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Ion Dependence of the Partially Purified Mitochondrial Dihydropyridine Ca^{2+} Antagonist Receptor

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SUMMARY

The mitochondrial inner membrane contains specific binding sites for dihydropyridine (DHP) Ca^{2+} antagonists that are associated with an inner mitochondrial membrane anion channel (IMAC) [*Mol. Pharmacol.* **38**:362–369 (1990)]. As in particulate preparations, binding of the DHP (±)-[^{3}H]nitrendipine ([^{3}H]NTR) to partially purified mitochondrial DHP receptors strongly depended on a variety of cations and inorganic as well as organic anions. Monovalent anions saturably stimulated [^{3}H]NTR binding with a potency rank order of $I^- > Br^- > Cl^- > F^-$. The potency rank order for monovalent cations was $Cs^+ > Rb^+ > Li^+ > K^+ > Na^+$. [^{3}H]NTR binding stimulation potency of the cations strikingly depended on their charge density, with EC_{50} values being 125 mM for K^+, 5 mM for Ca^{2+}, and 41 μM for La^{3+}. This selectivity order clearly differed from one predicted on the basis of a simple surface charge-screening effect of the cations. In general, allosteric ion effects were due to changes in [^{3}H]NTR affinity for the partially purified mitochondrial DHP receptor. SCN^- and NO_3^-, known permeators of the IMAC [*J. Biol. Chem.* **262**:15085–15093 (1987)], stimulated [^{3}H]NTR binding with EC_{50} values of 26 mM and 96 mM, respectively. The IMAC permeators butylmalonate^{2-} and 1,2,3-benzenetricarboxylate^{3-} were ineffective when given alone but dose-dependently inhibited 500 mM NaCl-stimulated [^{3}H]NTR binding, as did $PO_4^{1.5-}$ and SO_4^{2-}. Gluconate$^-$, which was reported not to permeate the IMAC, qualitatively behaved as a partial agonist with respect to Cl^-. Glucuronate$^-$ was without effect on [^{3}H]NTR binding to the partially purified mitochondrial DHP receptor. These results point to the existence of rather large ion-binding domains. The cation-binding site was estimated to have a minimum diameter of 0.67 nm. The anion-binding domain could accommodate either spherical ligands with diameters of up to 0.6 nm or molecules with a flat backbone with dimensions of approximately 0.9 nm × 0.7 nm × 0.3 nm.

The mitochondrial inner membrane contains specific binding sites for DHP (1–3), PAA (4, 5), and benzothiazepine Ca^{2+} antagonists (6). The DHP Ca^{2+} antagonist sites have been shown to be associated with an IMAC (6). By inhibiting the IMAC, certain Ca^{2+} antagonists might indirectly prevent mitochondrial Ca^{2+} overload in ischemically compromised tissue (Ref. 6; for a review of other putative mitochondrial Ca^{2+} antagonist target structures, see Ref. 7). The existence of such an anion channel has so far been demonstrated by functional experiments only (8–12). Using mitochondrial swelling experiments, Beavis and Garlid (9) have demonstrated that the IMAC displays a very broad substrate specificity, including organic anions of considerable size, among them specific inhibitors for a variety of other anion-transporting systems (e.g., butylmalonate or 1,2,3-benzenetricarboxylate). The broad substrate specificity and the high molecular weight cutoff is reminiscent of the voltage-dependent anion channel of the outer mitochondrial membrane (cutoff, 3400) (13) and the bacterial porins (14). Since the first patch-clamp studies on cuprizone-induced giant mitochondria, which revealed single-channel activities with a 4.5-fold anion versus cation selectivity (Cl^- versus K^+) (12), a number of high-conductance channels in the inner mitochondrial membrane with slight anion or cation selectivity and different sensitivities for a variety of inhibitors have been described (e.g., Refs. 15–18). To our knowledge, no unequivocal differentiation between these channels is available so far. From the reported 107-pS single-channel conductance in symmetrical 150 mM KCl (12), a channel pore diameter of 0.8 nm can be calculated, using the algorithm given in Ref. 19.

Using postreversible binding of [^{3}H]NTR, a DHP Ca^{2+} antagonist, as a quality control, we report the at least partial biochemical purification of the mitochondrial Ca^{2+} antagonist receptors, thus allowing tentative conclusions about the struc-

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ABBREVIATIONS: DHP, 1,4-dihydropyridine; B_{max}, maximal density of receptor sites; IMAC, inner mitochondrial membrane anion channel; EC_{50}, concentration causing 50% of maximal stimulation; Hill coefficient, H; IC_{50}, concentration causing 50% of maximal inhibition; $K_{0.5}$, concentration causing 56% of maximal effect; K_d, equilibrium dissociation constant; [^{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.

ture of the receptor-associated polypeptide complex. Furthermore, by systematically investigating the anion and cation dependence of the partially purified DHP Ca^{2+} antagonist binding site, certain predictions about the anion- and cation-binding domains of the purified receptor-IMAC complex can be made. To that end, we have made use of the selectivity pattern analyses of Jenny (20), Bungenberg de Jong (21), Eisenman and Horn (22, 23), and Diamond and Wright (24). These selectivity pattern analyses have already been successfully used for a variety of biological systems (for reviews, see Refs. 23–25). Preliminary data have been presented (5, 26–28).

Experimental Procedures

Materials. [^{3}H]NTR (87 Ci/mmol) was obtained from New England Nuclear (Vienna, Austria). Sources for other Ca^{2+} antagonists are given elsewhere (1). Servacel ECTEOLA 23-cellulose anion exchange gel was from Serva (Heidelberg, FRG); CM Bio-Gel A cation exchange gel (50–200 mesh) was from Bio-Rad (Vienna, Austria). All other chemicals were from Sigma (Munich, FRG) or Merck (Darmstadt, FRG) and of the highest purity available.

