Br. J. Pharmacol. (1991), **102**, 446–452 © Macmillan Press Ltd, 1991

Very high affinity interaction of DPI 201-106 and BDF 8784 enantiomers with the phenylalkylamine-sensitive Ca^{2+}-channel in *Drosophila* head membranes

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1 Piperazinylindoles (DPI 201-106, BDF 8784), drugs known to act on voltage-dependent Na^+-channels, bind with very high affinity to a Ca^{2+}-channel-associated phenylalkylamine receptor in *Drosophila melanogaster* head membranes. These compounds and (+)-tetrandrine, a naturally occurring Ca^{2+}-antagonist, were the most selective inhibitors for phenylalkylamine-labelled *Drosophila* Ca^{2+}-channels compared to mammalian L-type Ca^{2+}-channels.

2 Replacement of the cyano group by a methyl group in (+)-DPI 201-106 ((+)-BDF 8784) increases the IC_{50} value for inhibition of phenylalkylamine labelling of *Drosophila* Ca^{2+}-channels from 0.29 to 2.1 nM but decreases the IC_{50} value for inhibition of phenylalkylamine labelling of mammalian skeletal muscle Ca^{2+}-channels from 3480 to 49 nM.

3 DPI 201-106 enantiomers completely block (at 0.1 μM) phenylalkylamine photolabelling of a 136 K polypeptide in *Drosophila* head membranes whereas 10 μM aconitine or lidocaine are without effect.

4 Assessment of the Ca^{2+}-antagonist effects of the substituted DPI 201-106 enantiomers in K^+-depolarized taenia strips from guinea-pig caecum yielded pA_2 values of 6.33 ± 0.07 for (−)-BDF 8784 and 6.99 ± 0.17 for (+)-BDF 8784, respectively.

5 Piperazinylindoles, previously believed to act nonspecifically on voltage-dependent mammalian L-type Ca^{2+}-channels, therefore have stereoselectivity for a novel binding site and chemical selectivity unrelated to local anaesthetic activity.

6 It is proposed that a very high affinity piperazinylindole-selective site is coupled to the phenylalkylamine receptor of *Drosophila* Ca^{2+}-channels. These sites are still present on mammalian L-type Ca^{2+}-channels but have lower affinity and/or are less tightly coupled to phenylalkylamine receptors on the α_1-subunit.

Introduction

Drosophila melanogaster head membranes contain at least eight distinct voltage-regulated Ca^{2+}-channels after reconstitution in phospholipid bilayers (Pelzer *et al.*, 1989). There is a Ca^{2+} channel, permeable to Ba^{2+} (13 pS conductance) which is extremely sensitive to phenylalkylamines (transient stimulation and subsequent block by nM concentration of gallopamil or (−)-desmethoxyverapamil) but is completely insensitive to 1,4 dihydropyridines. As *Drosophila melanogaster* head membranes also contain 1,4 dihydropyridine-sensitive Ca^{2+}-channels (with 21 and 31 pS conductances, respectively), that are insensitive to phenylalkylamines, in addition to Ca^{2+} channels that are not blocked by either class of Ca^{2+} antagonists, they offer a unique system to study the evolution of drug receptor domains and the phylogeny of Ca^{2+}-channels. The mammalian L-type Ca^{2+}-channel, e.g. that from skeletal muscle transverse tubule membranes, possesses several distinct but allosterically coupled drug receptors (e.g. for 1,4 dihydropyridines, phenylalkylamines, benzothiazinones, benzothiazepines, diphenylbutylpiperidines and indolizinsulfones) which are all localized on the α_1-subunit as shown by photolabelling (see e.g. Hosey & Lazdunski, 1988; Schmid *et al.*, 1989; Glossman & Striessnig, 1990). The precise location of these drug domains within the primary amino acid sequence of the α_1-subunit is not yet known but the possibility exists that mammalian L-type Ca^{2+}-channels evolved by gene duplication and fusion, assembling 1,4-dihydropyridine and phenylalkylamine receptors on one continuous peptide chain.

High-affinity phenylalkylamine binding sites are found in *Drosophila melanogaster* head membranes (Pauron *et al.*, 1987; Pelzer *et al.*, 1989; Greenberg *et al.*, 1989). These sites are not coupled (as are mammalian L-type Ca^{2+}-channel phenylalkylamine receptors) to 1,4 dihydropyridine sites (Pauron *et al.*, 1987; Greenberg *et al.*, 1989) and can be specifically photolabelled by the arylazide phenylalkylamine [N-methyl-^{3}H]-LU 49888 ((−)-5-[(3-azido-phenethyl)-[N-methyl-^{3}H]methyl-amino-2-(3,4,5-trimethoxy-phenyl)-2-isopropylvalero-nitrile)]. [N-methyl-^{3}H]-LU 49888 photolabels a 135 K polypeptide in *Drosophila* head membranes (Pauron *et al.*, 1987; Greenberg *et al.*, 1989; Pelzer *et al.*, 1989) with a sedimentation coefficient of 12 S upon sucrose gradient centrifugation after solubilization with digitonin (Greenberg *et al.*, 1989). The *Drosophila melanogaster* phenylalkylamine-sensitive Ca^{2+}-channel seems to be unique, as Ca^{2+}-antagonists from other chemical classes (benzothiazepines e.g. (+)-*cis*-diltiazem; 1,4 dihydropyridines e.g. nitrendipine) interacted only very weakly or not at all with the radiolabelled receptor (Pauron *et al.*, 1987; Greenberg *et al.*, 1989). DPI 201-106 (4-3(4-diphenyl-methyl-1-piperazinyl)-2-hydroxy- propoxy-1H-indole-2-carbonitrile) is a new cardiotonic agent (Scholtysik *et al.*, 1985). The two enantiomers of DPI 201-106 have opposite effects on the voltage-dependent Na^+-channel in neuronal or heart cells, but allosterically inhibit with apparently equal affinity, [^{3}H]-batrachotoxinin A20-α-benzoate binding (Romey *et al.*, 1987). (S)- and (R)-DPI 201-106 seem to interact at distinct receptor sites on the voltage-dependent Na^+-channel (Wang *et al.*, 1989). A methyl-indole derivative of DPI 201-106 (BDF 8784, 2-methyl-4-3′-(4″-benzhydryl-1″-piperazinyl) - 2′ - hydroxypropoxy-1H-indole) is not cardiotonic and both enantiomers

