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A 75-kDa polypeptide, located primarily at the plasma membrane of carrot cell-suspension cultures, is photoaffinity labeled by the calcium channel blocker LU 49888

(signal perception/signal transduction/second messenger/photolabeling/subcellular localization)

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ABSTRACT **Calcium channel blockers of the phenylalkylamine family bind specifically to membranes and inhibit calcium uptake in carrot protoplasts. LU 49888, an azido derivative of phenylalkylamine, behaves as its unmodified homolog in terms of affinity and specificity and therefore allows us to probe the receptor by photoaffinity labeling. Upon UV irradiation, a 75-kDa peptide was specifically labeled. Incubation of microsomes with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, a zwitterionic detergent, led to the solubilization of the LU 49888-binding protein. Electrophoretic analysis under denaturing conditions and gel filtration of the solubilized "receptor–ligand" complex show a 75-kDa peptide mainly located at the plasma membrane. Consequently the LU 49888-binding protein in plants differs significantly from its animal counterpart by its size and may be a primary target for external signal molecules.**

Pharmacologically active drugs, referred to as calcium channel agonists or antagonists, may control various physiological processes in plants. Biochemical evidence has been obtained concerning the ability of membranes derived from plant cells to specifically bind calcium channel blockers (1–6). Moreover, it has been shown (*i*) that the site occupancy of the receptor by the ligands results in the inhibition of calcium uptake in carrot protoplasts (1) and (*ii*) that the receptor of calcium channel blockers may be a primary target of some phytotoxins (2). Together, these data suggest that functional calcium channels occur in plants, but little is known of their structure.

A membrane-bound protein retaining the ability to bind verapamil derivatives has been solubilized from maize coleoptile membranes (3). However, the verapamil-binding protein has not been clearly identified. The development of photoaffinity probes has allowed considerable progress in the field of receptor and carrier isolation. Auxin-binding proteins, putative auxin carriers, and receptors have been covalently labeled with azido auxin derivatives (7, 8). In animal systems, photoaffinity labeling has led not only to the molecular analysis of a number of hormone receptors but also to the isolation of calcium channel components (9–13). Such a strategy has been chosen to probe the receptor of the calcium channel antagonist in membranes from carrot cell-suspension cultures and detergent-solubilized proteins.

Since phenylalkylamine derivatives have been shown to be the best ligands for carrot membranes and protoplasts (1, 2), we have used LU 49888 (Fig. 1) as a photoaffinity probe.

Here, we report on the specific labeling of a 75-kDa peptide that is primarily located at the plasma membrane and retains its ability to bind calcium channel antagonists upon solubilization.

MATERIALS AND METHODS

Plant Material, Membrane Preparation, and Storage. Details on the carrot cell-suspension cultures (*Daucus carota* L.) have been extensively described (1). Microsomes were prepared according to a published procedure (1, 2) and were either used fresh or stored at −80°C.

Solubilization of Membrane-Bound Proteins. Microsomes (5 mg of protein per ml) were diluted 2-fold with 20 mM Tris·HCl, pH 7.5, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS), and 10% (vol/vol) glycerol and stirred for 30 min at 0°C. When indicated, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM N^{α}-(*p*-tosyl)lysine chloromethyl ketone, antipain (1 μg/ml), leupeptin (1 μg/ml), and pepstatin (1 μg/ml) were added.

Binding Experiments and Photoactivation. The routine binding assay described by Graziana *et al.* (1) was used except that the incubations were performed at 0–4°C in the dark. LU 49888 was irreversibly bound by photoactivation with a UV lamp (T UV 30 W; Philips; 254 nm) from a distance of 10 cm at −196°C for 1 min. The irradiance was approximately 1 mW/cm^2 at the sample level. When solubilized proteins were used as a source of binding proteins, 100 μg of protein was incubated in 1 ml of 20 mM Tris·HCl at pH 7.5 containing 1% CHAPS, 5% glycerol, and (−)-[^{3}H]LU 49888 and was irradiated.

