Tiapride binds to mouse striatal tissue pre-exposed to dopamine stimulation

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Agents from the substituted benzamide series have been in routine clinical use for some 15 years and have proven action in psychiatry (sulpiride ameliorates psychotic behaviour), neurology (tiapride antagonizes dyskinetic motor phenomena), gastroenterology (metoclopramide increases gastric motility) and in diagnostic neuroendocrinology (many benzamides can be used to elevate prolactin secretion), yet this breadth of clinical usage is equalled by our ignorance of their mechanisms of action. Whilst the benzamide derivatives are generally classified as 'neuroleptics' their actions can be easily distinguished from those of 'classical' neuroleptic agents such as the phenothiazines and butryophenones. Thus, at a behavioural level, sulpiride and tiapride have little or no ability to mimic the classical actions of neuroleptic agents to induce catalepsy or antagonize stereotyped behaviour and, at a biochemical level, fail to mimic the actions of classical agents to displace labelled neuroleptic ligands or inhibit dopamine-sensitive adenylate cyclase (Costall et al 1978a; Jenner et al 1978). Indeed, this latter difference between benzamide and classical neuroleptic action on adenylate cyclase has prompted a classification of dopamine receptors into D-1 (adenylate cyclase linked and blocked by classical neuroleptics) and D-2 (antagonized by benzamide agents independent of cyclase inhibition) (Kebabian & Calne 1979; see also Jenner & Marsden 1979). Whilst the different types of neuroleptic agent may finally be shown to inhibit different types of dopamine receptors, it is of singular note that in those clinical and experimental situations where the benzamide derivatives do inhibit dopamine function, this reflects an inhibitory action on raised or tonic dopamine activity. Hence, taking the actions of tiapride as example, this agent does not, in reasonable dosage, reduce motor performance in normal animals or induce extrapyramidal disturbance in man (Costall et al 1978a; Lhermitte et al 1977; Price et al 1978). It does, however, incisively inhibit dyskinesias induced by dopamine agonists in laboratory animals (Costall et al 1977) and can reduce the dyskinesias caused by L-dopa in the clinic (Lhermitte et al 1977; Price et al 1978). Further, recent studies have shown that tiapride more effectively antagonizes denervated (rendered supersensitive) than normal striatal dopamine receptors subject to apomorphine stimulation (Costall et al 1979). Thus, we have hypothesized that a dopamine antagonist action for tiapride may only effectively be shown in situations which reflect increased dopamine receptor sensitivity or stimulation. In the present studies we investigate this hypothesis in an in vitro situation by assessing the characteristics of tiapride binding to both 'normal' striatal tissue and striatal tissue pre-exposed to dopamine stimulation.

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[^{3}H]Tiapride (specific activity 21 Ci m mol^{-1}) (SESIF, France) was custom tritiated by the Radiochemical Centre, Amersham, from whom [^{3}H]spiperone (20 Ci mmol^{-1}) was purchased. The purity of each ligand was 98% as judged by thin layer and paper chromatography. Tiapride hydrochloride (SESIF, France), (+)- and (−)-butaclamol hydrochloride (Ayerst Laboratories), and dopamine hydrochloride (Koch-Light) were dissolved in 0.1% ascorbic acid with subsequent dilution in the appropriate incubation buffer.

Male B.K.W. mice (20–25 g) were killed by cervical dislocation and their brains rapidly removed. The striata were dissected out over ice, homogenized (polytron PT-10 setting 5 for 5 s) in 100 vol of ice-cold 50 mM Tris-HCl buffer, and centrifuged twice at 40 000 *g* for 10 min with rehomogenization in fresh buffer. Final pellets were resuspended in 100 vol 50 mM Tris-HCl buffer containing 0·1% ascorbic acid, 5 mM Na_2EDTA and 12·5 μM nialamide. The optimum buffer solutions used were pH 7·4 and pH 7·6 for [^{3}H]spiperone and [^{3}H]tiapride binding respectively. These tissue suspensions were then subjected to primary incubation for 30 min at 37 °C in the absence or presence of dopamine (10^{-10}–10^{-4} M). After centrifugation and washing, to remove the added dopamine, resuspension was in the appropriate Tris-HCl buffer solution.