Purification of mitochondrial Ca^{2+} antagonist receptors. Mitochondria from guinea pig liver were prepared by differential centrifugation (29, 30), as described elsewhere (1), except that the following protease inhibitors were always present during preparation and subsequent purification procedures (all performed at 4°): 1 mM iodoacetamide, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, and 1 μM pepstatin A. After solubilization of the mitochondrial membranes in 2% (w/v) digitonin in Tris buffer (20 mM Tris-HCl, pH 7.0 at 4°, plus protease inhibitors), at a ratio of 1.5 mg of detergent/mg of protein [determined according to the method of Bradford (31)], for 15 min, the mixture was centrifuged at 100,000 × *g* for 60 min. The clear supernatant was diluted in Tris buffer to a final concentration of 0.2% (w/v) digitonin (D buffer), and an aliquot, 100–150 mg of solubilized protein, was applied to 80 ml of swollen Servacel ECTEOLA 23-cellulose anion exchange gel preequilibrated with 4 volumes of D buffer (4-cm column diameter). Mitochondrial Ca^{2+} antagonist receptors (assessed by reversible [^{3}H]NTR-binding) were eluted with 2 volumes of E buffer (D buffer supplemented with 50 mM NaCl). Pooled peak fractions (19–21 ml) were layered on four continuous 5–20% (w/v) sucrose density gradients in E buffer. After centrifugation at 100,000 × *g* for 105 min, gradients were recovered in 2-ml fractions. The sucrose density gradient fractions containing the highest reversible [^{3}H]NTR binding were routinely identified by their typical protein profile and pooled. In a third purification step, the pooled sucrose density gradient peak fractions were adjusted with acetic acid to a pH of 4.9 at 4°, applied to 10 ml of CM Bio-Gel A cation exchange gel preequilibrated with 10 volumes of C buffer (20 mM acetic acid-NaOH, pH 4.9 at 25°, plus protease inhibitors), and eluted with 2 volumes of C buffer containing 100 mM NaCl. Elution quality was routinely assessed by postreversible [^{3}H]NTR binding.

Reversible [^{3}H]NTR binding. For determination of reversible binding of [^{3}H]NTR to solubilized mitochondrial Ca^{2+} antagonist receptors, 0.6–60 μg of protein [determined according to the method of Bradford (31)] were incubated with 1.0–2.8 nM [^{3}H]NTR for 120 min at 37°, in 500 μl of a Tris buffer (50 mM Tris-HCl, pH 7.4) supplemented with various concentrations of the ions listed below. Specific binding to the solubilized membranes was linear up to 100 μg (determined in the presence of 500 mM NaCl; data not shown). [^{3}H]NTR binding is expressed as pmol of [^{3}H]NTR/mg of protein and has been normalized for a total radioligand concentration of 1 nM, with the relationship between [^{3}H]NTR binding and total radioligand being linear at least up to 17 nM [^{3}H]NTR (data not shown). Base-line specific binding, i.e., [^{3}H]NTR binding in the presence of 50 mM Tris·HCl and 30–40 mM NaCl, from the cation exchange elution buffer containing the partially purified mitochondrial receptors (150–200 μl of C buffer with 100 mM NaCl, in a total binding assay volume of 500 μl), was 0.15 ± 0.03 pmol/mg (47 experiments; range, 0–1.02 pmol/mg), corresponding to only 5% of the maximal Tris effect (2.83 pmol/mg) (see Table 2) or 8% of the maximal NaCl effect (2 pmol/mg) (see Table 2). Drugs were diluted as described (6). For equilibrium saturation analysis, the specific activity of [^{3}H]NTR was varied from 87 to 0.003 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 (32). Nonspecific binding was determined in the presence of 10 μM nicardipine. The final digitonin concentration in the binding assays ranged from 0.04 to 0.08% (w/v). Under these conditions, no pseudo-specific binding occurred (data not shown). In the cases where nonspecific binding was increased by the added ion (KF, >100 mM; NaF, >175 mM; $LaCl_3$, >0.1 mM), care was taken to determine its exact value at each ion concentration used. Most of the stimulatory ions decreased [^{3}H]NTR binding again at very high concentrations (usually >500 mM for monovalent ions); for calculations of EC_{50} values for [^{3}H]NTR binding stimulation, these data points were discarded.

For particulate mitochondrial membrane preparations, routine comparisons of the protein determination methods according to the methods of Bradford (31) and Lowry *et al.* (33), over 2 years, gave the following relationship: μg of $protein_{Lowry}$ · 2.51 ± 0.20 = μg of $protein_{Bradford}$ (mean ± standard error, 19 experiments). This conversion factor was used whenever results of this study were compared with previously published results of our group (1).

Data analysis. Binding parameters of single experiments (performed in duplicate) were obtained using the GRAPHPad (ISI, Philadelphia, USA) computer package or nonlinear algorithms described in Ref. 34. Binding parameters obtained from single experiments (dose-response curves fitted to four to nine data points obtained from duplicate determinations) were averaged, and data are given as means ± standard errors of *n* experiments. Unless indicated otherwise, statistical comparison of binding parameters was done using Student's *t* test (unpaired).

Unhydrated radii of monovalent cations and anions were taken from the work of Pauling (35) (cited in Ref. 25). For the estimation of the dimensions of organic anions, a commercially available molecular model-building kit was used (MINIT-Molekülbaukasten; Verlag Chemie, Weinheim, FRG).

