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EFFECTS OF PROTEOLYTIC ENZYMES AND MONOVALENT IONS DEMONSTRATE PROTEASE-SENSITIVE AND PROTEASE-INSENSITIVE STEREOSPECIFIC BINDING SITES ON DOPAMINERGIC RECEPTORS IN RAT STRIATUM*

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Abstract—Rat striatal membranes from different subcellular fractions were treated with various proteolytic and other enzymes and the binding of a dopamine agonist ([^{3}H]NPA) and of an antagonist ([^{3}H]haloperidol) was assayed in several conditions. In membranes of striatal microsomal and mitochondrial fractions, stereospecific binding of both [^{3}H]NPA and [^{3}H]haloperidol assayed in a monovalent ion-poor buffer was potently and rapidly inhibited by trypsin and certain related proteases. The enzymes did not affect the binding of the ligands when assayed in a buffer containing monovalent ions (≥40 mM NaCl or KCl or a physiological mixture of electrolytes). The inhibition, seen in the monovalent ion-poor buffer, was dependent on the enzyme concentration. The endoproteases (trypsin, α-chymotrypsin, papain, ficin) showed nanomolar IC_{50}-values for inhibition of both [^{3}H]NPA and [^{3}H]haloperidol binding. The inhibition occurred very rapidly at 0° and was different from the slow proteolytic inactivation seen by prolonged incubation at 37°. It was demonstrated that monovalent ions did not themselves interfere with the interaction between the proteases and the membranes. The observations provide evidence for two different types of stereospecific dopaminergic binding sites which are differentially exposed for ligand binding depending on the concentration of monovalent ions. These sites are protease-sensitive sites, labelled in monovalent ion-poor media and protease-insensitive sites, labelled in media with higher concentrations of monovalent ions. Both types of binding sites bind dopamine agonists and dopamine antagonists with high affinity, but some differences were noted in the binding properties and the drug binding selectivity of the sites. It is argued that both sites form part of the same dopamine receptor macromolecular complex. The findings corroborate the hypothesis that dopamine receptors are composed of different sub-unit binding sites, but these are not distinct agonist and antagonist specific sites. The mechanism by which the protease-sensitive sites are rapidly inactivated by particular proteases, is probably a complexation between the enzymes and certain essential peptide moieties of the receptor sites involved.

The study of the dopamine receptors using *in vitro* binding techniques, has been approached in many different ways by numerous investigators. Various different hypotheses concerning the localization, the multiplicity and the regulation of the receptor sites have been put forward (for review see [1, 3]). The perennial problem is whether dopamine agonist and antagonist ligands label the same, partly the same or distinct receptor-linked binding sites. Comparison between studies by various investigators is hampered by the differences in binding assay conditions. In a previous study, we pointed out that alterations in the composition of the buffers used for the binding assays markedly influence the binding results and that effects are different when using catechol-like ^{3}H-agonists and ^{3}H-antagonists. It was shown that using ^{3}H-agonists, the stereospecific binding to dopaminergic receptors was optimally detectable in a Tris–HCl buffer containing EDTA. The EDTA was necessary to prevent massive complexation of the catechols to metal-ions incorporated in membranes, which gave rise to high non-specific binding. Using ^{3}H-antagonist, stereospecific binding was optimally detectable in Tris buffer containing physiological concentrations of electrolytes. In that buffer the lowest non-specific binding was obtained and the analysis of the stereospecific binding yielded linear Scatchard plots [4]. These optimal conditions for measuring ^{3}H-agonist and ^{3}H-antagonist binding were used in investigations of the equilibrium binding kinetics and in studies of the cellular and subcellular distribution of the binding sites [4–6]. It was found that the sites labelled by the dopamine agonists in the Tris buffer containing EDTA and the sites labelled by the antagonists in the Tris buffer with physiological salt concentrations occurred concomitantly in different subcellular fractions and on different neurones. This gave rise to the hypothesis of a unitary dopamine receptor which was probably composed of different sub-unit sites involved in dopamine agonist and antagonist binding.

In the study which we report now, we investigated whether the supposed sub-unit binding sites could be differentiated by enzymatic treatment of the membranes; and whether the different sub-unit sites could be reconciled with agonist and antagonist binding sites.

* Part of this work was presented at the Colloquium *Protides of the Biological Fluids*, in Brussels, 4–7 May 1981.

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Rat striatal membranes from various subcellular fractions were treated with proteolytic enzymes and other enzymes which are not directly related to membrane digestion. The effects of this on the binding of a potent dopamine agonist ([^{3}H]NPA)* and of a selective dopamine antagonist ([^{3}H]haloperidol) was investigated, and special emphasis was laid on the use of several assay conditions, in which both ligands were tested.

MATERIALS AND METHODS

Tissue preparation. Female Wistar rats (150 g) were decapitated, striata and other brain areas were rapidly dissected and freshly used. Subcellular fractions were obtained by differential centrifugation of tissue homogenates in 0.25 M sucrose (15 v/w) prepared with a Potter–Elvehjem homogenizer: the nuclear fraction was centrifuged at 745 g × 10 min and washed by resuspension in 0.25 M sucrose and centrifugation; from combined supernatants the mitochondrial fraction was centrifuged and washed at 30,000 g × 10 min; from the combined remaining supernatants the microsomal fraction was obtained by centrifugation at 251,800 g × 45 min. Subsequently, the membranes from the isolated subcellular fractions were lysed and extensively washed by suspension and centrifugation (40,000 g × 20 min), 2 × in Tris–EDTA buffer (see below) followed by 2 × in Tris buffer (see below). The average protein contents (estimated with the Lowry method [7]) of the final washed membrane preparations were 12 mg protein/g wet wt tissue for the microsomal fraction and 32 mg protein/g wet wt tissue for the mitochondrial fraction.

Trypsin pretreatment of membranes. Washed membranes were suspended in the indicated buffer (11.4 mg wet weight tissue/ml) at 0°. Trypsin was added to the ice-cold membrane suspension followed by either immediate centrifugation at 0° at 40,000 g × 10 min, or by incubation at 37°, and subsequent centrifugation. Thereafter membranes were routinely washed once by resuspension in Tris buffer and centrifugation. The membrane pellet was then divided and suspended in the various buffers for assaying the binding. In some experiments the proteolytic activity was stopped by adding soybean inhibitor followed by immediate assaying of the binding in the preincubation medium. In a third series of experiments the enzymes were directly added into the binding assay medium.

Controls were run systematically through a similar pretreatment procedure without trypsin. The proteolytic degradation of N^{α}-benzoyl-L-arginin-ethylester hydrochloride by trypsin in the Tris–EDTA buffer amounted to 0.066 μmoles/min · U at 25°. 1 U trypsin represented 4.1 μg protein.

Binding assay. Pretreated membrane pellets were suspended in Tris buffer of varying composition and assayed for total and non-specific ^{3}H-ligand binding. Incubation mixtures were composed of 2 ml membrane suspension (11.4 mg wet wt tissue/ml), 0.1 ml ^{3}H-ligand in 10% ethanol, 0.1 ml 10% ethanol (for total binding) or (+)-butaclamol at 1000-fold excess over the ^{3}H-ligand (for non-specific binding). Incubations were run for 30 min at 25° or 37°, stopped by rapid filtration through Whatman GF/B glass fibre filters and rinsed 3 × with 5 ml ice-cold Tris buffer. Radioactivity on the filters was counted in an Intertechnique Liquid scintillation counter equipped with a built-in computer system. Data were expressed in dpm using an external standard and calibrated against quench curves set up in experimental conditions. For both ^{3}H-ligands, stereospecific binding was taken as the difference between binding in the absence and the presence of 1000-fold excess (+)-butaclamol.

