Solubilized proteins from carrot (Daucus carota L.) membranes bind calcium channel blockers and form calcium-permeable ion channels
(voltage-dependent Ca\(^{2+}\) channel/signal transduction/cytosolic calcium/plant ion channel/liposome)

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ABSTRACT Calcium channels have been suggested to play a major role in the initiation of a large number of signal transduction processes in higher plant cells. However, molecular components of higher plant Ca\(^{2+}\) channels remain unidentified to date. Calcium channel blockers of the phenylalkylamine family and bepridil specifically inhibit Ca\(^{2+}\) influx into carrot (Daucus carota L.) cells. By using a phenylalkylamine azido derivative, a 75-kDa carrot membrane protein had been previously identified. Here we have partially purified this Ca\(^{2+}\) channel blocker-binding protein by lectin-affinity and ion-exchange chromatographies. The protein fraction containing the 75-kDa binding protein was incorporated into giant liposomes. Single-channel patch-clamp studies on these proteoliposomes showed the presence of Ca\(^{2+}\)-permeable channel currents. These Ca\(^{2+}\)-permeable channels were not stable. Recordings after durations of 2–10 min showed the appearance of nonselective ion channels with a permeability to calcium and chloride ions. These nonselective Ca\(^{2+}\)-permeable ion channels, in contrast, were stable and were recorded for extended durations. The addition of the Ca\(^{2+}\) channel-blocker bepridil (10 \(\mu\)M) led to the inhibition of these nonselective Ca\(^{2+}\)-permeable channels by reducing the probability of channel opening. These results suggest that the 75-kDa Ca\(^{2+}\) channel blocker-binding protein from carrot cells plays a role in channel sensitivity to Ca\(^{2+}\) channel inhibitors and may constitute one of the components of Ca\(^{2+}\) channels in higher plants.

A signal-induced increase in the cytosolic Ca\(^{2+}\) concentration is considered to be among the most important intracellular messengers for initiation of metabolic and developmental events in higher plants. Calcium controls various processes, including cellular organization, ion channel gating and enzyme activities, during physiological responses to external stimuli (1–5). The influx of calcium into the cytosol of plant cells is mediated by Ca\(^{2+}\)-permeable signal-regulated ion channels which traverse the plasma membrane and vacuolar membrane (refs. 6 and 7; for reviews, see refs. 8 and 9).

In spite of the demonstration that Ca\(^{2+}\) influx systems are involved in physiological processes in plants, the molecular structure of Ca\(^{2+}\) channel components remains unidentified in higher plant cells. Pharmacological agents, referred to as Ca\(^{2+}\) channel blockers or antagonists, are known to interfere with a variety of plant processes (1, 4, 9). High-affinity membrane receptors for these compounds have been characterized in different plants (10–13). Site occupancy of these receptors results in the inhibition of Ca\(^{2+}\) uptake into carrot protoplasts (11, 14). The most potent antagonists of Ca\(^{2+}\) influx found to date for higher plants are phenylalkylamines and bepridil (11, 14).

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A plasma membrane protein of 75-kDa molecular mass that binds with high affinity to a phenylalkylamine azido derivative, (—)-5-(3-azidophenylethyl)\([\text{H}]\)methylamino)-2-(3,4,5-trimethoxyphenyl)-2-isopropylvaleronitrile \([\text{H}]\)LU 49888, has been identified in carrot cells (13). This protein retains its ability to bind Ca\(^{2+}\) channel antagonists upon solubilization by a non-denaturating detergent, 3-[(3-cholamidopropyl)dime-thylammonio]-1-propanesulfonate (CHAPS) (13). In the present study, we investigated, using reconstitution experiments and the patch-clamp technique, whether membrane protein fractions enriched with the 75-kDa binding protein from carrot cells contribute to Ca\(^{2+}\)-permeable ion channel function and sensitivity to Ca\(^{2+}\) channel blockers. This approach allows biochemical insight into components contributing to the structure of Ca\(^{2+}\) channels of higher plants.

MATERIALS AND METHODS

Plant Material, Membrane Preparation, and Storage. Suspension cultures of carrot (Daucus carota L.) cells were prepared as described previously (11). Total membranes were prepared according to a published procedure (11). Membranes were used directly after preparation or after storage at −80°C. Protein concentrations were measured by the bicinchoninic acid assay (15).

