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Photoaffinity labelling of the phenylalkylamine receptor of the skeletal muscle transverse-tubule calcium channel

Jörg Striessnig, Hans-Günther Knaus, Manfred Grabner, Kurt Moosburger, Werner Seitz+, Helmut Lietz* and Hartmut Glossmann

Institut für Biochemische Pharmakologie, Peter-Mayr Str. 1, A-6020 Innsbruck, Austria, +*Hauptlaboratorium der BASF AG, D-6700 Ludwigshafen* and **Forschung und Entwicklung, Knoll AG, D-6700 Ludwigshafen, FRG*

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The tritiated arylazido phenylalkylamine (–)-5-[(3-azidophenethyl)[*N-methyl*-^{3}H]methylamino]-2-(3,4,5-trimethoxyphenyl)-2-isopropylvaleronitrile was synthesized and used to photoaffinity label the phenylalkylamine receptor of the membrane-bound and purified calcium channel from guinea-pig skeletal muscle transverse-tubule membranes. The photoaffinity ligand binds reversibly to partially purified membranes with a K_d of 2.0 ± 0.5 nM and a B_{max} of 17.0 ± 0.9 pmol/mg protein. Binding is stereospecifically regulated by all three classes of organic calcium channel drugs. A 155 kDa band was specifically photolabelled in transverse-tubule particulate and purified calcium channel preparations after ultraviolet irradiation. Additional minor labelled polypeptides (92, 60 and 33 kDa) were only observed in membranes. The heterogeneous 155 kDa region of the purified channel was resolved into two distinct silver-stained polypeptides after reduction (i.e. 155 and 135 kDa). Only the 155 kDa polypeptide carries the photoaffinity label and it is concluded that the 135 kDa polypeptide (which migrates as a 165 kDa band under alkylating conditions) is not a high-affinity drug receptor carrying subunit of the skeletal muscle transverse-tubule L-type calcium channel.

Ca^{2+} channel; Purification; Phenylalkylamine; Photoaffinity label

1. INTRODUCTION

The skeletal muscle transverse-tubule membrane is the richest mammalian source of calcium channel drug receptors [1]. Purification of the calcium channel from rabbit and guinea-pig skeletal muscle has been achieved employing radiolabelled 1,4-dihydropyridines such as [^{3}H]nitrendipine [2] and (+)-[^{3}H]PN 200-110 [3–6]. The functional reconstitution of the purified preparations has also been reported [5,7]. Three major polypeptides with molecular masses of 135–170, 50–65 and 30–35 kDa are usually co-purified together with 1,4-dihydropyridine [2–6] or phenylalkylamine [8] binding activity. Photoaffinity labelling experiments with the 1,4-dihydropyridine photoaffinity probe [^{3}H]azidopine identified the 155 kDa polypeptide region of the purified calcium channel from guinea-pig skeletal muscle as the 1,4-dihydropyridine receptor carrying component [6]. In order to answer the question as to which of the polypeptides carries the phenylalkylamine receptor, a potent, optically pure arylazidophenylalkylamine was synthesized. Here we report the reversible binding characteristics of the novel ligand and the specific photolabelling of the phenylalkylamine receptor of the membrane-bound and purified guinea-pig skeletal muscle calcium channel.

Correspondence address: H. Glossmann, Institut für Biochemische Pharmakologie, Peter-Mayr Str. 1, A-6020 Innsbruck, Austria

2. MATERIALS AND METHODS

2.1. *Materials*

(–)-5-[(3-Azidophenethyl)-[*N-methyl*-^{3}H]meth-

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ylamino]-2-(3,4,5-trimethoxyphenyl)-2-isopropylvaleronitrile ([*N-methyl*-^{3}H]LU49888) was synthesized by condensation of 3-azidophenethylamine with (−)-5-chloro-2-(3,4,5-trimethoxyphenyl)-2-isopropylvaleronitrile and subsequent *N*-methylation using [^{3}H]methyl iodide. After purification by HPLC, the optically pure [*N-methyl*-^{3}H]LU49888 with a specific activity of 85 Ci/mmol and a radiochemical purity > 98% was obtained. LU49888 was selected out of a group of azido derivatives of verapamil on the basis of its potent calcium antagonistic activity as determined by inhibition of K^+-induced contraction of rat aortic strips and of (−)-[^{3}H]desmethoxyverapamil binding (Raschack, M. and Unger, L., personal communication). The structure of the ligand is shown in fig.1.