Free-Flow Electrophoresis. The photolabeled microsomes were centrifuged for 30 min at 45,000 × *g*, and the pellet was resuspended in running buffer (30 mM Tris and 10 mM boric acid at pH 8.3) containing 0.3 M sorbitol, 0.5 mM $MgCl_2$, and 5 mM KCl. The microsomes were washed and resuspended in running buffer. Unlabeled microsomes were added to a final concentration of 2 mg of protein per ml. The membranes were separated by free-flow electrophoresis at 4°C with a Vap-22 electrophoresis unit (Bender and Hobein, Munich) using 100 mM Tris and 100 mM boric acid at pH 8.3 as the electrode buffer. The running conditions were as follows: current, 110 mA (about 1200 V); buffer flow, 3 ml per hr per fraction; sample injection rate, 2 ml per hr. The separated fractions were collected by centrifugation, and marker enzyme activities were determined (14).

NaDodSO$_4$/PAGE, Fluorography, and Size-Exclusion Chromatography. The photolabeled membranes were pelleted, and proteins were solubilized by CHAPS. The suspension was centrifuged for 60 min at 150,000 × *g*, the supernatant was concentrated by using a microconcentrator Cen-

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Abbreviation: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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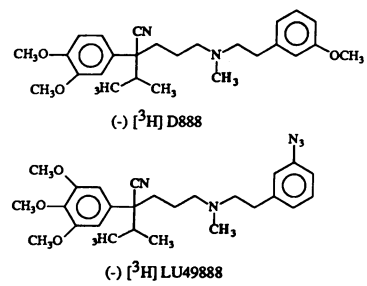

FIG. 1. Structures of D888 [desmethoxyverapamil; 2,7-dimethyl-3-(3,4-dimethoxyphenyl)-3-cyan-7-aza-9-(3-methoxyphenyl)nonane] and LU 49888 {5-[(3-azidophenylethyl)methylamino]-2-(3,4,5-trimethoxyphenyl)-2-isopropylxaleronitrile}. The systematic name for D600 (methoxyverapamil) is α-[3-[[2-(3,4-dimethoxyphenyl) ethyl]methylamino]propyl]-3,4,5-trimethoxy-α-(1-methylethyl)-benzeneacetonitrile (structure not shown).

tricon 10 (Amicon), and proteins were analyzed by $NaDodSO_4$/PAGE (12.5% acrylamide) (15). After electrophoresis, gels were prepared for fluorography (16) and exposed at −80°C to Hyperfilm MP (Amersham) for 3–5 weeks. Solubilized binding proteins were photolabeled and concentrated as described above. The samples were suspended in running buffer and analyzed by fast protein liquid chromatography by size exclusion on a calibrated Superose 6 column (Pharmacia). The column was equilibrated with 20 mM Tris·HCl at pH 7.5 containing 0.2% CHAPS and 5% glycerol, and the chromatography was performed at 8°C. Fractions were collected, and the radioactivity was determined.

Protein Measurements. Proteins were measured by the bicinchoninic acid assay (17).

Chemicals and Radiochemicals. Chemicals and radiochemicals were analytical grade when available. Bepridil was a gift from N. Busch (Laboratoire Cerm, Riom, France), (−)-[^{3}H]LU 49888 (3.15 TBq/mmol; 1 μCi = 37 kBq; radiochemical purity >98%) was a gift from M. Traut (Knoll AG, Ludwigshafen, F.R.G.), and verapamil derivatives (D600 and D888; see Fig. 1) were a gift from M. Lazdunski (Université de Nice, France). Protease inhibitors were purchased from Sigma and (−)-[*N-methyl*-^{3}H]D888 (3.03 TBq/mmol) was from Amersham.

