Photoaffinity labelling of the cardiac calcium channel

(–)-[³H]Azidopine labels a 165 kDa polypeptide, and evidence against a [³H]-1,4-dihydropyridine-isothiocyanate being a calcium-channel-specific affinity ligand

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The arylazide 1,4-dihydropyridine (–)-[³H]azidopine binds to a saturable population of sites in guinea-pig heart membranes with a dissociation constant (K_D) of 30 ± 7 pM and a density (B_{max}) of 670 ± 97 fmol/mg of protein. This high-affinity binding site is assumed to reside on voltage-operated calcium channels because reversible binding is blocked stereoselectively by 1,4-dihydropyridine channel blockers and by the enantiomers of Bay K 8644. A low-affinity (K_D 25 ± 7 nM) high-capacity (B_{max} 21.6 ± 9 pmol/mg of protein) site does not bind (–)- or (+)-Bay K 8644, but is blocked by high concentrations (> 500 nM) of dihydro-2,6-dimethyl-4-(2-isothiocyanatophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester (1,4-DHP-isothiocyanate) or, e.g., (±)-nicardipine. (–)-[³H]Azidopine was photoincorporated covalently into bands of 165 ± 8 , 39 ± 2 and 35 ± 3 kDa, as determined by SDS/polyacrylamide-gel electrophoresis. Labelling of the 165 kDa band is protected stereoselectively by 1,4-dihydropyridine enantiomers at low (nM) concentrations and by (–)- and (+)-Bay K 8644, whereas the lower- M_r bands are not. Thus, only the 165 kDa band is the calcium-channel-linked 1,4-dihydropyridine receptor. Photolabelling of the 39 or 35 kDa bands was only blocked by $10 \mu\text{M}$ -1,4-DHP-isothiocyanate or $50 \mu\text{M}$ -(±)-nicardipine but not by $10 \mu\text{M}$ -(–)-Bay K 8644. [³H]-1,4-DHP-isothiocyanate binds to guinea-pig heart membranes with a K_D of 0.35 nM and dissociates with a k_{-1} of 0.2 min^{-1} at 30°C . [³H]-1,4-DHP-isothiocyanate irreversibly labels bands of 39 and 35 kDa which are protected by $> 10 \mu\text{M}$ -(±)-nicardipine or unlabelled ligand but not by $10 \mu\text{M}$ -(–)-Bay K 8644. Thus, [³H]-1,4-DHP-isothiocyanate is not an affinity probe for the calcium channel.

INTRODUCTION

The slow inward current of heart, which is carried by calcium, is selectively blocked by low concentrations of the 1,4-dihydropyridine nifedipine (Fleckenstein, 1977). [³H]Nifedipine (Holck *et al.*, 1982) and chiral radio-labelled 1,4-dihydropyridines have been employed as probes to characterize the calcium channel in particulate heart membranes (Bellemann *et al.*, 1981; Ehlert *et al.*, 1982; Glossmann *et al.*, 1982; De Pover *et al.*, 1983; Glossmann & Ferry, 1985; Ferry *et al.*, 1985a) and after solubilization (Rengasamy *et al.*, 1984; Ruth *et al.*, 1986). The cardiac calcium channel has been claimed to be photoaffinity labelled with (±)-[³H]nitrendipine (Campbell *et al.*, 1984) or ¹²⁵I-labelled 1,4-dihydropyridines (Sarmiento *et al.*, 1985) and affinity labelled with 1,4-DHP-isothiocyanate (Venter *et al.*, 1983; Horne *et al.*, 1984); polypeptides of 32–35 kDa were identified. However, in skeletal muscle transverse tubule membranes the arylazide 1,4-dihydropyridine [³H]azidopine photoincorporates into a band of 145–158 kDa (Ferry *et al.*, 1984, 1985b).

In the present paper we report on the photoaffinity labelling of the cardiac calcium channel with the arylazide 1,4-dihydropyridine probe (–)-[³H]azidopine and examine [³H]-1,4-DHP-isothiocyanate as a putative calcium channel affinity probe.

MATERIALS AND METHODS

Materials

Sources of unlabelled calcium channel ligands have been previously given (Glossmann & Ferry, 1985). The optical enantiomers of the calcium channel agonist Bay K 8644 (Franckowiak *et al.*, 1985) were from Bayer AG, Wuppertal, Germany. (–)-[³H]Azidopine was prepared as previously described (Ferry *et al.*, 1984), except that the (–) enantiomer of 1,4-dihydro-2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinecarboxylic acid 2-(aminoethyl)ethyl ester was coupled to [³H]succinimidyl-4-azidobenzoate (45 Ci/mmol; NEN, Dreieich, Germany). (+)-[³H]PN 200-110 (85 Ci/mmol) was from Amersham International. [³H]-1,4-DHP-isothiocyanate (76.6 Ci/mmol) and unlabelled 1,4-DHP-isothiocyanate were from NEN. Pepstatin A, soya bean trypsin inhibitor, leupeptin and lima bean trypsin inhibitor were from Sigma. Lysine-Sepharose was from Pharmacia.

Membrane preparation

Guinea-pig hearts were placed in ice-cold 50 mM-Tris/HCl (pH 7.4) containing 0.1 mM-phenylmethanesulphonyl fluoride, 0.1 mM-benzamide, $1 \mu\text{M}$ -EDTA, $1 \mu\text{g}$ of pepstatin A/ml and $1 \mu\text{g}$ of soya bean trypsin

Abbreviations used: 1,4-DHP-isothiocyanate, dihydro-2,6-dimethyl-4-(2-isothiocyanatophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester; SDS/PAGE, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis; K_D , dissociation constant; B_{max} , maximum density of binding sites; K_i , binding inhibition constant; K_H , high-affinity binding inhibition constant for a two-site model; K_L , low-affinity binding inhibition constant for a two-site model.