The sources for the unlabelled calcium channel drugs are given in [6,9]. Econofluor® and Protosol® were from New England Nuclear, Dreieich.

2.2. *Membrane and calcium channel preparation*

Guinea-pig partially purified transverse-tubule membranes were prepared as described [10]. Calcium channels were purified from the membranes employing a rapid two-step purification procedure [6].

2.3. *Binding assays and data calculation*

Reversible binding of [*N-methyl*-^{3}H]LU49888 to membrane-bound and purified calcium channels was measured in 50 mM Tris-HCl buffer (pH 7.4) at 25°C as described for (−)-[^{3}H]desmethoxyverapamil [6,9] in the absence of ultraviolet light. Nonspecific binding was determined in the presence of 1 μM (membranes) or 3 μM (−)-desmethoxyverapamil (purified calcium channel) and was subtracted from total binding to yield specific binding. The association rate constant was calculated using the pseudo-first-order rate equation. Dissociation of the [*N-methyl*-^{3}H]LU49888-receptor complex at equilibrium was initiated by 80-fold dilution of the incubation mixture with buffer. The dissociation rate constant was calculated by linear methods as in [11]. Means ± SD from at least three independent experiments are given. Binding-inhibition data were fitted with non-linear methods [11].

2.4. *Photoaffinity labelling experiments*

Photoaffinity labelling experiments with membrane-bound calcium channels were carried out as described for [^{3}H]azidopine [12,13] employing 2.3–14.5 nM [*N-methyl*-^{3}H]LU49888 and 0.4–0.7 mg membrane protein. Photoaffinity labelling experiments with purified calcium channels were carried out in a total volume of 1.5 ml employing 15–18 nM [*N-methyl*-^{3}H]LU49888 and 4–6 μg purified protein as described for (−)-[^{3}H]azidopine [6]. Nonspecific incorporation was defined as labelling in the presence of 1 μM (membranes) or 3 μM (−)-desmethoxyverapamil (purified calcium channel). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described with either 10 mM *N*-ethylmaleimide (alkylating conditions) or 10 mM dithiothreitol (reducing conditions) in the sample buffer [13]. To quantitate the amount of incorporated [*N-methyl*-^{3}H]LU49888, gels were cut into 2 or 3 mm slices and radioactivity determined after extraction with Econofluor® containing 3% Protosol®.

3. RESULTS

3.1. *Reversible [*N-methyl-^{3}*H]LU49888 binding to partially purified skeletal muscle transverse-tubule membranes*

Reversible specific binding of [*N-methyl*-^{3}H]LU49888 was linear with respect to membrane protein up to a concentration of 0.060 mg/ml. No saturable binding of the ligand to glass fiber filters was observed under our assay conditions. Binding was pH-dependent with a binding optimum between pH 7.0 and 8.0 (not shown). The association reaction of [*N-methyl*-^{3}H]LU49888 was rapid at 25°C, reaching a steady state within 20 min when 2–3 nM ligand and 0.35–0.70 nM drug receptor were employed. Using the first-order rate equation a k_{+1} of 0.072 ± 0.006 $nM^{-1} \cdot min^{-1}$ (n = 3) was calculated. Dissociation kinetics of the [*N-methyl*-^{3}H]LU49888-receptor complex after dilution were mono-exponential with a $t_{1/2}$ of 3.9 min at 25°C (k_{-1} = 0.179 ± 0.021 min^{-1}; n = 3). The saturation isotherm of [*N-methyl*-^{3}H]LU49888 was monophasic with a K_d of 2.0 ± 0.5 nM and a B_{max} of 17.0 ± 0.9 pmol/mg protein (means ± SD from 4 experiments) at 25°C. A representative experiment is shown in fig.1. The K_d (2.5 nM), calculated from the kinetic constants, was in fair agreement