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block in a noncompetitive manner the activation of Na^+-channels by DPI 201-106 (Honerjäger *et al.*, 1988; Armah *et al.*, 1989). In the following study we show that these piperazinylindoles interact with very high affinity at the *Drosophila* phenylalkylamine receptors. These compounds define a previously unrecognized class of drug receptors that are linked to the phenylalkylamine-sensitive Ca^{2+}-channel in *Drosophila* head membranes. Piperazinylindoles also define a novel drug receptor domain on mammalian L-type Ca^{2+}-channels that is allosterically coupled to 1,4-dihydropyridine-, phenylalkylamine- and benzothiazepine-selective sites, but with lower affinity than the *Drosophila* Ca^{2+}-channel.

Methods

Membrane isolation

Membrane extracts from Canton-S *Drosophila melanogaster* heads were prepared as described (Greenberg *et al.*, 1989). Briefly, 100 mg (wet weight) of fly heads per ml ice-cold 0.2 M sucrose, 10 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride, 50 mM HEPES (pH 7.0) were homogenized in a motor-driven glass-teflon homogenizer (30 strokes at 1700 r.p.m.). The homogenate was centrifuged (3000 g, 10 min, 4°C), the supernatant saved and the pellet re-homogenized as above. After repeating the centrifugation, the pooled supernatants were centrifuged at 48,000 g for 40 min and the upper, lighter portion of the pellet resuspended by homogenization (4 strokes in 50 mM HEPES, pH 7.0) at a final head membrane protein concentration of 4 mg ml^{-1}. Membranes were flash frozen and stored in liquid nitrogen until use. Partially purified transverse tubule membranes from guinea-pig skeletal muscle and guinea-pig cerebral cortex membranes were isolated as described by Glossmann & Ferry (1985).

Binding assays

Na^+-channel labelling [^{3}H]-batrachotoxinin A 20-α-benzoate (6.2–6.5 nM final concentration) was incubated for 60 min at 37°C with 0.14 to 0.23 mg ml^{-1} guinea-pig cerebral cortex membrane protein in 50 mM Tris-HCl (pH 7.4), 65 mM choline chloride, 0.2 mg ml^{-1} bovine serum albumin, 0.1 mM phenylmethylsulphonylfluoride (PMSF) and 1.6 mg ml^{-1} of crude *L. quinquestratus* toxin. The assay volume was 0.125 or 0.25 ml. The blank binding definition was 0.4 mM aconitine. Drugs were dissolved and diluted in dimethylsulphoxide (DMSO) and 2 or 5 μl added to the assay tubes as described (Boer *et al.*, 1989). The final DMSO concentration never exceeded 2% (v/v).

Ca^{2+}-channel labelling Interaction of drugs with the phenylalkylamine-selective domain of the L-type Ca^{2+}-channel in guinea-pig membranes was investigated with (−)-[^{3}H]-desmethoxyverapamil (Goll *et al.*, 1984). The ligand concentrations varied between 0.66 to 1.2 nM, and the definition of nonspecific binding was by 2 μM (±)-desmethoxyverapamil. The assay volume was 0.5 ml. Incubations were at 25°C for 60 min in 50 mM Tris-HCl, pH 7.4 supplemented with 0.1 mM PMSF. Skeletal muscle transverse tubule membrane protein was 0.04 to 0.12 mg ml^{-1}, cerebral cortex membrane protein varied from 0.07 to 0.16 mg ml^{-1}. Drugs were added in DMSO as above (5 μl); the final DMSO concentration never exceeded 1% (v/v). Saturation analysis with (−)-[^{3}H]-desmethoxyverapamil and skeletal muscle membranes was performed and data analysed as described (Knaus *et al.*, 1990) in the absence and presence of different concentrations of (+)-BDF 8784, to yield dissociation constants (K_D values) and maximal densities of sites (B_{max}). Interaction of drugs with the 1,4-dihydropyridine-selective domain of L-type Ca^{2+}-channels in guinea-pig membranes was investigated with (+)-[^{3}H]-isradipine. Cerebral cortex membrane protein (0.07–0.08 mg ml^{-1}) was incubated with 0.2–0.25 nM radioligand for 60 min at 25°C; for skeletal muscle membranes 0.03 to 0.06 mg ml^{-1} protein and 0.53–0.89 nM radioligand was employed and the incubation time was 30 min at 25°C. The assay volume, drug additions in DMSO and buffers were as above; the definition of nonspecific binding was 1 μM (±)-isradipine. Dissociation experiments to assess the type of interaction of the (+)-BDF 8784 enantiomer were performed as described by Knaus *et al.* (1990). Briefly, skeletal muscle membranes (0.005 mg protein ml^{-1}) were incubated at 37°C with 3 μM (+)-*cis* dilitazem and 50–90 pM (+)-[^{3}H]-isradipine for 90 min. Dissociation was initiated by action of unlabelled (±)-isradipine (3 μM), (+)-BDF 8784 (5 μM) or by simultaneous addition of both drugs and followed for 30 min by measuring specifically bound radioligand in duplicates at 1 min intervals for 15 min and at 20, 25 and 30 min. Dissociation data from 3 independent experiments was fitted by Graphpad™ to the monoexponential decay function to yield dissociation rate constants.