Materials. ^{3}H-Ligands (concns used in binding assays; sp. radioactivity): [^{3}H]NPA (0.5 nM; 58.5 Ci/mmole, New England Nuclear Boston, MA); [^{3}H]haloperidol (2 nM; 12 Ci/mmole, IRE, Fleurus, Belgium). (+)-Butaclamol was kindly donated by Ayerst Laboratories (Canada). Enzymes (mol.wt†, isoelectric point‡, source): Trypsin, EC 3.4.4.4 (24,000, > 9.5, bovine pancreas—Aldrich-Europe); α-Chymotrypsin, EC 3.4.4.5 (25,000, −, bovine pancreas—Aldrich-Europe); Chymotrypsinogen-A (22,500, −, bovine pancreas—Aldrich-Europe); Ficin, EC 3.4.22.3 (26,000, > 9.6, ficus carica—Boehringer-Germany); Papain, EC 3.4.4.10 (23,000, 8.75, carica papaya latex—Merck, Darmstadt, West Germany); Collagenase, EC 3.4.4.19 (109,000 8.6, Cl-histolyticum—Aldrich-Europe); Carboxypeptidase-A, EC 3.4.12.2 (33,000, 6.0, bovine pancreas—Boehringer-Germany) Pepsin, EC 3.4.4.1 (35,000, < 1.0, porcine stomach mucosa—Merck); Lactoperoxidase, EC 1.11.1.7 (80,000, 9.6, cow milk—Boehringer-Germany); Catalase, EC 1.11.1.6 (24,000, 5.8, bovine liver—Boehringer-Germany); Aldolase, EC 4.1.2.13 (158,000 6-1, rabbit muscle—Sigma Chemical Co., St. Louis, MO); Hyaluronidase, EC 3.2.1.35 (43,000, −, bovine testes—Aldrich-Europe).

Buffers: Tris–EDTA: Tris–HCl 15 mM, pH 7.5, 1 mM Na_2 EDTA, 0.01% ascorbic acid; 'Tris': Tris–HCl, 50 mM, pH 7.6; 'Tris–SALT': Tris–HCl 50 mM, pH 7.6, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% ascorbic acid, 1 μM pargyline.

RESULTS

Pretreatment of striatal membranes with trypsin and investigation of [^{3}H]NPA and [^{3}H]haloperidol binding in washed membranes

Membranes from the nuclear, mitochondrial and microsomal fractions of striatum were briefly pretreated with trypsin in Tris–EDTA buffer at 0°, washed and then tested for [^{3}H]NPA binding in Tris–EDTA buffer and for [^{3}H]haloperidol binding in Tris–SALT buffer. As outlined in the introduction, these conditions were proven to yield optimal binding for the ^{3}H-ligands respectively [4]. Results are shown in Table 1. Trypsin pretreatment caused a marked decrease of stereospecific binding of

* Abbreviations used: NPA = *N*,*n*-propyl-norapomorphine; APO = apomorphine.

† Literature and manufacturer data.

‡ Measured by thin layer isoelectrofocusing, showing for all enzyme preparations a single main band.

Table 1. ^{3}H-Ligand binding (fmoles/mg tissue) to membranes of subcellular fractions of rat striatum after brief trypsin pretreatment in Tris–EDTA buffer at 0°, centrifugation and washing of the membranes

Tissue fraction	Trypsin pretreatment (U/ml)	[^{3}H]NPA (0.5 nM) binding (Tris–EDTA, 25°) Total	Non-specific	[^{3}H]Haloperidol (2 nM) binding (Tris–SALT, 37°) Total	Non-specific
Nuclear	0	2.6	2.2	5.9	4.8
	0.03	5.2	5.6		
	0.3			8.8	6.4
Mitochondrial	0	9.8	2.9	14.2	6.9
	0.03	4.8	2.6		
	0.3			15.1	6.5
Microsomal	0	9.2	2.1	15.4	4.1
	0.03	3.6	1.5		
	0.3			14.1	5.0

[^{3}H]NPA to the membranes of the mitochondrial and the microsomal fraction. In membranes of the nuclear fraction, which do not contain stereospecific binding sites, the non-specific [^{3}H]NPA binding was doubled after trypsin pretreatment. In contrast, for [^{3}H]haloperidol, neither stereospecific nor non-specific binding, assayed in Tris–SALT buffer, was altered by trypsin pretreatment in any of the subcellular fractions.

To further explore the effects of trypsin pretreatment on dopaminergic receptors, the binding of both [^{3}H]NPA and [^{3}H]haloperidol was examined in various buffers, using striatal microsomal membranes which were pretreated in various conditions. The membranes were pretreated with 0.01 and 0.1 U/ml trypsin during 0, 30 and 60 min at 37° in Tris–EDTA buffer without NaCl, or in this buffer with 120 mM NaCl. After centrifugation and washing, membrane aliquots were assayed for [^{3}H]NPA and [^{3}H]haloperidol binding in Tris–EDTA, or Tris–EDTA + 120 mM NaCl or in Tris–SALT buffer. Results are shown in Table 2. The binding of both ^{3}H-ligands, assayed in Tris–EDTA buffer was inhibited in a trypsin concentration-dependent way. The highest inhibition in binding was observed after pretreatment in the absence of NaCl, but a significant inhibition was also noted after pretreatment in the presence of NaCl. The most prominent inhibition of binding occurred very rapidly during brief pretreatment at 0°; prolonged pretreatment at 37° caused only a slight further trypsin-dependent decrease in binding. In contrast, when the binding of both ^{3}H-ligands was assayed in Tris–SALT buffer or in a Tris–EDTA buffer containing 120 mM NaCl, no rapidly occurring inhibition of binding by trypsin was obtained. Hence, the most prominent trypsin effect occurred very rapidly at 0°, and affected the stereospecific binding of both [^{3}H]NPA and [^{3}H]haloperidol only when measured in the absence of high concentrations of monovalent ions. The presence of NaCl only during the pretreatment, slightly hampered but did not prevent the rapid inactivation by trypsin of the stereospecific binding measured subsequently in a monovalent ion-poor medium.

Data in Table 3 demonstrate that the rapidly occurring trypsin inhibition of [^{3}H]NPA binding observed in Tris–EDTA buffer could not be reversed by repeated extensive washing of the membranes with different buffer.

Pretreatment of striatal microsomal membranes with trypsin and reaction blockade by soybean inhibitor; effects of trypsin concentration and time of pretreatment on binding

The foregoing observations suggested that the immediate effects of trypsin on the binding in the absence of monovalent ions were distinct from inactivation of binding due to proteolysis of the membranes. However, the whole centrifugation procedure for separating the membranes from the solubilized trypsin lasts about 15 min and it is possible that proteolytic activity is not completely blocked at low temperature. In the following experiments, proteolytic activity was stopped by addition of soybean inhibitor, after preincubation of the microsomal membranes with trypsin in Tris–EDTA buffer for various time periods. Subsequently, the binding was assayed by adding the ^{3}H-ligand directly into the preincubation mixture. It was experimentally verified that mixing an equal amount of soybean inhibitor protein and trypsin protein was largely sufficient to block all proteolytic activity. Findings on [^{3}H]NPA binding are presented in Fig. 1. A marked, immediate, and trypsin concentration-dependent inhibition of stereospecific binding in Tris–EDTA was observed; 50% inhibition was reached at about 0.01 U/ml trypsin, corresponding to 0.041 μg protein/ml or 1.7 nM trypsin. Also in these experiments it appeared that prolonged preincubation of the membranes in the presence of trypsin at 37° caused only a slow, further decrease in binding.