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inhibitor/ml (buffer A) and chopped with scissors. The heart fragments were then homogenized by 3×30 s of half-speed Ultra-turrax treatment, suspended in buffer A at a wet wt./vol. ratio of 1:20 and centrifuged at 600 *g* for 10 min. The supernatant was kept, the pellet resuspended in buffer A and then centrifuged at 600 *g* for 10 min. The supernatants were pooled and centrifuged at 48000 *g* for 10 min and the resulting pellet resuspended in buffer A. This procedure was repeated and membranes were stored in buffer A at a protein concentration of 1–2 mg/ml in liquid N_2 .

Reversible binding experiments

These were performed in buffer A, except for experiments with the 1,4-DHP-isothiocyanate which were performed in 25 mM-boric acid/NaOH buffer (pH 7.4) without benzamidine (buffer B). This is because the compound reacts avidly with amino groups (our batches of labelled and unlabelled 1,4-DHP-isothiocyanate covalently incorporated to $95 \pm 3\%$ into lysine-Sepharose; results not shown). Buffer B did not alter (–)-[3 H]azidopine or (+)-[3 H]PN 200-110 binding constants. For drug competition experiments, 200–400 pM (–)-[3 H]azidopine with 15–25 μ g of heart membrane protein were incubated in a volume of 0.25 ml with 10 concentrations of unlabelled drug for 90 min at 30 °C before separating bound and free ligand by filtration through Whatman GF/C filters as described (Glossmann & Ferry, 1985). Saturable binding to calcium-channel-linked receptors was defined by 10 μ M (–)-Bay K 8644 or 100 nM (+)-PN 200-110. In experiments to determine the affinities of 1,4-dihydropyridines for low-affinity sites the assay was performed in the presence of 1 μ M (–)-Bay K 8644. K_i values of competing drugs were calculated according to Linden (1982), taking into account the receptor concentration. Saturation experiments with (–)-[3 H]azidopine were performed in the same manner, employing 30–1500 pM free ligand, except that 2 h at 30 °C was allowed before separation of bound and free ligand. At a K_D concentration of (–)-[3 H]azidopine 80% of filter-retained radioactivity is specifically bound. To determine the affinity of (–)-[3 H]azidopine for the low-affinity sites, the specific activity was titrated with unlabelled (–)-azidopine and blanks were defined by 10 μ M (±)-nicardipine.

Photoaffinity labelling with (–)-[3 H]azidopine

In general, 0.15–1.5 mg of heart membrane protein (in 10 ml of buffer A) was incubated with 0.15–0.6 pM of (–)-[3 H]azidopine with and without drug additions at 30 °C for 60 min in polycarbonate centrifuge tubes in the absence of u.v. light. Aliquots (0.05 ml) were removed to determine the quantity of specifically bound ligand. The tubes were then stored once for 15 min until the assay mixture reached 4 °C and were centrifuged at 40000 *g* for 15 min. The pellets were resuspended in 10 ml of buffer A and then irradiated at 10 cm distance on ice in 8 cm Petri dishes with a 40 W/08 'black light' lamp (Phillips) for 5 min. The samples were then centrifuged at 40000 *g* for 15 min and the pellets taken up in 100 μ l of 10 mM-Tris/HCl, containing 10 mM-EDTA, 100 μ g of leupeptin/ml, 1.5 mg of lima bean trypsin inhibitor/ml, 1 mg of phenylmethanesulphonyl fluoride/ml, 1 mg of benzamidine/ml and 10 μ g of pepstatin A/ml. Non-reducing boiling stop solution (200 μ l; for composition see Ferry *et al.*, 1984) was then added together with

Fig. 1. Saturation analysis with (–)-[3 H]azidopine and (+)-[3 H]PN 200-110 in guinea-pig heart membranes

(a) Heart membranes (8 μ g of protein) were incubated at 30 °C for 2 h in a volume of 0.25 ml in buffer A before separation of bound and free ligand was performed. (–)-[3 H]Azidopine (12–1220 pM; ●) or (+)-[3 H]PN 200-110 (80–1920 pM; ○) were employed. Blanks defined by 10 μ M (–)-Bay K 8644 have been subtracted. The saturation curves were run in parallel. For (–)-[3 H]azidopine the K_D value is 9.7 pM and B_{max} 29.8 pM, which corresponds to 930 fmol/mg of membrane protein. For (+)-[3 H]PN 200-110 the K_D is 47 pM and B_{max} 28.3 pM which corresponds to 884 fmol/mg of membrane protein. The data are shown in the Hofstee transformation. (b) As for panel (a) except that 25 μ g of heart membrane protein (from a different membrane preparation than in (a)) were incubated with 70–1500 pM of (–)-[3 H]azidopine for 2 h at 30 °C in triplicate. Blanks were defined by either 10 μ M (–)-Bay K 8644 (●) or 10 μ M (±)-nicardipine (□). At 1500 pM-ligand the total binding was 1884 ± 92 d.p.m. ($n = 3$) and the blank binding defined by (–)-Bay K 8644 623 ± 38 d.p.m. ($n = 3$). The K_D of (–)-[3 H]azidopine [with blanks defined by (–)-Bay K 8644] is 29.9 pM and the B_{max} 49.1 pM, which corresponds to 491 fmol/mg of membrane protein. The saturation data obtained with 10 μ M (±)-nicardipine as blank was subjected to non-linear curve fitting and gave K_{D1} 20 ± 8 pM and K_{D2} 18 ± 10 nM, with corresponding B_{max} values of 41 ± 11 pM and 1660 ± 340 pM, respectively.