The benzothiazepine-selective domain of the Ca^{2+}-channel in skeletal muscle membranes was probed at 4°C and 30°C with (+)-*cis*-[^{3}H]-diltiazem as follows: 0.06–0.17 mg ml^{-1} membrane protein was incubated with and without drugs (added as above) with 0.5–1.75 nM radioligand and incubated either for 60 min (30°C) or 12 h (4°C) in an assay volume of 0.5 ml in 50 mM Tris-HCl, pH 7.4, 0.1 mM PMSF with 10 μM (+)-*cis*-diltiazem used for definition of nonspecific binding.

Binding-inhibition experiments with the phenylalkylamine receptor in *Drosophila* membranes were performed as described by Greenberg *et al.* (1989), with assay volumes of 0.5 or 1.0 ml and 1 μM (±)-desmethoxyverapamil used for definition of nonspecific binding. [N-methyl ^{3}H]-LU 49888 (0.52–0.84 nM) or (−)-[^{3}H]-desmethoxyverapamil (0.66–1.21 nM) were used in 50 mM Tris-HCl (pH 7.4) buffer, supplemented with 0.1 mM PMSF and 0.051 to 0.091 mg ml^{-1} membrane protein. The incubation time at 25°C was 60 min. Drugs (with the exception of heparin) were added in DMSO as above.

Separation of bound and free ligand After incubation, bound and unbound radioligands were separated (after dilution with 3.5 ml ice-cold 10% polyethylene glycol 6000, 10 mM Tris HCl, 10 mM $MgCl_2$ (pH 7.4)) by rapid filtration over Whatman GF/C glass fibre filters followed by two washes (3.5 ml each). Dried filters were counted for radioactivity in a liquid scintillation counter. Specific binding was defined as the differences between radioligand bound in the absence and presence, respectively of the appropriate blank definition.

Photoaffinity labelling

For photoaffinity labelling of the phenylalkylamine receptor in *Drosophila* head membranes, 0.12 to 0.13 mg ml^{-1} protein was incubated with 3.0–3.5 nM [N-methyl ^{3}H]-LU 49888 (with or without added drugs) in 50 mM Tris-HCl (pH 7.4) buffer at 25°C in the dark for 60 min. Incubation mixtures were transferred to Petri dishes on ice, irradiated for 30 min with ultraviolet light as described by Greenberg *et al.* (1989) and centrifuged for 10 min (4°C) at 40,000 g. Membrane pellets were solubilized (at 56°C) in electrophoresis sample buffer (10 mM dithiothreitol, reducing conditions) in the presence of a protease inhibitor mix (Vaghy *et al.*, 1988) and electrophoresed on 9% SDS polyacrylamide gels. Fixed gels were either incubated with Amplify™ (Amersham) and fluorography performed (Greenberg *et al.*, 1989) or radioactivity was quantified by cutting the gel into 3 mm slices, extracted with Econofluor™, containing 3% (v/v) Protosol™ (both New England Nuclear) and liquid scintillation counting.

Data analysis

Binding-inhibition curves were analysed by fitting the experimental data to the general dose-response equation (De Lean *et al.*, 1978), yielding maximal inhibition (as %), IC_{50} value and Hill slopes (nH).

Assessment of Ca^{2+} antagonist effects

Ca^{2+}-antagonism of the BDF 8784 enantiomers was assessed as described by Spedding (1982). Briefly, strips of taenia obtained from the caecum of male guinea-pigs (300–350 g) (1–2 mm diameter, relaxed length 1.8–2.5 cm) were set up in an organ bath (30 ml) containing the K^+-depolarized Tyrode solution (Spedding, 1982) at 37°C and gassed with 95% O_2 and 5% CO_2. Cumulative concentration-response curves were obtained to $CaCl_2$ (0.03–1 mM) by increasing the Ca^{2+} concentration at 3–5 min intervals in logarithmic increments. Drugs were preincubated for 2 h and dose-ratios calculated by the method of Arunlakshana & Schild (1959).

Drugs and chemicals

DPI 201-106 enantiomers were a gift from Prof. Scholtysik (Sandoz AG Basle, Switzerland), (+)-tetrandrine was obtained from Dr Kaczorowski (Merck Sharp and Dohme, Rahway N.J., U.S.A.), (R)-(+) BDF 8784 (BDF 9158) (called (+)-BDF 8784 here) and (S)-(−)-BDF 8784 (BDF 9157) (named (−)-BDF 8784 here) were provided by one of us (B.I.A.), HOE-166 (R-(±)-3,4-dihydro-2-isopropyl-4-methyl- 2-[2-[4-[4-[2-(3,4,5-tri-methoxyphenyl)ethyl]piperazinyl]- butoxy]phenyl]- 2H-1, 4-benzothiazin-3-on- dihydrochloride) and its (S)-enantiomer (HOE-167) were gifts from Prof. Schölkens (Hoechst AG, Frankfurt, F.R.G.), *trans*-diclofurime was a gift from Dr Spedding, (Syntex Lab, Edinburg, UK), (−)-cyanopindolol a gift from Dr Engel, Sandoz AG, Basle, Switzerland. Crude *L. quinquestratus* toxin and high molecular weight heparin (average molecular weight: 13600) were from Sigma (Munich, F.R.G.). (+)-[^{3}H]-isradipine (70 Ci mmol^{-1}), (−)-[^{3}H]-desmethoxyverapamil (76 Ci mmol^{-1}), [^{3}H]-batrachotoxinin A 20-α-benzoate (44 or 42.7 Ci mmol^{-1}) and (+)-*cis*-[^{3}H]-diltiazem (136 Ci mmol^{-1}) were from Amersham, Vienna, Austria. [N-methyl ^{3}H]-LU 49888 (85 Ci mmol^{-1}) as well as the optically pure enantiomers of the phenylalkylamines were gifts from Knoll AG (Ludwigshafen, F.R.G.).

