Photoaffinity-labelling of the calcium-channel-associated 1,4-dihydropyridine and phenylalkylamine receptor in guinea-pig hippocampus

A 195 kDa polypeptide carries both drug receptors and has similarities to the α_1 subunit of the purified skeletal-muscle calcium channel

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This study identifies calcium-antagonist-receptor-carrying polypeptides of calcium channels in guinea-pig hippocampus membranes. The arylazide ligands (–)-[³H]azidopine and [*N*-methyl-³H]LU49888 {(–)-5-[(3-azidophenethyl)][*N*-methyl-³H]methylamino]-2-(3,4,5-trimethoxyphenyl)-2-isopropylvaleronitrile} were used to selectively label 1,4-dihydropyridine and phenylalkylamine receptors respectively. In the absence of u.v. light, both ligands reversibly bound to a single class of high-affinity receptors with a calcium-channel-typical pharmacological profile. [*N*-methyl-³H]LU49888 bound to the extent of 849 ± 188 fmol/mg of protein (mean $\pm$ S.D., $n = 3$) with a dissociation constant (K_d) of 1.4 ± 0.3 nM. Under identical assay conditions (–)-[³H]azidopine labelled to the extent of 562 ± 132 fmol/mg of protein with a K_d of 0.096 ± 0.024 nM. After u.v. irradiation of the [*N*-methyl-³H]LU49888- and (–)-[³H]azidopine-labelled membranes, both photoaffinity probes were found to be incorporated specifically into a 190–195 kDa band as shown by SDS/polyacrylamide-gel electrophoresis (SDS/PAGE). Photoincorporation occurred with a protection profile identical with that produced by reversible binding-inhibition. [*N*-methyl-³H]LU49888, but not (–)-[³H]azidopine, specifically labelled an additional 265 kDa band. Both photolabelled bands had an identical electrophoretic mobility on SDS/PAGE, irrespective of pretreatment either with 10 mM-*N*-ethylmaleimide or 10 mM-dithiothreitol. The electrophoretic properties of the 195 kDa polypeptide and the lability of receptor-incorporated (–)-[³H]azidopine to nucleophilic agents resemble those of the previously described drug-receptor-carrying α_1 subunit of the purified skeletal-muscle calcium channel. The data suggest that this polypeptide carries both the high-affinity 1,4-dihydropyridine as well as the phenylalkylamine receptor of neuronal calcium channels in guinea-pig hippocampus and is a component of the L-type calcium channel.

INTRODUCTION

To reveal the structure of voltage-sensitive calcium channels, calcium-antagonist drug receptors have been purified from guinea-pig and rabbit skeletal-muscle transverse-tubule (T-tubule) membranes (Curtis & Catterall, 1984; Borsotto *et al.*, 1985; Striessnig *et al.*, 1986; Flockerzi *et al.*, 1986; Leung *et al.*, 1987; Morton & Froehner, 1987; Nakayama *et al.*, 1987). Purified preparations consist of four major non-covalently associated polypeptides, termed α_1 -, α_2 -, β - and γ -subunit (Takahashi *et al.*, 1987; Vaghy *et al.*, 1987a), and form functional calcium channels after reconstitution into artificial membranes (Curtis & Catterall, 1986; Flockerzi *et al.*, 1986; Glossmann *et al.*, 1987a,b; Smith *et al.*, 1987). Photoaffinity-labelling and phosphorylation experiments have recently proven that the α_1 -subunit (155–170 kDa, apparent M_r not changed on reduction of disulphide bonds) carries the receptors for calcium-antagonist drugs and phosphorylation sites for the cyclic AMP-dependent protein kinase (Flockerzi *et al.*, 1986; Vaghy *et al.*,

1987a; Sieber *et al.*, 1987). The primary structure of this polypeptide has been deduced from the cDNA sequence (Tanabe *et al.*, 1987). It shows homology with the voltage-dependent sodium channel and might serve as a voltage sensor for excitation-contraction coupling as well as a calcium channel in skeletal-muscle T-tubules.

By contrast, the α_2 -polypeptide does not carry high-affinity 1,4-dihydropyridine (1,4-DHP) or phenylalkylamine receptors. It is heavily glycosylated and covalently attached to smaller (20–30 kDa) polypeptides via disulphide bridges, thus changing its apparent M_r upon reduction from 160–170 kDa to 135–145 kDa (Schmid *et al.*, 1986a,b; Vandaele *et al.*, 1987; Vaghy *et al.*, 1987a; Takahashi *et al.*, 1987a,b; Sieber *et al.*, 1987). The functional role of the α_2 , β (55–65 kDa) and γ -subunit (33 kDa) is not known.

Antibodies directed against the α -polypeptides from skeletal muscle immunoprecipitate reversible 1,4-DHP and phenylalkylamine binding from several tissues, including heart and brain (Schmid *et al.*, 1986a,b; Norman *et al.*, 1987; Takahashi & Catterall, 1987a,b).

Abbreviations used: B_{max} , maximal binding capacity; DHP, dihydropyridine; K_d , dissociation constant; K_i , inhibition constant; k_{+1} , association rate constant; k_{-1} , dissociation rate constant; [*N*-methyl-³H]LU49888, (–)-5-[(3-azidophenethyl)][*N*-methyl-³H]methylamino]-2-(3,4,5-trimethoxyphenyl)-2-isopropylvaleronitrile; PMSF, phenylmethanesulphonyl fluoride; PAGE, polyacrylamide-gel electrophoresis; T-tubule, transverse tubule; IC_{50} , concentration causing half-maximal inhibition; 1,4-DHP, 1,4-dihydropyridine.

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However, these studies were unable to demonstrate a polypeptide similar to the receptor carrying the α_1 -subunit of skeletal-muscle calcium channels in heart and brain. Instead, they identified polypeptides immunologically and structurally related to the α_2 subunit.

In the present study we used the photoaffinity-labelling technique to unequivocally identify the calcium-channel-associated 1,4-DHP- and phenylalkylamine-receptor-carrying polypeptides. We provide evidence that, in mammalian brain, these receptor domains are located on a 190–195 kDa polypeptide which does not change its M_r upon reduction and thus resembles the α_1 -subunit of the purified skeletal-muscle calcium channel.