Binding was determined by incubating 500 μl of tissue suspension with varying concentrations of [^{3}H]tiapride (2·5–40 nM) or [^{3}H]spiperone (0·25 nM) in the presence or absence of dopamine (10^{-5} M) or 2-amino-6,7-dihydroxytetrahydronaphthalene (6,7-di-OHATN, ADTN) (10^{-5} M) at 37 °C for 1 h or 15 min respectively. The total incubate volume, including either buffer or the agent under investigation, was 1·1 ml. [^{3}H]Spiperone samples were rapidly filtered under vacuum through Whatman GF/B filters and washed with two 5 ml rinses of ice-cold buffer. Bound [^{3}H]tiapride was separated by centrifugation at 50 000 *g* for 6 min, the supernatant discarded, and the pellet rapidly and lightly rinsed with ice-cold buffer. In each study the filters or pellets were vigorously shaken for 30 min in Insta-gel (Packard Instruments) and the radioactivity measured by liquid scintillation spectrometry (efficiency 50%). Protein was determined by the method of Lowry et al (1951).

The density of binding sites of 0·25 mM [^{3}H]spiperone (a subsaturating concentration) as defined by the excess over blanks in the presence of 6,7-diOHADTN (10^{-5} M) (Quik & Iversen 1978) was 77·4 ± 12·6 fmol mg^{-1} protein. This specific binding was reduced by addition of relatively high concentrations of tiapride (10^{-7}–10^{-4} M) into the incubates. The dissociation constant of the competing drug, determined by application of the formula $K_I = IC50/(1 + L/K_D)$ (Cheng & Prusoff 1973) was $1{\cdot}86 \times 10^{-6}$ M.

The affinity of tiapride was further investigated using the labelled cogener. Addition of [^{3}H]tiapride (2·5–40 nM) to striatal tissue suspensions produced a linear increase in total tissue binding (Fig. 1), although this may be considered to reflect non-specific binding since

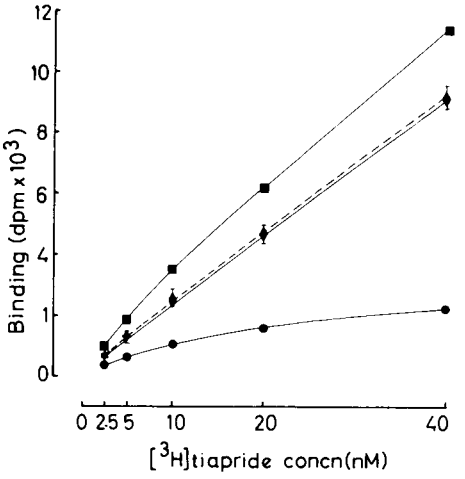

FIG. 1. Total binding of [^{3}H]tiapride (2·5–40 nM) to mouse striata following in vitro pre-exposure to control buffer solution (▲– – –▲) or 10^{-6} M dopamine (■——■) for 30 min at 37 °C. The residual (▼) and specific (●) binding of [^{3}H]tiapride to sensitized tissue suspensions were judged by displacement with dopamine (10^{-5} M). There was no displacement element of control pre-incubated tissue binding. Each point is the mean value of three experiments performed in triplicate. Standard errors, generally obscured by the symbols, were less than 12·1% of the mean.

it was non-displaceable by dopamine (10^{-5} M). However, when the striatal tissue was pre-exposed to dopamine (10^{-6} M) during the primary incubation phase an increase in [^{3}H]tiapride binding was apparent (full details of the development of this methodology are to be published elsewhere; Fortune, in preparation). Dopamine (10^{-5} M) decreased the elevated element of [^{3}H]tiapride binding to tissue, pre-exposed to dopamine (10^{-6} M), revealing the presence of a saturable, displaceable component (Fig. 1). Resolution of this specific component by Scatchard analysis indicated a maximum number of [^{3}H]tiapride binding sites of 263·7 ± 9·9 fmol mg^{-1} protein and a dissociation constant (K_D) of 25·8 ± 3·3 nM (Fig. 2). Further analysis of this data using a Hill plot yielded a slope of 1·01, not significantly deviating from unity, indicating that the specific [^{3}H]tiapride binding obeyed classic mass action laws. For displacement experiments a non-saturating concentration of [^{3}H]tiapride of 10 nM was used. Under these conditions specific binding was 1084 ± 52 d min^{-1}, constituting 30·6% of the total binding.

The specific binding of [^{3}H]tiapride (10 nM) induced by pre-exposure of the striatal tissue suspension to dopamine (10^{-6} M), was displaced in a concentration dependent manner by unlabelled tiapride (10^{-10}–