When trypsin was mixed with soybean inhibitor prior to its addition to the microsomal membranes, the inactivation of [^{3}H]NPA or [^{3}H]haloperidol binding in Tris–EDTA buffer gradually diminished with an increasing amount of soybean inhibitor per amount of trypsin protein. In Fig. 2 it is shown that inactivation of binding no longer occurred when the soybean inhibitor and trypsin were previously mixed in a 1:1 ratio of protein content.

Influence of the ionic composition of the binding assay medium on the direct inhibition of binding by trypsin

Trypsin was directly added into the incubation medium for the binding assay, for investigation of its influence on [^{3}H]NPA binding in assay media with and without EDTA, monovalent ions (NaCl, KCl)

Table 2. (a) [^{3}H]NPA (0.5 nM) and (b) [^{3}H]haloperidol (2 nM) stereospecific binding to rat striatal membranes in various assay conditions subsequent to trypsin pretreatment of the membranes at 37° for various time periods in two different buffers*

Binding assay conditions:	Stereospecific binding in fmoles/mg tissue for controls (no trypsin) (mean ± S.E.M., $n = 2$) and in % of matched controls for trypsin pretreated membranes							
	Tris–EDTA (25°)			Tris–EDTA + 120 mM NaCl (25°)	(37°)		Tris–SALT (37°)	
Pretreatment time:	0 min	30 min	60 min	0 min	0 min	0 min	30 min	60 min
Pretreatment buffer								
(a) Tris–EDTA								
No trypsin	6.6 ± 0.9	4.9 ± 0.7	4.1 ± 0.8	4.1 ± 0.7	3.7 ± 0.2	3.9 ± 0.2	2.6 ± 0.6	2.6 ± 0.1
0.01 U/ml	41%	31%	22%	105%	116%	103%	77%	62%
0.1 U/ml	18%	14%	12%	85%	70%	92%	62%	44%
Tris–EDTA + 120 mM NaCl								
No trypsin	6.0 ± 1.0	6.0 ± 0.6	4.5 ± 0.8			6.3 ± 0.5	4.9 ± 0.4	4.8 ± 0.8
0.01 U/ml	83%	65%	78%			99%	110%	100%
0.1 U/ml	33%	40%	44%			94%	102%	79%
(b) Tris–EDTA								
No trypsin	21.1 ± 0.8	18.5 ± 0.1	17.8 ± 1.6	10.8 ± 1.2	10.1 ± 1.0	9.5 ± 1.4	7.9 ± 0.6	7.8 ± 0.4
0.01 U/ml	64%	37%	38%	111%	104%	104%	94%	67%
0.1 U/ml	37%	25%	16%	103%	102%	105%	80%	54%
Tris–EDTA + 120 mM NaCl								
No trypsin	17.9 ± 0.2	17.6 ± 0.4	16.7 ± 0.2			9.5 ± 1.4	9.2 ± 1.2	8.3 ± 0.7
0.01 U/ml	87%	72%	72%			106%	104%	105%
0.1 U/ml	50%	43%	48%			113%	99%	106%

* The pretreatment was stopped by centrifugation and one washing of the membranes.

Table 3. [^{3}H]NPA stereospecific binding (fmoles/mg tissue) in Tris–EDTA to the rat striatal microsomal membranes briefly pretreated with 0.1 U/ml trypsin in Tris–EDTA buffer followed by different washings*

Buffer for membrane washing	One washing		Two washings		Three washings	
	Control	Trypsin	Control	Trypsin	Control	Trypsin
Tris–EDTA	7.2 ± 0.5	1.4 ± 0.2	5.9 ± 0.2	1.1 ± 0.1	5.5 ± 0.3	0.9 ± 0.1
Tris–EDTA + 120 mM NaCl	6.2 ± 0.3	2.3 ± 0.1	5.5 ± 0.1	2.0 ± 0.2	5.6 ± 0.2	2.0 ± 0.1
Tris–EDTA + 120 mM KCl	5.2	1.6	5.4	2.3	4.7	2.3

* One washing consisted of suspending the membrane pellet in 50 vol. buffer per g wet wt of tissue, incubation for 5 min at 37° and centrifugation for 15 min at 40,000 *g* at 0°.

Table 4. Direct inhibition by trypsin of [^{3}H]NPA (0.5 nM) stereospecific binding to the rat striatal microsomal membranes in various assay conditions

Trypsin (U/ml)	Tris–EDTA (25%)			Tris (37°)			
	No additive	KCl 120 mM	NaCl 120 mM	No additive	KCl 120 mM	NaCl 120 mM	SALT
fmoles/mg tissue							
None	8.6	7.4	4.1	5.4	6.7	3.2	5.0
% Stereospecific binding							
0.001	80	85	98	90	101	122	100
0.003	53	103	102	69	99	131	92
0.01	38	100	115	35	106	134	98
0.1	22	100	115	22	96	116	112

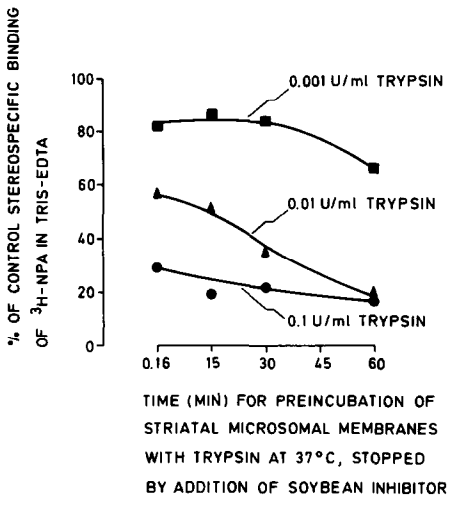

Fig. 1. Striatal microsomal membranes suspended in Tris–EDTA buffer were preincubated with trypsin at 37°. The preincubation was stopped by adding soybean inhibitor (amount of soybean inhibitor protein equal to the amount of trypsin protein) immediately followed by addition of [^{3}H]NPA (0.5 nM). The samples were further incubated for 30 min at 25° and assayed for binding as described in Materials and Methods. Control stereospecific binding of [^{3}H]NPA amounted to 7.5 fmoles/mg tissue without preincubation and to 6.6 fmoles/mg tissue after 60 min preincubation. Points shown are mean values of 2 independently performed experiments with duplicate assays, standard deviations of means did not exceed 5% of the mean values.

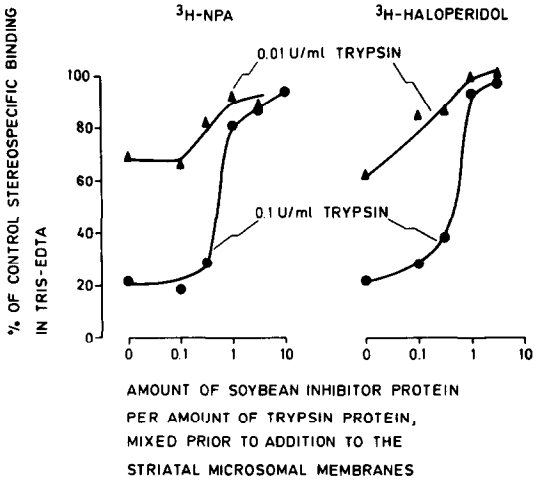

Fig. 2. [^{3}H]NPA (0.5 nM) and [^{3}H]haloperidol (2 nM) binding to striatal microsomal membranes in Tris–EDTA buffer (30 min incubation at 25°) in the presence of trypsin and soybean inhibitor–trypsin complex. The soybean inhibitor–trypsin complex was formed prior to its addition to the binding assay medium, by incubating the mixture of soybean inhibitor and trypsin protein in Tris–EDTA buffer for 10 min at 25°. Control stereospecific binding amounted to 5.2 fmoles/mg tissue for [^{3}H]NPA and to 29.3 fmoles/mg tissue for [^{3}H]haloperidol. Points shown are mean values of 2 independent experiments with duplicate assays, standard deviations of means did not exceed 5% of the mean values. Similar observations were made with aprotinin, another peptide-like protease inhibitor.