Results

Enrichment of solubilized mitochondrial DHP Ca^{2+} antagonist receptors in a three-step purification procedure. Table 1 shows that postreversible [^{3}H]NTR binding to

TABLE 1

Purification of the mitochondrial [^{3}H]NTR binding site

Data are means ± standard errors of three independent experiments (performed in a buffer containing 500 mM NaCl, 50 mM Tris·HCl, pH 7.4, and protease inhibitors; see Experimental Procedures) (triplicate determinations each). Guinea pig liver mitochondrial membranes (131 ± 11 mg of protein) were solubilized and applied to 80 ml of ECTEOLA 23 (anion exchange) gel. Pooled peak [^{3}H]NTR-binding fractions (19–21 ml) were separated by sucrose density gradient centrifugation. Finally, 23–24 ml of pooled peak [^{3}H]NTR-binding fractions of the sucrose gradient were applied to 10 ml of BioGel A (cation exchange). Six to 24 ml of the eluted fractions showed peak [^{3}H]NTR binding. Specific [^{3}H]NTR binding was normalized to a total of 1 nM radioligand used. Under these conditions, approximately 0.17% of the receptors, corresponding to 0.24 pmol/mg of protein [determined according to the method of Bradford (31)], are labeled in particulate mitochondrial membrane preparations (1). A comparison of particulate mitochondrial membranes with the cation exchange peak fractions thus yields a 11-fold [^{3}H]NTR binding purification.

Purification step	Protein		Specific [^{3}H]NTR binding	Purification
	mg	%	*pmol/mg*	*fold*
Digitonin extract	99 ± 22	100	0.12 ± 0.04	1
Anion exchange	7.5 ± 1.5	8	0.96 ± 0.35	8
Sucrose gradient	1.27 ± 0.03	1	2.88 ± 1.41	24
Cation exchange	0.34 ± 0.04	0.3	2.75 ± 0.83	23

the digitonin-solubilized mitochondrial sites, when determined at a radioligand concentration (1 nM) far below receptor saturation, can be enriched 24-fold in a two-step purification procedure (anion exchange chromatography and sucrose density gradient centrifugation). SDS-PAGE analysis of the two-step-purified mitochondrial DHP binding sites consistently showed a very prominent double band in the 50-kDa region, a 34-kDa band, a double band in the 25-kDa region, and bands in the 12-kDa range (Fig. 1). In a third purification step (cation exchange chromatography), the relative contribution of the 12-kDa-range polypeptides became larger, whereas the relative amount of the 34-kDa polypeptide routinely decreased (Fig. 1). Although there was considerable interexperimental variation in the staining intensity of the different polypeptide bands (compare *lanes SP* and *CM* with *SP2* and *CM2* of Fig. 1), SDS-PAGE analysis (Fig. 2A) and postreversible [^{3}H]NTR binding experiments performed on the same fractions of a 0–1 M NaCl gradient elution of the cation exchange column clearly showed that the distribution of [^{3}H]NTR binding correlated with the staining intensity of the 12-kDa, 25-kDa, and 50-kDa polypeptides (Fig. 2B), whereas it differed from the distribution of the 34-kDa band (Fig. 2B). Overall, [^{3}H]NTR binding could not be enriched by the third purification step (Table 1). [^{3}H]NTR saturation binding experiments yielded a K_d of 662 ± 63 and a B_{max} of 0.16 ± 0.01 nmol/mg of protein (n = 6) for the digitonin extract and a K_d of 293 ± 40 nM and a B_{max} of 0.53 ± 0.19 nmol/mg of protein (n = 6) for the cation exchange chromatography peak fractions. This represents a 2-fold decrease in K_d and a 4-fold increase in B_{max} of the three-step-purified receptors, compared with particulate preparations (1) [protein determination

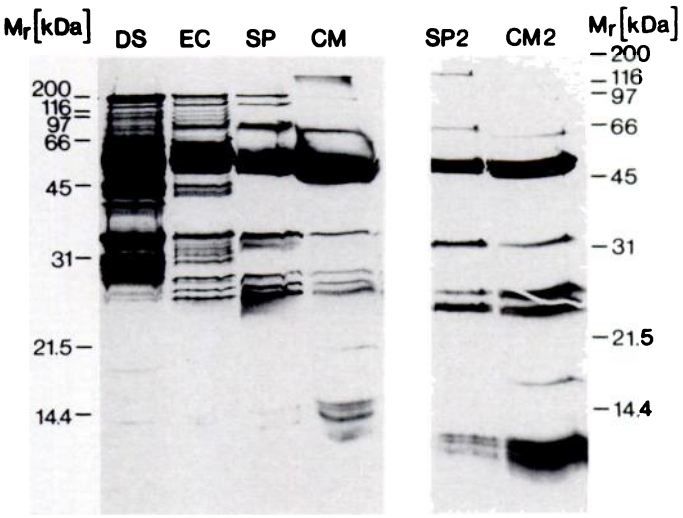

Fig. 1. SDS-PAGE analysis [according to the method of Laemmli (46)] of the polypeptide pattern of the three-step-purified mitochondrial DHP receptors. To 150 μl of the different fractions, 50 μl of stop solution (15% SDS, 3 M urea, 313 mM Tris·HCl, pH 6.8, 0.5 mM phenylmethylsulfonyl fluoride, 3% glycerol) were added, together with 10 μl of 2-mercaptoethanol (reducing conditions). Samples were denatured at 95° for 5 min, separated on 15% polyacrylamide slab gels, and stained with $AgNO_3$. *M_r*, relative molecular mass of marker proteins; *DS*, 46 μg of digitonin extract [protein determined according to the method of Bradford (31)]; *EC*, 40 μg of anion exchange column (ECTEOLA 23 column) eluate showing peak [^{3}H]NTR binding; *SP* and *SP2*, 33 and 22 μg of sucrose density gradient peak [^{3}H]NTR-binding fractions; *CM* and *CM2*, 51 and 34 μg of eluate from the cation exchange column (BioGel A) eluate with the highest specific [^{3}H]NTR binding. SP and SP2, as well as CM and CM2, were taken from the same preparation but were separated and stained on different days.

