striatal membrane fraction. From saturation experiments the following K_d- and B_{max}-values were obtained: 0.72 ± 0.18 nM and 540 ± 80 fmol mg^{-1} in 50 mM ammonium formiate, 1.1 ± 0.1 nM and 1300 ± 100 fmol mg^{-1} in 50 mM Tris-HCl and 1.5 ± 0.1 nM and 870 ± 110 fmol mg^{-1} in 1 mM EDTA, 120 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$ and 50 mM Tris-HCl (Table 1).

Table 1: Inhibition of [^{3}H] SCH 23390 binding to porcine striatal membranes by Dopamine antagonists in different buffers

	Ammonium Formiate K_i [nM]	Tris-HCl K_i [nM]	Tris-HCl + salts K_i [nM]
(+)-Butaclamol	5.4 ± 1.9	14 ± 3	49 ± 1
Chlorpromazine	300 ± 40	580 ± 90	2500 ±
100Haloperidol	110 ± 2	160 ± 20	670 ± 20
Pimozide	2500 ± 500	2000 ± 300	7000 ± 600
SKF83566	2.7 ± 0.6	1.3 ± 0.1	2.6 ± 0.1
(*S*)-Sulpiride	> 10000	> 10000	> 10000
Trifluoperazine	215 ± 20	290 ± 90	410 ± 30

References

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