Table 5. Influence of the NaCl concentration on stereospecific binding of [^{3}H]NPA to striatal microsomal membranes in Tris–EDTA buffer at 25° and on the direct inhibition of binding by trypsin

NaCl concn (mM)	Stereospecific binding (fmoles/mg tissue)	% Stereospecific binding with 0.01 U/ml trypsin
1.2	6.4	36
4	6.5	37
12	5.7	58
40	4.4	98
120	4.0	90

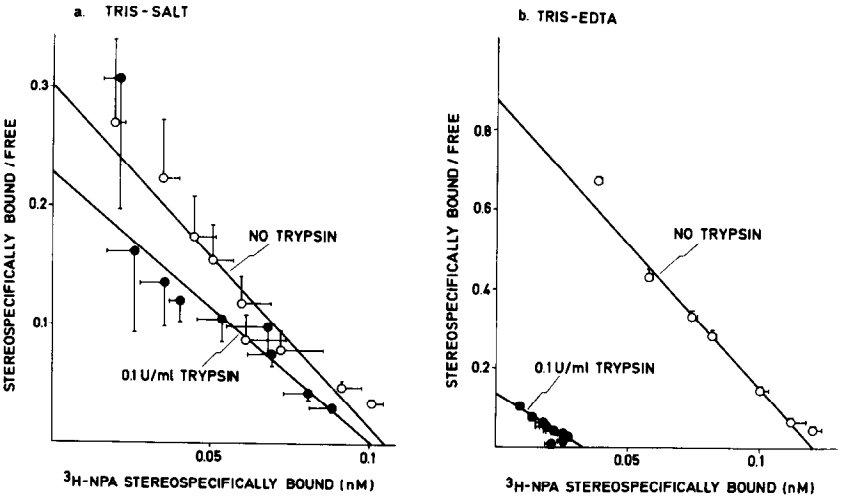

Fig. 3. Scatchard plots of stereospecific [^{3}H]NPA binding to membranes of the rat striatal microsomal fraction assayed in (a) Tris–SALT buffer at 37° and (b) Tris–EDTA buffer at 25° in the absence of trypsin (○) and the presence of 0.1 U/ml trypsin (●). The tissue concn was 10.33 mg original wet wt/ml with a protein content of 11.8 ± 1.6 mg protein/g tissue. Stereospecific binding was taken as the difference between total binding and non-specific binding measured in the presence of 1000-fold excess (+)-butaclamol. The free ligand concn was calculated as the difference in concentration of added ligand and totally bound. Binding parameters were derived from regression lines through linear Scatchard plots of individual experiments and averaged. (a) in Tris–SALT: K_D = 0.37 ± 0.15 nM (no trypsin) and 0.66 ± 0.47 nM (0.1 U/ml trypsin). B_{max} = 10.2 ± 0.6 fmoles/mg tissue (no trypsin) and 11.0 ± 1.5 fmoles/mg tissue (0.1 U/ml trypsin). (b) in Tris–EDTA: K_D = 0.14 ± 0.01 nM (no trypsin) and 0.25 ± 0.04 nM (0.1 U/ml trypsin) and B_{max} = 11.7 ± 0.4 fmoles/mg tissue (no trypsin) and 3.1 ± 0.3 fmoles/mg tissue (0.1 U/ml trypsin). The presented Scatchard plots were computed from the following original mean data ± S.E.M.

	[^{3}H]NPA concn (pM)	No trypsin		0.1 U/ml Trypsin	
		Binding [fmoles/ml (mean ± S.E.M.)] Total	Non-specific	Binding [fmoles/ml (mean ± S.E.M.)] Total	Non-specific
(a)	100 ± 4	25 ± 3	5.3 ± 0.1	27 ± 5	5.6 ± 0.1
	205 ± 7	45 ± 5	9.9 ± 0.2	36 ± 9	10 ± 1
	319 ± 4	59 ± 7	14.7 ± 0.1	50 ± 10	17 ± 2
	400 ± 20	69 ± 7	18.9 ± 0.9	62 ± 4	22 ± 1
	600 ± 10	90 ± 10	28.4 ± 0.3	80 ± 10	31 ± 2
	810 ± 20	100 ± 10	39 ± 1	110 ± 10	43 ± 0.2
	1040 ± 10	120 ± 10	48 ± 3	122 ± 6	53 ± 2
	2110 ± 70	188 ± 5	97 ± 1	180 ± 10	101 ± 3
	3200 ± 200	244 ± 7	143 ± 4	248 ± 6	160 ± 1
(b)	100 ± 4	42 ± 1	3.4 ± 0.1	11 ± 1	2.0 ± 0.1
	205 ± 7	64 ± 1	6.1 ± 0.1	18 ± 1	3.7 ± 0.3
	319 ± 4	83 ± 2	8.5 ± 0.1	23 ± 3	5.4 ± 0.1
	400 ± 20	93 ± 1	11.8 ± 0.5	27 ± 3	8.3 ± 0.6
	600 ± 10			33 ± 2	10.4 ± 0.6
	810 ± 20	121 ± 3	20.7 ± 0.2	40 ± 1	14 ± 1
	1040 ± 10			46 ± 1	18.6 ± 0.1
	2110 ± 70	160 ± 1	48 ± 5	62 ± 2	36.1 ± 0.4
	3200 ± 200	195 ± 5	75 ± 8	77 ± 1	56.1 ± 0.9

All values are means ± S.E.M. of two independently performed experiments in duplicate.

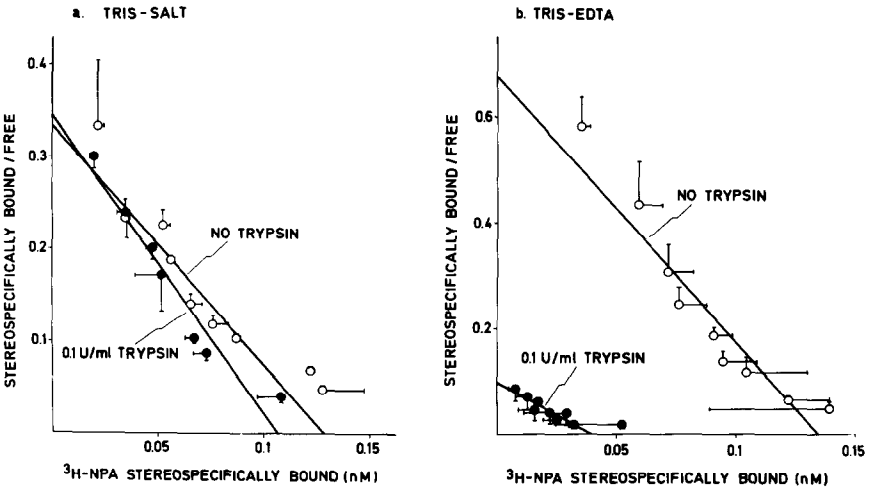

Fig. 4. Scatchard plots of stereospecific [^{3}H]NPA binding to membranes of the rat striatal mitochondrial fraction. The tissue concentration was 10.33 mg original wet wt/ml with a protein content of 34.6 ± 0.1 mg protein/g tissue. Experimental design, symbols and calculations are as in legend to Fig. 1. Binding parameters were derived from linear Scatchard plots. (a) in Tris–SALT: K_D = 0.46 ± 0.16 nM (no trypsin) and 0.29 ± 0.02 nM (0.1 U/ml trypsin) and B_{max} = 14 ± 3 fmoles/mg tissue (no trypsin) and 10 ± 1 fmoles/mg tissue (0.1 U/ml trypsin). (b) in Tris–EDTA; K_D = 0.20 ± 0.07 nM (no trypsin) and 0.33 ± 0.01 nM (0.1 U/ml trypsin) and B_{max} = 13 ± 5 fmoles/mg tissue (no trypsin) and 3.5 ± 1.5 fmoles/mg tissue (0.1 U/ml trypsin). The presented Scatchard plots were computed from the following original mean data ± S.E.M.