method-adjusted (see Experimental Procedures) B_{max} values, i.e., 0.14 ± 0.02 nmol/mg], corresponding to an overall increase of saturable binding by a factor of 8. This is in good agreement with the 11-fold increase in [^{3}H]NTR binding (three-step-purified receptors versus particulate preparations) routinely determined during the purification procedures (Table 1). Accordingly, direct comparison of the starting material (i.e., the digitonin extract) and the end product of the three-step purification yielded a 2.3-fold increase in [^{3}H]NTR affinity and a 3.3-fold increase in B_{max}, resulting in a >7.6-fold increase in saturable [^{3}H]NTR binding. As for the difference between this roughly 8-fold increase determined in saturation binding experiments and the 23-fold increase found in binding experiments using radioligand concentrations far below its K_d, it should be emphasized that the saturation binding experiments are fraught with a possible error due to the high dilutions of [^{3}H]NTR specific activity (up to 30,000-fold; see Experimental Procedures), which, however, are necessary for a system displaying a dissociation constant in the submicromolar range.

Ion dependence of [^{3}H]NTR binding to the partially purified mitochondrial receptors. Table 2 summarizes the effects on [^{3}H]NTR binding to the partially purified mitochondrial DHP receptors of group VIIa anions, group Ia cations, divalent and trivalent cations, and several organic ions that either have been reported to be good permeators of the IMAC (e.g., butylmalonate or benzenetricarboxylate) or have been shown not to be able to cross the mitochondrial membrane through the IMAC (gluconate or glucuronate) (9).

The rank order of monovalent cation affinities (given as EC_{50} values for [^{3}H]NTR binding stimulation by the chloride salts) was $Cs^+ > Rb^+ > Li^+ > K^+ > Na^+$; the affinity rank order of the monovalent anions (potassium salts) was $I^- > Br^- > Cl^- > F^-$. $Tris^+$ also proved to be an effective stimulator of [^{3}H]NTR binding to the partially purified mitochondrial DHP receptor.

Increasing the charge density of the cation dramatically increased its affinity, with the rank order of EC_{50} values being K^+ (125 mM) > Ca^{2+} (5 mM) ≫ La^{3+} (41 μM) (or 1:0.04:0.0003) (Table 2).

$SCN^- > NO_3^- >$ gluconate$^-$ stimulated [^{3}H]NTR binding to the partially purified receptor complex, whereas butylmalonate^{2-} and 1,2-3-benzenetricarboxylate^{3-} did not stimulate [^{3}H]NTR binding when given alone (Table 2). In the presence of 500 mM NaCl, however, butylmalonate^{2-} and 1,2,3-benzenetricarboxylate^{3-} dose-dependently inhibited [^{3}H]NTR binding (Table 3). Phosphate > sulfate also inhibited [^{3}H]NTR binding to the partially purified receptor complex in the presence of 500 mM NaCl (Table 3). They were ineffective when given alone (data not shown). Qualitatively, gluconate$^-$ behaved as a partial agonist with respect to the full agonist Cl^-; when tested alone, sodium gluconate increased [^{3}H]NTR binding from 0.46 ± 0.11 pmol/mg of protein (n = 4; normalized for 1 nM radioligand) to 1.65 ± 0.47 pmol/mg of protein (see also Table 2). In the presence of 500 mM NaCl, however, sodium gluconate decreased [^{3}H]NTR binding in the same preparations from 1.87 ± 0.51 pmol/mg of protein to 1.15 ± 0.41 pmol/mg of protein, which corresponded to an inhibition of 37 ± 15% of binding in the presence of 500 mM NaCl. It should be emphasized, however, that the full agonist Cl^- shifted the $K_{0.5}$ of gluconate$^-$ only slightly, from 290 ± 75 mM to 301 ± 51 mM. Glucuronate$^-$ was ineffective when given either alone or in the presence of 500 mM NaCl (Tables 2 and 3).