Results

Pharmacological profile of the phenylalkylamine receptor in Drosophila *head membranes*

In previous studies (Pauron *et al.*, 1987; Greenberg *et al.*, 1989) the high affinity and selectivity of the (13 pS) Ca^{2+}-channel-associated receptor (Pelzer *et al.*, 1989) in *Drosophila* membranes for phenylalkylamines was noted. The data in Table 1 and the examples shown in Figure 1, however illustrate that drugs from different chemical classes, including compounds that are not classified as L-type Ca^{2+}-channel blockers, can inhibit phenylalkylamine (e.g. (−)-[^{3}H]-desmethoxyverapamil and [N-methyl ^{3}H]-LU 49888) binding to *Drosophila* membranes. Most notable was the very high affinity interaction of the piperazinylindoles. The (+)-enantiomer of DPI 201-106 was 3 times more potent than the (−)-enantiomer. An eudismic ratio around unity was found for the methylindole-substituted DPI 201-106 derivatives, (+) BDF 8784 and (−) BDF 8784. The importance of the side chain substitution of the piperizinylindoles is underlined by (−)-cyanopindolol which inhibited phenylalkylamine binding with an IC_{50} value three orders of magnitude higher than (+)-DPI 201-106. (+)-Tetrandrine, an L-type Ca^{2+}-channel blocker, which binds in a competitive manner (K_i = 550 nM at 25°C) to the (+)-*cis* [^{3}H]-diltiazem-labelled benzothiazepine-selective domain in mammalian heart membranes (King *et al.*, 1988) interacted with an IC_{50} value of 9.5 nM. The diltiazem diastereomers were inhibitory with Hill slopes smaller than unity, indicating an inhibitory mechanism that was not simple

Table 1 Pharmacological profile of phenylalkylamine receptors in *Drosophila melanogaster* head membranes in comparison to mammalian skeletal muscle transverse tubule membranes

Chemical class	Drosophila melanogaster *Drug*	*IC_{50} [nM]*	*nH*	*Mammalian skeletal muscle IC_{50} (nM)*	*Ratio*
Piperazinylindoles[1]	(+)-DPI 201-106	0.29 ± 0.003	0.90 ± 0.16	1230	4240
	(−)-DPI 201-106	0.72 ± 0.1	0.97 ± 0.12	3480	4833
	(+)-BDF 8784	2.1 ± 0.5	0.97 ± 0.12	49.4	23.5
	(−)-BDF 8784	3.1 ± 0.3	1.02 ± 0.11	1790	577
Phenylalkylamines	(−)-Verapamil	0.66 ± 0.12	1.1 ± 0.21	40.2	61
	(+)-Verapamil	16.7 ± 1.8	0.93 ± 0.14	70.4	4.2
	(−)-Gallopamil	0.96 ± 0.07	0.89 ± 0.12	12.5	13.0
	(+)-Gallopamil	20.5 ± 3.7	1.0 ± 0.08	55.3	2.7
	(−)-Desmethoxyverapamil	0.66 ± 0.09	0.97 ± 0.08	4.0	6.1
	(+)-Desmethoxyverapamil	5.9 ± 1.2	0.94 ± 0.12	34.7	5.9
	(−)-Emopamil	5.4 ± 1.3	0.84 ± 0.13	37.2	6.9
	(+)-Emopamil	7.8 ± 1.6	1.17 ± 0.28	33.5	4.3
Benzothiazinones[2]	HOE 167	30.1 ± 2.5	0.89 ± 0.06	5.94	0.2
	HOE 166	88.4 ± 5.9	1.14 ± 0.08	1.04	0.01
Benzothiazepines	(+)-*cis*-Dilitiazem	750 ± 133	0.63 ± 0.11	164	0.22
	(−)-*cis*-Diltiazem	146 ± 80	0.71 ± 0.14	9140	62.6
Diphenylbutylpiperidines[3]	Fluspirilene	15.8 ± 5.0	0.75 ± 0.16	0.4	0.02
	Pimozide	84.4 ± 1.4	1.3 ± 0.30	2.0	0.02
Miscellaneous[4]	(+)-Tetrandrine	9.5 ± 1.8	1.3 ± 0.3	>10000	>100
	(−)-Cyanopindolol	330.4 ± 69.4	0.8 ± 0.11	ND	ND
	trans-Diclofurime	35 ± 6.8	0.93 ± 0.14	39.1	1.1
	Heparin	>10000		64	<0.01

IC_{50} values are means from 4–5 experiments (±s.d.) each carried out with 8 to 11 different concentrations of drugs in duplicates employing [N-methyl ^{3}H]-LU 49888 as ligand for *Drosophila* phenylalkylamine receptors, except for the benzothiazinone data which were obtained with (−)-[^{3}H]-desmethoxyverapamil. All drugs inhibited binding to *Drosophila* membranes by 100%. 'Ratio' defined as: IC_{50} value for mammalian skeletal muscle divided by the IC_{50} value for the *Drosophila* phenylalkylamine receptor. Data for skeletal muscle Ca^{2+}-channel-linked phenylalkylamine receptors were obtained as described in Methods. (ND = not determined).

