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Photoaffinity Labeling of the Partially Purified Mitochondrial Phenylalkylamine Calcium Antagonist Receptor

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SUMMARY

Mitochondria contain specific Ca^{2+} antagonist binding sites that are associated with an inner mitochondrial membrane anion channel. These mitochondrial Ca^{2+} antagonist receptors can be solubilized with digitonin and partially purified [as assessed by postreversible (±)-[^{3}H]nitrendipine binding] using ion exchange chromatography and sucrose density gradient centrifugation. In the present study, reversible binding of the phenylalkylamine Ca^{2+} antagonist [^{3}H]ludopamil, an optically pure photoaffinity analog of verapamil, to the partially purified mitochondrial Ca^{2+} antagonist receptor complex (K_d, 9 ± 4 μM; B_{max}, 1.2 ± 0.5 nmol/mg of protein) depended on $NaNO_3$ and was inhibited by the 1,4-dihydropyridine niludipine and by ATP. Accordingly, the unlabeled racemic analog of [^{3}H]ludopamil, (±)-LU 47781, dose-dependently inhibited the binding of the 1,4-dihydropyridine (±)-[^{3}H]nitrendipine to the purified mitochondrial receptors (IC_{50}, 2.1 ± 0.1 μM). After UV irradiation, [^{3}H]ludopamil specifically incorporated into two polypeptides of 12.7 ± 0.1 kDa and 11.7 ± 0.1 kDa, with the pharmacological profile of [^{3}H]ludopamil photoincorporation stimulation and protection being identical to that of reversible binding.

The mitochondrial inner membrane contains specific binding sites for DHP (1–3), PAA (4–6), and benzothiazepine (7) Ca^{2+} antagonists. The mitochondrial DHP binding is associated with an inner mitochondrial anion channel (7). By inhibiting this channel, certain Ca^{2+} antagonists might indirectly prevent Ca^{2+} overload of mitochondria in ischemically compromised tissue (for a detailed discussion, see Refs. 6 and 7 and references cited therein). The mitochondrial receptors have been at least partially purified using ion exchange chromatography and sucrose density gradient centrifugation (8). Because the partially purified mitochondrial DHP Ca^{2+} antagonist receptor consists of several polypeptides in the 60-kDa, 50-kDa, 34-kDa, 25-kDa, and 12-kDa regions, unequivocal identification of the binding domain(s) has not yet been achieved (8), although postreversible [^{3}H]-NTR binding correlated only with the amount of protein found in the 50-kDa, 25-kDa, and 12-kDa regions (see Fig. 2 of Ref. 8). Using [^{3}H]LU, a PAA Ca^{2+} antagonist photoaffinity label that has already been successfully used for the identification of the PAA binding domain of the L-type Ca^{2+} channel (9–12) and that also specifically interacts with the mitochondrial PAA binding sites (4–6), we report specific photoaffinity labeling of two polypeptides in the 12-kDa region of the partially purified mitochondrial DHP and PAA Ca^{2+} antagonist binding site complex, thus opening the way for the structural identification of the mitochondrial PAA-binding domains.

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Experimental Procedures

Materials. [^{3}H]LU, (specific activity, 85 Ci/mmol), (±)-LU 47781 (unlabeled racemic LU 49888), and (±)-gallopamil were synthesized by Knoll AG (Ludwigshafen, Germany) as described (9). Niludipine was a gift from Bayer AG (Wuppertal, Germany). [^{3}H]N-TR (87 Ci/mmol) was obtained from New England Nuclear (Vienna, Austria). Sources for other Ca^{2+} antagonists are given elsewhere (1). High and low molecular weight markers for SDS-PAGE were obtained from Bio-Rad (Vienna, Austria). All other chemicals were from Sigma (Munich, Germany) or Merck (Darmstadt, Germany) and of the highest purity available.