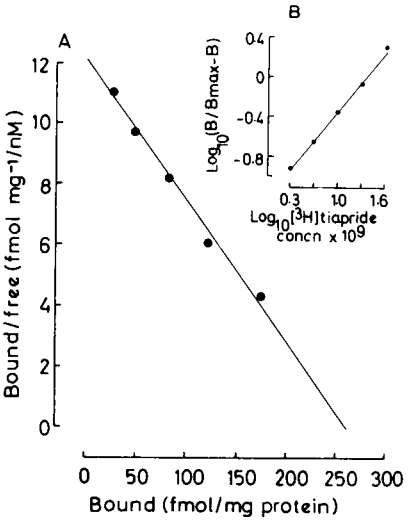

FIG. 2. Characteristics of the specific binding of [^{3}H]tiapride to mouse striatal preparations pre-exposed to dopamine (10^{-6} M) ,as judged by displacement with dopamine (10^{-5} M). A. Scatchard plot analysis, $K_D = 25{\cdot}8 \pm 3{\cdot}3$ nM, $B_{max} = 263{\cdot}7$ fmol mg^{-1} protein. ıB. Hill plot analysis, slope = 1·01, r = 0·99. Data was derived from three experiments in triplicate.

10^{-6} M) or dopamine (10^{-9}–10^{-4} M). The stereospecificity of this saturable component was demonstrated by the finding that the (+)-enantiomer of butaclamol was approximately 750 times more active than (−)-butaclamol in causing displacement (Table 1).

The essential finding of the present studies is that whilst tiapride has no demonstrable affinity for 'specific' binding sites on 'normal' striatal membranes, pre-exposure of the membranes to dopamine allows the subsequent development of a 'specific' binding of [^{3}H]tiapride to dopamine sites. Under these conditions, the presence of a saturable component of [^{3}H]tiapride binding indicates the presence of a limited number of specific binding sites. The saturability of these sites by relatively low concentrations of dopamine and cold tiapride, and the stereospecificity of displacement by

Table 1. Dissociation constants (K_i values) of drugs displacing [^{3}H]tiapride (10 nM) binding to mouse striatal preparations pre-exposed to dopamine (10^{-6} M). Each value is the mean result of three experiments performed in quadruplicate, and was obtained by application of the formula $K_i = IC50/(1 + L/K_D)$ (Cheng & Prusoff 1973). IC50 values were assessed as the concentration of drug causing a half-maximal displacement of [^{3}H]tiapride binding.

Displacing drug	Concn drug (M)	K_i (nM)
Tiapride	10^{-10}–10^{-6}	13·5
Dopamine	10^{-9}–10^{-4}	78·0
(+)-Butaclamol	10^{-10}–10^{-6}	1·02
(—)-Butaclamol	10^{-8}–10^{-5}	766

the more pharmacologically active isomer of butaclamol (Humber et al 1975) suggests that these receptors may have a pharmacological importance.

The observation of increased neuroleptic binding following dopamine stimulation is not, in itself, unique. Thus McManus et al (1978) showed a modest increase in [^{3}H]haloperidol binding to calf caudate tissue following pre-incubation with dopamine and, in an in vivo study, Baudry et al (1977) showed striatal pimozide binding to be modestly increased by concomitant amphetamine administration (considered by the authors that the enhanced release of dopamine caused by amphetamine modified the binding sites for the neuroleptic). Further, Howlett & Nahorski (1979) have similarly reported that a single acute injection of amphetamine results in a small increase in [^{3}H]-spiperone binding sites in rat striatum. However, [^{3}H]haloperidol and [^{3}H]spiperone normally exhibit excellent binding to striatal tissue per se. The distinguishing feature of tiapride binding is that it can only be observed after dopamine treatment.

The data obtained in the present in vitro design is in line with our original hypothesis that tiapride may interact more effectively with dopamine receptors subject to enhanced dopamine stimulation. Any attempt to correlate these in vitro biochemical observations with the functional changes induced by tiapride in vivo is necessarily conjectural. Nevertheless, the selective binding of tiapride after dopamine incubation may in part explain the action of tiapride to inhibit the dyskinesias caused by enhanced striatal dopamine receptor stimulation (see introductory paragraph) and perhaps the potent inhibitory action against amphetamine hyperactivity (Costall et al 1978b), and apomorphine circling (Costall et al 1979). It is also possible that the dopamine facilitated tiapride binding may be relevant to the antagonism of L-dopa induced dyskinesias in the clinic, which are generally associated with an increased striatal dopamine receptor sensitivity (Klawans et al 1979; Seeman et al 1978).