[^{3}H]NPA concn (pM)	No trypsin		0.1 U/ml Trypsin	
	Binding [fmoles/ml (mean ± S.E.M.)]		Binding [fmoles/ml (mean ± S.E.M.)]	
	Total	Non-specific	Total	Non-specific
100 ± 4	33 ± 2	11.6 ± 0.5	32 ± 1	11.7 ± 0.5
205 ± 7	58 ± 1	23 ± 2	58 ± 6	23 ± 1
319 ± 4	86 ± 6	34 ± 3	83 ± 4	35 ± 1
400 ± 20	101 ± 5	45 ± 3	103 ± 9	51 ± 4
600 ± 10	130 ± 10	66 ± 4		
810 ± 20	160 ± 10	85 ± 5	155 ± 5	87 ± 1
1040 ± 10	192 ± 8	105 ± 9	190 ± 10	116 ± 8
2110 ± 70	310 ± 20	190 ± 20		
3200 ± 200	440 ± 40	310 ± 20	420 ± 5	310 ± 20
100 ± 4	40 ± 4	5.7 ± 0.5	13 ± 2	6.0 ± 0.1
205 ± 7	70 ± 10	10.1 ± 0.9	24 ± 5	11.6 ± 0.1
319 ± 4	90 ± 10	14.4 ± 0.1	34 ± 3	17 ± 2
400 ± 20	90 ± 10	18.7 ± 0.5	43 ± 8	27 ± 1
600 ± 10	116 ± 9	26 ± 1	60 ± 10	37 ± 1
810 ± 20	130 ± 20	35 ± 2	80 ± 10	50 ± 2
1040 ± 10	150 ± 30	45 ± 1	91 ± 5	66 ± 1
2110 ± 70	200 ± 20	75 ± 2	159 ± 9	130 ± 10
3200 ± 200	270 ± 50	131 ± 3	230 ± 30	175 ± 1

All values are means ± S.E.M. of two independently performed experiments in duplicate.

and SALT. Data in Table 4 show that a concentration-dependent trypsin inhibition of [^{3}H]NPA binding to striatal microsomal membranes occurred in Tris buffer with and without EDTA, measured at 25° and 37°, provided that no other salts were added. In the presence of 120 mM NaCl or KCl or also a physiological SALT mixture no inhibition of binding by trypsin was observed.

From the data in Table 5 it appears that when the NaCl concentration in the binding assay medium was gradually increased, the stereospecific binding measured at 0.5 nM [^{3}H]NPA decreased and at the same time the inhibition caused by trypsin was diminished. Decrease in stereospecific binding and a concomitantly smaller inhibition by trypsin started at 12 mM NaCl; the trypsin inhibition had completely disappeared at 40 mM NaCl.

Influence of trypsin on K_D- *and* B_{max}-*values of* [^{3}H]*NPA and* [^{3}H]*haloperidol binding in Tris–EDTA and Tris–SALT buffer*

Using striatal microsomal and mitochondrial membranes, ligand concentration binding isotherms were measured in Tris–EDTA (25°) and Tris–SALT (37°) buffer in the absence and the presence of tryp-

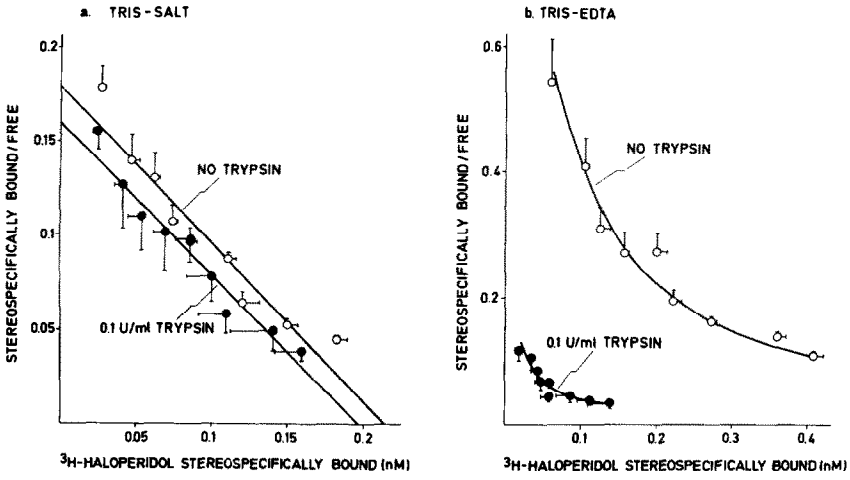

Fig. 5. Scatchard plots of [^{3}H]haloperidol binding to membranes of the rat striatal microsomal fraction. The tissue concentration was 10.33 mg original wet weight/ml with a protein content of 12.4 ± 1 mg protein/g tissue. Experimental design, symbols and calculations were as in legend to Fig. 1. Binding parameters derived from linear Scatchard plots are: in Tris–SALT: K_D = 1.25 ± 0.16 nM (no trypsin) and 1.30 ± 0.05 nM (0.1 U/ml trypsin) and B_{max} = 21 ± 1 fmoles/mg tissue (no trypsin) and 20 ± 3 fmoles/mg tissue (0.1 U/ml trypsin). The presented Scatchard plots were computed from the following original mean data ± S.E.M.

[^{3}H]Haloperidol concn (pM)	No trypsin		0.1 U/ml Trypsin	
	Binding [fmoles/ml (mean ± S.E.M.)] Total	Non-specific	Binding [fmoles/ml (mean ± S.E.M.)] Total	Non-specific
(a) 189 ± 5	36 ± 1	9 ± 2	32 ± 2	8 ± 1
400 ± 10	61 ± 4	14 ± 2	56 ± 5	14 ± 1
570 ± 30	79 ± 6	16 ± 4	73 ± 9	19 ± 2
800 ± 20	98 ± 6	23 ± 4	90 ± 10	25 ± 3
1004 ± 2	116 ± 3	30 ± 6	120 ± 20	30 ± 5
1440 ± 20	160 ± 10	50 ± 10	150 ± 20	47 ± 8
2060 ± 20	180 ± 10	60 ± 10	170 ± 30	62 ± 9
3080 ± 30	230 ± 10	80 ± 20	210 ± 40	70 ± 10
4400 ± 100	280 ± 20	100 ± 10	260 ± 30	100 ± 10
(b) 189 ± 5	77 ± 7	16 ± 2	27 ± 3	9 ± 1
400 ± 10	136 ± 8	31 ± 5	53 ± 7	17 ± 2
570 ± 30	170 ± 20	41 ± 8	60 ± 10	21 ± 4
800 ± 20	210 ± 20	50 ± 10	90 ± 10	31 ± 4
1004 ± 2	270 ± 20	67 ± 9	100 ± 20	42 ± 9
1440 ± 20	320 ± 20	90 ± 10	120 ± 20	70 ± 10
2060 ± 20	390 ± 30	120 ± 20	170 ± 30	80 ± 10
3080 ± 30	520 ± 30	160 ± 20	220 ± 40	110 ± 30
4400 ± 100	620 ± 50	210 ± 50	280 ± 60	140 ± 30