Purification of mitochondrial Ca^{2+} antagonist receptors. Purification was performed exactly as described in detail previously (8). Briefly, mitochondria were prepared by differential centrifugation and solubilized at a ratio of 1.5 mg of digitonin/mg of protein [determined according to the method of Bradford (13)]. The solubilized mitochondrial Ca^{2+} antagonist receptors were purified using a two- or three-step procedure (step 1, anion exchange chromatography; step 2, sucrose density gradient centrifugation; step 3, cation exchange chromatography). Throughout all procedures, the following protease inhibitors were

ABBREVIATIONS: DHP, 1,4-dihydropyridine; B_{max}, maximal density of receptor sites; IC_{50}, concentration causing 50% of maximal inhibition; K_d, equilibrium dissociation constant; [^{3}H]LU, [^{3}H]ludopamil [[^{3}H]LU 49888, (−)-5-[(3-azidophenethyl)[*N-methyl*-^{3}H]methylamino]-2-(3,4,5-trimethoxyphenyl)-2-isopropylvaleronitrile]; (±)-LU 47781, (±)-5-[(3-azidophenethyl)methylamino]-2-(3,4,5-trimethoxyphenyl)-2-isopropylvaleronitrile; [^{3}H]NTR, (±)-[^{3}H]nitrendipine, [(±)-[5-*methyl*-^{3}H]3,5-ethylmethyl-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine dicarboxylate]; PAA, phenylalkylamine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

present: 1 mM iodoacetamide, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, and 1 μM pepstatin A. Integrity of the purified receptors was assessed by postreversible [^{3}H]-NTR binding, which could be enriched 8-fold (assessed by equilibrium [^{3}H]-NTR saturation binding) to 23-fold [assessed at 1 nM [^{3}H]-NTR, which is far below receptor saturation] (8). Because the third purification step did not increase overall [^{3}H]-NTR binding and because the yield of the third purification step was only about 30% (see Table 1 of Ref. 8), only two-step-purified mitochondrial Ca^{2+} antagonist receptors were used in the [^{3}H]LU photoaffinity labeling experiments.

Reversible [^{3}H]LU binding. For determination of reversible binding of [^{3}H]LU to solubilized mitochondrial Ca^{2+} antagonist receptors, 0.6–18 μg of protein were incubated with 1.1–1.6 nM [^{3}H]LU for 120 min at 37° in 500 μl of Tris buffer (50 mM Tris·HCl, pH 7.4 at 25°, plus the protease inhibitors given above) containing 500 mM $NaNO_3$. Drugs were diluted as described (7). For equilibrium saturation analysis, the specific activity of [^{3}H]LU was varied from 85 to 0.007 Ci/mmol by addition of the unlabeled racemic compound. Bound and free radioligand were separated by polyethylene glycol precipitation in the presence of carrier protein (2 mg of bovine serum albumin and γ-globulin, respectively) as described (8). Nonspecific binding was determined in the presence of 500 μM (±)-gallopamil.

Photoaffinity labeling with [^{3}H]LU. Pooled sucrose density gradient peak fractions were concentrated in a Micro-ProDiCon chamber model MPDC-15 (ProDiMem dialysis membrane PA-10; cutoff, 10 kDa; Tris-buffer, 4°) to 0.6–1.6 mg of protein/ml and were immediately used for photoaffinity labeling experiments. To that end, 0.3–0.9 mg/ml concentrated protein was incubated with 26–68 nM [^{3}H]LU for 120 min in a volume of 220–660 μl of a buffer containing 500 mM $NaNO_3$ and 10 mM Tris·HCl (pH 7.4 at 25°). Aliquots (200 μl) were transferred to plastic wells (1.5-cm diameter) on ice and irradiated from a distance of 10 cm with either a Philips 38W/TL black light lamp or a GTE Sylvania G105T8 germicidal lamp, for the times indicated. Near the end of the irradiation procedure, 5-μl aliquots of residual nonirradiated samples were assayed for reversible binding and total radioligand concentration. After irradiation, 50 μl of stop solution (15% SDS, 313 mM Tris·HCl, pH 6.8, 0.5 mM phenylmethylsulfonyl fluoride, 3% glycerol) were added to the 200-μl sample together with 10 μl of 2-mercaptoethanol (reducing conditions) or 10 μl of 200 mM *N*-ethylmaleimide (alkylating conditions). Samples were denatured at 95° for 5 min and separated on 15% polyacrylamide slab gels. To determine the incorporated radioactivity, gels were cut into 3-mm slices, heated overnight at 50° with 1 ml of H_2O_2 and 50 μl of NaOH (25%, v/v) (14), and counted in 4 ml of Emulsifier Safe scintillation liquid (Packard-Canberrra, Vienna, Austria). Alternatively, gels were impregnated with Entensify, dried, and exposed to Kodak X-OMAT AR film at −70° for the times indicated. The estimation of the molecular weights of the photolabeled polypeptides was based on a linear regression of (mm traveled in the SDS-polyacrylamide gel) versus the logarithm of the molecular weight of the marker proteins.