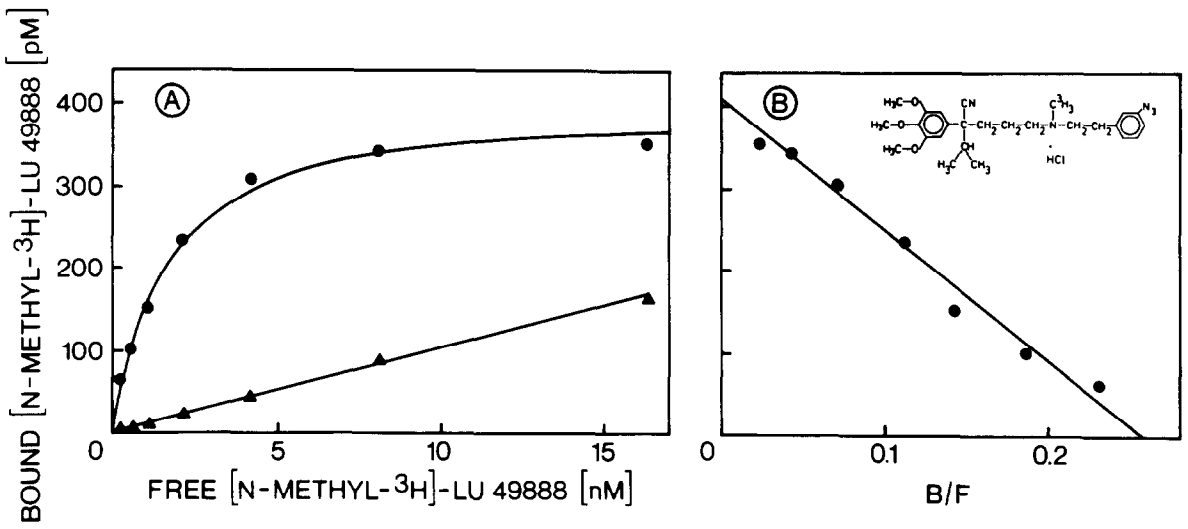

Fig.1. Saturation isotherm of [*N-methyl*-^{3}H]LU49888 binding to guinea-pig partially purified skeletal muscle membranes. (A) Increasing concentrations (0.1–18 nM) of [*N-methyl*-^{3}H]LU49888 were incubated with 6.3 μg membrane protein at 25°C in a total assay volume of 0.25 ml for 45 min. Binding in the presence of 1 μM (−)-desmethoxyverapamil (▲, nonspecific binding) was subtracted from total binding to yield specific binding (●). (B) Hofstee transformation of the equilibrium binding data. The correlation coefficient was 0.99, the B_{max} = 404.3 nM (= 16 pmol/mg protein) and the K_d = 1.6 nM. Means from triplicate determinations. (Inset) Structural formula of [*N-methyl*-^{3}H]LU49888.

with the K_d determined by equilibrium saturation analysis. [*N-methyl*-^{3}H]LU49888 binding is stereoselectively regulated by all three classes of organic calcium channel blockers (fig.2A–C) in a manner very similar to (−)-[^{3}H]desmethoxyverapamil binding [9]. Reversible binding is also inhibited by Cd^{2+} and Ca^{2+} (fig.2D) and by La^{3+} (IC_{50} = 32.0