10 mM-*N*-ethylmaleimide and samples were immediately applied to 4–15% gradient polyacrylamide gels. After electrophoresis gels were either cut wet into 3 mm slices and counted for 3 H as previously described (Ferry *et al.*, 1984) or fixed, impregnated with Amplify and then

Fig. 2. Pharmacological profile of unlabelled 1,4-dihydropyridine derivatives as competitors of $(-)$ - $[^3\text{H}]$ azidopine binding to guinea pig heart membranes

Guinea-pig heart membranes (30 μg of protein) were incubated with 0.4 nM $(-)$ - $[^3\text{H}]$ azidopine for 2 h at 30 °C in the absence or presence of added drugs at the indicated concentrations. Points are means of duplicates from a representative experiment. The competing drugs are $(\pm)$ -nicardipine, in the absence of $(-)$ -Bay K 8644 ($\circ$) and in the presence of 1 μM $(-)$ -Bay K 8644 ($\bullet$), and $(-)$ -Bay K 8644 alone ($\triangle$). The results are shown as d.p.m. collected on the filters; no blanks have been subtracted. Binding constants derived from non-linear curve fitting are given in Table 1.

exposed to Kodak X-Omat film at -80 °C for 30–40 days. By using ^{14}C -labelled proteins as standards, molecular masses were calculated by linear interpolation (Ferry *et al.*, 1985b).

Affinity labelling with $[^3\text{H}]$ -1,4-DHP-isothiocyanate

These experiments were conducted in buffer B using 3 nM $[^3\text{H}]$ -1,4-DHP-isothiocyanate and were performed as for $(-)$ - $[^3\text{H}]$ azidopine photoaffinity labelling experiments, except that after 1 h incubation at 30 °C samples were centrifuged as above and then immediately

solubilized in the boiling stop solution and applied to 4–15% gradient polyacrylamide gels.

Data analysis and statistics

Dose-response curves were fitted to one- or two-site models on a computer and the goodness of fit determined by comparing the error mean sum of squares for the two models. Dose-response curves which fit better to two-site models have high-affinity binding constant (K_H) and a low-affinity binding constant (K_L), which are then corrected to account for receptor and $(-)$ - $[^3\text{H}]$ azidopine concentration according to Linden (1982). All means are given $\pm$ S.E.M. for $n = 3$ experiments; if more experiments have been performed this is indicated.

RESULTS

Reversible $(-)$ - $[^3\text{H}]$ azidopine binding

In the absence of u.v. light and with $(-)$ -Bay K 8644 as a definition of nonspecific binding, $(-)$ - $[^3\text{H}]$ azidopine binds to 670 ± 97 ($n = 4$) fmol/mg of membrane protein with a K_D value of 30 ± 7 pM (see Fig. 1). The density of $(-)$ - $[^3\text{H}]$ azidopine-labelled sites is essentially identical to the density of sites labelled by $(+)$ - $[^3\text{H}]$ PN 200-110. The latter binds to 697 ± 106 fmol/mg of protein with a K_D of 51 ± 11 pM ($n = 5$). At 30 °C the k_{+1} for $(-)$ - $[^3\text{H}]$ azidopine is $0.086 \text{ nm}^{-1} \cdot \text{min}^{-1}$. Addition of 200 nM $(+)$ -PN 200-110 at equilibrium causes monophasic dissociation of $(-)$ - $[^3\text{H}]$ azidopine with a k_{-1} of 0.0083 min^{-1} (results not shown). The K_D derived from the kinetic constants is 97 pM, in reasonable agreement with the equilibrium binding data. However, if blanks are defined with 10 μM $(\pm)$ -nicardipine (see below), $(-)$ - $[^3\text{H}]$ azidopine labels a lower-affinity high-capacity binding site in addition to the high-affinity site. The B_{max} was 21.6 ± 9 pmol/mg of membrane protein, the K_D 25 ± 7 nM ($n = 4$).

Pharmacological profile of $(-)$ - $[^3\text{H}]$ azidopine binding

To determine the specificity of $(-)$ - $[^3\text{H}]$ azidopine as a calcium channel probe in heart membranes, de-

Table 1. Binding constants of unlabelled 1,4-dihydropyridines as competitors of $(-)$ - $[^3\text{H}]$ azidopine and $[^3\text{H}]$ -1,4-DHP-isothiocyanate

Examples of the inhibition curves are shown in Figs. 2 and 6. In control binding-inhibition experiments, $(\pm)$ -nicardipine, the 1,4-DHP-isothiocyanate, $(+)$ -PN 200-110, $(-)$ -PN 200-110 and $(\pm)$ -nitrendipine displacement data fitted better to a two-site model. K_H and K_L have been corrected for $(-)$ - $[^3\text{H}]$ azidopine concentration according to Linden (1982) and the affinity of $(-)$ - $[^3\text{H}]$ azidopine at the respective sites. In the presence of 1 μM $(-)$ -Bay K 8644 binding-inhibition curves fitted better to a one-site model. For the two-site fits shown, K_H was 75% of all sites and K_L 25%.