Photolabeling. The presence of the calcium channel blocker-binding protein was verified by photoaffinity labeling with \([\text{H}]\)LU 49888 as described previously (13). The non-specific component of the binding was determined in the presence of the unlabeled calcium channel blocker (—)-bepridil at 50 \(\mu\)M. The photolabeled microsomes were used as starting material to purify the \([\text{H}]\)LU 49888-binding protein complex. Bepridil was from Laboratoire Cerm (Riom, France), and \([\text{H}]\)LU 49888 (3.15 TBq/mmol) was from Knoll (Ludwigshafen, F.R.G.).

Solubilization and Purification of the Calcium Channel Blocker-Binding Protein. Membrane proteins were solubilized as described (13). The solubilized proteins were diluted 10-fold with 20 mM Tris·HCl, pH 7.5–5/50% (vol/vol) glycerol and loaded onto a concanavalin A (Con A)-Sepharose column (Pharmacia LKB) equilibrated in 20 mM Tris·HCl, pH 7.5/50% glycerol/0.1% CHAPS (buffer A). The column was washed with buffer A and the calcium channel blocker-binding protein was eluted with 0.25 M methyl a-D-mannopyranoside in buffer A and loaded onto a DEAE-trisacryl column (IBF) equilibrated in buffer A. Unbound materials were washed with 100 mM NaCl in buffer A, and the proteins retained by the ion exchanger were eluted by a

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

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gradient 100–300 mM NaCl in buffer A. The fractions containing the binding protein (eluted at 200 mM NaCl) were pooled, diluted 2-fold with buffer A, and loaded onto a second Con A-Sepharose column, which was equilibrated with 100 mM NaCl in buffer A. The column was washed with 100 mM NaCl in buffer A and the binding protein was eluted with 0.25 M methyl-α-d-mannoside/100 mM NaCl in buffer A. The final extract (partially purified extract) was desalted by using a PD-10 Sephadex G-25M column (Pharmacia), equilibrated in 20 mM Tris-HCl, pH 7.5/5% glycerol/1% CHAPS and was concentrated by using a Centricon 10 microconcentrator (Amicon). All procedures were performed at 4°C.

Reconstitution and Formation of Large Liposomes. For reconstitution experiments, solubilization and purification of the calcium channel blocker-binding protein was performed according to the procedure described above without previous treatment with [3H]LU 49888.

Total solubilized proteins or partially purified extracts were mixed with l-a-lecithin lipids from soybean (type II-S, Sigma) dissolved in 20 mM Tris-HCl, pH 7.5/5% glycerol/1% CHAPS (protein-to-lipid ratio: 1 mg/100 mg). The reconstitution was performed at 4°C by removing the detergent by dialysis for 72 hr against three 1-liter batches of 20 mM Tris-HCl, pH 7.5. Subsequently, the proteoliposomes were transformed into large liposomes, suitable for patch-clamp studies, by using the dehydration-rehydration technique (16, 17). The rehydration was carried out with 20 mM Hepes-Tris, pH 6.5/90 mM CaCl2.

**Patch-Clamp Recording and Data Analysis.** All experiments were performed at room temperature (21 ± 2°C). For patch-clamp measurements, 2.5 μl of large liposomes was diluted in 1.5 ml of 20 mM Hepes-Tris, pH 6.5/90 mM CaCl2 (bath solution), resembling the solution used for identification of the molecular components contributing to skeletal muscle Ca2+ channel function (18, 19). Single-channel currents were measured by patch-clamp techniques (20). Kima-51 glass pipettes (Kimble Glass, Vineland, NJ) were filled with 20 mM Hepes-Tris, pH 6.5/9 mM CaCl2 containing 200 mM d-mannitol to adjust the osmolality of the pipette solution to the same osmolality as the bath solution (240 mmol·kg−1). After the pipette was sealed, withdrawal of the pipette from the membrane surface and quickly passing the tip through the air/buffer interface resulted in an excised membrane patch ("inside-out configuration"; see ref. 20). Single-channel currents were measured and recorded with an Axopatch 200 amplifier (Axon Instruments, Burlingame, CA). Membrane potentials are referred to as the potential on the interior of the pipette with respect to the bath electrode, which was held to virtual ground. Ion channel recordings were stored and analyzed after pulse code modulation and subsequently digitized by using an Axolab TL-1 DMA interface (Axon Instruments) as described previously (21). Recorded single-channel data were filtered at 0.5 kHz, digitized at a sampling interval of 100 μsec, and analyzed by using the program pClamp (Axon Instruments). For all experiments corrections for ionic activities (22) and liquid junction potentials (23) were determined and incorporated into the analysis. Amplitude histograms were measured by determining the amplitude of all samples digitized by the computer and therefore reveal single-channel conductances and changes in open probabilities (20).