[1] see Table 4 for details.

[2] Benzothiazinone data for *Drosophila* are taken from Grassegger *et al.* (1989).

[3] Fluspirilene and pimozide IC_{50} values for mammalian skeletal muscle are taken from Gallizi *et al.* (1986).

[4] Heparin inhibition data for mammalian skeletal muscle are taken from Knaus *et al.* (1990) using the average molecular weight given by the manufacturer.

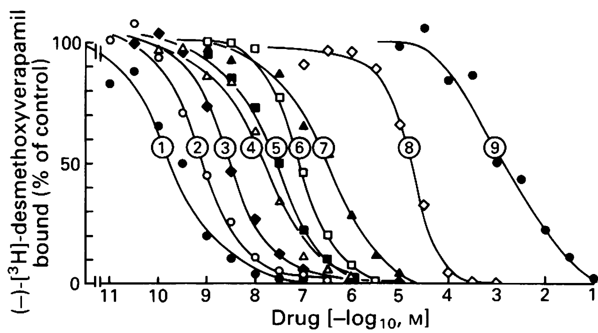

Figure 1 Binding-inhibition profile of (−)-[^{3}H]-desmethoxy-verapamil-labelled phenylalkylamine receptors in *Drosophila melanogaster* head membranes. The figure shows representative experiments where points are means from duplicate determinations. Under the experimental conditions (see Methods) the concentration of total bound ligand varied from 48.9 to 66.9 pM, nonspecifically bound ligand from 4.8 to 6.8 pM. (1) (+)-DPI 201-106; (2) (−)-DPI 201-106; (3) (+)-BDF 8784; (4) fluspirilene; (5) HOE-167; (6) HOE-166; (7) (−)-cyanopindolol; (8) Ca^{2+}; (9) La^{3+}.

competition. Compared to the mammalian phenylalkylamine receptors, the stereoselectivity is reversed for these benzothiazepines as was reported for the benzothiazinones previously (Grassegger *et al.*, 1989). *Trans*-diclofurime, suggested to interact preferentially with the benzothiazepine-selective site of L-type Ca^{2+}-channels (Mir & Spedding, 1987; Spedding *et al.*, 1987) inhibited phenylalkylamine binding to *Drosophila* membranes with an IC_{50} value of 35 nM. For comparison, IC_{50} values for inhibition of the phenylalkylamine-selective domain of the Ca^{2+}-channel from mammalian skeletal muscle membranes are shown in Table 1 together with a calculated ratio of the IC_{50} values.

Photoaffinity labelling experiments

The fluorogram in Figure 2 shows that 0.1 μM (+)- or (−)-DPI 201-106 completely inhibited photolabelling of the 135 K phenylalkylamine receptor in *Drosophila* head membranes,

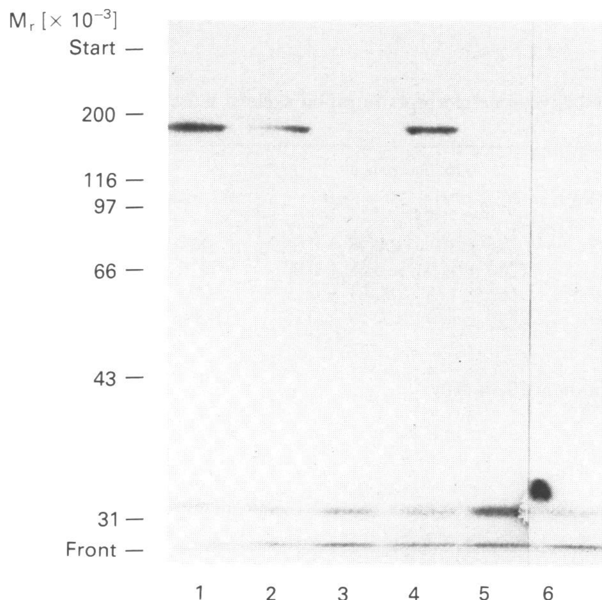

Figure 2 Photoaffinity labelling of *Drosophila* head membranes. The fluorogram of the SDS-gel shows in lane (1) control photoincorporation, (2) lidocaine 10 μM present, (3) 0.1 μM (+)-DPI 201-106 present, (4) 10 μM aconitine present, (5) 0.1 μM (−)-DPI 201-106 present, (6) 0.3 μM (−)-desmethoxyverapamil present. Membrane protein concentration for photolabelling was 0.13 mg ml^{-1}, [N-methyl ^{3}H]-LU 49888 was 3.1 nM and the film exposure time of the gel was 2 weeks at −80°C. The mobility of marker proteins is shown on the left side.