RESULTS

Microsomes from Carrot Cells Bind Labeled D888 and Its Azido Derivative, LU 49888. Since the binding to detergent-solubilized proteins was less effective at 20°C than at 4°C (data not shown), all the experiments were performed at 4°C. In the dark (no UV light), the kinetic parameters of the binding of LU 49888 and its unmodified homolog are comparable (Fig. 2 *a–c*). The B_{max} values were practically the same (48 and 50 pmol/mg of protein), whereas the K_d for LU 49888 was lower than that for D888 (14 nM and 57.9 nM, respectively) (Fig. 2*c*). Moreover, the order of potency of the competitors was identical to that already described (1). The (−) stereoisomers were the most effective (Fig. 2*d*).

In the dark, LU 49888 binding to membranes was inhibited (time 0) or reversed (120 min) when the unlabeled competitor was added (Fig. 3). Irradiation with UV light at −196°C led to the irreversible binding of LU 49888, which was inhibited by incubation with (−)-bepridil (Fig. 3*a*). The incorporation of LU 49888 was maximal after 1 min of irradiation and remained stable after 2 further min of photoactivation (data not shown). Addition of the unlabeled competitor after irradiation had no effect on the binding (Fig. 3*b*). Therefore, the ligand was covalently bound to the membranes. In routine experiments, 15–20% of the photolabeled probe was incorporated in the membrane fraction. However, after photoactivation, the nonspecific binding component reached 55% of the total binding instead of 10% (no UV control) (Fig. 3). Therefore the labeled microsomes were incubated with 1% CHAPS, resulting in a selective solubilization of the LU