EXPERIMENTAL

Materials

(-)-[^3H]Azidopine was synthesized by coupling (-)-1,4-dihydro-2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinecarboxylic acid 2-aminoethyl ethyl ester with [^3H]N-hydroxysuccinimidyl 4-azidobenzoate (36 Ci/mmol; Amersham International) as described by Ferry *et al.* (1984a). [N -methyl- ^3H]LU49888 (85 Ci/mmol) and the unlabelled enantiomers of desmethoxyverapamil and ($\pm$)-D619 were from Knoll A.G. (Ludwigshafen, Germany). The enantiomers of PN200-110 were from Sandoz A.G. (Basel, Switzerland), D- and L-*cis*-diltiazem were from Gödecke A.G. (Freiburg, Germany). Proteinase inhibitors and bovine serum albumin were purchased from Sigma (Munich, Germany). Econofluor and Protosol were from NEN, Vienna, Austria. Protein M_r standards for SDS/PAGE were obtained from Bio-Rad.

Reversible binding

Guinea-pig hippocampus membranes were prepared as described by Ferry *et al.* (1984b). For binding-inhibition studies, 0.2–0.4 nM(-)-[^3H]azidopine or 0.8–1.3 nM-[N -methyl- ^3H]LU49888 were incubated at 25 °C with membrane protein (0.045–0.1 mg/ml) for 90 min in 0.25–0.5 ml of assay buffer [50 mM-Tris/HCl (pH 7.4)/0.1 mM-PMSF]. Bound and free ligand were separated by filtration over GF/C Whatman glass-fibre filters as described by Glossmann & Ferry (1985). Non-specific binding was determined in the presence of 100 nM-(+)-PN200-110 or 1 μM -(-)-desmethoxyverapamil respectively. Non-specific binding was subtracted from total binding to yield specific binding. Binding-inhibition data from three experiments were fitted to the general dose-response equation (De Lean *et al.*, 1978) by non-linear methods. The K_d and B_{max} were calculated by linear-regression analysis after Scatchard transformation of saturation analysis data. Kinetic experiments were carried out as described (Glossmann & Ferry, 1985; Striessnig *et al.*, 1987). Dissociation was induced by blocking the forward reaction with 1 μM -(+)-PN200-110 or 1 μM -(-)-desmethoxyverapamil respectively after equilibrium was reached. k_{+1} was calculated by using the second-order rate equation as described by Striessnig *et al.* (1985). k_{-1} was calculated by plotting $\ln[(\text{ratio of specifically bound ligand at various times after starting the addition})/(\text{specifically bound ligand at equilibrium})]$ against time. k_{-1} is the negative slope of the regression line.

Data for equilibrium binding and kinetic constants are given as means $\pm$ S.D. for at least three experiments.

Photoaffinity-labelling experiments

Guinea-pig membranes (0.5–1.2 mg) were incubated in assay buffer at 25 °C with 0.26–0.42 nM(-)-[^3H]azidopine or 3.5–4.8 nM-[N -methyl- ^3H]LU49888 in the absence (total binding) or presence (non-specific binding) of 100 nM-(+)-PN200-110 or 1 μM -(-)-desmethoxyverapamil respectively in a total volume of 20 ml. After 90 min of incubation, 0.2 ml aliquots were assayed for reversible binding. The labelled membranes were centrifuged at 40000 *g* (4 °C) for 10 min and resuspended in 6 ml of ice-cold assay buffer. The suspension was irradiated for 30 min with u.v. light on ice as described by Striessnig *et al.* (1987). The membranes were collected by centrifugation and resuspended in 0.05 ml of assay buffer containing proteinase inhibitors [5 mM-EDTA, 0.5 mM-PMSF, benzamidine (0.1 mg/ml), leupeptin (0.1 mg/ml), Trasylol (0.02 mg/ml), 10 μM -pepstatin A, soya-bean trypsin inhibitor (0.5 mg/ml)]. After 15 min the membranes were solubilized for SDS/PAGE as described by Ferry *et al.* (1985) in the presence of a final concentration of 10 mM-*N*-ethylmaleimide (alkylating conditions) or 10 mM-dithiothreitol (reducing conditions). High- and low- M_r marker proteins were run on separate lanes. The Coomassie Blue-stained gel was cut into 3 mm slices. A portion (3.5 ml) of 3% (v/v) Protosol in Econofluor was added, heated for 12 h at 55 °C and counted for radioactivity after 36 h with a counting efficiency of 44%. M_r values were calculated by linear interpolation (Ferry *et al.*, 1985). Protein concentrations were determined by using the Lowry *et al.* (1951) assay. BSA was used as the standard.

Precipitation of purified (-)-[^3H]azidopine-photolabelled 1,4-DHP receptors

Calcium channels from guinea-pig skeletal muscle were purified and photolabelled with (-)-[^3H]azidopine as described by Striessnig *et al.* (1986). Purified polypeptides were precipitated with chloroform/methanol in the presence of carrier protein (0.1 mg of bovine serum albumin) as described by Wessel & Flügge (1984). The final pellet was dried and was counted for radioactivity after solubilization in 10% (w/v) SDS.

Antibody production and radioimmunoassay

For antibody production, New Zealand White rabbits were immunized along the back with 1,4-dihydro-2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinecarboxylic acid 2-aminoethyl ethyl ester covalently coupled to human serum albumin at a molar ratio of 0.7:1 with carbodi-imide. Binding of (-)-[^3H]azidopine to the antiserum (pa 8885/7) was determined as follows. (-)-[^3H]Azidopine at 0.2–2.0 nM was incubated with antiserum (final dilution 1:1600) for 120 min at ambient temperature (or at 4 °C overnight) in 1 ml of TBS [20 mM-Tris/HCl (pH 7.4)/150 mM-NaCl]. Free (-)-[^3H]azidopine was separated from antiserum-bound ligand by the addition of 0.05 ml of a dextran-coated charcoal suspension (1 g/100 ml in TBS; 25 mg of dextran/g of charcoal), followed by centrifugation. The remaining radioactivity in the supernatant was determined by liquid-scintillation counting. For saturation studies the specific radioactivity of (-)-[^3H]azidopine was decreased by the addition of increasing concentrations of unlabelled azidopine.