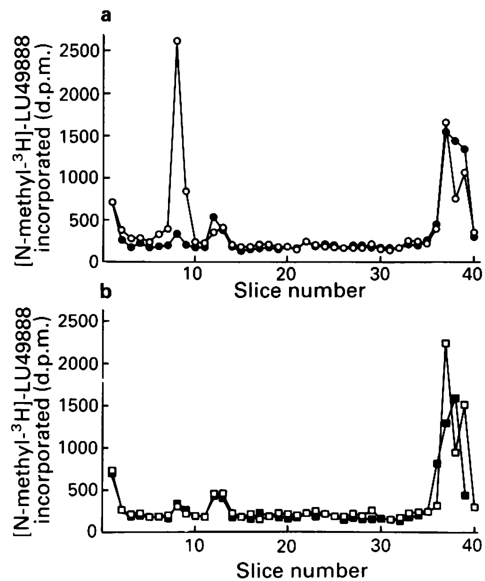

Figure 3 Quantitation of photoincorporated radioactivity in *Drosophila* head membranes. (a) Photolabelling was as in Figure 2 except that 0.11 mg ml^{-1} of membrane protein and 2.8 nM [N-methyl ^{3}H]-LU 49888 was used. Gels were processed as described in Methods. Open symbols: control photoincorporation (no drug added); closed symbols: photoincorporation in the presence of 0.3 μM (−)-desmethoxyverapamil. (b) As above but with (+)-DPI 201-106 (open symbols) or with closed symbols (−)-DPI 201-106 present (each at 0.1 μM).

whereas lidocaine or aconitine were without effect. *Drosophila* head membranes also contain low affinity, high capacity phenylalkylamine sites which are not stereoselective but are photolabelled by [N-methyl ^{3}H]-LU 49888 (Greenberg *et al.*, 1989). Photoincorporation into this 27 K polypeptide can be suppressed by high (μM) concentrations of (+)- or (−)-verapamil (Greenberg *et al.*, 1989). Quantitative analysis of photoincorporated radioactivity (Figure 3) shows that the DPI 201-106 enantiomers do not inhibit photolabelling of the 27 K band.

Interaction of piperazinylindoles with mammalian Na^+- and Ca^{2+}-channel associated drug receptors

Table 2 confirms for guinea-pig cerebral cortex membranes results previously reported for rat brain synaptosomes (Romey *et al.*, 1987), namely that both DPI 201-106 enantiomers inhibit specific [^{3}H]-batrachotoxin A 20-α-benzoate binding with similar IC_{50} values. The BDF 8784 enantiomers were slightly less potent. When the piperazinylindoles were diluted in buffer (instead of DMSO) as described by Glossmann & Ferry (1985), IC_{50} values were higher and apparent Hill slopes ($n = 3$) significantly greater than unity: (±)-BDF 8784 (IC_{50}: 460 ± 55 nM, nH 1.5 ± 0.2), (+)-BDF 8784 (IC_{50}: 510 ± 66 nM, nH = 1.88 ± 0.33), (−)-BDF 8784 (IC_{50}:

Table 2 Interaction of piperazinylindoles with [^{3}H]-batrachotoxinin A 20-α-benzoate labelled Na^+-channels in guinea-pig cerebral cortex membranes

Drug	*IC_{50}* (nM)	*nH*	*Maximal inhibition* (%)
(−)-DPI 201-106	90.7 ± 23.8	1.0 ± 0.2	100
(+)-DPI 201-106	93.8 ± 14.2	1.1 ± 0.17	100
(+)-BDF 8784	149.3 ± 28.9	0.99 ± 0.17	100
(−)-BDF 8784	200.6 ± 42.8	1.1 ± 0.25	100

$n = 3$. Values are means ± s.d. For details, see Methods.

692 ± 83 nM, nH 1.89 ± 0.34), (±)-DPI 201-106 (IC_{50}: 458 ± 31 nM, nH 1.6 ± 0.15). It was recently shown that DPI 201-106 interacted allosterically with the three classical Ca^{2+}-antagonist drug receptor domains to downregulate 1,4-dihydropyridine, phenylalkylamine and benzothiazepine binding. However, because the inhibition was not stereoselective it was suggested that DPI 201-106 binds to a local anaesthetic-like site, common to both Ca^{2+}- and Na^{+}-channels (Siegl *et al*., 1988). From the results shown in Table 2 we expected that the BDF 8784 enantiomers would have similar affinities for Ca^{2+}-antagonist drug receptors. Data summarized in Tables 3, 4 and 5 show that this is clearly not the case. In general the DPI 201-106 enantiomers (with the exception of (+)-DPI 201-106 and phenylalkylamine labelling) were more potent inhibitors of brain Ca^{2+}-channel labelling than in skeletal muscle. The reverse was observed for the BDF 8784 enantiomers. Enantioselectivity was marginal or absent for the DPI 201-106 enantiomers when the 1,4-dihydropyridine-selective site was studied but (−)-DPI 201-106 was 3.7 times more potent than (+)-DPI 201-106 in inhibiting the brain phenylalkylamine receptor. Clear evidence for enantioselectivity was obtained when the phenylalkylamine-selective site of the skeletal muscle Ca^{2+}-channel was investigated with the BDF 8784 enantiomers. (+)-BDF 8784 was 36 times more potent than the (−)-enantiomer. The racemate inhibited with an IC_{50} value approximately twice that obtained for the eutomer. The piperazinylindoles were very potent inhibitors at the benzothiazepine-selective domain of skeletal muscle Ca^{2+}-channels. (+)-BDF 8784 was 4 (at 30°C) or 7 (at 4°C) times more potent than the (−)-enantiomer. Although the IC_{50} value of the BDF eutomer is in the range observed for (+)-*cis*-diltiazem (see Table 5) the steep Hill slope and kinetic experiments performed with (+)-BDF 8784 (Glossmann & Striessnig, 1990) clearly prove that the inhibition is allosteric and not occurring by direct binding to the (+)-*cis*-[^{3}H]-diltiazem labelled receptor domain, confirming for skeletal muscle Ca^{2+}-channels previous findings with heart Ca^{2+}-channel labelling (Siegl *et al*., 1988). Study of dissociation kinetics is a powerful tool to distinguish competitive from allosteric inhibition (Romey *et al*., 1987). When the 1,4-dihydropyridine-selective domain of the skeletal muscle membrane Ca^{2+}-antagonist receptors was labelled with (+)-[^{3}H]-PN 200-110 in the presence of 3 μM (+)-cis diltiazem the following dissociation rate constants (K_{-1} values) were obtained: isradipine, 0.11 ± 0.006 min^{-1}; (+)-BDF 8784, 0.24 ± 0.007 min^{-1}; isradipine plus (+)-BDF 8784: 0.24 ± 0.008 min^{-1}. Clearly this methylindole-substituted DPI 201-106 enantiomer interacts allosterically with the dihydropyridine-selective site as previously shown for (±)-DPI 201-106 (Siegl *et al*., 1988). Saturation analysis of the phenylalkylamine-selective domain of Ca^{2+}-antagonist receptors in skeletal muscle membranes with (−)-[^{3}H]-desmethoxyverapamil (performed in the absence and presence of different concentrations of (+)-BDF 8784) demonstrated a concentration-dependent reduction of B_{max}