Ligand	Drug	Control		Bay K 8644 (1 μM) present K_i (nM)
		K_H (nM)	K_L (nM)	
$[^3\text{H}]$ Azidopine	$(\pm)$ -Nicardipine	0.31	898	923
	1,4-DHP-isothiocyanate	0.37	868	527
	$(+)$ -PN 200-110	0.06	7600	7400
	$(-)$ -PN 200-110	7.40	3000	2800
	$(\pm)$ -Nitrendipine	0.95	4600	6500
	$(-)$ -Bay K 8644	5.6	—	—
	$(+)$ -Bay K 8644	13.9	—	—
	$(+)$ -PN 200-110	0.05	—	—
$[^3\text{H}]$ 1,4-DHP-isothiocyanate	$(-)$ -PN 200-110	7.90	—	—

Fig. 3. Stereoselectivity of $(-)$ - $[^3\text{H}]$ azidopine photoaffinity labelling of guinea-pig heart membranes

Guinea-pig heart membranes (0.81 mg of protein) were incubated with 160 pM $(-)$ - $[^3\text{H}]$ azidopine in a volume of 10 ml for 60 min in the absence of u.v. light at 30 °C, in the absence of added drug ($\circ$) or presence of 20 nM $(+)$ -PN 200-110 ($\bullet$), 20 nM $(-)$ -PN 200-110 ($\triangle$) or 200 nM $(-)$ -PN 200-110 ($\blacktriangle$). Prior to u.v. light exposure, the control assay mixture bound 5320 d.p.m. of $(-)$ - $[^3\text{H}]$ azidopine/ml, in the presence of 20 nM $(+)$ -PN 200-110 2340 d.p.m./ml, in the presence of 20 nM $(-)$ -PN 200-110 4340 d.p.m./ml and in the presence of 200 nM $(-)$ -PN 200-110 2960 d.p.m./ml. After photoaffinity labelling, the pelleted assay mixtures were applied to SDS/PAGE, and the gels cut and counted for radioactivity (first 20 slices only); 592 d.p.m. were incorporated into the 165 kDa peak (slices 12 and 13) in the control lane. The 39 and 35 kDa bands (not shown here; see Fig. 4) incorporated 17500 $\pm$ 1500 d.p.m. under all conditions.

tailed competition experiments were performed. $(+)$ -PN 200-110 up to 100 nM or $(-)$ -Bay K 8644 up to 10 μM displaced $(-)$ - $[^3\text{H}]$ azidopine binding equally. However, $(\pm)$ -nicardipine exhibits a biphasic displacement curve and at concentrations $> 1 \mu\text{M}$ this 1,4-dihydropyridine displaced below the level defined by either 100 nM $(+)$ -PN 200-110 or 10 μM $(-)$ -Bay K 8644 (Fig. 2). In Fig. 2 a $(\pm)$ -

Fig. 4. Photoaffinity labelling of guinea-pig heart membranes: effects of hexobendine and nicardipine

$(-)$ - $[^3\text{H}]$ azidopine (0.54 nM) was employed with 1.5 mg of membrane protein in 10 ml. Total binding prior to u.v. irradiation was 11680 d.p.m./ml (control), in the presence of 10 nM $(+)$ -PN 200-110 6740 d.p.m./ml, with 5 μM -hexobendine 11800 d.p.m./ml and with 50 μM $(\pm)$ -nicardipine 2340 d.p.m./ml. Parallel tracks were sliced and counted for incorporated radioactivity. In the control experiments 1890 $\pm$ 210 d.p.m. ($n = 3$) were incorporated into the 165 kDa band, in the presence of 5 μM -hexobendine 1740 $\pm$ 340 d.p.m. were recovered, with 10 nM $(+)$ -PN 200-110 present 110 $\pm$ 50 d.p.m. were found and with 50 μM $(\pm)$ -nicardipine 88 $\pm$ 28 d.p.m. were recovered in the 165 kDa region. The 39 and 35 kDa bands were cut out together and for the control, 10 nM $(+)$ -PN 200-110 and 5 μM -hexobendine conditions identical d.p.m. (52000 $\pm$ 3800, 51000 $\pm$ 4900 and 48000 $\pm$ 7000) were recovered. In the presence of 50 μM $(\pm)$ -nicardipine 2800 $\pm$ 850 d.p.m. were recovered in the 39–35 kDa region. In the autoradiograph of the gel, spillover from the hexobendine track is in the 39–35 kDa region of the nicardipine track.

nicardipine inhibition experiment is shown, when high-affinity $(-)$ - $[^3\text{H}]$ azidopine binding was either blocked by 1 μM $(-)$ -Bay K 8644 or in the absence of $(-)$ -Bay K 8644. As can be seen by inspection the IC_{50} of nicardipine for the low-affinity site is approx. 1 μM , but is in the low nM range for high-affinity $(-)$ - $[^3\text{H}]$ azidopine binding.

Table 1 summarizes the results obtained with a series of 1,4-dihydropyridines as competitors for $(-)$ - $[^3\text{H}]$ azidopine binding in the absence and presence of

1 μM -(−)-Bay K 8644, respectively. All the 1,4-dihydropyridines with alkyl substituents at the 3' position of the 1,4-dihydropyridine ring compete for the low-affinity (−)-[^3H]azidopine site with K_i value between 0.5 and 7 μM . The nucleoside transport inhibitors hexobendine and nitrobenzylthioguanosine [which block 1,4-dihydropyridine binding to the nucleoside transporter (Striessnig *et al.*, 1985)] had no effect on (−)-[^3H]azidopine binding at concentrations up to 10 μM (results not shown). Note that K_L [determined from displacement studies where 10 μM -(±)-nicardipine defined blanks] correlates very closely with the K_i values in the presence of 1 μM -(−)-Bay K 8644. The correlation coefficient between K_L and K_i was 0.96 with a slope of 1.07, determined by linear regression analysis. In conclusion, (−)-[^3H]azidopine labels, in addition to a high-affinity low- B_{max} site (which displays stereoselectivity), a low-affinity high-capacity site which does not bind the enantiomers of Bay K 8644 and does not discriminate the enantiomers of, e.g., PN 200-110.