Photoaffinity-labelling of anti-(–)-[³H]azidopine immunoglobulins

Immunoglobulins from 1 ml of immune serum were precipitated with (NH₄)₂SO₄, dialysed, and resuspended in 1 ml of TBS. A portion (0.05 ml) of immunoglobulins was incubated with 0.4–0.6 nM-(–)-[³H]azidopine in the absence (total binding and incorporation) or presence (non-specific binding and incorporation) of 10 μM-(+)-PN200-110 as described above and irradiated for 30 min with u.v. light. Preimmune serum was carried through the whole procedure and used as a control. The samples were freeze-dried and separated on 5–15 %-(w/v)-polyacrylamide gradient gels in the presence of 10 mM-*N*-ethylmaleimide or 10 mM-dithiothreitol in the sample buffer. The incorporated radioactivity was revealed by fluorography (Striessnig *et al.*, 1986) or quantified by liquid-scintillation counting of 3 mm slices.

RESULTS

Reversible binding of [*N*-methyl-³H]LU49888

The arylazido phenylalkylamine derivative [*N*-methyl-³H]LU49888 binds reversibly to guinea-pig hippocampus membranes as previously shown for the structurally related (–)-[³H]desmethoxyverapamil (Ferry *et al.*, 1985). At 25 °C the association constant (k_{+1}) for [*N*-methyl-³H]LU49888 was $3.03 \pm 1.0 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$ (Fig. 1a). The ligand dissociated slowly at 4 °C, the temperature used throughout the photoaffinity-labelling procedure. Dissociation of the [*N*-methyl-³H]LU49888 receptor complex [induced by adding a saturating concentration of unlabelled (–)-desmethoxyverapamil (1 μM) to the incubation mixture after equilibrium was reached] followed a first-order decay, with a half-life of 60 min ($k_{-1} 0.012 \pm 0.0004 \text{ min}^{-1}$) (Fig. 1b). Saturation-

Fig. 1. Kinetic and saturation studies with (–)-[³H]azidopine and [*N*-methyl-³H]LU49888 in guinea-pig hippocampus membranes

Association and dissociation kinetics are shown for the temperatures employed in the photoaffinity labelling experiments (25 °C for association, 4 °C during washing and irradiation). (a) Association kinetics of [*N*-methyl-³H]LU49888. A 0.063 mg portion of membrane protein and 1.2 nM-[*N*-methyl-³H]LU49888 were employed; $k_{+1} = 3.74 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$. (b) Dissociation of [*N*-methyl-³H]LU49888. Dissociation of the [*N*-methyl-³H]LU49888–receptor complex at equilibrium was induced by addition of 1 μM-(–)-desmethoxyverapamil at zero time; $k_{-1} = 0.011 \text{ min}^{-1}$. B_t is specifically bound ligand at time t ; B_e is specifically bound ligand at equilibrium. (c) Scatchard transformation of a saturation experiment with [*N*-methyl-³H]LU49888. Incubation of membrane protein (0.1 mg/ml) with [*N*-methyl-³H]LU49888 (0.246–10.5 nM) was carried out for 60 min at 25 °C; $K_d = 1.63 \text{ nM}$; $B_{\text{max}} = 108.9$ (1.02 pmol/mg of protein). (d) Association kinetics of (–)-[³H]azidopine (25 °C). A 0.068 mg portion of membrane protein and 0.94 nM-(–)-[³H]azidopine were employed; $k_{+1} = 1.41 \times 10^8 \text{ M}^{-1} \cdot \text{min}^{-1}$. (e) Dissociation kinetics of (–)-[³H]azidopine (37 °C). Dissociation of the (–)-[³H]azidopine–receptor complex was induced by addition of a final concentration of 1 μM-(+)-PN200-110 to the incubation mixture at zero time; $k_{-1} = 0.011 \text{ min}^{-1}$. (f) Scatchard transformation of a saturation experiment with (–)-[³H]azidopine. Incubation of membrane protein (0.106 mg/ml) with (–)-[³H]azidopine (0.055–2.3 nM) was carried out for 90 min at 25 °C; $K_d = 0.071 \text{ nM}$; $B_{\text{max}} = 69.7 \text{ pM}$ (0.655 pmol/mg of protein).

Table 1. Stereospecific regulation of reversible [*N*-methyl-³H]LU49888 and (–)-[³H]azidopine binding to guinea-pig hippocampus membranes by different classes of calcium antagonist drugs

A portion (0.045–0.1 mg/ml) of membrane protein was incubated at 25 °C with 0.2–0.4 nM (–)-[³H]azidopine or 0.8–1.3 nM [*N*-methyl-³H]LU49888 in the absence or presence of other calcium antagonists. Binding–inhibition data from three experiments were computer-fitted to the general dose–response equation. The following parameters are given $\pm$ asymptotic s.d.: *h*, apparent Hill coefficient; maximal inhibition {defined as $[(1 - B)/B_0] \times 100$, where B_0 is control binding and B is binding in the presence of the highest drug concentration}. K_i values in case of competitive inhibition (indicated by *) were calculated as described by Linden (1982). Abbreviation: n.e., no effect up to the concentration indicated.

Ligand or drug	IC ₅₀ or K_i (*) (nM)	<i>h</i>	Maximal inhibition (%)
[<i>N</i>-methyl-³H]LU49888			
+ (+)-PN200-110	1.2 $\pm$ 0.3	> 1.3	50.8 $\pm$ 2
+ (–)-PN200-110	11.5 $\pm$ 4.3	0.75 $\pm$ 0.17	56.9 $\pm$ 4
+ (–)-D888	3.4 $\pm$ 0.7*	0.89 $\pm$ 0.14	100 $\pm$ 4
+ (+)-D888	48.3 $\pm$ 9.9*	0.76 $\pm$ 0.11	100 $\pm$ 4
+ (±)-D619	5388 $\pm$ 1600*	0.80 $\pm$ 0.17	100 $\pm$ 7
+ D- <i>cis</i> -Diltiazem	132.9 $\pm$ 11.8	> 1.3	70.9 $\pm$ 2
+ L- <i>cis</i> -Diltiazem	1001.1 $\pm$ 185	0.69 $\pm$ 0.08	100 $\pm$ 3
(–)-[³H]Azidopine			
+ (+)-PN200-110	0.071 $\pm$ 0.02*	0.82 $\pm$ 0.14	100 $\pm$ 6
+ (–)-PN200-110	21.3 $\pm$ 6.8*	0.91 $\pm$ 0.22	100 $\pm$ 7
+ (–)-D888	5.7 $\pm$ 1.2	0.88 $\pm$ 0.14	76.4 $\pm$ 3
+ (+)-D888	109.1 $\pm$ 46.7	> 1.3	27.0 $\pm$ 2
+ (±)-D619	211.2 $\pm$ 167	0.59 $\pm$ 0.22	32.2 $\pm$ 5
+ D- <i>cis</i> -Diltiazem	420 $\pm$ 128	0.94 $\pm$ 0.23	50.9 $\pm$ 2
+ L- <i>cis</i> -Diltiazem	n.e. (10 μ M)	–	–