Binding parameters were obtained using the GraphPad (ISI, Philadelphia, PA) computer package or nonlinear algorithms described in Ref. 15. Data are given as means ± standard errors of *n* determinations.

Results and Discussion

Throughout the purification steps, postreversible [^{3}H]LU binding coeluted and cosedimented with postreversible [^{3}H]NTR binding (data not shown). Furthermore, [^{3}H]NTR binding to the two-step-purified mitochondrial sites was dose-dependently inhibited by LU 47781, the unlabeled racemic analog of [^{3}H]LU, displaying an IC_{50} of 2.1 ± 0.1 μM and a Hill slope of 1.06 ± 0.05 (n = 4). Finally, [^{3}H]LU saturably bound to sucrose density gradient peak fractions with a K_d of 8.6 ± 3.8 μM and a B_{max} of 1.17 ± 0.54 nmol/mg of protein (n = 3), corresponding to a 7-fold increase in K_d and a 13-fold increase in B_{max}, compared with particulate preparations (5) [B_{max} values were adjusted for protein, determined according to the method of Bradford (13), i.e., 0.09 ± 0.03 nmol/mg]. However, it must be emphasized that due to the very low signal-to-noise ratio of [^{3}H]LU binding to the sucrose density gradient peak fractions (range of 1.2:1 to 1.4:1) and the saturation binding method used (i.e., only 1.1–1.3 nM total [^{3}H]LU used and dilution of its specific activity from 85 to 0.007 Ci/mmol, procedures used due to the scarcity of available radioligand), the saturation binding parameters represent only a rough approximation. In comparison, saturable [^{3}H]NTR binding to the cation exchange chromatography peak fractions yielded a K_d of 293 ± 40 nM and a B_{max} of 0.53 ± 0.19 nmol/mg of protein (n = 6) (8), corresponding to a 2-fold decrease in K_d and a 4-fold increase in B_{max}, compared with particulate preparations (8).

Fig. 1 shows that specific photoaffinity labeling of a 12.8- and a 11.6-kDa polypeptide of the two-step-purified mitochondrial Ca^{2+} antagonist receptor complex with [^{3}H]LU became apparent only after addition of 500 mM $NaNO_3$, an allosteric stimulator of reversible [^{3}H]LU binding in particulate as well as solubilized mitochondrial membranes (see legend to Fig. 1; also see Ref. 5). Interestingly, gallopamil-mediated protection of [^{3}H]LU photoincorporation into the 50-kDa polypeptides that comprise the majority of the purified protein mass (see *lane CO* of Figs. 1 and 2) became weaker as the conditions for reversible [^{3}H]LU binding were improved by addition of