Photoaffinity labelling with (−)-[^3H]azidopine

In Figs. 3 and 4 it is evident that photoincorporated (−)-[^3H]azidopine is recovered in several bands (means: 165 ± 8 , 39 ± 2 and 35 ± 3 kDa, $n = 8$) after SDS/PAGE. In terms of radioactivity incorporated, that in the 165 kDa band is 5–10% (average of eight experiments) compared with the 39 kDa and 35 kDa

bands. We investigated the stereoselectivity of photoincorporation: 10 or 20 nM of (+)-PN 200-110 completely protected photolabelling of the 165 kDa band, whereas 20 nM-(−)-PN 200-110 was ineffective (see Fig. 3 for protection studies). As expected, 200 nM-(−)-PN 200-110 did block photoincorporation. Neither of the PN 200-110 enantiomers blocked photoincorporation into the 39 kDa (or 35 kDa) band at the concentrations employed. Whereas 5 μM -hexobendine was ineffective in blocking photoincorporation, 50 μM -nicardipine protected labelling of the 165 kDa as well as the 39 and 35 kDa bands (Fig. 4). The efficiency of (−)-[^3H]azidopine photoincorporation into the 165 kDa band (taken as the specifically bound radioactivity found by counting the gel divided by the equilibrium specific binding prior to u.v. irradiation) was $3 \pm 1\%$ ($n = 8$). From reversible binding experiments it was evident that certain 1,4-dihydropyridine derivatives inhibited (−)-[^3H]azidopine binding below the level to which 100 nM-(+)-PN 200-110 or 10 μM -(−)-Bay K 8644 displaced. The 1,4-DHP-isothiocyanate was among these. Because of the isothiocyanate substitution it was necessary to use an amine-free buffer without benzamidine for these experiments. The 1,4-DHP-isothiocyanate {when (−)-[^3H]azidopine or (+)-[^3H]PN 200-110 (results not shown) are the labelled ligands} clearly exhibits a biphasic inhibition profile with a K_i of 0.37 nM for the high-affinity component and 0.53 μM for the low-affinity

Fig. 5. Inhibition of (−)-[^3H]azidopine photoaffinity labelling by 1,4-DHP-isothiocyanate and (−)-Bay K 8644 and affinity labelling experiments with [^3H]-1,4-DHP-isothiocyanate

(−)-[^3H]Azidopine photoaffinity labelling is shown in (a)–(f). Guinea-pig heart membranes (0.85 mg of protein/10 ml) were incubated with 242 pM-(−)-[^3H]azidopine for 60 min at 30 °C in the absence of added drugs (a, ○) or in the presence of 100 nM-1,4-DHP-isothiocyanate (DHP-NCS) (b, ●), 10 μM -1,4-DHP-isothiocyanate (c, ▲) or 10 μM -(−)-Bay K 8644 (d, ■). Under these conditions, the (−)-[^3H]azidopine bound to 3560 ± 520 , 2100 ± 260 , 1950 ± 180 and 1620 ± 200 d.p.m./ml, respectively. Membranes were photoaffinity labelled, centrifuged and pellets applied to SDS/PAGE (0.85 mg of protein/track). Tracks were sliced and counted for radioactivity as described in the Materials and methods section. In (e) and (f) slices 22–40 are shown with a different ordinate scale. (g) and (h) show an affinity labelling experiment with 3 nM-[^3H]-1,4-DHP-isothiocyanate which was incubated with 0.85 mg of heart membranes in a volume of 10 ml for 60 min at 30 °C in the absence of added drugs (○), in the presence of 50 nM-(±)-nicardipine (●), 1 μM -(−)-Bay K 8644 (△) or 50 μM -(±)-nicardipine (▲).

Fig. 6. Properties of [^3H]-1,4-DHP-isothiocyanate binding to guinea-pig heart membranes, and properties of the unlabelled 1,4-dihydropyridine isothiocyanate as a competitor of (–)-[^3H]azidopine and (+)-[^3H]PN 200-110 binding