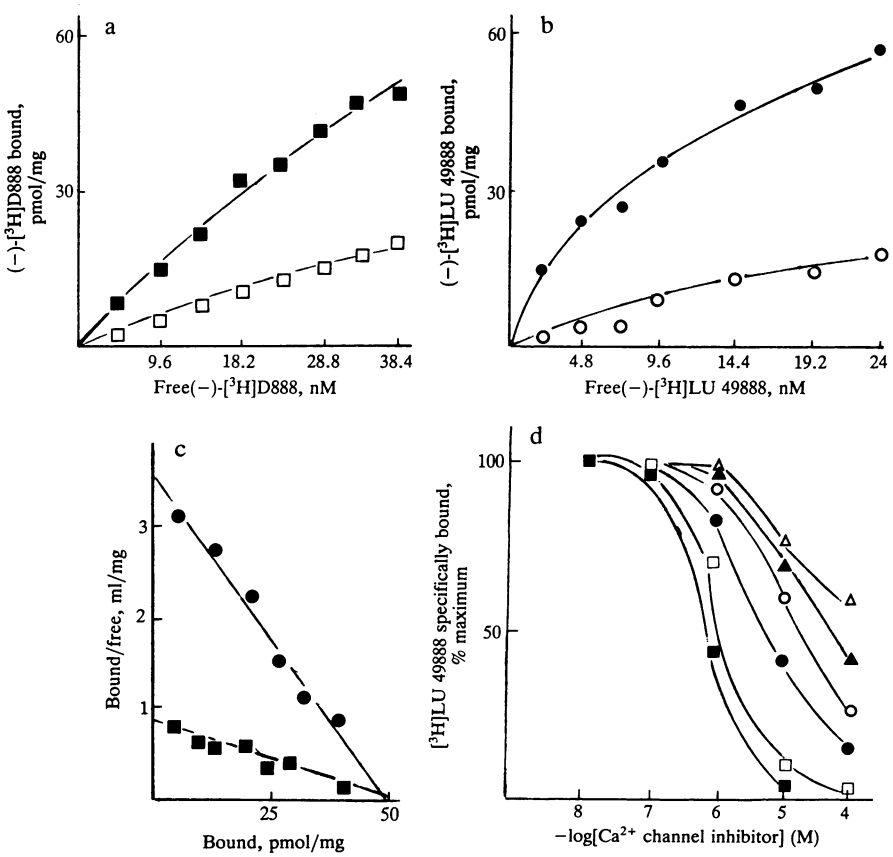

FIG. 2. Equilibrium binding of (−)-[^{3}H]D888 and (−)-[^{3}H]LU 49888 to carrot microsomes and inhibition of binding by various calcium channel inhibitors. Equilibrium binding was measured by using increasing concentrations of (−)-[^{3}H]D888 (at 82 Ci/mmol) or (−)-[^{3}H]LU 49888 (at 85 Ci/mmol) and 50 μg of microsomal proteins per ml in 50 mM Tris·HCl at pH 7.5 for 120 min at 4°C in the dark. (*a*) Binding of (−)-[^{3}H]D888 in the presence (□) or absence (■) of 50 μM (−)-bepridil as a competitor. (*b*) Binding of [^{3}H]LU 49888 in the presence (○) or absence (●) of 50 μM (−)-bepridil. (*c*) Scatchard plots for the specific (−)-[^{3}H]D888 (■) and [^{3}H]LU 49888 (●) binding components. (*d*) Inhibition of (−)-[^{3}H]LU 49888 binding to carrot microsomes at equilibrium by calcium channel inhibitors. The specific binding of radiolabeled ligand (2.4 nM) to microsomes (50 μg/ml) was measured in the presence of increasing concentrations of (+)-bepridil (□), (−)-bepridil (■), (+)-D888 (○), (−)-D888 (●), (+)-D600 (△), and (−)-D600 (▲) after a 120-min incubation in the dark.

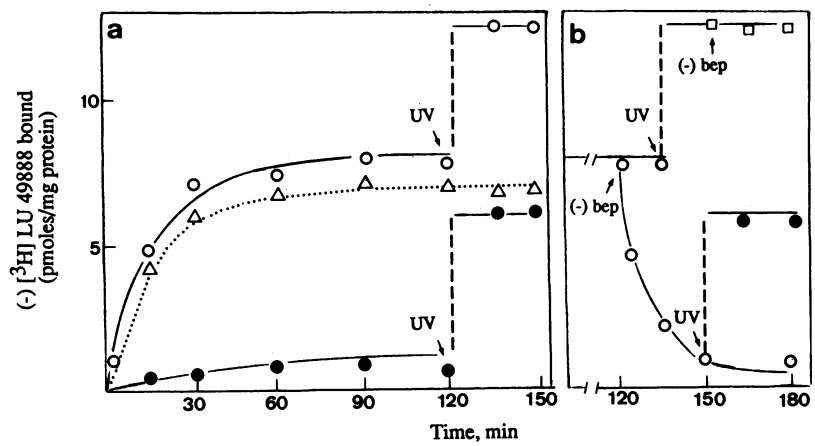

FIG. 3. Effect of UV irradiation on the binding of (−)-[^{3}H]LU 49888 to carrot microsomes. (−)-[^{3}H]LU 49888 (2.4 nM) was incubated with microsomes (50 μg of protein per ml) in the absence (○) or the presence (●) of 50 μM (−)-bepridil in the dark for 120 min. (*a*) Five hundred microliters of the incubation media was removed at the indicated times, filtered, and rinsed, and the radioactivity remaining on the filters was determined. After a 120-min incubation, the samples were frozen at −196°C, UV irradiated for 1 min, and allowed to thaw. Five hundred microliters was processed as described for the nonirradiated samples. △, Specific component of the binding. (*b*) After a 120-min incubation, an aliquot of the assay done in the absence of bepridil was supplemented with 50 μM (−)-bepridil and either maintained in the dark throughout (○) or for 30 min before freezing and UV irradiation (●). Another aliquot was supplemented with 50 μM (−)-bepridil after UV irradiation and thawing of the sample (□). The radioactivity remaining on the membrane retained by the filters was determined. (−) bep, (−)-bepridil.

49888-binding protein. Thus, 70% of the specific binding component (vs. 50% of total membrane-bound proteins) was recovered in the solubilized fraction, whereas most of the nonspecific component remained membrane bound (Table 1).