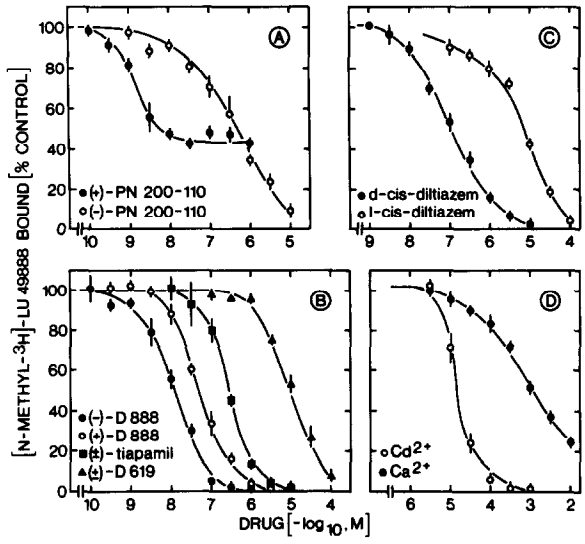

Fig.2. Binding-inhibition studies. 0.8–1.6 nM [*N-methyl*-^{3}H]LU49888 were incubated with 4.2–6.8 μg membrane protein in the absence or presence of drugs or divalent cations (triplicate or duplicate assays). Specific binding in the presence of added drug was normalized with respect to control binding (100%). Pooled binding-inhibition data from 3 independent experiments are shown. Points are means ± SD. Binding parameters are given ± asymptotic SD. K_i values for competitive inhibitors were calculated according to Linden [23]. IC_{50} values are given for noncompetitive inhibitors. (A) 1,4-Dihydropyridines: (+)-PN 200-110: IC_{50} = 1.43 ± 0.18 nM, n_H = 1.70 ± 0.33, maximal inhibition to 46 ± 4% of control binding at this ligand concentration; (−)-PN 200-110: IC_{50} = 489 ± 105 nM, n_H = 0.63 ± 0.07. (B) Phenylalkylamines: (−)-desmethoxyverapamil (D-888): K_i = 5.7 ± 1.0 nM, n_H = 0.98 ± 0.15; (+)-desmethoxyverapamil: K_i = 24.4 ± 3.2 nM, n_H = 1.05 ± 0.12; (±)-tiapamil: K_i = 146 ± 17 nM, n_H = 1.41 ± 0.21; (±)-D619: K_i = 5.6 ± 0.61 μM, n_H = 0.98 ± 0.09. (C) Benzothiazepines: d-*cis*-diltiazem: IC_{50} = 163.6 ± 27.5 nM, n_H = 0.79 ± 0.10; l-*cis*-diltiazem: IC_{50} = 9.14 ± 0.12 μM, n_H = 1.04 ± 0.12. (D) Divalent cations: Cd^{2+}: IC_{50} = 15.9 ± 1.10 μM, n_H = 1.89 ± 0.19; Ca^{2+}: IC_{50} = 820 ± 270 μM; n_H = 0.66 ± 0.09, maximal inhibition to 10 ± 8% of control binding.

± 9 μM). Depletion of divalent cations from membranes by incubation with increasing concentrations of EDTA at 25°C caused only partial inhibition (30%) of binding at EDTA > 10 mM (not shown).

3.2. *Properties of reversible [*N-methyl-$_3$*H]-LU49888 binding to purified calcium channels*

The dissociation constant for (−)-[^{3}H]-desmethoxyverapamil increases 15–35-fold upon calcium channel purification [8]. A similar loss of affinity was observed when [*N-methyl*-^{3}H]LU49888 binding to purified channel preparations was investigated. The K_d (at 25°C) was 62 ± 11 nM (n = 3). (−)-Desmethoxyverapamil was a competitive inhibitor (K_i = 40 ± 12 nM) and twice as potent as (±)-desmethoxyverapamil (K_i = 94 ± 20 nM). The pharmacologically less potent phenylalkylamine (±)-D619 [9] was only a weak competitor of reversible [*N-methyl*-^{3}H]LU49888 binding in purified preparations (IC_{50} = 44 ± 11 μM). The interaction of the ligand with the purified receptor is stereoselectively regulated by the enantiomers of the benzodadiazole 1,4-dihydropyridine PN 200- 110. (+)-PN 200-110 allosterically stimulates reversible binding (EC_{50} = 21 ± 5 nM, maximal stimulation to 238 ± 61% of control binding) whereas the respective (−)-enantiomer was without effect up to 1 μM (not shown).