All values are means ± S.E.M. of three independently performed experiments in duplicate.

sin, (0.1 U/ml directly added into the binding assay medium). Scatchard plots of mean stereospecific binding are shown in Figs. 3–6, original data on ligand concentration, total binding and non-specific binding in each condition are presented in the legends to the figures. Scatchard plots of [^{3}H]NPA binding (Figs. 3 and 4) were virtually linear in a concentration range of 0.1 to 2 or 3 nM in all conditions. In the Tris–SALT buffer, the Scatchard plots of binding in the absence and the presence of trypsin completely overlapped, using membranes of both microsomal and mitochondrial fractions. In the Tris–EDTA buffer the stereospecific binding measured in the presence of 0.1 U/ml trypsin was likewise reduced at all [^{3}H]NPA concentrations, by 77 ± 1% in microsomal membranes and by 74 ± 2% in membranes from the mitochondrial fraction (calculated from the data in legends to Figs. 3 (b) and 4 (b). As a result, Scatchard plots in the presence of trypsin were markedly shifted towards the origin. The K_D-values were 2- to 3-fold higher in the Tris–SALT buffer (K_D's about 0.4 nM), than in the Tris–EDTA buffer (K_D's about 0.2 nM). K_D-Values were virtually similar in the corresponding buffers using membranes of microsomal and mitochondrial fractions. B_{max}-Values amounted to 10–12 fmoles/mg tissue in the microsomal and 10–14 fmoles/mg tissue in the mitochondrial fraction for assays in the Tris–SALT buffer with and without trypsin and in the Tris–EDTA buffer without trypsin. In the Tris—EDTA buffer in the presence of trypsin the B_{max}-values were reduced by 73% in both subcellular fractions. It is

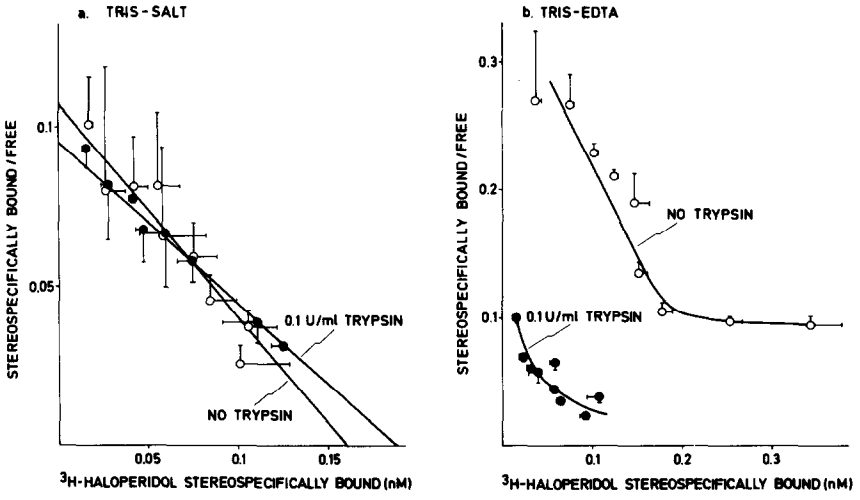

Fig. 6. Scatchard plots of [^{3}H]haloperidol binding to membranes of the rat striatal mitochondrial fraction. The tissue concn was 10.33 mg original wet wt/ml with a protein content of 29 ± 6 mg protein/g tissue. Experimental design, symbols and calculations were as in legend to Fig. 1. Binding parameters derived from linear Scatchard plots are: in Tris–SALT: K_D = 1.23 ± 0.03 nM (no trypsin) and 1.95 ± 0.06 nM (0.1 U/ml trypsin) and B_{max} = 14 ± 4 fmoles/mg tissue (no trypsin) and 18 ± 3 fmoles/mg tissue (0.1 U/ml trypsin). The presented Scatchard plots were computed from the following original mean data ± S.E.M.

[^{3}H]Haloperidol concn (pM)	No trypsin		0.1 U/ml Trypsin	
	Binding [fmoles/ml (mean ± S.E.M.)] Total	Non-specific	Binding [fmoles/ml (mean ± S.E.M.)] Total	Non-specific
(a) 194 ± 3	28 ± 3	12 ± 1	25 ± 2	9 ± 1
400 ± 20	50 ± 10	20 ± 1	46 ± 3	18 ± 1
598 ± 4	72 ± 9	30 ± 1	62 ± 1	21 ± 1
790 ± 40	90 ± 10	37 ± 1	79 ± 7	31 ± 3
1006 ± 2	110 ± 10	60 ± 10	100 ± 20	41 ± 3
1420 ± 10	140 ± 20	69 ± 6	130 ± 20	60 ± 10
2050 ± 20	180 ± 20	94 ± 7		
3070 ± 50	220 ± 20	118 ± 1	200 ± 20	93 ± 1
4200 ± 100	280 ± 20	174 ± 4	250 ± 10	125 ± 5
(b) 194 ± 3	59 ± 5	23 ± 1	29 ± 2	12 ± 1
400 ± 20	113 ± 1	38 ± 1	48 ± 1	23 ± 1
598 ± 4	153 ± 4	52 ± 1	68 ± 6	35 ± 6
790 ± 40	194 ± 2	69 ± 2	90 ± 10	49 ± 6
1006 ± 2	230 ± 10	86 ± 1	112 ± 5	50 ± 10
1420 ± 10	280 ± 20	130 ± 10	135 ± 7	78 ± 7
2050 ± 20	350 ± 20	174 ± 8	184 ± 6	119 ± 7
3070 ± 50	470 ± 10	218 ± 2	240 ± 30	140 ± 10
4200 ± 100	640 ± 20	300 ± 20	300 ± 20	210 ± 10

All values are means ± S.E.M. of two independently performed experiments in duplicate.

to be noted that, in the microsomal and the mitochondrial fraction the B_{max}-values appeared to be the same when expressed per mg wet weight of tissue, but calculated per mg protein, the binding site density was 2.3 times higher in the microsomal fraction than in the mitochondrial fraction (see Materials and Methods). The Scatchard plots of stereospecific [^{3}H]haloperidol binding (Figs. 5 and 6) are linear for binding measured in the Tris–SALT buffer but obviously curvilinear for binding measured in the Tris–EDTA buffer. Similar observations were made using membranes of both subcellular fractions. Again, Scatchard plots in the Tris–SALT buffer were completely superimposable for binding in the absence and the presence of trypsin. Binding parameters derived from the linear Scatchard plots are indicated in the legend to Figs. 5 (a) and 6 (a). In the Tris–SALT buffer, using membranes from the microsomal fraction the B_{max}-value of [^{3}H]-haloperidol binding is two times greater than the B_{max}-value of [^{3}H]NPA binding, whereas in membranes from the mitochondrial fraction there is no significant difference in the number of sites labelled by both ligands. In the Tris–EDTA buffer containing

Table 6. Inhibitory potency of enzymes (IC_{50}, nM) for direct inhibition of stereospecific ^{3}H-ligand binding to the rat striatal microsomal membranes