(a) [^3H]-1,4-DHP-isothiocyanate (DHP-NCS) at the indicated concentrations was incubated with 26 μg of guinea-pig heart membranes for 30 min in a volume of 0.25 ml at 30 $^{\circ}\text{C}$ in the absence of added drug (total binding, $\bullet$) and in the presence of 10 μM (–)-Bay K 8644 (blank binding, $\circ$). Saturable binding (Δ) is obtained by subtracting blank binding from total binding. Scatchard analysis of the saturable component gave a K_D of 475 pM and a B_{max} of 27.4 pM, which corresponds to 261 fmol/mg of protein. (b) Pooled data from two independent dissociation experiments of [^3H]-1,4-DHP-isothiocyanate binding. Guinea-pig heart membranes (26 μg of protein) were incubated with 1.0 nM [^3H]-1,4-DHP-isothiocyanate for 30 min before 10 μM (–)-Bay K 8644 was added for the indicated times. Exponential regression analysis revealed a dissociation rate constant, k_{-1} , of 0.22 min^{-1} . (c) Competition of unlabelled 1,4-DHP-isothiocyanate with (–)-[^3H]azidopine binding to guinea-pig heart membranes. Guinea-pig heart membranes (30 μg of protein) were incubated with 0.45 nM (–)-[^3H]azidopine for 90 min at 30 $^{\circ}\text{C}$ in a volume of 0.25 ml in the absence of Bay K 8644 ($\bullet$) and in the presence of 1 μM (–) Bay K 8644 ($\circ$) with the indicated concentrations of 1,4-DHP-isothiocyanate. (d) Guinea-pig heart membranes (8 μg of protein) were incubated with buffer ($\circ$) or 30 nM unlabelled 1,4-DHP-isothiocyanate ($\bullet$) for 1 h at 30 $^{\circ}\text{C}$. (+)-[^3H]PN 200-110 was then added in the concentration range 190–4360 pM for 2 h, before bound and free radioactivity were separated. Blanks defined by 1 μM (–) Bay K 8644 have been subtracted. Non-linear curve fitting of the data gave a K_D for (+)-[^3H]PN 200-110 of 55 ± 19 pM in the absence of 1,4-DHP-isothiocyanate, with a B_{max} of 9.9 ± 0.4 pM. For membranes preincubated with 30 nM 1,4-DHP-isothiocyanate, the K_D is 1993 ± 670 pM and the B_{max} 13.5 ± 1.7 pM.

component (Table 1). Photoaffinity labelling with (–)-[^3H]azidopine was then performed in the presence of 100 nM- and 10 μM -1,4-DHP-isothiocyanate and for comparison in the presence of 10 μM (–)-Bay K 8644 (Fig. 5). Clearly 100 nM-1,4-DHP-isothiocyanate and 10 μM (–)-Bay K 8644 blocked incorporation into the

165 kDa band but not into the lower- M_r bands. However, 10 μM -1,4-DHP-isothiocyanate inhibits photo-incorporation into both the 165 kDa and the lower- M_r bands.

Properties of unlabelled and [^3H]-labelled 1,4-DHP-isothiocyanate

To investigate if the isothiocyanate-substituted 1,4-dihydropyridine bound irreversibly to cardiac calcium channels, membranes were preincubated with 30 nM of the unlabelled 1,4-DHP-isothiocyanate for 1 h at 30 $^{\circ}\text{C}$ in amine-free buffer. Saturation analysis with (+)-[^3H]PN 200-110 was then performed. Fig. 6 shows that the unlabelled compound acts as a competitive inhibitor with apparent K_i 1.1 nM and does not reduce the B_{max} . The conclusion is that no irreversible modification of the calcium-channel-linked 1,4-dihydropyridine receptors occurred under these conditions.

The [^3H]-labelled 1,4-DHP-isothiocyanate [when blanks are defined by 10 μM (–)-Bay K 8644] bound to a saturable population of sites with a K_D value of 0.35 ± 0.1 nM (Fig. 6) which is close to the K_H determined for unlabelled 1,4-DHP-isothiocyanate as a competitor of (–)-[^3H]azidopine binding. (+)-PN 200-110 exhibited a K_i value of 50 ± 5 pM and (–)-PN 200-110 a K_i value of 7.8 ± 0.2 nM. (–)-Bay K 8644 at 10 μM , added at equilibrium, led to a monophasic dissociation of the labelled ligand with a k_{-1} of 0.2 ± 0.05 min^{-1} (Fig. 6); 100% of specifically bound ligand dissociated. No attempt was made to characterize the low-affinity binding sites for 1,4-dihydropyridines with the labelled 1,4-DHP-isothiocyanate; from competition studies (see Table 1) the dissociation constant must be around 1 μM for this site.

[^3H]-1,4-DHP-isothiocyanate was then used in an attempt to affinity label cardiac membranes. Ligand at 3 nM ($9 \times K_D$ for the high-affinity site) was incubated with membranes for 60 min at 30 $^{\circ}\text{C}$ in the absence of added drugs and in the presence of 50 nM (–)-nicardipine, 50 μM (–)-nicardipine and 10 μM (–)-Bay K 8644. The labelled ligand was covalently incorporated into bands of 39 and 35 kDa as seen after SDS/PAGE. Neither of these bands was protected by 50 nM (–)-nicardipine or 10 μM (–)-Bay K 8644 but the 39 kDa band is partially protected by 50 μM (–)-nicardipine. The results are shown in Fig. 5 to allow a comparison with the photolabelling experiments.

Photoaffinity labelling of chick and rat heart calcium channels with (–)-[^3H]azidopine

Chick and rat heart membranes bound (–)-[^3H]azidopine with K_D values of 40 ± 5 ($n = 3$) and 48 ± 4 ($n = 4$) pM, respectively, when nonspecific binding was defined by 10 μM (–)-Bay K 8644 (results not shown). The B_{max} in chick heart membranes was 1200 ± 110 fmol/mg of protein and in rat heart 450 ± 80 fmol/mg of protein. (–)-[^3H]Azidopine was photoincorporated into bands of 165 kDa in rat and chick heart membranes (Fig. 7). These bands are completely protected by 1 μM (–)-Bay K 8644. Bands of 39 and 35 kDa were also photoaffinity labelled, but not protected by (–)-Bay K 8644 (Fig. 7). In chick and rat heart the photoincorporation into the 165 kDa and into the 39 kDa and 35 kDa bands is of roughly equal magnitude, in contrast with guinea-pig membranes. The efficiency of (–)-[^3H]azidopine photoaffinity labelling for the