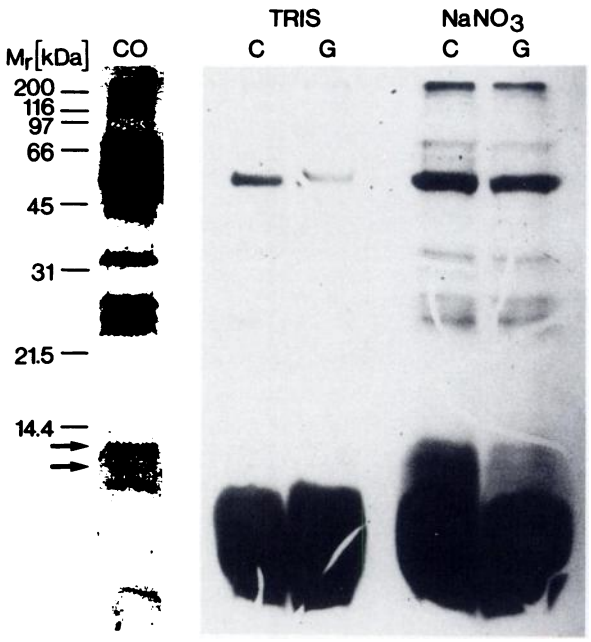

Fig. 1. Ion dependence of specific photoaffinity labeling of the partially purified mitochondrial Ca^{2+} antagonist site by [^{3}H]LU. Two-step-purified mitochondrial Ca^{2+} antagonist receptors (500 μg/ml protein) were incubated with 26–28 nM [^{3}H]LU in either Tris buffer alone (*TRIS*) or Tris buffer supplemented with 500 mM $NaNO_3$ (*$NaNO_3$*). Specific [^{3}H]LU binding was 0.5–0.6 pmol/mg of protein in Tris buffer alone and 1.2–2.0 pmol/mg in the presence of $NaNO_3$. After 80 min of UV irradiation (black light), 100-μg aliquots were denatured under reducing conditions and separated by SDS-PAGE. For conditions of separation and detection of photoincorporated [^{3}H]LU, see Experimental Procedures. The gel cracks visible in the fluorograph developed during gel drying and are due to the high acrylamide concentration; they did not impair the resolution of the gel. X-ray film was exposed for 34 days. M_r, relative molecular mass of marker proteins; *arrows*, 12.8-kDa and 11.6-kDa polypeptides. *Lane CO*, Coomassie stain of Tris control lane; *lanes C*, fluorography of total [^{3}H]LU incorporation (control lanes); *lanes G*, [^{3}H]LU incorporation in the presence of 500 μM gallopamil.

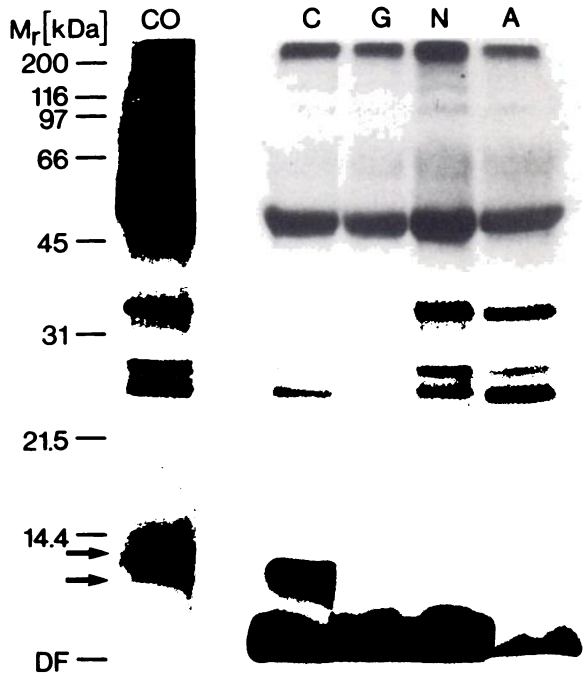

Fig. 2. Pharmacological protection profile of mitochondrial Ca^{2+} antagonist receptor photoaffinity labeling with [^{3}H]LU. Two-step-purified mitochondrial Ca^{2+} antagonist receptors (317 μg) were incubated with 62 nM [^{3}H]LU in 440 μl of N buffer either alone (*lane C*) or in the presence of 500 μM gallopamil (*lane G*), 30 μM niludipine (*lane N*), or 10 mM ATP (*lane A*). Steady state reversible [^{3}H]LU binding was (in pmol/mg; $n = 3$) 5.3 ± 0.3 (*lane C*), 1.3 ± 0.2 (*lane G*), 1.5 ± 0.1 (*lane N*), and 2.8 ± 0.1 (*lane A*). After 80 min of UV irradiation (black light), 144-μg samples were denatured under either reducing or alkylating (not shown) conditions and were separated by SDS-PAGE. M_r, relative molecular mass of marker proteins; *arrows*, 12.7-kDa and 11.7-kDa polypeptides; *DF*, dye front. *Lane CO*, Coomassie stain of control lane; *lane C*, fluorogram of control lane (total incorporated radioactivity, 9-day exposure); *lanes G, N,* and *A*, photoincorporation in the presence of gallopamil, niludipine, and ATP, respectively. The alkylated samples displayed the same photoincorporation protection pattern (not shown).