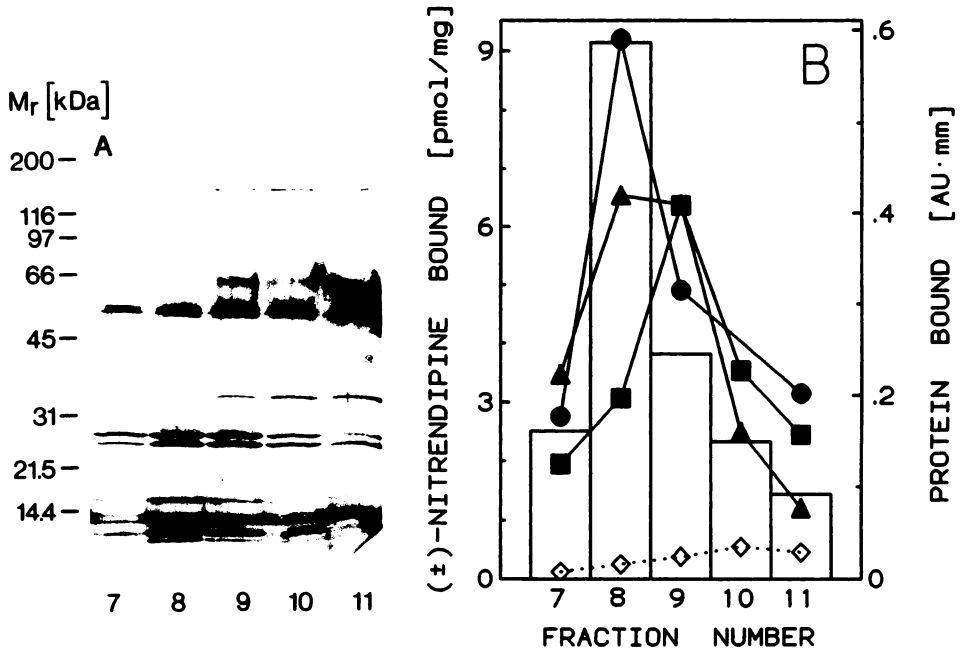

Fig. 2. Cation exchange chromatography: correlation between amount of protein and [^{3}H]NTR binding for different polypeptides of the partially purified mitochondrial DHP receptor complex. One hundred micrograms of protein of pooled sucrose density gradient peak [^{3}H]NTR-binding fractions were loaded on a 2-ml BioGel A cation exchange column (diameter, 1.5 cm). After washing of the column with 10 volumes of C buffer, the adsorbed protein was eluted in 1-ml fractions using a 0–1 M NaCl gradient in C buffer (1 ml/min flow, 40 min). A, M_r, relative molecular mass of marker proteins; *7–11*, polypeptide pattern of 130-μl aliquots of fractions 7–11 (5–15% polyacrylamide slab gel, $AgNO_3$ stain). B, The SDS-PAGE polypeptide pattern of fractions 7–11 was analyzed on a LKB Ultroscan XL densitometer. The amount of $AgNO_3$-stained polypeptides per fraction is given as absorbance units (*AU*)·mm, grouped according to the approximate molecular mass range of the major polypeptide constituents (■, 50 kDa; ◇, 34 kDa; ▲, 25 kDa; ●, 12 kDa). *Bars*, specific [^{3}H]NTR binding (pmol/mg of protein) of the same fractions (1.9 nM radioligand used, normalized to a total of 1 nM radioligand) (see also legend to Table 1). Nonspecific binding remained constant for all fractions. The estimated NaCl concentration of fractions 7–11 ranged from 150 mM to 275 mM in the eluate, contributing 30–55 mM NaCl in the final binding assay, which contained an additional 500 mM NaCl. Essentially identical results were obtained in two separate experiments.

TABLE 2

Stimulation of [^{3}H]NTR binding to the three-step-purified mitochondrial DHP receptor by various cations and anions

Data are means ± standard errors from *n* experiments performed in duplicate. Specific [^{3}H]NTR binding in the absence of ions was 0.15 ± 0.03 pmol/mg of protein (n = 47). EC_{50} values or maximal effects on [^{3}H]NTR binding of the different ions were compared with those of KCl (containing both reference ions, K^+ and Cl^-), using Student's *t* test (unpaired); *p* values are given for each comparison. Counterions are given in parentheses.

Ion	EC_{50}	*p*	Maximal stimulation	*p*	*H*	*n*
	mM		*pmol/mg at 1 nM*			
Group Ia cations (chloride salts)						
LiCl	81 ± 26	0.18	3.10 ± 0.40	0.17	4.5 ± 0.9	3
NaCl	222 ± 61	0.17	2.00 ± 0.52	0.59	3.3 ± 0.4	3
KCl	125 ± 8		2.34 ± 0.23		2.7 ± 0.3	3
RbCl	67 ± 6	0.02	2.64 ± 0.92	0.71	3.7 ± 0.5	2
CsCl	47 ± 12	0.01	2.39 ± 0.54	0.92	3.3 ± 2.3	2
Other cations (chloride salts)						
$MgCl_2$	6 ± 3	<0.001	1.44 ± 0.71	0.13	1.5 ± 0.5	3
$CaCl_2$	5 ± 1	<0.001	2.36 ± 0.91	0.99	2.1 ± 0.2	3
$LaCl_3$	0.041 ± 0.02	<0.001	2.79 ± 0.37	0.35	1.7 ± 0.7	3
Tris·HCl	182 ± 38	0.22	2.83 ± 0.52	0.43	4.5 ± 1.0	3
Group VIIa halides						
NaF	224 ± 57	0.16	2.93 ± 1.07	0.42	7.8 ± 1.8	3
KF	203 ± 65	0.36	5.68 ± 2.03	0.23	5.2 ± 2.4	4
KCl	125 ± 8		2.34 ± 0.23		2.7 ± 0.3	3
KBr	66 ± 9	0.01	2.24 ± 0.58	0.89	4.4 ± 2.2	3
KI	47 ± 7	0.002	4.48 ± 0.90	0.08	2.6 ± 0.3	3
Other anions						
KSCN	26 ± 5	0.003	3.74 ± 0.83	0.13	4.2 ± 2.3	2
KNO_3	96 ± 3	0.07	2.26 ± 0.20	0.82	2.1 ± 0.1	2
Gluconate (Na)	290 ± 75	0.095	1.65 ± 0.47	0.25	2.4 ± 0.8	3
Butylmalonate (K)			0.05 ± 0.03		(400 mM)	3
Benzenetricarboxylate (K)			0.09 ± 0.09		(400 mM)	2
Glucuronate (Na)			0.28 ± 0.04		(400 mM)	4

TABLE 3

Modulation of [^{3}H]NTR binding by different ions in the presence of 500 mM NaCl

Data are means ± standard errors from *n* experiments performed in duplicate. [^{3}H]NTR binding in the presence of 500 mM NaCl alone was 1.86 ± 0.31 (*n* = 21). Counterions are given in parentheses.