**RESULTS**

**Purification and Photolabeling.** Previous data demonstrated that carrot cell membranes could be specifically photolabeled by using the phenylalkylamine azido derivative [3H]LU 49888 (13). Therefore, a strategy, developed to purify the calcium channel from skeletal muscle transverse tubules, was used for this study (24). After photolabeling, the [3H]LU 49888-binding protein complex was purified by a combination of Con A-Sepharose and ion-exchange chromatographies, resulting in a final purification factor of 24.6-fold and 30% recovery (based on the starting soluble radioactive material) (Table 1). This purification procedure allowed solubilization and purification of the calcium channel blocker-binding protein from membranes which had not been photolabeled with [3H]LU 49888. The partially purified fraction contained several polypeptides (Fig. 1A, lane 1) among which only the 75-kDa peptide was specifically photolabeled (Fig. 1B).

NaDodSO4/PAGE and silver nitrate staining of the partially purified fraction showed the presence of four major polypeptides (Fig. 1A, lane 1). However, in control experiments, without loading carrot protein extracts onto the Con A-Sepharose column, three of these polypeptides (66, 32, and 16 kDa) were eluted from the column with the equilibration buffer and were therefore contaminants from the Con A-Sepharose column (Fig. 1A, lane 2). These polypeptides could be the result of the nonspecific elution of the Con A not covalently bound to the support column. The presence of these contaminating polypeptides in the purified sample indicates that our estimated final purification factor can be viewed as a minimal estimate. The protein preparation partially purified from carrot membranes was enriched with the 75-kDa peptide.

**Electrophysiological Studies: Control Experiments.** To exclude any nonspecific electrical activities, patch-clamp experiments were performed on different control liposomes. No channel activity was observed at pipette holding potentials ranging from −150 to +150 mV on giant liposomes made as described in Materials and Methods in the absence of carrot protein extracts (n = 10) and on giant liposomes reconstituted with thermally denatured crude (n = 10) or thermally denatured partially purified solubilized proteins (n = 5). In addition, patch-clamp analysis of liposomes reconstituted with the contaminating proteins eluted from the Con A-Sepharose complex was performed. The results obtained for a typical experiment are shown in Table 1.

**Table 1.** Purification of the complex of calcium channel blocker-binding protein with [3H]LU 49888 from membranes of carrot cells

<table>
<thead>
<tr>
<th>Step</th>
<th>Binding, *dpm × 10⁻⁵</th>
<th>Recovery, %</th>
<th>Protein, mg</th>
<th>Specific activity, dpm × 10⁻³/mg</th>
<th>Purification, fold</th>
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<tr>
<td>Solubilized proteins</td>
<td>11.2</td>
<td>100</td>
<td>1.31</td>
<td>8.5</td>
<td>1</td>
</tr>
<tr>
<td>Con A-Sepharose</td>
<td>8.51</td>
<td>76</td>
<td>0.1</td>
<td>85.1</td>
<td>10</td>
</tr>
<tr>
<td>DEAE-trisacryl</td>
<td>3.87</td>
<td>34.5</td>
<td>0.021</td>
<td>184.4</td>
<td>21.6</td>
</tr>
<tr>
<td>Con A-Sepharose</td>
<td>3.36</td>
<td>30</td>
<td>0.016</td>
<td>210.4</td>
<td>24.6</td>
</tr>
</tbody>
</table>

*The membranes (200 μg) were photolabeled with 5 nM [3H]LU 49888 in the absence (assay) or the presence of 50 μM bepridil (control). Subsequently, from both photolabeled samples, the "LU 49888 receptor" was solubilized and purified as described in Materials and Methods. At each step of the purification, the radioactivity associated with the different protein fractions was determined and the presence of the complex was calculated by the difference (specific binding) between the radioactivity measured in the assay and the one found in the control. The dpm values were automatically determined and calculated by the electronic counter, which was calibrated for [3H].

arose with the equilibrium buffer (Fig. 1 A, lane 2) showed that no current was detectable at pipette holding potentials ranging from $-150$ to $+150$ mV ($n = 8$).