3.3. *Photolabelling of the phenylalkylamine receptor*

Fig.3A,B shows the quantitative analysis of a representative [*N-methyl*-^{3}H]LU49888 photolabelling experiment with transverse-tubule membranes. Four polypeptides with apparent molecular masses of 155 ± 3, 92 ± 2, 60 ± 3 and 33 ± 3 kDa (means ± SD from 5 experiments), termed a–d, are photolabelled. The incorporation of [*N-methyl*-^{3}H]LU49888 into these bands was inhibited by 1 μM (−)-desmethoxyverapamil but not by 1 μM (±)-D619. 3 μM d-*cis*-diltiazem caused only partial inhibition. Thus, the protection profile shows the same pharmacological regulation by dif-

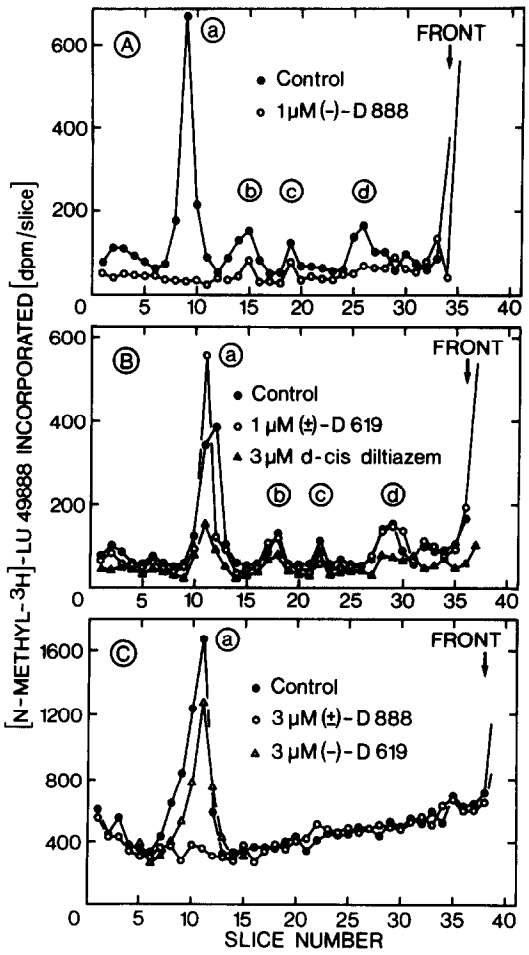

Fig.3. Photoaffinity labelling of the membrane-bound (A,B) and purified calcium channel (C) with [*N-methyl*-^{3}H]LU49888. Photoaffinity labelling experiments and SDS-PAGE were carried out as described [13]. 2.42 nM [*N-methyl*-^{3}H]LU49888 was incubated with 0.62 mg membrane protein for 45 min at 25°C. (A) 4.3 pmol [*N-methyl*-^{3}H]LU49888 were reversibly bound in the absence (●, total binding lane) and 0.27 pmol in the presence (○, nonspecific binding lane) of (−)-desmethoxyverapamil prior to irradiation. (B) 3.9 pmol [*N-methyl*-^{3}H]LU49888 were reversibly bound in the absence (●) of added drug, 3.8 pmol in the presence of 1 μM (±)-D619 (○) and 2.1 pmol in the presence of 3 μM d-*cis*-diltiazem (▲) prior to irradiation. (C) 17.8 nM [*N-methyl*-^{3}H]LU49888 was incubated with 4.7 μg purified calcium channel protein for 45 min at 25°C and irradiated as described in [6]. 2.6 pmol [*N-methyl*-^{3}H]LU49888 were specifically bound in the absence of added drug (●, total binding lane), 0.57 pmol in the presence of 3 μM (−)-desmethoxyverapamil (○, nonspecific binding lane) and 2.1 pmol in the presence of 3 μM (±)-D619 (△) prior to irradiation.