Fig. 7. Photoaffinity labelling of rat and chick heart membranes

Rat and chick heart membrane experiments were performed in parallel, employing 0.57 nM-($-$)-[3 H]azidopine and 1.5 mg of protein/10 ml and 0.42 mg of protein/10 ml, respectively. For rat heart membranes the total binding prior to u.v. irradiation was 7340 d.p.m./ml (track 2) and in the presence of 1 μ M-($-$)-Bay K 8644 was 2140 d.p.m./ml (track 1). Parallel tracks were sliced and counted for radioactivity and revealed 1240 d.p.m. of covalent incorporation in the 165 kDa band, which was blocked by 1 μ M-($-$)-Bay K 8644 (68 d.p.m.). For chick heart membranes the total binding prior to u.v. irradiation was 5760 d.p.m./ml (track 4) and in the presence of 1 μ M-($-$)-Bay K 8644 was 1330 d.p.m./ml (track 3). Parallel tracks were sliced and counted for radioactivity and revealed 824 d.p.m. of covalent incorporation into the 165 kDa band, which was blocked by 1 μ M-($-$)-Bay K 8644 (78 d.p.m.).

165 kDa band is in the range 2–3% for the chick and rat heart membranes, which is close to the value of 3% found in guinea-pig heart membranes.

DISCUSSION

The specific density of 1,4-dihydropyridine-labelled calcium channels in guinea-pig heart membranes of approx. 600 fmol/mg of protein is nearly 100-fold lower than in skeletal muscle transverse-tubule membranes (Fosset *et al.*, 1983; Glossmann *et al.*, 1983). In addition, guinea-pig heart membranes (in contrast with skeletal muscle) have abundant low-affinity sites for radiolabelled

1,4-dihydropyridines which have no obvious relationship to calcium channels (Bellemann *et al.*, 1981). In the present paper we report that the enantiomers of Bay K 8644 (or the racemic mixture) are selective agents which block reversible binding of the photoaffinity probe ($-$)-[3 H]azidopine to high-affinity low-capacity sites (presumably linked to calcium channels) but do not inhibit binding to the high-capacity low-affinity sites previously identified with racemic [3 H]nitrendipine (Bellemann *et al.*, 1981). Because of its high density and the virtual absence of high-capacity low-affinity binding sites for 1,4-dihydropyridines, the skeletal muscle transverse tubule calcium channel has been much easier to solubilize and purify close to homogeneity with conventional purification protocols (Glossmann & Ferry, 1983; Curtis & Catterall, 1984; Borsotto *et al.*, 1985). With all reported procedures a polypeptide of 155–170 kDa predominates in the most purified fractions. Smaller peptides of 50 kDa (Curtis & Catterall, 1984) and 32 kDa (Borsotto *et al.*, 1985) have also been found. A band of 145–158 kDa is specifically photoaffinity labelled by ($\pm$)-[3 H]azidopine in transverse tubule membranes from guinea-pig, rabbit, chick and frog skeletal muscle (Ferry *et al.*, 1985b). In contrast, in the single paper on the claimed purification of the chick heart calcium channel, bands of 67, 43 and 32 kDa in the most purified fractions are reported (Rengasamy *et al.*, 1984). In apparent support of this, the 1,4-dihydropyridine ($\pm$)-[3 H]nitrendipine, when irradiated with canine heart membranes, photoincorporates into a band of 32 kDa. However, 10 nM-($\pm$)-[3 H]nitrendipine was employed ($30 \times K_D$), and incorporation into this band is only partially protected (70%) by 20 μ M-nitrendipine (Campbell *et al.*, 1984). In addition, the 1,4-DHP-isothiocyanate affinity ligand (at 10 nM or approx. $30 \times K_D$) incorporates into bands of 52, 42 and 32 kDa in canine heart membranes (Horne *et al.*, 1984). Labelling of these bands was only partially protected by 50 μ M-($\pm$)-nicardipine or 10 μ M unlabelled 1,4-DHP-isothiocyanate. Sarmiento *et al.* (1985) have employed a [125 I]-labelled 1,4-dihydropyridine [1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic acid iodoethyl ester] to photoaffinity label the 1,4-dihydropyridine calcium channel binding site in cardiac, skeletal and smooth muscle. A 32–35 kDa band was photoaffinity labelled in the three tissues. Thus, one could get the impression that the heart calcium channel 1,4-dihydropyridine receptors, in contrast with the skeletal muscle, consist of polypeptides much smaller than the major component of the skeletal muscle calcium channel. Apparently (in contrast with the evidence obtained by specific photoaffinity probes and purification), labelled 1,4-dihydropyridines, when irradiated with high-intensity u.v. light, photoincorporate non-specifically into low- M_r membrane proteins.

In our experiments with guinea-pig heart membranes ($-$)-[3 H]azidopine photoincorporated into three bands, as judged by SDS/PAGE. The proportion of radioactivity incorporated into the 165 kDa band was minor (5–10%) and the 39 and 35 kDa bands predominated (90%) in the guinea-pig heart. Photoincorporation into the 165 kDa band is stereoselectively blocked by the enantiomers of the 1,4-dihydropyridine channel blocker PN 200-110 and by ($-$)-Bay K 8644.