Table 3 Interaction of piperazinylindoles with Ca^{2+}-channel-linked 1,4 dihydropyridine receptors in mammalian skeletal and guinea-pig brain cortex membranes labelled with (+)-[^{3}H]-isradipine

	Skeletal muscle			*Brain cortex*		
Drug	IC_{50} (nM)	*nH*	*MI* (%)	IC_{50} (nM)	*nH*	*MI* (%)
(−)-DPI 201-106	3410 ± 1330	0.98 ± 0.2	79	1810 ± 380	0.98 ± 0.17	87
(+)-DPI 201-106	5210 ± 1370	0.94 ± 0.2	67	1970 ± 540	1.14 ± 0.3	92
(+)-BDF 8784	155 ± 28.4	0.92 ± 0.14	99	1080 ± 390	1.13 ± 0.08	95
(−)-BDF 8784	492 ± 140	0.58 ± 0.15	88	960 ± 280	0.81 ± 0.16	89

n = 4. Values are mean ± s.d. MI = maximal inhibition at 10 μM drug.

Table 4 Interaction of piperazinylindoles with Ca^{2+}-channel-linked phenylalkylamine receptors in rabbit skeletal muscle and guinea-pig brain cortex membranes labelled with (−)-[^{3}H]-desmethoxyverapamil

	Skeletal muscle			*Brain cortex*		
Drug	IC_{50} (nM)	*nH*	*MI* (%)	IC_{50} (nM)	*nH*	*MI* (%)
(−)-DPI 201-106	3480 ± 810	0.99 ± 0.19	75	346 ± 36	0.46 ± 0.08	96
(+)-DPI 201-106	1230 ± 330	0.98 ± 0.21	91	1280 ± 130	0.68 ± 0.09	97
(+)-BDF 8784	49.4 ± 8	1.41 ± 0.2	100	666 ± 135	1.1 ± 0.19	100
(−)-BDF 8784	1790 ± 110	0.89 ± 0.2	100	2840 ± 290	0.59 ± 0.10	100
(±)-BDF 8784	97.9 ± 26	0.97 ± 0.2	100		ND	

n = 3; values are means ± s.d. ND = not determined. MI = maximal inhibition at 10 μM drug.

Table 5 Interaction of piperazinylindoles with Ca^{2+}-channel-linked benzothiazepine receptors in rabbit skeletal muscle membranes labelled with (+)-*cis*-[^{3}H]-diltiazem

	30°C			4°C		
Drug	IC_{50} (nM)	*nH*	*MI* (%)	IC_{50} (nM)	*nH*	*MI* (%)
(−)-DPI 201-106	61.1 ± 11	0.72 ± 0.07	100	ND		
(+)-DPI 201-106	43.1 ± 15	0.80 ± 0.2	100	ND		
(+)-BDF 8784	56.5 ± 5.9	1.5 ± 0.2	100	24.5 ± 7	1.3 ± 0.2	100
(−)-BDF 8784	228 ± 46	1.0 ± 0.2	100	184 ± 26	0.96 ± 0.2	100
(+)-*cis*-Diltiazem	57 ± 13	0.9 ± 0.1	100	44 ± 8	1.0 ± 0.1	100

n = 4. Values are means ± s.d. MI = maximal inhibition at 10 μM drug. ND = not determined.

values and increases in apparent K_D values by the drug (results not shown).

Ca^{2+} antagonist effects of the BDF 8784 enantiomers

Having obtained evidence for enantioselective interactions of the BDF 8784 compounds with L-type Ca^{2+} channel-associated drug receptors we were prompted to study their effects in K^+ depolarized smooth muscle. Using taenia strips from guinea-pig caecum the following apparent pA_2 values ($n = 4$) were determined: (+)-BDF 8784, 6.99 ± 0.17 (slope 0.89 ± 0.07); (−)-BDF 8784, 6.33 ± 0.07 (slope 1.03 ± 0.04).