The precise nature and location of the tiapride binding site, and the mechanism by which dopamine can facilitate binding remain to be elucidated. Whilst tiapride will displace [^{3}H]spiperone or [^{3}H]cis (Z)-flupenthixol, the concentrations required are high (Jenner et al 1978; Hyttel 1980), and the importance of this interaction at a 'classical' neuroleptic receptor site to any pharmacological effect of tiapride remains speculative, particularly since [^{3}H]tiapride failed to show saturable binding to normal striatal tissue. In this context [^{3}H]tiapride binding differs notably from that of another substituted benzamide derivative, sulpiride, which only weakly inhibits [^{3}H]spiperone binding (Jenner et al 1978) but exhibits a more potent and unique spectrum of specific binding when used as a ligand in 'normal' striatal tissue (Theodorou et al 1979; Hall et al 1980). Therefore, even within the benzamide series there may exist differing propensity to influence different membrane sites. The tiapride binding site may be a distinct entity and the use of further dopamine agonists and other benzamide compounds, differing incubation conditions and tissues, is required to establish the specificity of the tiapride-dopamine interaction. Such studies have important implications for the design of agents which, like tiapride, may exhibit selective binding to dopamine receptors of modified sensitivity.

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Leukotriene B: a potential mediator of inflammation

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Mediators of acute inflammation have been classified into those agents affecting vascular permeability and those with leucotactic properties (Ward 1974). Substances that either mediate or modulate changes in vascular permeability include vasoactive amines, kinins and prostaglandins. Leucotactic agents include the complement derived peptide C5a, bacterial chemotactic factors, as exemplified by the synthetic peptide F-met-leu-phe and monohydroxyeicosatetraenoic acids derived from arachidonic acid (Gallin & Quie 1978; Goetzl & Sun 1979). The results of the present work suggest that to this category must be added leukotriene B (5,12-dihydroxy 6,8,10,14-eicosatetraenoic acid).

Rat and human polymorphonuclear leucocytes (PMNs) when exposed to the calcium ionophore A23187 release a product of the lipoxygenase pathway which causes the aggregation and chemokinesis of fresh PMN suspensions (Bray et al 1980). This product has been identified as leukotriene B and is active over the concentration range 10 pg to 5 ng ml^{-1} (Ford-Hutchinson et al 1980). It shows comparable activity on a molar basis in vitro to that observed for C5a and F-met-leu-phe and is between 100 and 1000 times

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Table 1. The effects of leukotriene B on the chemotaxis of human PMN in vitro. Chemotaxis of human PMNs prepared from dextran sedimented venous blood was assessed using the agarose plate technique described by Nelson et al (1975). Results are calculated as a chemotactic ratio A/B, where A is the distance migrated towards the stimulant and B the distance migrated towards the control well. The concentrations of leukotriene B required to produce positive chemotaxis appear to be higher than those required for maximal chemokinetic response (Ford-Hutchinson et al 1980) but this reflects dilution of the leukotriene B along the chemotactic gradient rather than a difference in sensitivity. Results are expressed as means ± s.e.m. ** $P < 0.005$. n = no of determinations.

Concn of leukotriene B in outer well, ng ml^{-1}	A/B	n =
0	0·92 ± 0·07	10
0·3	0·92 ± 0·09	5
1	1·08 ± 0·03	4
3	1·07 ± 0·02	5
10	1·66 ± 0·13**	5
30	1·6 ± 0·14**	5
100	1·71 ± 0·08**	5
300	1·84 ± 0·02**	5
1000	1·52 ± 0·05**	5

Table 2. Effects of leukotriene B and F-met-leu-phe on the accumulation of leucocytes into the guinea-pig peritoneum in vivo. Leucocyte movement in vivo was assessed following intraperitoneal injection into 200–250 g Dunkin Hartley guinea-pigs of leukotriene B or F-met-leu-phe (Sigma Chemical Co., Poole, Dorset, U.K.) dissolved in 0·4 ml of Hank's balanced salt solution. Cells were harvested 1 and 5 h after injection as previously described (Goetzl et al 1979) and total and differential counts performed. Results obtained after 5 h have been expressed above as mean total cell counts per animal $\times 10^6$ ± s.e.m. In a parallel experiment no increase in leucocyte migration was observed after 1 h incubation. * $P < 0.05$. ** $P < 0.01$.

Treatment	Dose per animal	n	Total white cell count	Macrophages	Polymorphonuclear leucocytes	Eosinophils
Vehicle alone	—	8	9·5 ± 0·6	6·1 ± 0·5	1·1 ± 0·2	2·2 ± 0·3
Leukotriene B	1 μg	7	30·8 ± 4·4**	14·0 ± 3·2*	13·1 ± 3·9**	3·6 ± 0·7
Leukotriene B	100 ng	9	16·9 ± 3·0**	7·7 ± 1·0	7·8 ± 1·9**	2·2 ± 0·5
F-met-leu-phe	1 μg	4	15·5 ± 2·5*	11·1 ± 1·8**	0·8 ± 0·4	3·5 ± 1·0
F-met-leu-phe	100 ng	4	11·4 ± 2·0	6·6 ± 1·5	1·0 ± 0·1	3·7 ± 1·0*