It is well known that the pharmacological action of chiral 1,4-dihydropyridine channel blockers exhibits

stereoselectivity. The 4*S*-enantiomers [(−)-nitrendipine, (−)-nimodipine, (+)-PN 200-110] are considerably more potent than their respective 4*R*-enantiomers (Towart *et al.*, 1981; Bolger *et al.*, 1983; Hof *et al.*, 1985). In reversible binding and photoaffinity labelling experiments stereoselectivity is therefore an essential criterion for identification of calcium channel-linked receptors (Ferry & Glossmann, 1982; Bolger *et al.*, 1983; Lee *et al.*, 1984; Ferry *et al.*, 1985b; Glossmann & Ferry, 1985). Previous studies employing [³H]-1,4-DHP-isothiocyanate as a potential calcium-channel affinity probe (Horne *et al.*, 1984; Venter *et al.*, 1983) used 10 μM unlabelled ligand (Venter *et al.*, 1983) or 50 μM-(±)-nicardipine (Horne *et al.*, 1984) to define 'channel-specific' binding. In our experiments the 1,4-dihydropyridines 1,4-DHP-isothiocyanate, (±)-nicardipine, (±)-nitrendipine and (−)-PN 200-110 displaced significantly more (−)-[³H]azidopine binding than did saturating concentrations (10 μM) of (−)- or (+)-Bay K 8644. As (−)-Bay K 8644 is an 'agonist' and activates voltage-operated calcium channels (Schramm *et al.*, 1983) it could be argued that this phenomenon is related to the channel-activating properties of this compound. However, (+)-Bay K 8644 is a potent channel blocker (Franckowiak *et al.*, 1985); nevertheless, it selectively interacted with the high-affinity binding site found by us in guinea-pig heart membranes in reversible binding experiments with (−)-[³H]azidopine. Using 1 μM-(−)-Bay K 8644 as a 'selective' inhibitor of high-affinity calcium-channel-specific (−)-[³H]azidopine binding, the *K_i* values of 1,4-dihydropyridines were determined for the low-affinity high-capacity site. High concentrations of 1,4-DHP-isothiocyanate or (±)-nicardipine (which saturate the low-affinity binding site) protected the 39 and 35 kDa bands from photoaffinity labelling by (−)-[³H]azidopine. Lower concentrations (up to 100 nM) selectively prevented photoincorporation into the 165 kDa band but did not inhibit photolabelling of the lower-*M_r* bands.

We conclude that the low-affinity binding site for (−)-[³H]azidopine in guinea-pig cardiac membranes is most likely the photolabelled 39 kDa band and/or the 35 kDa band. Interestingly, in rat and chick membranes these bands are photolabelled to a much lesser extent than in the guinea-pig. It has not been investigated if this phenomenon is related to the *B_{max}* of the low-affinity 1,4-dihydropyridine binding sites in these tissues.

In amine-free buffer, unlabelled 1,4-DHP-isothiocyanate was a pure competitive (e.g. reversible) inhibitor of (+)-[³H]-PN 200-110 binding. Experiments with [³H]-1,4-DHP-isothiocyanate showed that (+)-PN 200-110 inhibited binding with a *K_i* values of 50 pM, which is the same as the *K_D* value for (+)-[³H]PN 200-110 and very close to the *K_i* value of 60 pM (*K_H*) determined by non-linear regression analysis of (+)-PN 200-110 as an inhibitor of (−)-[³H]azidopine binding. Also, at 30 °C, 100% of the label bound to calcium channels dissociated with a half-life of 4.5 min. The covalent incorporation of [³H]-1,4-DHP-isothiocyanate into guinea-pig heart membranes shows similarities with the results of Venter *et al.* (1983) and Horne *et al.* (1984). Bands of 39 and 35 kDa were identified in our guinea-pig heart membranes, and the 39 kDa band was partially protected by 50 μM- (±)-nicardipine or 10 μM unlabelled 1,4-DHP-isothiocyanate; however, 10 μM-(−)-Bay K 8644 was completely ineffective.

Presumably Venter *et al.* (1983) and Horne *et al.* (1984) measured incorporation into similar low-affinity high-capacity 1,4-dihydropyridine-binding sites which are not obviously associated with the calcium channel. The same criticism applies to Campbell *et al.* (1984) and to Sarmiento *et al.* (1985) who identified lower-*M_r* bands after photolabelling with non-arylazide 1,4-dihydropyridines. This leads to the conclusion that 1,4-DHP-isothiocyanate does not have access to an amino group when it binds to the high-affinity 1,4-dihydropyridine receptor of the calcium channel, but does have access to amino groups on the smaller proteins it covalently modifies, which have no relationship to calcium channels. However, we have not shown that the low-affinity sites photolabelled by (−)-[³H]azidopine are identical with the sites affinity labelled by the [³H]-1,4-DHP-isothiocyanate.

In conclusion, photoaffinity labelling with (−)-[³H]azidopine has shown for the first time that the 1,4-dihydropyridine receptor of the cardiac calcium channel in different species is a large (165 kDa) polypeptide, as was previously found in skeletal muscle. Radiation-inactivation data also support the hypothesis that the calcium channels in different tissues have a similar polypeptide composition, as virtually identical target sizes (180–210 kDa) are found in heart (Doble *et al.*, 1985), skeletal muscle (Norman *et al.*, 1983; Ferry *et al.*, 1983a; Goll *et al.* 1983) and brain (Ferry *et al.*, 1983b). Our results advise caution as to the specificity of 1,4-DHP-isothiocyanate (Venter *et al.*, 1983; Horne *et al.*, 1984) and (±)-[³H]nitrendipine (Campbell *et al.*, 1984) as affinity or photoaffinity probes for the calcium channel.

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