binding isotherms (at 25 °C) were monophasic, with a K_d of 1.4 ± 0.3 nM and a B_{max} of 849 ± 188 fmol/mg of hippocampus membrane protein (Fig. 1c). Binding of the radioligand was modulated by all three classes of calcium-antagonist drugs (see Table 1) with a pharmacological profile characteristic of calcium-channel-associated phenylalkylamine receptors. The rank order of potency for competitive inhibitors was (–)-desmethoxyverapamil > (+)-desmethoxyverapamil $\gg$ (±)-D619, a result in good accord with their calcium-antagonistic activity (Mannhold, 1978) or the ability of these phenylalkylamines to inhibit (–)-[³H]desmethoxyverapamil binding (Ferry *et al.*, 1984b).

Reversible binding of (–)-[³H]azidopine

In the absence of u.v. light the arylazide photoaffinity ligand (–)-[³H]azidopine labelled a single class of receptor sites in hippocampus membranes with high affinity. At receptor and radioligand concentrations similar to those employed in photoaffinity experiments, binding equilibrium was reached after 60–80 min of incubation at 25 °C (Fig. 1d). The k_{+1} was $1.4 \pm 0.01 \times 10^8$ M^{–1}·min^{–1}. The (–)-[³H]azidopine–receptor complex showed no significant dissociation at 4 °C within 4 h. However, binding of (–)-[³H]azidopine was reversible, and a monophasic dissociation (Fig. 1e) with a k_{-1} of 0.010 ± 0.002 min^{–1} was observed at incubation temperature of 37 °C. In saturation experiments (Fig. 1f) (–)-[³H]azidopine labelled 562 ± 132 fmol/mg of membrane protein with a K_d of 0.096 ± 0.024 nM. The density of (–)-[³H]azidopine-labelled sites is similar to the density of sites labelled by (+)-[³H]PN200-110 (B_{max} = 390 ± 123 fmol/mg of protein; K_d = 0.075 ± 0.008 nM, $n = 3$) at 37 °C in the same preparation. Binding–

inhibition studies with all three classes of calcium-antagonist drugs revealed a calcium-channel-typical pharmacological profile (Table 1).

Photoaffinity labelling with [*N*-methyl-³H]LU49888

Fig. 2 shows that photoincorporation of [*N*-methyl-³H]LU49888 occurs into several protein bands [apparent molecular masses 265 ± 19 , 195 ± 9 , 95 ± 4 , 60 ± 7 and 35 ± 5 kDa (mean $\pm$ s.d., $n = 5$)]. The apparent M_r of these labelled bands was the same under both alkylating and reducing conditions of SDS/PAGE. In some experiments the 265 kDa band was only poorly resolved from the 195 kDa band, resulting in a broad peak in the high- M_r region (see, e.g., Fig. 2b). The overall recovery of incorporated label was similar, or sometimes slightly higher, when samples were solubilized in the presence of dithiothreitol before electrophoresis. Only the incorporation into the 265 and 195 kDa bands was specific, as it was not observed in the presence of 1 μ M (–)-desmethoxyverapamil. The labelling efficiency [% of specifically incorporated radioactivity (d.p.m.) compared with the specifically bound radioactivity (d.p.m.) before photoaffinity labelling] was $1.7 \pm 0.2\%$ (mean $\pm$ s.d., $n = 5$). The specificity of the photoaffinity labelling was investigated by including either the potent phenylalkylamine (–)-desmethoxyverapamil ($K_i = 3.4$ nM) at 300 nM or the > 1000-fold-less-active phenylalkylamine (±)-D619 ($K_i = 5.4$ μ M; see Table 1) at 300 nM in the incubation mixture before photolysis (Fig. 3). In agreement with reversible binding, 300 nM (–)-desmethoxyverapamil, but not 300 nM (±)-D619, protected incorporation into the 265 and 195 kDa bands. Non-specific incorporation into the lower- M_r bands was unaffected.

Fig. 2. Photoaffinity labelling of guinea-pig hippocampus membranes with $[N\text{-methyl-}^3\text{H}]\text{LU49888}$

A 0.93 mg portion of membrane protein was incubated for 90 min at 25 °C with 3.8 nM $[N\text{-methyl-}^3\text{H}]\text{LU49888}$ and photoaffinity-labelled as described in the Materials and methods section. Before photolysis 409 075 d.p.m. were bound in the absence (●), and 269 450 d.p.m. in the presence (○), of 1 μM $(-)\text{-desmethoxyverapamil}$. Reversibly bound label was determined by filtration of 0.2 ml aliquots of the incubation mixture over GF/C filters as described in the Materials and methods section. Means from duplicate determinations varying less than 10% are given (see also the legends to Figs. 3 and 4). Separation of the labelled bands in SDS/PAGE was carried out either under (a) alkylating (10 mM N -ethylmaleimide in the sample buffer) or (b) reducing conditions (10 mM-dithiothreitol).