Compound	EC_{50} or IC_{50}	Maximal stimulatory or inhibitory effect of added ion	*H*	*n*
	mM	*% of binding*		
Stimulatory				
Fluoride (Na)		38 ± 17 (500 mM)		6
Inhibitory				
Gluconate (Na)	301 ± 51	37 ± 15	2.4 ± 0.5	3
Butylmalonate (Na)	93 ± 17	100 ± 0.3	2.0 ± 0.2	4
Benzenetricarboxylate (Na)	15 ± 15	61 ± 19	1.4 ± 0.4	3
Sulfate (Na)	167 ± 68	39 ± 9	14.0 ± 9.2	2
Phosphate (Na)	71 ± 15	56 ± 3	2.0 ± 0.2	5
Noneffective				
Glucuronate (Na)		11 ± 40 (500 mM)		6

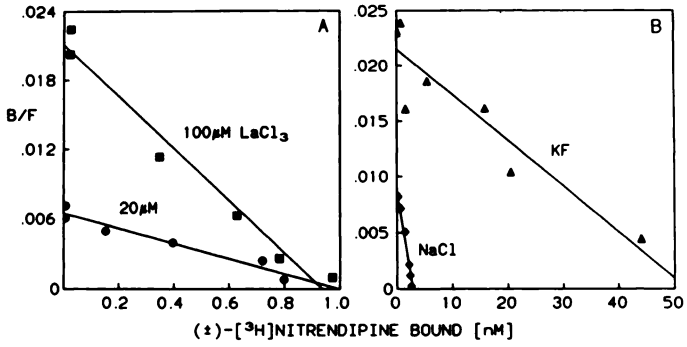

Fig. 3. Saturation isotherm of [^{3}H]NTR binding to partially purified mitochondrial DHP receptors under different ionic conditions. A, Protein (4.4 μg) was incubated with 1.2–1000 nM [^{3}H]NTR (specific activity, 87–0.09 Ci/mmol; nonspecific binding defined by 10 μM nicardipine), in 500 μl of Tris buffer supplemented with either 20 μM or 100 μM $LaCl_3$, for 2 hr at 37°. Scatchard analysis yielded a K_d value of 154 nM and a B_{max} value of 0.114 nmol/mg of protein (n = 6, r = −0.97) in the presence of 20 μM $LaCl_3$ (●). In the presence of 100 μM $LaCl_3$ (■), K_d was 45 nM and B_{max} was 0.107 nmol/mg of protein (n = 6, r = −0.99). B, Protein (8.9 μg) was incubated with 0.9–5000 nM [^{3}H]NTR (specific activity, 87–0.02 Ci/mmol), in 500 μl of Tris buffer supplemented with either 500 mM NaCl (♦) or 500 mM KF (▲), for 2 hr at 37°. Scatchard analysis of [^{3}H]NTR equilibrium binding by linear regression yielded a K_d of 340 nM and a B_{max} of 2.9 nM (0.16 nmol/mg of protein) in the presence of 500 mM NaCl (n = 8, r = −0.98). In the presence of 500 mM KF, the K_d was 2435 nM and the B_{max} was 52.3 nM (2.94 nmol/mg of protein; n = 8, r = −0.93).

Scatchard analysis of equilibrium [^{3}H]NTR binding in the presence of two different concentrations of the same stimulatory cation (Fig. 3A) showed that the cation-mediated [^{3}H]NTR binding stimulation was due to an increase in affinity; in the presence of 20 μM $LaCl_3$ (nonspecific binding, 23 ± 8% of total binding; n = 4), [^{3}H]NTR saturably bound with a K_d of 109 ± 23 nM (n = 4) and a B_{max} of 0.332 ± 0.023 nmol/mg of protein, whereas the same partially purified mitochondrial DHP receptor preparations yielded a K_d of 48.8 ± 2.3 nM (n = 4; p = 0.04) and a B_{max} of 0.209 ± 0.048 nmol/mg (p = 0.79) in the presence of 100 μM $LaCl_3$ (nonspecific binding, 14 ± 3% of total binding). Thus, overall binding was increased 2.1-fold, which is in good agreement with a calculated 1.8-fold [^{3}H]NTR binding increase for 20 μM versus 100 μM $LaCl_3$, obtained from the dose-response curve of the pooled $LaCl_3$ data (not shown). A comparison of $LaCl_3$ and NaCl (both at near-maximally stimulating concentrations) also showed that the differences in [^{3}H]NTR binding stimulation were mainly due to changes in the affinity of [^{3}H]NTR for the mitochondrial sites; in the presence of 500 mM NaCl, the K_d of [^{3}H]NTR was increased to 239 ± 40 nM (n = 6; p = 0.001, compared with 100 μM $LaCl_3$), whereas its B_{max} was increased to 0.53 ± 0.19 nmol/mg of protein (p = 0.22). Comparison of NaCl and KF, however, gave a different picture. Fig. 3B shows that the 2.8-fold stronger maximal [^{3}H]NTR binding stimulation observed with KF, compared with NaCl, that can be seen at very low radioligand concentrations (Table 2) was due to an 18-fold increase in the number of available [^{3}H]NTR binding sites (in the same preparation), whereas the affinity of [^{3}H]NTR was decreased 7-fold, corresponding to an overall 2.6-fold increase in binding (see Table 2). Despite the fact that addition of 500 mM KF resulted in a considerable increase in nonspecific binding (47 ± 7% of total binding; n = 8), compared with NaCl (18 ± 2% of total binding; n = 6), thus adding to the possible error caused by the high dilutions of the specific activity of [^{3}H]NTR (see above), the findings shown in Fig. 3A were confirmed in a larger experimental series. Replacing the 500 mM NaCl with 500 mM KF increased the B_{max} value to 10.8 ± 6.6 nmol/mg (n = 8; p = 0.20, with respect to NaCl), while increasing the K_D to 4980 ± 1770 μM (p = 0.04). In conclusion, comparison of either two concentrations of the same cation (i.e., La^{3+}) or comparison of near-maximally stimulating concentrations of different cations (i.e., La^{3+}, Na^+, and K^+) showed that only the ion effects on the affinity of [^{3}H]NTR were of statistical significance and, with the exception of KF, they corresponded to the ion effects seen at very low radioligand concentrations (Table 2). It should be noted, however, that the counterion (i.e., Cl^-) remained the same only in the case of La^{3+} and Na^+.