Enzymes	[^{3}H]NPA (0.5 nM) binding in: Tris–EDTA	Tris–SALT	[^{3}H]Haloperidol (2 nM) binding in: Tris–EDTA	Tris–SALT
Trypsin	1.3	>1000	3.0	>1000
Ficin	3.0	>1000	2.8	>1000
Papain	16	600	20	>1000
α-Chymotrypsin	20	>1000	32	>1000
Chymotrypsinogen-A	2000	>10000	3000	>10000
Pepsin	>1000	>10000	>1000	>1000
Collagenase	>1000	>1000	>1000	>1000
Carboxypeptidase-A	>1000	>1000	>1000	>1000
Lactoperoxidase	200	140	>500	>500
Catalase	>1000	>1000	>1000	>1000
Aldolase	>500	>500	>500	>500
Hyaluronidase	6000	17000	17000	>10000

trypsin, the curvilinear Scatchard plot was markedly shifted towards the origin. From the original data in the legends to Figs. 5 (b) and 6 (b) the calculated reduction in stereospecific binding due to the presence of trypsin is similar over the entire ligand concentration range: 69 ± 1% and 64 ± 2% for binding to membranes from microsomal and mitochondrial fractions respectively. Binding parameters have not been derived from the curvilinear plots for reasons discussed below. It is worth noting that [^{3}H]haloperidol binding (total, non-specific and stereospecific) in the absence of trypsin is considerably higher in the Tris–EDTA than in the Tris–SALT buffer.

Effects of various proteolytic and non-proteolytic enzymes on [^{3}H]NPA and [^{3}H]haloperidol binding

Besides trypsin, various proteolytic and other enzymes were investigated for inhibition of [^{3}H]NPA and [^{3}H]haloperidol binding, upon direct addition in the binding assay medium. The enzymes were checked for purity by thin layer isoelectrofocussing. They all showed a single main band and the isoelectric point corresponded to literature and manufacturer data (see Materials and Methods). Molar concentrations of the enzymes were calculated from weighed out dry protein or from protein determinations with the Lowry method [7]. The nanomolar IC_{50}-values, derived from enzyme inhibition curves for [^{3}H]NPA and [^{3}H]haloperidol binding in Tris–EDTA and Tris–SALT buffer are shown in Table 6. Only the serine-proteases trypsin and α-chymotrypsin and the sulfhydryl-proteases ficin and papain inhibited at nanomolar concentrations the binding of both ^{3}H-ligands in Tris–EDTA buffer. They were ineffective on binding in Tris–SALT in agreement with the findings for trypsin. For these enzymes the inhibition curves in the various conditions are shown in Fig. 7 (a) and (b). The inhibitory potency and the paths of the inhibition curves for the [^{3}H]NPA and the [^{3}H]haloperidol binding were virtually similar for each enzyme. As shown in Table 6, the pro-protease, chymotrypsinogen-A, and other proteases such as pepsin, collagenase and carboxypeptidase-A did not cause any inhibition of binding at concentrations up to 1 μM. Amongst some non-proteolytic enzymes, only the very basic lactoperoxidase slightly inhibited [^{3}H]NPA binding in both the Tris–EDTA and the Tris–SALT buffer.

DISCUSSION

The main finding in this study is that trypsin and certain related proteolytic enzymes potently and rapidly inactivate the stereospecific binding sites on rat striatal membranes which are labelled by [^{3}H]NPA and [^{3}H]haloperidol in binding assay media which do not contain high concentrations of monovalent ions. In contrast, no rapidly occurring effect of the enzymes on the binding of either ligand is observed when the binding is assayed in the presence of monovalent ions (≥40 mM NaCl, 120 mM KCl, SALT). The rapid inactivation by the proteases of the binding observed in monovalent ion-poor media appears to be different from a much slower proteolytic effect. The inhibition of binding in the monovalent ion-poor buffer was observed after trypsin pretreatment irrespective of whether ions were present during pretreatment. Binding in that medium was not restored when the membranes were extensively washed with buffers containing 120 mM NaCl or KCl following the trypsin pretreatment. The findings suggest that two different types of stereospecific dopaminergic binding sites exist: one which is inhibited by certain proteolytic enzymes and which binds dopamine agonists and antagonists in a monovalent ion-poor medium and a second one which is not inhibited by the enzymes and which is occupied by dopamine agonists and antagonists in the presence of higher concentrations of monovalent ions. The two different types of binding sites, which are further denoted as protease-sensitive and protease-insensitive sites, seem to become differentially exposed for binding depending on the concentration of monovalent ions. Both sites display the important common features of being stereospecific and of binding both with high affinity, a dopamine agonist (NPA) and an antagonist (haloperidol). Hence, our previous hypothesis is only in part corroborated: different sub-unit sites are demonstrated, but these sites do not differentiate between agonists and antagonists. The present findings are not in accordance with the hypothesis that sodium ions would mediate agonist and antagonist states of the receptor, which has been proposed for the opiate receptor [9].

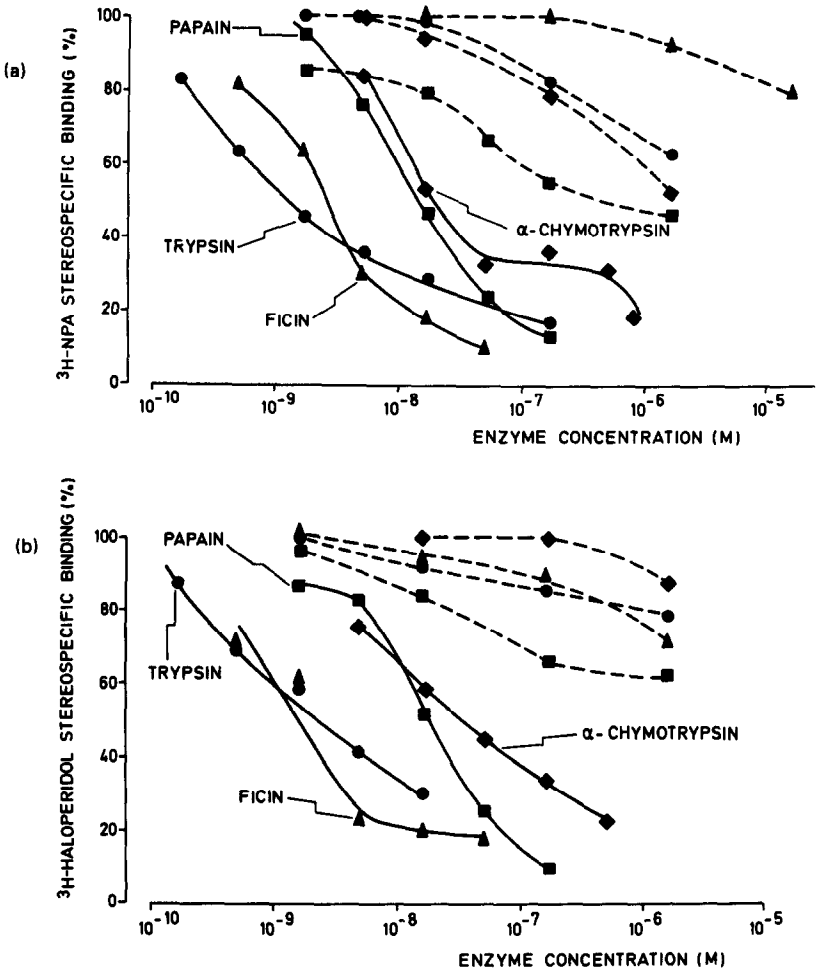

Fig. 7. Direct inhibition by proteolytic enzymes of (a) stereospecific [^{3}H]NPA (0.5 nM) binding, (b) stereospecific [^{3}H]haloperidol (2 nM) binding to washed membranes of the rat striatal microsomal fraction, assayed in Tris–EDTA buffer at 25° (full lines) and in a Tris–SALT buffer at 37° (dotted lines).