Discussion

Our interest in investigating the interaction of piperazinylindoles with *Drosophila* Ca^{2+}-channels, characterized by radiolabelled phenylalkylamines, originated from the observation that (+)-DPI 201-106 was 10 times more potent in inhibiting phenylalkylamine labelling of Ca^{2+}-channels in mammalian brain than in skeletal muscle membranes. Furthermore, having available DPI 201-106 derivatives (i.e. the BDF 8784 enantiomers) we realized that the postulate of a low affinity, nonspecific, local anaesthetic site (Siegl *et al.*, 1988) for the piperazinylindoles on L-type Ca^{2+}-channels could not be upheld. Replacement of the cyano group in DPI 201-106 by a methyl group dramatically increased the potency of (+)-DPI 201-106 to inhibit 1,4-dihydropyridine and phenylalkylamine binding in skeletal muscle whereas modest increases (1,4-dihydropyridine binding-inhibition in guinea-pig cerebral cortex membranes) or decreases (phenylalkylamine binding-inhibition in guinea-pig cerebral cortex membranes) were observed for the methyl-substituted (−)-DPI 201-106. The investigated piperazinylindoles are among the most potent agents to inhibit allosterically (+)-*cis*-[^{3}H]-diltiazem labelling of the skeletal muscle Ca^{2+}-channels. The BDF derivatives had eudismic ratios of 4 or 7, respectively, depending on temperature. An eudismic ratio of 4.5 was also established for Ca^{2+} antagonism in a well established functional assay system (Spedding, 1982). Taken together this pointed to a well defined domain on L-type Ca^{2+}-channels which discriminated between optical antipodes and displayed chemical specificity. The chemical specificity was unrelated to local anaesthetic properties which were similar or even weaker for the methyl-substituted DPI 201-106 enantiomers as shown by batrachotoxinin A 20-α-benzoate labelling of the voltage-dependent Na^+-channel. It should be noted that the piperazinylindoles as well as some other compounds listed in Table 1 exhibited very steep Hill slopes and higher IC_{50} values when diluted in a conventional manner (see Glossmann & Ferry, 1985) in plastic tubes with aqueous buffer. However, when drugs were dissolved and diluted in DMSO and glass tubes, inhibition-potency increased and Hill slopes decreased, exactly as previously described by Boer *et al.* (1989) for hydrophobic 1,4-dihydropyridines. This was strong evidence for partitioning of the drugs into plastic upon dilution in buffer, henceforth all experiments reported above were performed with the DMSO dilution protocol.

The discovery of a (13 pS) voltage-dependent Ca^{2+}-channel (Pelzer *et al.*, 1989) in *Drosophila* head membranes, where stereoselective phenylalkylamine receptors have higher affinity than observed for the mammalian L-type Ca^{2+}-channel (Pauron *et al.*, 1987; Greenberg *et al.*, 1989), prompted us to search for compounds which are allosteric regulators of phenylalkylamine binding to the mammalian L-type channel and display some preference for the neuronal subtype. As mentioned above, the piperazinylindoles were promising candidates and much to our surprise they were among the most potent drugs. It was beyond the scope of the present investigation to characterize the mechanism of inhibition (competitive vs allosteric) in *Drosophila* membranes. With the exception of the phenylalkylamines themselves, all drugs in Table 1 are allosteric inhibitors for the phenylalkylamine-selective domain of the L-type Ca^{2+}-channel. Thus the ratios in Table 1 are not ratios of true affinities. Nevertheless the data are consistent with the assumption that a drug receptor domain for piperazinylindoles which is coupled to a phenylalkylamine-selective domain is present on both mammalian L-type Ca^{2+}-channels and (with very high affinity or perhaps much tighter coupling) on (13 pS) *Drosophila* Ca^{2+}-channels. For the latter we could demonstrate that the DPI 201-106 enantiomers at very low concentration prevent photolabelling of a 135 KDa polypeptide. This polypeptide has a pharmacological profile (Pauron *et al.*, 1987; Greenberg *et al.*, 1989) which strongly suggests that it is a subunit of the reconstituted phenylalkylamine-sensitive 13 pS Ca^{2+}-channel (Pelzer *et al.*, 1989). We believe that this is further evidence for the structural relationship of mammalian L-type and the *Drosophila* Ca^{2+}-channels.

Interestingly, the only naturally occurring Ca^{2+} antagonist that we investigated, (+)-tetrandine, was a very potent inhibitor at the *Drosophila* phenylalkylamine receptor. This alkaloid is isolated from the Chinese medicinal plant *Stephania tetrandra* and we suggest that it could perhaps belong to the family of plant insecticides as do the pyrethroids. Heparin, on the other hand, which is a very potent allosteric regulator of Ca^{2+}-antagonist receptor labelling in skeletal muscle from carp or rabbit (Knaus *et al.*, 1990) was completely ineffective at the *Drosophila* receptor. This indicates that the heparin binding domain is either not present or not coupled to the phenylalkylamine receptors of *Drosophila* head Ca^{2+}-channels.

Beside its proven interaction with the voltage-dependent Na^+-channel, DPI 201-106 has well-documented (but weak) Ca^{2+}-channel blocking activity (Hof & Hof, 1985; Takahashi *et al.*, 1987; Siegl *et al.*, 1988; Holck & Osterrieder, 1988). Direct evidence for inhibition of reconstituted Ca^{2+}-channels in *Drosophila* brain membranes by DPI 201-106 is missing. However, we have documented stereospecific Ca^{2+} antagonist effects of the methylindole substituted DPI 201-106 derivatives. Our findings may give clues for development of novel insecticides, Ca^{2+}-channel blocking agents or new high affinity labels for Ca^{2+}-channels in *Drosophila* head membranes. In addition probes such as the piperazinylindoles may be useful to study the evolution of drug receptor domains on Ca^{2+}-channels.

The authors would like to thank their colleagues from other drug companies for supplying them with compounds. H.G. was supported by grants (S45-01, S45-02) from Fonds zur Förderung der wissenschaftlichen Forschung (Austria) and by a grant from Bundesministerium für Wissenschaft und Forschung (Austria).

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(Received July 6, 1990
Revised September 24, 1990
Accepted October 17, 1990)