Photoaffinity labelling with $(-)\text{-}^3\text{H}$ azidopine

In order to investigate whether one of the specifically $[N\text{-methyl-}^3\text{H}]\text{LU49888}$ -labelled bands also carries the receptor for 1,4-DHPs in hippocampus membranes, $(-)\text{-}^3\text{H}$ azidopine was employed as a 1,4-DHP-receptor-selective photoaffinity probe. After irradiation the $(-)\text{-}^3\text{H}$ azidopine-labelled membranes were solubilized in sample buffer containing 10 mM N -ethylmaleimide (Fig. 4a) and separated in 5–15%-(w/v)-polyacrylamide gradient gels. In contrast with $[N\text{-methyl-}^3\text{H}]\text{LU49888}$, only one band, with an apparent M_r of 191 ± 20 kDa, was labelled specifically. Non-specific incorporation occurred into 95 ± 7 kDa, 56 ± 10 kDa and 33 ± 2 kDa (mean $\pm$ S.D., $n = 5$) bands. The amount of specific photo-incorporation into the 191 kDa band and the labelling efficiency (0.4–2%) for $(-)\text{-}^3\text{H}$ azidopine showed higher inter-experimental variation compared with that for $[N\text{-methyl-}^3\text{H}]\text{LU49888}$.

The protection profile of $(-)\text{-}^3\text{H}$ azidopine photo-labelling was also investigated. Incorporation was completely prevented by 3 nM $(+)\text{-PN200-110}$, but not by

Fig. 3. Pharmacological specificity of $[N\text{-methyl-}^3\text{H}]\text{LU49888}$ photoaffinity labelling

A 0.7 mg portion of membrane protein was incubated for 90 min at room temperature with 3.6 nM of $[N\text{-methyl-}^3\text{H}]\text{LU49888}$ in the absence (a) or presence (b and c) of the indicated concentrations of phenylalkylamines and photolysed as described in the text. Under these conditions reversible binding of $[N\text{-methyl-}^3\text{H}]\text{LU49888}$ was 264 400 d.p.m. (control), 140 400 d.p.m. [300 nM $(-)\text{-desmethoxyverapamil}$ present] and 274 000 d.p.m. [300 nM $(\pm)\text{-D619}$ present] before photolysis. Separation was carried out under alkylating conditions of SDS/PAGE. The results of one out of two similar experiments are shown.

a 3 nM concentration of the 150-fold-less-potent $(-)\text{-enantiomer}$. Increasing the concentration of $(-)\text{-PN200-110}$ to 1 μM also completely protected labelling of the band, in accordance with the reversible-binding experiments (Table 1). The electrophoretic mobility of the specifically labelled band in the 195 kDa region was identical when the $(-)\text{-}^3\text{H}$ azidopine- and $[N\text{-methyl-}^3\text{H}]\text{LU49888}$ -photoaffinity-labelled membranes were separated on parallel lanes in SDS/PAGE. Thus the small difference (191 as against 195 kDa; mean for five experiments) for $(-)\text{-}^3\text{H}$ azidopine and $[N\text{-methyl-}^3\text{H}]\text{LU49888}$ labelling of this band is not significant and is due to variations in gel calibration.

Specific but not non-specific photoaffinity labelling by $(-)\text{-}^3\text{H}$ azidopine was sensitive to dithiothreitol upon solubilization for SDS/PAGE (Fig. 4b), in clear contrast with $[N\text{-methyl-}^3\text{H}]\text{LU49888}$ labelling (Fig. 2). The remaining, apparently dithiothreitol-insensitive, specifically incorporated azidopine label was < 10% compared with samples treated with 10 mM N -ethylmaleimide (Fig. 4). This sensitivity to dithiothreitol has been observed for $(-)\text{-}^3\text{H}$ azidopine photolabelled membrane-bound (Ferry *et al.*, 1985) and purified 1,4-DHP receptors (Vaghy *et al.*, 1987a) from skeletal muscle. We further characterized this phenomenon by employing

Fig. 4. Photoaffinity labelling of guinea-pig hippocampus membranes with (–)-[³H]azidopine

A 0.93 mg portion of membrane protein was incubated for 90 min at 25 °C with 0.5 nM (–)-[³H]azidopine and photolysed as described in the text. The result of separation of the labelled polypeptides under alkylating (a) as well as reducing (b) conditions is shown. Before photolysis 66475 ± 694 d.p.m. of (–)-[³H]azidopine were reversibly bound in the absence (●), and 25550 ± 2428 d.p.m. in the presence (○) of 100 nM-(+)-PN200-110.

purified skeletal-muscle calcium channels as a rich source of labelled 1,4-DHP receptors and a polyclonal anti-serum recognizing (–)-[³H]azidopine with high affinity.

Fig. 5(a) illustrates the effect of increasing concentrations of dithiothreitol on purified and (–)-[³H]azidopine photoaffinity-labelled 1,4-DHP receptors from guinea-pig skeletal muscle. A portion (1–2 µg; 0.1 ml) of (–)-[³H]azidopine-photoaffinity-labelled 1,4-DHP-receptor protein was preincubated with either 10 mM-*N*-ethylmaleimide (control) or increasing concentrations of dithiothreitol for 15 min at 57 °C and then precipitated with chloroform/methanol. Analysis of the same sample in alkylating SDS/PAGE followed by gel slicing revealed the incorporation of (–)-[³H]azidopine into a single 155 kDa polypeptide. Photoincorporated (–)-[³H]azidopine is firmly bound, since it remains associated with the receptor-carrying polypeptide during SDS/PAGE and upon heating to 95 °C, and cannot be removed by treatment with organic solvents (upon precipitation with chloroform/methanol, impregnation for fluorography, staining procedures) or precipitation by 15% (w/v) trichloroacetic acid. Increasing concentrations of dithiothreitol in the incubation buffer before precipitation caused a dose-dependent release of the incorporated label into the supernatant. In all, 84–86% (*n* = 3) of the specific binding was lost at the highest dithiothreitol concentration (10 mM), the effect being half-maximal at 0.1 mM (Fig. 5a). Heat treatment during preincubation (15 min at 95 °C) did not affect the results. β-Mercaptoethanol at 0.5 M causes loss of label (86%) similar to that produced by dithiothreitol; 0.2 M-

hydroxylamine, a nucleophile without reducing properties, also releases photolabel (27% loss of specific binding). Therefore nucleophilic attack rather than reduction of disulphide bonds seems to be important for removal. Even prolonged incubation (e.g. 60 min) at high (> 10) or low pH (1.0) had no effect whatsoever on the stability of the incorporated label (results not shown). In reducing SDS/PAGE, dithiothreitol caused a similar decrease of the recovery of total incorporated (–)-[³H]azidopine from the 155 kDa polypeptide (alkylating: 3572 ± 297 d.p.m.; reducing: 394 ± 250 d.p.m.; means ± S.D., *n* = 3) as in precipitation experiments.