Changing the ionic environment also affected the affinity of allosteric inhibitors of [^{3}H]NTR binding for the partially purified mitochondrial Ca^{2+} antagonist receptors, as well as the degree of allosteric coupling. (±)-Gallopamil dose-dependently inhibited [^{3}H]NTR binding in the presence of all three cationic [^{3}H]NTR binding stimulators tested (i.e., 500 mM NaCl, 500 mM KF, and 30 μM $LaCl_3$). In the presence of 500 mM NaCl, its IC_{50} was 13.4 ± 1.6 μM (n = 3), the maximal inhibition was 96 ± 4% of specific binding, and the Hill slope was 0.92 ± 0.09. In the presence of 500 mM KF, the IC_{50} was 11.8 ± 5.1 μM (n = 3), the maximal inhibition was 80 ± 14%, and H was 0.91 ± 0.36. In the presence of 30 μM $LaCl_3$, the IC_{50} was 59.4 ± 17.2 μM, the maximal inhibition was 93 ± 7%, and H was 1.22 ± 0.56.

Discussion

The solubilized mitochondrial DHP receptor complex has been enriched 8–23-fold (depending on the method of postreversible [^{3}H]NTR binding assays; see Results) using anion exchange chromatography, sucrose density gradient centrifugation, and cation exchange chromatography (three-step procedure; Table 1). Although the last purification step, i.e., cation exchange chromatography, did not result in further enrichment of overall [^{3}H]NTR binding (Table 1), a 34-kDa protein still present after the sucrose density gradient centrifugation (Fig. 1) (see also Ref. 5) could be at least partially removed. Furthermore, comparison of the densitometer scans of SDS-gels from different fractions of a NaCl gradient eluate from the cation exchange column with postreversible [^{3}H]NTR binding

from the same fractions showed that only the amount of protein found in the polypeptide bands in the 50-kDa, 25-kDa, and 12-kDa regions, and not that of the 34-kDa polypeptide, correlated with [^{3}H]NTR binding (Fig. 2). Therefore, the 30–40-kDa-range polypeptides that had been specifically photolabeled by (±)-[^{3}H]azidopine (a DHP photoaffinity analog) in particulate mitochondrial membrane preparations (36) apparently do not belong to the purified mitochondrial DHP receptor but represent different and as yet unidentified structures. Interestingly, the rather nonspecific cation exchange procedure removed no other polypeptide (Fig. 1). All this evidence suggests that the remaining polypeptides could all be part of one polymeric receptor complex. It should be noted, however, that further experimental work will be needed to allow for both unequivocal identification of the polypeptide(s) carrying the Ca^{2+} antagonist receptor and the assignment of functions for the other associated polypeptides.

The considerable ion dependence of DHP and PAA binding that can be observed in particulate mitochondrial membrane preparations (1, 5) might reflect both specific interactions with the mitochondrial Ca^{2+} antagonist receptor complex and interactions of the ions with the surrounding lipid phase. Indeed, nitrate, the most effective stimulator of [^{3}H]NTR as well as [*N-methyl*-^{3}H]LU 49888 binding in particulate membranes (1, 5), is known for its strong chaotropic effect (37). By removing most of the surrounding native lipids during digitonin solubilization and purification procedures (Table 1), we tried to minimize this source of possible artifacts. Additionally, by using a solubilized and partially purified preparation, we can minimize interactions of the mitochondrial Ca^{2+} antagonist receptors with other mitochondrial proteins that might occur in particulate preparations. Overall, the purification procedures decreased the percentage of nonspecific [^{3}H]NTR binding assessed in the presence of 500 mM NaCl roughly 2-fold [22 ± 2% of total binding in three-step-purified preparations ($n = 24$) versus approximately 50% of total binding in particulate membranes; data not shown]. Thus, both [^{3}H]NTR binding to the partially purified mitochondrial DHP receptor and its regulation by ions should reflect specific interactions with the DHP- and the ion binding domains in the receptor protein complex.

In general, the potencies for the monovalent ions (groups Ia and VIIa) in modulating [^{3}H]NTR binding to the partially purified receptor are rather low, ranging from 47 to 224 mM. This is not surprising, because electrophysiological studies on other monovalent ion pores yielded apparent dissociation constants for equilibrium binding ranging from 19 to 54 mM for the K^+ channel of the sarcoplasmic reticulum (38) and ranging from 7 to 456 mM for the frog skeletal muscle endplate channel (39). Furthermore, it has been shown for a variety of other, well documented, channel structures that permeant ions influence binding of toxins or drugs to the channel; see Lombet *et al.* (40) for the Na^+ channel, Vazquez *et al.* (41) for the high conductance Ca^{2+}-activated K^+ channel, or Glossmann and Striessnig (42) for a review of data on Ca^{2+} channels obtained from several groups. By analogy, it might very well be that the ions investigated in our binding studies are permeators of the protein structures described in this study.