The "Ligand–Binding-Protein Complex" Occurs as a 75-kDa Molecular Entity. The detergent-solubilized samples were analyzed by NaDodSO$_4$/PAGE, and the radiolabeled peptides were identified by fluorography (Fig. 4). Of the many polypeptides, only a 75-kDa polypeptide was labeled with the photoactivable probe. The presence of exogenous (−)-bepridil prevented the incorporation of LU 49888 into the polypeptide, showing that the photolabeling was specific. Labeling did not proceed without photolysis or when LU 49888 was photolyzed prior to the immediate addition of the membranes, suggesting that the nitrene species generated by the irradiation is short lived. The specific label was associated with the 75-kDa polypeptide regardless of the denaturing conditions. Therefore, the polypeptide does not contain an interdisulfide linkage. Inclusion of protease inhibitors during the overall process did not change the size of the labeled polypeptide.

The Detergent-Solubilized Polypeptide Is Able to Bind LU 49888. Incubation of microsomes with CHAPS led to the solubilization of membrane-bound proteins retaining their ability to bind LU 49888. As determined by size-exclusion chromatography in the presence of CHAPS (Fig. 5), the radiolabel was associated with a 75-kDa molecule. UV irradiation of the detergent-solubilized polypeptides prior to incubation with [^{3}H]LU 49888 and photoactivation did not change the molecular size of the labeled molecule, suggesting that there is no photogenerated proteolysis (18). There was no label in the fractionation range of the column when unlabeled (−)-bepridil was added prior to UV irradiation or if the photoactivable probe was photolyzed prior to the addition of the solubilized proteins. NaDodSO$_4$/PAGE and fluorography of the concentrated samples showed a labeled 75-kDa polypeptide (Fig. 5*b*). Consequently, it appears that

Table 1. CHAPS solubilization of [^{3}H]LU 49888 bound to its putative receptor from photolabeled carrot microsomes

	Bound ^{3}H, dpm × 10^{-3}		
Binding	UV-irradiated microsomes	CHAPS-insoluble fraction	CHAPS-solubilized fraction
Total	2520	755.22	1226.22
Nonspecific	1386	752.14	356.20
Specific	1134	3.08	870.02

Microsomes (2.5 mg of protein) were incubated in the dark with [^{3}H]LU 49888 (2.4 nM) in the absence (total binding) or the presence (nonspecific binding) of 50 μM (−)-bepridil. After a 120-min incubation, the samples were irradiated. The photolabeled membranes were supplemented with 1% CHAPS and 5% glycerol and stirred for 30 min at 0°C. The samples were centrifuged 60 min at 150,000 × *g*, and the radioactivity associated with the pellet and the supernatant was determined.