To test whether the nucleophile-sensitive bond is also formed between photoactivated (–)-[³H]azidopine and other high-affinity binding sites for this ligand, antibodies which bound azidopine with nanomolar *K_d* values were photolabelled. The immunoglobulins specifically bound (–)-[³H]azidopine with a *K_d* of 19 nM (mean for two experiments whose results differed by less than 10%) in the absence of u.v. light. Other 1,4-DHPs (e.g. (+)-PN200-110 [*IC*₅₀ > 250 nM], nifedipine [*IC*₅₀ > 3 µM]) were of lower affinity. Preimmune serum drawn before immunization with the albumin-coupled 1,4-DHP did not specifically recognize (–)-[³H]azidopine or other radiolabelled 1,4-DHPs (results not shown). Fig. 5(b) shows that (–)-[³H]azidopine specifically photoincorporates into 885/7 immunoglobulins, labelling both the heavy and light chains. Reduction by dithiothreitol (or exposure to other nucleophilic agents; results not shown) does not lead to a loss of photoincorporated radioactivity. This was verified in parallel experiments, the incorporated label being quantified by gel slicing (see the legend to Fig. 5b).

DISCUSSION

In the present paper we introduce the photoaffinity ligands [*N*-methyl-³H]LU49888 and (–)-[³H]azidopine as highly specific probes to label reversibly and irreversibly calcium-channel-associated 1,4-DHP and phenylalkylamine receptors in mammalian brain. In previous studies, both labels were extremely valuable tools to identify the receptor-carrying channel polypeptide in skeletal-muscle T-tubule and heart membranes (Ferry *et al.*, 1984a,b, 1985, 1987; Glossmann *et al.*, 1987b; Striessnig *et al.*, 1987) and purified skeletal-muscle preparations (Striessnig *et al.*, 1986, 1987; Vaghy *et al.*, 1987a; Takahashi *et al.*, 1987). Despite a more than 25–100-fold lower density of receptor sites for 1,4-DHPs and phenylalkylamines in hippocampus (Ferry *et al.*, 1984b; Reynolds *et al.*, 1986) compared with purified skeletal-muscle T-tubule membranes (Gallizzi *et al.*, 1984, 1986; Goll *et al.*, 1984; Vaghy *et al.*, 1987b), we were successful with both (–)-[³H]azidopine and [*N*-methyl-³H]LU49888 in specifically photolabelling a 190–195 kDa polypeptide. The apparent *M_r* on SDS/PAGE is somewhat higher than those of specifically labelled bands in skeletal muscle (155–170 kDa) or heart (165 kDa). Analysis of the electrophoretic behaviour of the 190–195 kDa band clearly demonstrates that it is not linked to lower-*M_r* polypeptides via disulphide bridges, as it does not change its *M_r* analysed under reducing conditions of SDS/PAGE. Thus our data do not support results from immunochemical studies, which concluded that a polypeptide with the

Fig. 5. Effect of reduction on specific (—) $[^3\text{H}]\text{azidopine}$ photoaffinity labelling of purified guinea-pig skeletal-muscle 1,4 DHP receptors or anti-1,4-DHP immunoglobulin pa 885/7

(a) 1,4-DHP receptor purified from guinea-pig skeletal muscle was photolabelled with (—) $[^3\text{H}]\text{azidopine}$ in the absence (●, total incorporation) or presence (○, non-specific incorporation) of $1 \mu\text{M}$ -(+)-PN200-110. A 0.1 ml portion of the labelled protein (1–2 μg) was preincubated for 15 min at 57°C with 10 mM-*N*-ethylmaleimide (NEM) (control) or increasing concentrations of dithiothreitol (DTT) and precipitated with chloroform/methanol in the presence of carrier protein. The radioactivity recovered in the pellet is plotted against the final concentration of dithiothreitol in the preincubation mixture. Each point is the mean of duplicate determinations varying less than 10%. (b) Anti-1,4-DHP immunoglobulins were photoaffinity-labelled as described in the Materials and methods section and separated on SDS/5–15%-(w/v)-polyacrylamide gradient gels under both alkylating and reducing conditions. An autoradiograph from one out of two identical experiments is shown. The migration of non-reduced control γ -globulin (160 kDa) and of the heavy (54 kDa) and light (25 kDa) chains after reduction is indicated. A separate experiment was used for quantification of the amount of (—) $[^3\text{H}]\text{azidopine}$ recovered in the gels before and after reduction by gel slicing (3 mm/slice); 3239 d.p.m. (100%) were incorporated into the 160 kDa band (alkylating conditions). After reduction 89% of the radioactivity was found in the 54 kDa band (1513 d.p.m., 47%) and 25 kDa band (1348, 42%) respectively. T, total incorporation; NS, non-specific incorporation in the presence of $10 \mu\text{M}$ -(+)-PN200-110.

characteristics of the α_2 -subunit (see the Introduction) of the purified skeletal-muscle calcium channel carries the 1,4-DHP receptor. Such a polypeptide was identified by immunochemical methods in several electrically excitable tissues known to possess 1,4-DHP-sensitive voltage-dependent calcium channels (Schmid *et al.*, 1986a,b; Norman *et al.*, 1987; Takahashi & Catterall, 1987a,b). Our photoaffinity-labelling studies in skeletal muscle and, as shown here, for brain, rule out the possibility that it functions as a high-affinity drug receptor site for calcium-channel drugs in these tissues. However, as it is co-distributed with calcium-antagonist (e.g. 1,4-DHP)-binding sites in mammalian hippocampus and cerebral cortex (Schmid *et al.*, 1987a), it could be closely associated with the drug-receptor-carrying polypeptide, as was shown for skeletal muscle (Takahashi *et al.*, 1987a,b; Vaghy *et al.*, 1987a).