ferent calcium channel drugs as reversible binding. Specifically incorporated radioactivity was distributed as follows: a = 62 ± 10, b = 12 ± 1%, c = 14 ± 10, d = 12 ± 3% (means ± SD from 3 experiments). No difference in the apparent molecular mass of the labelled bands was observed when the samples were separated on SDS-PAGE under reducing conditions (not shown). The efficiency of specific photoincorporation (as a percentage of reversibly bound ligand was 0.2 ± 0.04%, somewhat lower than that of the 1,4-dihydropyridine photoaffinity label [^{3}H]azidopine [13].

In the purified calcium channel preparation three polypeptides with apparent molecular masses of 155, 65 and 32 kDa were identified after SDS-PAGE under alkylating conditions [6]. Only the 155 kDa polypeptide region was specifically labelled by [*N-methyl*-^{3}H]LU49888. Photolabelling was completely inhibited by 3 μM (−)-desmethoxyverapamil but not by 3 μM (±)-D619 (fig.3). The 155 kDa polypeptide region is a broad diffuse silver-stained band under alkylating conditions of electrophoresis in 5–15% gradient gels [2,5,6]. However, we observed that in 8% polyacrylamide gels the heterogeneity of this region is more apparent (see fig.4) and two polypeptides with apparent molecular masses of 155 ± 2 and 165 ± 3 kDa (n = 5) can be partly resolved under alkylating conditions. Under reducing conditions of SDS-PAGE two distinct migrating polypeptides of 155 ± 3 and 135 ± 2 kDa (n = 5) can be clearly identified by silver staining (fig.4). To determine exactly which of these polypeptides carried the irreversibly incorporated ligand, purified channels were separated after photolabelling under reducing and alkylating conditions by SDS-PAGE. Gels were stained and radioactivity in the stained bands was quantitated. It was found that a 155 ± 3 kDa (n = 3) polypeptide which does not alter its mobility upon reduction contained more than 90% of the incorporated label. A representative experiment is shown in fig.4. The extent of photoincorporation was 0.8 ± 0.1% (n = 3) in the purified preparation.