$NaNO_3$, although total [^{3}H]LU photoincorporation into the 50-kDa polypeptides was increased. Overall, in the presence of 500 mM $NaNO_3$, more polypeptides were nonspecifically photolabeled with [^{3}H]LU. In only two small polypeptides of 12.8 and 11.6 kDa, total photoincorporation as well as incorporation protection by gallopamil became stronger (Fig. 1). Indeed, only the polypeptides of the 12-kDa region showed gallopamil-preventable photoincorporation, which was both proportional to the amount of purified protein used (60–175 μg) and the irradiation time, regardless of whether UV irradiation of high (germicidal lamp, 1–5 min) or low (black light, 20–80 min) intensity was used (data not shown). The increase in nonspecific [^{3}H]LU photoincorporation into the other polypeptides might be caused by the chaotropic activity of the nitrate ion (16) and/or by charge neutralization of the positively charged (at a pH of 7.4) PAA molecule (17) by nitrate.

Fig. 2 shows the pharmacological profile of [^{3}H]LU incorporation protection by gallopamil, niludipine (a DHP Ca^{2+} antagonist), and ATP. Dihydropyridines and ATP are allosteric inhibitors of [^{3}H]LU binding in particulate membranes (4–6). Accordingly, the PAA (±)-LU 47781 (the unlabeled racemic analog of [^{3}H]LU) proved to be an effective inhibitor of (±)-[^{3}H]-NTR (DHP) binding to the partially purified mitochondrial site (see above). As can be seen in the legend to Fig. 2, gallopamil, niludipine, and ATP effectively inhibited reversible [^{3}H]LU binding. Furthermore, [^{3}H]LU photoincorporation only into two polypeptides of 12.7 and 11.7 kDa was prevented by all three inhibitors (Fig. 2). Thus, identity of the pharmacological profiles of reversible and irradiation-induced irreversible labeling, a prerequisite for unequivocal identification of a specific binding site by photoffinity labeling (for review, see Ref. 18), can be demonstrated only for the two polypeptides in the 12-kDa region. The calculated relative molecular masses of the two specifically photolabeled polypeptides were 12.7 ± 0.1 kDa and 11.7 ± 0.1 kDa ($n = 3$) and remained unchanged under reducing or alkylating conditions. The efficiency of specific photoincorporation into the two bands (given as the percentage of reversibly bound ligand) was 3.5–7% [84,280 dpm of [^{3}H]LU reversibly bound to a total of 144 μg of purified protein; amount of recovered photoincorporated [^{3}H]LU in the 12-kDa region, 5900 dpm (alkylating conditions), 5280 dpm (reducing conditions), and 2980 dpm (untreated sample)].

The 50-kDa doublet (which has not been resolved under the SDS-PAGE conditions used in this study; but see Fig. 4 of Ref. 4) might correspond to the [^{3}H]LU-photolabeled 55-kDa peak identified in sliced SDS gels of rabbit microsomal membranes by Wernet *et al.* (12); this structure was found to be of very low PAA affinity and unrelated to the high affinity [^{3}H]LU binding domain of the L-type Ca^{2+} channel (12).

In conclusion, [^{3}H]LU specifically photolabeled two polypeptides of the partially purified mitochondrial Ca^{2+} antagonist receptor complex, with molecular masses of about 12 kDa. With this experimental evidence, determination of the structure of both polypeptides becomes feasible.

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