The drug receptors for the different chemical classes of calcium-channel-active drugs and their reciprocal allosteric coupling mechanisms are similar in skeletal muscle and brain, as revealed by radioligand binding studies (Glossmann *et al.*, 1985; Glossmann & Ferry, 1985). Radiation-inactivation experiments also suggest an identical target size for the 1,4-DHP receptor in both tissues (Norman *et al.*, 1983; Glossmann *et al.*, 1985; Glossmann & Ferry, 1985). Our results with (—) $[^3\text{H}]\text{azidopine}$ photoaffinity labelling also indicate that the 1,4-DHP-binding domain of the skeletal-muscle and

brain calcium channel share another important structural feature: channel-specific incorporation of (—) $[^3\text{H}]\text{azidopine}$ in brain and skeletal muscle involves to a major extent the formation of covalent bonds which are sensitive to nucleophilic agents. This leads to a significant loss of (calcium-channel) attached label when SDS/PAGE is carried out under reducing conditions (Ferry *et al.*, 1985; Vaghy *et al.*, 1987a). As neither non-specific incorporation of (—) $[^3\text{H}]\text{azidopine}$ nor photolabelling of specific immunoglobulins was sensitive to nucleophilic attack, we believe that this feature presents additional biochemical evidence of having photoaffinity-labelled a receptor-carrying polypeptide in brain which is similar to the α_1 -subunit in skeletal muscle. Sensitivity to nucleophilic attack of covalently attached photolabels derived from arylazides has been observed previously (Abercrombie *et al.*, 1982; Mas *et al.*, 1980). At present there is limited knowledge about the nature of bonds involved [see Mas *et al.* (1980) and Nielsen & Burchardt (1982) for a general discussion], but at least in one case a tyrosine residue was identified as the photoaffinity-labelled amino acid (Abercrombie *et al.*, 1982). Notably, the incorporated phenylalkylamine was completely resistant to nucleophiles in both skeletal muscle (Striessnig *et al.*, 1987) and brain.

With [*N*-methyl- ^3H]LU49888 a 265 kDa polypeptide was specifically photolabelled which was not found in skeletal muscle (Striessnig *et al.*, 1987) and is not

recognized by $(-)-[{}^3\text{H}]\text{azidopine}$ in brain. Photolabelling of this polypeptide was blocked by other phenylalkylamines in a calcium-channel-typical profile. The additional labelling of this previously unrecognized high-affinity phenylalkylamine receptor could explain the higher B_{max} values always obtained with reversible phenylalkylamine binding [$[N\text{-methyl-}{}^3\text{H}]\text{LU49888}$: $B_{\text{max}} = 849 \text{ fmol/mg}$ of membrane protein; $(-)-[{}^3\text{H}]\text{-desmethoxyverapamil}$: $B_{\text{max}} = 870 \text{ fmol/mg}$ of protein; see Ferry *et al.*, 1984b] as compared with 1,4-DHP binding [$(-)-[{}^3\text{H}]\text{azidopine}$: $B_{\text{max}} = 560 \text{ fmol/mg}$ of protein; $(+)-[{}^3\text{H}]\text{PN200-110}$: $B_{\text{max}} = 390 \text{ fmol/mg}$ of protein].

The existence of different species of voltage-dependent calcium channels is the most simple explanation for the 265 kDa polypeptide photoaffinity-labelled by [$N\text{-methyl-}{}^3\text{H}]\text{LU49888}$. Indeed, by electrophysiological techniques at least two different types of voltage-dependent calcium channels, both of which are sensitive to phenylalkylamines, were identified in mammalian central neurons (Yaari *et al.*, 1986). One of them exhibited several properties of L-type calcium channels [activation at high voltage (-30 to -20 mV), slowly inactivating, time-dependent 'run-down' of activity, selectivity for Ba^{2+} over Ca^{2+}], the second current those of T-type calcium channels [activation at low voltage ($< -50 \text{ mV}$), no 'run-down' of activity, fast inactivation]. Both currents were inhibited by $100 \mu\text{M}$ -verapamil to a similar extent. As only L-type calcium channels are sensitive to 1,4-DHPs, the 265 kDa polypeptide labelled by [$N\text{-methyl-}{}^3\text{H}]\text{LU49888}$ could be part of a T-type calcium channel.

Molecular-biological techniques may provide more information about the existence of structurally distinct calcium-channel proteins as shown, e.g., for the voltage-dependent sodium channel. Distinct sodium-channel species in excitable tissues were first postulated from reversible binding studies (e.g. in adult rat cardiac muscle; Lombet *et al.*, 1981, 1983), and recently the existence of distinct sodium-channel mRNAs in rat brain has been described (Noda *et al.*, 1986). On the other hand, non-calcium-channel-related high-affinity phenylalkylamine receptors may be located on the 265 kDa polypeptide.

Taken together our experiments unequivocally identify a 190–195 kDa polypeptide in brain which carries both 1,4-DHP and phenylalkylamine receptors. The co-existence of both drug-receptor domains on the same polypeptide is convincing evidence that it is part of an L-type calcium channel. The relationship of the 265 kDa polypeptide (labelled exclusively by [$N\text{-methyl-}{}^3\text{H}]\text{LU49888}$) to neuronal calcium channels remains to be investigated.

We thank Dr. M. Holtzhauer and Dr. F. Fahrenholtz for stimulating discussions, Ms. C. Eiter, Ms. A. Koza, Ms. H. Fluch and Mr. S. Daubenmerkl for competent help in the laboratory, and Mr. C. Trawöger for preparing the graphs. This work was supported by the Fonds zur Förderung der wissenschaftlichen Forschung, Deutsche Forschungsgemeinschaft and the Dr. Legerlotz Foundation.