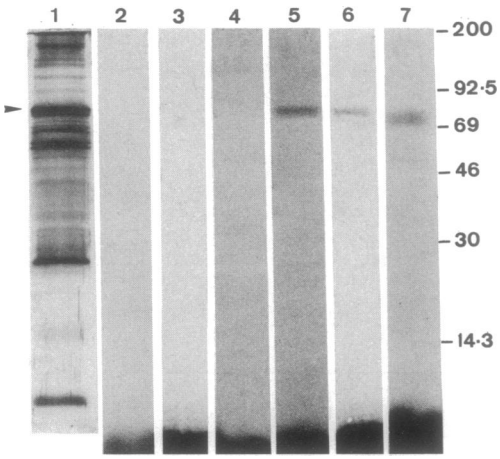

FIG. 4. Molecular size of the LU 49888-binding protein. The microsomes photolabeled under the specified conditions were incubated with 1% CHAPS and 5% glycerol (final concentration) for 30 min at 4°C. The soluble fraction was concentrated and analyzed by NaDodSO$_4$/PAGE and fluorography (12.5% acrylamide gels). Lane 1, silver nitrate staining of the CHAPS-solubilized polypeptides (10 μg of protein); lanes 2–6, fluorography of labeled proteins; lane 2, nonirradiated sample (no UV control; 75 μg of protein); lane 3, membranes added immediately into the reaction chamber after a 1-min photolysis of the photoaffinity probe without the membranes (75 μg of protein); lane 4, incubation done in the presence of 50 μM (−)-bepridil (75 μg of protein); lanes 5–7, incubation and photolabeling in the absence of bepridil; lane 5, experiments done in the presence of a protease inhibitor mixture (75 μg of protein); lane 6, protein (50 μg) denatured under nonreducing conditions (10 mM *N*-ethylmaleimide); lane 7, protein (75 μg) denatured under reducing conditions (10 mM dithiothreitol).

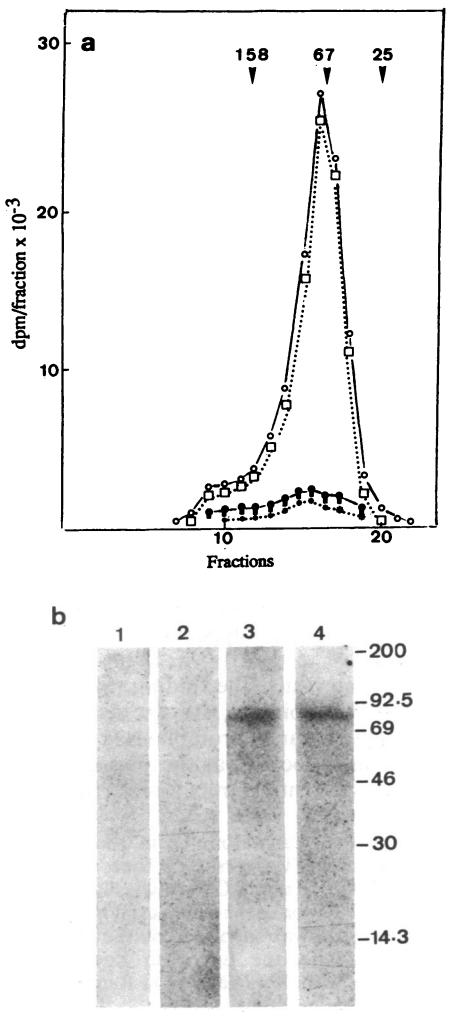

FIG. 5. Size-exclusion chromatography of the solubilized LU 49888-binding protein. Proteins were detergent-solubilized as described for Fig. 4, and a sample was irradiated for 1 min at 0–4°C. Samples were incubated with 2.4 nM (−)-[^{3}H]LU 49888 for 120 min in the dark at 4°C in the presence (■, irradiated proteins; ●, nonirradiated proteins) or the absence (□, irradiated proteins; ○, nonirradiated proteins) of 50 μM (−)-bepridil, frozen at −196°C, and UV irradiated. (*a*) The photolabeled peptides (200 μg) were fractionated by size-exclusion chromatography (Superose 6 column). Only the radioactivity eluted in the fractionation range of the column is shown. *, LU 49888 photolyzed prior to the addition of the proteins. ▼, Elution volumes of standard proteins of known molecular size. (*b*) NaDodSO$_4$/PAGE and fluorography of the LU 49888-binding protein recovered after size-exclusion chromatography. Lane 1, LU 49888 photolyzed prior to the addition of protein; lane 2, photoaffinity labeling performed in the presence of 50 μM (−)-bepridil; lane 3, protein irradiated before photoaffinity labeling; lane 4, photoaffinity labeling of untreated protein.