**Patch-Clamp Studies on Reconstituted Liposomes with the LU 49888-Binding Protein.** Patch-clamp analysis was performed on proteoliposomes reconstituted with either crude (i.e., total solubilized proteins) or partially purified proteins (Fig. 1 A, lane 1) from unlabeled membranes. In both cases, we observed Ca$^{2+}$-permeable single-channel currents (Fig. 2), in asymmetrical solutions with a 10-fold gradient of CaCl$_2$ (Fig. 2 A Inset). Single-channel currents measured by patch-clamp recordings from liposomes reconstituted with crude ($n = 11$) and partially purified ($n = 15$) proteins were similar (compare Fig. 2 A and B). These channels showed reproducible conductances of 49 $\pm$ 7 pS ($\pm$ SD, $n = 5$) and 38 $\pm$ 4 pS ($n = 4$), respectively (Fig. 2 C and D). The direction of single-channel currents reversed at a pipette potential of approximately $+25$ mV, which is the range of the equilibrium potential (Nernst potential) for Ca$^{2+}$ ($+24$ mV) under the imposed experimental conditions (Fig. 2 C and D). These data show that the measured single-channel currents were due to the passage of calcium ions through reconstituted Ca$^{2+}$-permeable ion channels.

However, these Ca$^{2+}$-permeable channels were not stable. In both types of reconstituted liposomes (i.e., reconstituted either with the solubilized extract or with the partially purified protein) Ca$^{2+}$ channels disappeared after the single-channel currents had been recorded for durations of 2–10 min, thus hampering detailed cationic selectivity measurements. In such experiments, an apparently new type of nonselective Ca$^{2+}$-permeable channel appeared after disappearance of the above-described channel currents ($n = 6$) (Fig. 3). In several experiments ($n = 7$) these nonselective ion channels were the first to appear in membrane patches. The reversal potential of these channel currents was $-10$ mV (Fig. 3 B and C), between the Nernst potential for Cl$^-$ ($-47$ mV) and the Nernst potential for Ca$^{2+}$ ($+24$ mV). Therefore, these channels displayed a permeability to both calcium and chloride ions. The permeability ratio for Ca$^{2+}$ with respect to Cl$^-$ ($P_{Ca^{2+}}/P_{Cl^-}$), calculated from the Goldman–Hodgkin–Katz equation for divalent ions over monovalent ions (25), was 0.7. The single-channel conductance of these currents was $67 \pm 10$ pS ($\pm$ SD, $n = 7$) (Fig. 3).

In contrast to the Ca$^{2+}$-selective channels, these nonselective Ca$^{2+}$- and Cl$^-$-permeable channels were more stable and could be recorded for durations of up to 60 min, allowing pharmacological studies. When the calcium channel blocker (−)-bepridil ($10 \mu$M) was added in the bath solution, single-channel currents through these nonselective channels were immediately blocked ($n = 6$) (Fig. 4). Bepridil led to a substantial reduction of the open-state probability of this channel (Fig. 4). The inhibition was reversed ($n = 5$) by perfusing the bath solution with the buffer devoid of bepridil (data not shown). An identical inhibition was also observed with the phenylalkylamine Ca$^{2+}$ channel blocker verapamil at $10 \mu$M ($n = 2$).

**DISCUSSION**

In higher plants, Ca$^{2+}$ channel activation by physiological stimuli such as plant hormones, light, and fungal elicitors has been suggested to play a primary role in the initiation of signal-transduction processes (1, 4, 6, 9). However, the molecular components of higher plant Ca$^{2+}$ channels remain unknown. In the present study, solubilization, partial purification, reconstitution of a membrane Ca$^{2+}$ channel blocker-binding protein from carrot cells, and patch clamp analysis have allowed us to address the question of whether this protein may contribute to Ca$^{2+}$ channel function.

Previous studies have shown that Ca$^{2+}$ channel blockers of the phenylalkylamine family block Ca$^{2+}$ influx into carrot cells (11) and that a 75-kDa Ca$^{2+}$ channel antagonist-binding protein solubilized from membranes of these cells is photo-labeled with the phenylalkylamine azido derivative [3H]LU 49888 (13). This property of the binding protein was used in this study to follow the polypeptide during purification and subsequently to establish a purification procedure.

The combination of membrane protein reconstitution techniques and patch-clamp studies has led to the identification and characterization of a large number of different ion channel proteins from mammalian systems (for review, see ref. 26) and more specifically the main ion channel conducting subunits of Ca$^{2+}$ channels (18, 19, 27–29). The reconstitution of membrane proteins into liposomes, as pursued in this study, is an approach which allows the association of biological functions to defined membrane proteins at a resolution level of the molecular dynamics of single proteins (26). In the present study reconstitution of the partially purified protein fraction containing the 75-kDa polypeptide into liposomes and subsequent patch-clamp analysis showed that the protein extract displayed Ca$^{2+}$-permeable single-channel currents (Figs. 2 and 3). Reconstitution of either contaminating proteins eluted from Con A-Sepharose columns (Fig. 1A, lane 2) or thermally denatured protein extracts did not show Ca$^{2+}$ channel activity.