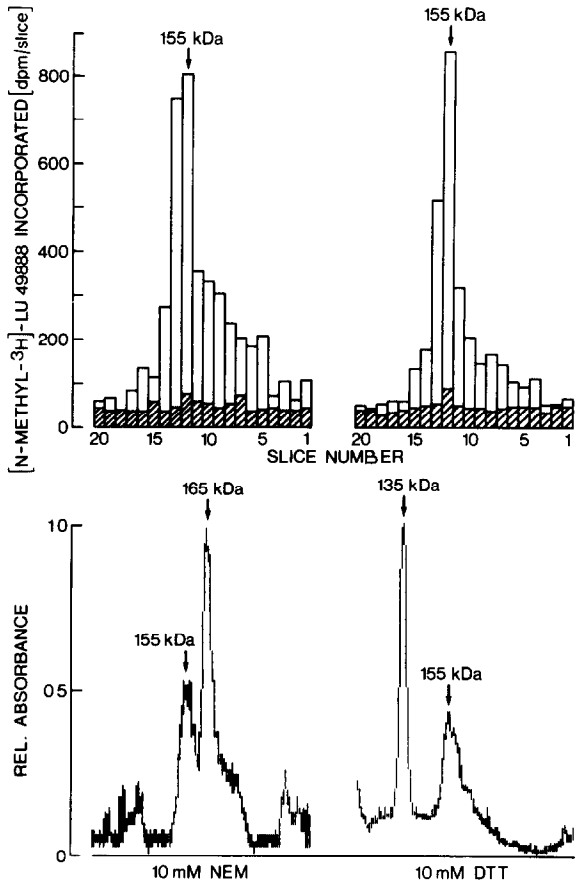

Fig.4. Identification of the [*N-methyl*-^{3}H]LU49888-labelled polypeptide in purified calcium channel preparations. Purified calcium channels (5.1 μg protein) were photolabelled, employing 15.1 nM [*N-methyl*-^{3}H]-LU49888. The irradiated samples were separated on an 8% polyacrylamide gel under alkylating (10 mM *N*-ethylmaleimide, NEM; left panel) and reducing (10 mM dithiothreitol, DTT; right panel) conditions. The gel was stained with silver and scanned on an LKB Ultroscan laser densitometer. Gel lanes were subsequently cut into 2 mm slices and the radioactivity determined by liquid scintillation counting. The figure shows the results from the top of the gel (slice no.1) to the 100 kDa region (slice no.20). Incorporated radioactivity in the absence (open columns) and presence (hatched columns) of 3 μM (−)-desmethoxyverapamil (upper panel) is shown in comparison with the densitometric scan (lower panel).

4. DISCUSSION

The arylazidophenylalkylamine, [*N-methyl*-^{3}H]LU49888, exhibits similar binding characteristics to (−)-[^{3}H]desmethoxyverapamil. The latter is a high-affinity, high-specific-activity ligand for calcium channel-linked phenylalkylamine receptors in skeletal muscle [9,14,15], brain [15,16] and heart [17,18]. Our data show that [*N-methyl*-^{3}H]-LU49888 labels phenylalkylamine receptors in skeletal muscle in the absence of ultraviolet light with a K_d value of 2.0 nM, almost identical with that of (−)-[^{3}H]desmethoxyverapamil [9]. After photolysis of [*N-methyl*-^{3}H]LU49888-labelled membranes, the ligand covalently incorporated into four bands (a–d) with apparent molecular masses of 155, 92, 60 and 33 kDa after separation by SDS-PAGE. The protection profile of incorporation into these bands was identical to the regulation of reversible [*N-methyl*-^{3}H]LU49888 binding. This is convincing evidence for the pharmacological specificity. 62% of the specifically incorporated radioactivity was recovered in the 155 kDa region. The irreversibly labelled 155 kDa polypeptide had identical electrophoretic mobility under reducing and alkylating SDS-PAGE conditions when the particulate calcium channel preparation was investigated. In contrast to the membrane-bound channel, specific photolabelling in the 92, 60 and 33 kDa region of the purified channel was not observed. Perhaps, these lower molecular mass polypeptides are proteolytic fragments of the drug-receptor carrying channel component which are lost upon purification. Similar findings have been reported for [^{3}H]azidopine which can also specifically photoincorporate to a minor extent into lower molecular mass membrane polypeptides [13] but only labels the 155 kDa polypeptide region in the purified channel preparation [6]. Employing reducing conditions in SDS-PAGE the 155 kDa region of the purified channel was clearly resolved into two distinct polypeptides. The 155 kDa polypeptide had identical electrophoretic mobility under both (alkylating and reducing) conditions and carries the specifically incorporated photoaffinity label observed in the purified preparation. The other polypeptide (165 kDa under alkylating conditions) changes its mobility, has an apparent molecular mass of 135 kDa after reduction and was not photolabelled. This polypeptide has similar electrophoretic properties to the protein identified by immunoblotting, claimed to carry the 1,4-dihydropyridine receptor [19,20]. As only L-type calcium channels in skeletal muscle preparations are sensitive to 1,4-dihydropyridines and phenylalkylamines [21,22], we conclude that the 155 kDa and not the 135 kDa polypeptide is the high-affinity phenylalkylamine receptor carrying subunit of the L-type calcium channel. We suggest that amino acid sequences from the 155 kDa [*N-methyl*-^{3}H]LU49888-photolabelled polypeptide of the purified channel are the most promising approach for the generation of oligonucleotides. These should be used for the screening of cDNA libraries in order to obtain the primary structure of the drug-receptor carrying calcium channel subunit.

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