REFERENCES

Abercrombie, D. M., McCormick, W. M. & Chaiken, I. M. (1982) *J. Biol. Chem.* **257**, 2274–2281

- Borsotto, M., Barhanin, J., Fosset, M. & Lazdunski, M. (1985) *J. Biol. Chem.* **260**, 14255–14263
- Curtis, B. M. & Catterall, W. A. (1984) *Biochemistry* **23**, 2113–2118
- Curtis, B. M. & Catterall, W. A. (1986) *Biochemistry* **25**, 3077–3083
- De Lean, A., Munson, P. J. & Rodbard, D. (1978) *Am. J. Physiol.* **4**, E97–E102
- Ferry, D. R., Rombusch, M., Goll, A. & Glossmann, H. (1984a) *FEBS Lett.* **169**, 112–118
- Ferry, D. R., Goll, A., Gadow, C. & Glossmann, H. (1984b) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **327**, 183–187
- Ferry, D. R., Kämpf, K., Goll, A. & Glossmann, H. (1985) *EMBO J.* **4**, 1933–1940
- Ferry, D. R., Goll, A. & Glossmann, H. (1987) *Biochem. J.* **243**, 127–135
- Flockerzi, V., Oeken, H.-J., Hofmann, F., Pelzer, D., Cavalie, A. & Trautwein, W. (1986) *Nature (London)* **323**, 66–68
- Gallizzi, J.-P., Fosset, M. & Lazdunski, M. (1984) *Eur. J. Biochem.* **144**, 211–215
- Gallizzi, J.-P., Borsotto, M., Barhanin, J., Fosset, M. & Lazdunski, M. (1986) *J. Biol. Chem.* **261**, 1393–1397
- Glossmann, H. & Ferry, D. R. (1985) *Methods Enzymol.* **109**, 521–550
- Glossmann, H., Ferry, D. R., Goll, A., Striessnig, J. & Zernig, G. (1985) *Drug Res.* **35**, 1917–1935
- Glossmann, H., Striessnig, J., Hymel, L. & Schindler, H. (1987a) *Ann. N.Y. Acad. Sci.*, in the press
- Glossmann, H., Ferry, D. R., Striessnig, J., Goll, A. & Moosburger, K. (1987b) *Trends. Pharmacol. Sci.* **8**, 95–100
- Goll, A., Ferry, D. R., Striessnig, J., Schober, M. & Glossmann, H. (1984) *FEBS Lett.* **176**, 371–377
- Leung, A. T., Imagawa, T. & Campbell, K. P. (1987) *J. Biol. Chem.* **262**, 7943–7946
- Linden, J. (1982) *J. Cyclic Nucleotide Res.* **8**, 163–172
- Lombet, A., Renaud, J.-F., Cicheportiche, R. & Lazdunski, M. (1981) *Biochemistry* **20**, 1279–1285
- Lombet, A., Kazazoglu, T., Delpont, E., Renaud, J.-F. & Lazdunski, M. (1983) *Biochem. Biophys. Res. Comm.* **110**, 894–901
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mannhold, R., Steiner, R., Haas, W. & Kaufmann, R. (1978) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **302**, 217–226
- Mas, M. T., Wang, J. K. & Hargrave, P. A. (1980) *Biochemistry* **19**, 684–692
- Morton, M. E. & Froehner, S. (1987) *J. Biol. Chem.* **262**, 11904–11907
- Nakayama, N., Kirley, T. L., Vaghy, P. L., McKenna, E. & Schwartz, A. (1987) *J. Biol. Chem.* **262**, 6572–6576
- Nielsen, P. E. & Burchardt, O. (1982) *Photochem. Photobiol.* **35**, 317–323
- Noda, M., Ikeda, T., Kayano, T., Suzuki, H., Takeshima, H., Kurasaki, M., Takahashi, H. & Numa, S. (1986) *Nature (London)* **320**, 188–192
- Norman, R. I., Borsotto, M., Fosset, M. & Lazdunski, M. (1983) *Biochem. Biophys. Res. Commun.* **111**, 878–883
- Norman, R. I., Burgess, A. J., Allen, E., Harrison, T. M. (1987) *FEBS Lett.* **212**, 127–132
- Reynolds, I. J., Snowman, A. D. & Snyder, S. H. (1986) *J. Pharmacol. Exp. Ther.* **237**, 731–738
- Schmid, A., Barhanin, J., Murre, C., Coppola, T., Borsotto, M. & Lazdunski, M. (1986a) *Biochem. Biophys. Res. Commun.* **139**, 996–1002
- Schmid, A., Barhanin, J., Coppola, T., Borsotto, M. & Lazdunski, M. (1986b) *Biochemistry* **25**, 3492–3495
- Sieber, M., Nastainczyk, W., Zubor, W., Wernet, W. & Hofmann, F. (1987) *Eur. J. Biochem.* **167**, 117–122

- Smith, I. S., McKenna, I. J., Ma, J., Vilven, J., Vaghy, P. L., Schwartz, A., Coronada, R. (1987) *Biochemistry* **26**, 7182–7188
- Striessnig, J., Zernig, G. & Glossmann, H. (1985) *Eur. J. Biochem.* **150**, 67–77
- Striessnig, J., Moosburger, K., Goll, A. & Glossmann, H. (1986) *Eur. J. Biochem.* **261**, 603–609
- Striessnig, J., Knaus, H.-G., Grabner, M., Moosburger, K., Seitz, W., Lietz, H. & Glossmann, H. (1987) *FEBS Lett.* **212**, 247–253
- Takahashi, M. & Catterall, W. A. (1987a) *Science* **236**, 88–91
- Takahashi, M. & Catterall, W. A. (1987b) *Biochemistry* **26**, 5518–5526
- Takahashi, M., Seagar, M. J., Jones, J. F., Reber, B. F. X. & Catterall, W. A. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5478–5482
- Tanabe, T., Takeshima, H., Mikami, A., Flockerzi, V., Takahashi, H., Kangawa, K., Kojima, M., Matsuo, H., Hirose, T. & Numa, S. (1987) *Nature (London)* **328**, 313–318
- Vaghy, P. L., Striessnig, J., Miwa, K., Knaus, H.-G., Itagaki, K., McKenna, E., Glossmann, H. & Schwartz, A. (1987a) *J. Biol. Chem.* **262**, 14337–14342
- Vaghy, P. L., Williams, J. S. & Schwartz, A. (1987b) *Am. J. Cardiol.* **59**, 9A–17A
- Vandaele, S., Fosset, M., Gallizzi, J.-P. & Lazdunski, M. (1987) *Biochemistry* **26**, 5–9
- Wessel, D. & Flüggé, U. I. (1984) *Anal. Biochem.* **138**, 141–143
- Yaari, Y., Hamon, B. & Lux, H. D. (1986) *Science* **232**, 680–682

Received 1 October 1987/21 January 1988; accepted 2 March 1988