Reconstituted ion channels selective for Ca$^{2+}$ over Cl$^-$ (Fig. 2) were unstable after seal formation, as these single-channel currents vanished after recording for durations of several minutes. Due to this instability, determination of permeability ratios for Ca$^{2+}$ with respect to other cations was hampered. The large single-channel conductance for Ca$^{2+}$ of the partially purified extract (Fig. 2C) in various studies showing block of Ca$^{2+}$ uptake into carrot cells by phenylalkylamines and bepridil (11) support the hypothesis that the partially purified protein plays a role in Ca$^{2+}$ influx. Single-
Fig. 2. Recordings of single Ca\(^{2+}\)-permeable ion channel currents in inside-out membrane patches excised from liposomes reconstituted with either solubilized proteins (crude extract) (A) or the partially purified calcium channel blocker-binding protein from carrot membranes (B). (A and B) Single-channel currents recorded at different pipette potentials, indicated to the right. Solutions were 20 mM Hepes-Tris, pH 6.5/9 mM CaCl\(_2\)/200 mM mannitol in the pipette and 20 mM Hepes-Tris, pH 6.5/90 mM CaCl\(_2\) in the bath (see Inset). Closed and open states of ion channels are indicated by C and O, respectively. (C) Current-voltage relationship of single Ca\(^{2+}\)-permeable channel currents recorded after reconstitution of the partially purified protein. Ca\(^{2+}\) channels were recorded on the same patch by four successive overlapping voltage ramps between -80 mV and +80 mV (ramp duration 0.4 sec). C and O indicate closed and open states. (D) Single-channel current amplitudes plotted as a function of the applied pipette potential from liposomes reconstituted with the solubilized proteins (o) or with the partially purified protein (●). The arrows indicate the theoretical reversal potentials for Ca\(^{2+}\) (+24 mV) and for Cl\(^-\) (−47 mV), after correction for ionic activities (22).

channel currents, appearing subsequently in these membrane patches, were nonselective, showing a permeability to both calcium and chloride ions (Fig. 3). One possible explanation for this modification of single ion channel currents may be a partial denaturation of the channel protein, thereby affecting ion selectivity. In studies which led to the identification of functional subunits of skeletal muscle Ca\(^{2+}\) channels, instability of channel function after insertion into lipid vesicles and

Fig. 3. Nonselective Ca\(^{2+}\)-permeable single ion channel currents were recorded in inside-out membrane patches excised from liposomes reconstituted with the partially purified calcium channel blocker-binding protein. (A) Single-channel recordings are shown at different pipette potentials, indicated to the right. (B) Current-voltage relationship of nonselective channel activity determined on the same patch by four successive overlapping voltage ramps between −80 mV and +80 mV (ramp duration 0.4 sec). (C) Amplitude of single-channel currents as a function of applied potential. The arrows indicate the theoretical reversal potential for Ca\(^{2+}\) (+24 mV) and for Cl\(^-\) (−47 mV). The pipette and bath solutions were identical to those in Fig. 2.
Further plants. The block of Ca2⁺ influx into carrot protoplasts (11) is direct recordings of inhibition of Ca2⁺ channels by blockers have not been obtained in higher plants to date. The only data allowing comparison to the mechanism of block come from animal systems. The block mechanisms of carrot (Fig. 4) and mammalian (19, 30) Ca2⁺ channels by calcium channel blockers are similar. Thus, it was observed that the antagonists did not lead to changes in the single channel current amplitudes through open Ca2⁺ channels but rather to blocker-induced reductions in the open-state probability of channels (Fig. 4; refs. 19 and 30).

In conclusion, the present study demonstrates that the 75-kDa binding polypeptide contributes to Ca2⁺ channel function after reconstitution and plays a role in the sensitivity to Ca2⁺ channel blockers. In addition, the presented findings provide evidence for a membrane protein that may constitute one of the molecular components of Ca2⁺ channels in higher plants. Further studies on the function and primary sequence of the Ca2⁺ channel blocker-binding protein and direct patch-clamp recordings of carrot cells may allow new insights to plant Ca2⁺ channel structure and regulation.

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