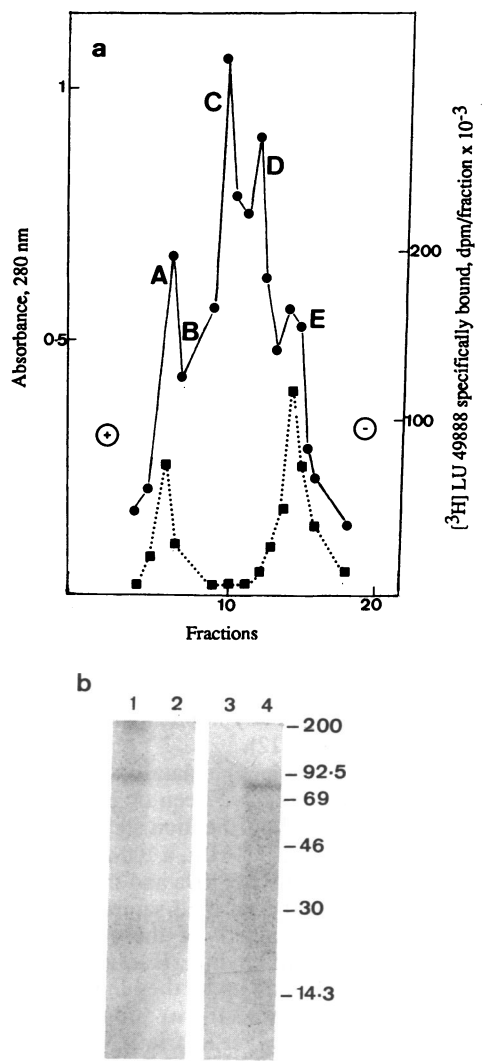

FIG. 6. Distribution of the (−)-[^{3}H]LU 49888 specifically bound to different membrane fractions after photolabeling of microsomes and subsequent separation by free-flow electrophoresis. (*a*) ●, Absorbance at 280 nm; ■, radioactivity corresponding to the specific binding component (e.g., total minus nonspecific binding). The distribution of marker enzymes among fractions A–E is given in Table 2. (*b*) Analysis by NaDodSO$_4$/PAGE and fluorography of peptides from fractions A [tonoplast; photolabeled in the absence (lane 1) or the presence (lane 2) of 50 μM (−)-bepridil] and E [plasma membrane; photolabeled in the absence (lane 4) or the presence (lane 3) of 50 μM (−)-bepridil].

the LU 49888-binding protein is a 75-kDa polypeptide that may be labeled either when located in membranes or after solubilization.

The Calcium Channel Blocker-Binding Protein Is Located Primarily at the Plasma Membrane. Microsomes photolabeled in the presence or absence of competitor were resolved by free-flow electrophoresis, and the nonspecific components were subtracted. Radioactivity corresponding to specific binding is shown in Fig. 6*a*. Seventy-three percent of the radioactivity was recovered in fraction E, and the remaining radioactivity was associated with fraction A. The distribution of marker enzymes among the different fractions (Table 2) indicates that the most active membrane is the plasma membrane (fraction E, based on glucan synthase II activity) followed by the tonoplast (fraction A, rich in pyrophosphatase). In both cases, a 75-kDa polypeptide was labeled (Fig. 6*b*). Other membranes located in fractions B, C, and D are essentially free of specific label.

DISCUSSION

In animal systems, the voltage-gated calcium channel is composed of four distinct subunits (for a review, see ref. 12). Photoaffinity labeling with the dihydropyridine [^{3}H]azidopine (10, 19), the azido derivative of phenylalkylamine, [^{3}H]LU 49888 (9, 19), or the benzothiazepine [^{3}H]azido diltiazem (13) has shown that the α_1 subunit (a 175-kDa polypeptide) contains the binding sites for all three of these classes of calcium channel blockers. LU 49888, by inhibiting K^+-induced contraction of rat aortic strips and (−)-[^{3}H]D888 binding, behaves as a true calcium channel blocker (20). Slow calcium current absent from cultured skeletal muscle cells

Table 2. Activities of marker enzymes associated with membrane fractions separated by free-flow electrophoresis