Binding characteristics of protease-sensitive and protease-insensitive sites

Some information can be derived from the analysis of Scatchard plots of stereospecific [^{3}H]NPA and [^{3}H]haloperidol binding in Tris–SALT buffer involving the protease-insensitive sites and in Tris–EDTA buffer involving the protease-sensitive sites. Regarding the binding to the protease-insensitive sites, it is clear that neither the K_D, nor B_{max}-values of [^{3}H]NPA or [^{3}H]haloperidol are affected by trypsin. NPA shows a 3-fold higher binding affinity for these sites than haloperidol. Findings were similar using membranes of the microsomal and the mitochondrial fraction, except for the peculiar observation that haloperidol labels twice as many protease-insensitive sites than NPA in the microsomal fraction, and an equal amount in the mitochondrial fraction. This is as yet difficult to explain. Experiments in Tris–EDTA buffer, show that NPA has a two times higher binding affinity for the protease-sensitive than for the protease-insensitive sites. The maximal number of the two types of sites labelled with [^{3}H]NPA appeared to be the same. The observation described in Table 5, of a decrease in stereospecific binding at 0.5 M [^{3}H]NPA when the NaCl concentration is increased, is caused by the lower binding affinity of [^{3}H]NPA for the protease-insensitive sites which become exposed at higher NaCl concentrations. The Scatchard plots of [^{3}H]haloperidol in Tris–EDTA are markedly curvilinear. The parallel shift of the line towards the origin when the binding is measured in the presence of trypsin and the same reduction in binding (~65%) at all [^{3}H]haloperidol concentrations, suggest again that trypsin reduces the number of binding sites and probably does not affect the binding affinity. For both [^{3}H]NPA and [^{3}H]haloperidol binding in Tris–EDTA buffer, findings were again similar using membranes from the microsomal or the mitochondrial fraction. It is beyond the scope of this study to extensively discuss the meaning of the curvilinear Scatchard plots. A frequently cited interpretation of such curved plots is the existence of high and low affinity sites. Sometimes, the possibility of the occurrence of cooperative

effects of binding is also proposed. We do not adhere to either hypothesis. In previous studies using [^{3}H]spiperone to label dopaminergic receptors we made the same observation of linear Scatchard plots for binding in Tris–SALT buffer and curvilinear plots in Tris–EDTA buffer [4]. When reporting that study, we pointed out the importance and implications of surface phenomena, which are inherent to interactions between solubilized ligands and membrane micelles in suspension. Therefore, we think it is inappropriate to draw speculative conclusions from the observation of curved Scatchard plots.

Studies are now in progress to investigate the drug binding selectivity of the protease-sensitive and protease-insensitive sites. Preliminary observations indicate that certain compounds, such as sulpiride, fluphenazine, spiperone and bromocryptin have 10–50 times lower IC_{50}-values for inhibiting [^{3}H]haloperidol binding in Tris–SALT buffer than in Tris–EDTA buffer. A number of other compounds, such as chlorpromazine, levopromazine, pipamperone, dopamine and apomorphine show approximately equal IC_{50}-values for assays in both buffers. The variations in IC_{50}-values of the above compounds are less pronounced when [^{3}H]NPA is used as ligand. The higher binding affinity of sulpiride in the Tris–SALT buffer is in agreement with the finding that Na^+-ions are required in order to obtain appreciable labelling of dopamine receptors using [^{3}H]sulpiride [10]. Hence certain compounds, including agonists and antagonists of different chemical series, seem to differentiate between the two types of binding sites. Further work is required to examine the pharmacological relevance of the protease-sensitive and protease-insensitive sites and how apparent differences in binding affinities of compounds for the sites are related to pharmacological effects.

Are protease-sensitive and protease-insensitive sites sub-units of a unitary dopamine receptor?

From the present study, it appears that the stereospecific binding of both NPA and haloperidol shows the same properties in membranes from microsomal and mitochondrial fractions and that the binding in both subcellular fractions is affected in the same manner by trypsin. From previous studies it appeared that in rat striatal tissue, stereospecific sites labelled with [^{3}H]APO in a monovalent ion-poor medium (involving protease-sensitive sites) and stereospecific sites labelled with [^{3}H]spiperone in the presence of monovalent ions (involving protease-insensitive sites), have the same subcellular distribution and are similarly affected by various types of neuronal lesions [5, 6]. Hence, both protease-sensitive and protease-insensitive sites occur concomitantly on different types of membranes and neurones. Therefore it is reasonable to hypothesize that they form part of a unitary macromolecular complex which constitutes the dopamine receptor.

Mechanism of the rapid inactivation of the protease-sensitive sites

The rapidly occurring inhibition of binding by the enzymes at 0° is clearly different from the slow proteolytic process observed at 37°. Amongst twelve different enzymes only the mammalian serine-proteases (trypsin and α-chymotrypsin) and the plant sulfhydryl-proteases (ficin and papain) were found to strongly inhibit the binding in an enzyme concentration-dependent way. Trypsin loses its inhibitory effect when it is pretreated with peptide-like protease inhibitors. Also pro-proteases (chymotrypsinogen-A), other proteolytic enzymes (pepsin, carboxypeptidase-A, collagenase) and various non-proteolytic enzymes are inactive. The endoproteases which inhibit the binding are all very basic proteins. However, their effect on the binding cannot be explained solely by a possible interaction between the positively charged enzyme proteins and the negative charges on the membranes, since the equally basic lactoperoxidase does not inactivate the binding in the same way. It seems that an active catalytic site of the proteolytic enzymes is required for the inhibition of the binding and it is known that the serine- and sulfhydryl-proteases show striking similarities in the essential groups of their catalytic site [11–13]. Therefore, it could be that these enzymes, via their catalytic site, rapidly cleave certain essential peptide moieties of the receptor binding site or that they remove some other essential constituents of it. This is compatible with the irreversibility of the receptor binding inactivation, but one would expect to see a fast progression of the effect especially at the lower enzyme concentrations where the initial inhibition amounts to about 20%. Progression of the effect upon prolonged incubation and also some degree of reversibility is expected if a reaction product of the proteolysis would interact with the receptor binding site, such as described by Lin and co-workers for the inactivation of opiate receptor binding by lipases [14]. We propose the alternative explanation that the enzymes bind very strongly to a certain area of the receptor macromolecule, as a result of which the protease-sensitive binding sites become inactivated. The formation of a tight complex between the enzymes and a peptide chain of the receptor is comparable with the well-known complexation between proteolytic enzymes and ubiquitous peptide-like protease inhibitors [15]. It is known that the catalytic sites of the enzyme are involved in the formation of these complexes. The complexes are rapidly formed, they are very stable and they only dissociate at acidic pH. Such a mechanism would be in agreement with all the presently made observations.

In conclusion, this study has demonstrated that two different types of stereospecific binding sites, with high binding affinity for dopamine agonists and antagonists exist on rat striatal membranes. They are called protease-sensitive and protease-insensitive sites. The sites show some differences in binding characteristics and they probably form part of the same dopamine receptor. *In vitro*, the exposure of the sites is regulated by monovalent ions.

Various questions remain to be answered. Which of the sites is functionally active and what governs the exposure of the sites *in vivo*? Does the existence of two types of sites play a role in phenomena of receptor super- and subsensitivity? Are both types of sites regulated by guanine nucleotides? Becoming aware of the complex composition of the dopamine

receptor may bring alternative approaches and explanations for *in vitro* and *in vivo* studies of the receptor.

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