Marker enzyme	Activity	Membrane fraction* A	B	C	D	E
Cytochrome *c* oxidase	Total†	0.98	1.87	5.0	0.99	0.55
	Specific‡	0.9	1.8	2.0	1.1	0.4
NADPH cytochrome *c* reductase	Total†	0.033	0.041	0.475	0.009	0.14
	Specific‡	0.03	0.04	0.19	0.01	0.10
Latent IDPase	Total§	176	572	575	180	153
	Specific¶	0.16	0.55	0.23	0.20	0.11
Glucan synthase II	Total‖	—	135	265	414	3874
	Specific**	ND	130	106	460	2787
Pyrophosphatase	Total††	18.09	4.16	—	—	—
	Specific‡‡	16.3	4.0	ND	ND	ND

ND, not detected; IDPase, Inosine diphosphatase.
*, Pooled fractions from free-flow electrophoresis (see Fig. 6*a*); †, OD units per min; ‡, OD units per min per mg of protein; §, nmol of P_i per hr; ¶, nmol of P_i per hr per μg of protein; ‖, dpm incorporated per hr; **, dpm incorporated per hr per mg of protein; ††, nmol of P_i per min; ‡‡, nmol of P_i per min per mg of protein.

from mice with muscular dysgeny is restored upon microinjection of an expression plasmid carrying the cDNA of the α_1 subunit (21). Consequently the α_1 subunit displays essentially all the properties of the channel such as binding of the channel blockers, ion conduction, and voltage sensing (21). The three other subunits are supposedly implicated in regulatory functions not yet elucidated (12).

In plants, the only molecular data on the "receptor" of calcium channel blockers have been obtained by Harvey *et al.* (3) in maize. The final purification step gave four stained bands corresponding to a 169-kDa, a 100-kDa, a 70-kDa, and a 66-kDa polypeptide. The 169-kDa and the 70-kDa polypeptides were enriched in the purified sample. Although it has not been demonstrated which of these bands is actually involved in the verapamil binding, Harvey *et al.* (3) suggest that the 169-kDa peptide might be the binding protein due to the similarity in the size with the α_1 subunit of the receptor in animal systems. However, various data have already suggested that plant systems may significantly differ from their animal counterpart. Thus, phenylalkylamines are the most efficient ligands for higher plants (1, 3, 6), whereas compounds of the dihydropyridine or diltiazem series are biologically active in mosses (4) and algae (5). In addition, monoclonal and polyclonal antibodies raised against the α_1 subunit from skeletal muscle do not cross-react with plant membrane proteins (1, 3).

Photoaffinity labeling has allowed us to address directly the questions of the molecular size of the LU 49888-binding protein and its subcellular localization. Binding was effective on membranes and detergent-solubilized proteins, resulting in the specific and photodependent labeling by short-lived nitrene intermediates of a 75-kDa polypeptide located primarily at the plasma membrane. A 75-kDa peptide has also been reported to occur as a proteolytic fragment of the native protein from frozen skeletal muscle (19). Such a situation may occur in plant systems; however, (*i*) photolabeling of fresh microsomes prepared and processed in the presence of several protease inhibitors and (*ii*) UV irradiation of CHAPS-solubilized proteins before photoaffinity labeling do not change the molecular size of the binding protein. In contrast, pretreatment of membranes or solubilized proteins with trypsin led to the loss in LU 49888-binding capacity (data not shown). Therefore, the 75-kDa peptide is not a photolytic product of a bigger protein or a proteolytic fragment generated by nonspecific proteases. From our data, we conclude that the LU 49888-binding protein from carrot membrane differs from the animal counterpart by its specificity for phenylalkylamine derivatives, its immunological properties, and its molecular size. At present, it cannot be determined if the 75-kDa peptide from carrot shares any homology with either the 169-kDa or the 70-kDa peptide from maize, since none of these polypeptides has been shown to bind verapamil.

Of particular interest is the finding that (*i*) the best competitors for LU 49888-binding sites are also the best inhibitors of calcium uptake by carrot protoplasts (1) and (*ii*) the binding protein is primarily located at the plasma membrane, suggesting that it is a potential target for external signals (2, 3, 6).

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