Although the EC_{50} values for the monovalent ions are rather low, most of them are significantly different (at the $p < 0.05$ level) from the EC_{50} value of the reference salt, potassium chloride (see p values of Table 2). Furthermore, changes in charge density of the cations vastly increase these differences (discussed below). Conversely, the maximal degree of [^{3}H]NTR binding stimulation did not differ significantly among the ions tested (see p values of Table 2). Therefore, our discussion of the possible underlying molecular mechanisms for ionic regulation of [^{3}H]NTR binding to the partially purified mitochondrial receptor complex will be based on the different potencies of the ions for [^{3}H]NTR binding stimulation; the maximal degree of [^{3}H]NTR binding stimulation will not be considered further. For most ions, we also observed a decline in [^{3}H]NTR binding stimulation at concentrations that were higher than those necessary for maximal [^{3}H]NTR binding stimulation (see Experimental Procedures). For the DHP binding domain of the L-type Ca^{2+} channel in brain, it has been shown that only divalent cations known as Ca^{2+} channel blockers displayed a similar biphasic response (for a review, see Ref. 42); because we do not have any pertinent electrophysiological data yet, we cannot discuss this phenomenon further. Saturation equilibrium binding analyses of either two concentrations of the same stimulatory cation (i.e., La^{3+}) or comparison of near-maximally stimulating concentrations of different cations (i.e., La^{3+}, Na^+, and K^+) showed that the ion effects on [^{3}H]NTR binding in general are due to changes in the affinity of [^{3}H]NTR for the partially purified mitochondrial DHP sites.

Cation stimulation of [^{3}H]NTR binding to the partially purified receptor complex strikingly depended on charge density. Thus, the rank order of EC_{50} values was K^+ (125 mM) > Ca^{2+} (5 mM) ≫ La^{3+} (41 μM) (or 1:0.04:0.0003; Table 2). If one assumes that these ion effects are simply due to a "screening," i.e., nonspecific charge-neutralizing, effect on fixed negative surface charges (on the partially purified protein and/or remaining surrounding phospholipids) by the cations in the surrounding medium, the mathematical model of Gouy (43) and Chapman (44) for diffuse double layers (see Ref. 45) predicts that the ratio of concentrations necessary to obtain a surface potential of −60 mV would be around 1:0.1:0.01 for monovalent:divalent:trivalent cations [110 mM:10 mM:1 mM for a fixed charge density of −1/300 A^2 (phospholipids in particulate preparations) or 11 μM:1 μM:0.1 μM for a fixed charge density of −1/30,000 A^2, assuming that 99% of the native lipids have been removed during the solubilization procedure]. This concentration ratio is clearly different from that observed for the mitochondrial DHP receptor (Table 2). If one assumes that the screening effect should depress the surface potential of the purified receptor-detergent complexes to −1 to −10 mV, the ratio of mono-:di-:trivalent cation becomes around 1:0.25:0.1 (e.g., for −1 mV and −1/300 A^2, 602 M:150 M:60 M; for −1 mV and 1/30,000 A^2, 60 mM:15 mM:6 mM; for −10 mV and 1/300 A^2, 5.9 M:1.4 M:0.6 M), differing even more from the observed values regarding both the concentration ratio and the absolute concentration values (Table 2). Furthermore, the EC_{50} values for [^{3}H]NTR binding stimulation of the monovalent cation group alone differ by factors of up to 4.7, which is not to be expected in the case of a purely screening effect (45). All this evidence points to the existence of a specific cation-binding domain on the purified mitochondrial DHP receptor. We cannot exclude, however, the possibility that, beside the interaction with specific binding sites, nonspecific surface charge-neutralizing effects might still be of importance for the ion effects. Unfortunately, the calculation of the contribution of the two effects to the overall ion effect would require the dissociation

constants of the ions themselves (45), the measurement of which lies beyond the scope of this article.

The potency (i.e., $1/EC_{50}$) rank order for monovalent cations was $Cs^+ > Rb^+ > Li^+ > K^+ > Na^+$, reminiscent of the Eisenman sequence I for group Ia cations (23) but with a distinct Li^+ inversion (23, 24). This strongly indicates that the cation-binding domain on the mitochondrial receptor complex is rather large (favoring larger cations), with the cation-binding domain interaction being more strongly governed by the polarizability of the binding domain than by purely coulombic interaction of two nonpolarizable point charges (23, 24). Furthermore, a plot of (1/unhydrated cation radius, in 1/Å) versus log ($1/EC_{50}$, in 1/mM) yielded a upwardly concave selectivity curve (data not shown), another indication of polarizability governing the ion-binding domain interaction (23). Similar results were obtained with the monovalent anions, with their potency rank order being $I^- > Br^- > Cl^- > F^-$ (Table 2), reminiscent of the selectivity sequence I of Diamond and Wright (24), indicative of a large anion-binding domain. Again, the upwardly concave plot (data not shown) of (1/unhydrated halide radius) versus log ($1/EC_{50}$) points to a polarizability sequence (23). Thus, the binding domains for cations and anions seem to be flexible structures capable of charge-induced conformation change. The assumption that both cation- and anion-binding domains of the mitochondrial DHP receptor are rather large is further strengthened by the fact that cations ($Tris^+$) and anions (NO_3^-, SCN^-, gluconate$^-$, 1,2,3-benzenetricarboxylate^{3-}, and butylmalonate^{2-}) of considerable size affect [^{3}H]NTR binding efficiently. The approximate diameter of $Tris^+$ is reported to be 0.67 nm (14), which might correspond to the minimum diameter of the cation-binding domain. With the aid of approximative three-dimensional molecular models (MINIT-Molekülbaukasten; Verlag Chemie), the following tentative conclusions about the anion-binding domain can be drawn. Flexible organic molecules (gluconate$^-$ and butylmalonate^{2-}) with negatively charged head groups of 0.6–0.7-nm diameter efficiently interact with the binding domain, with their hydrocarbon tails measuring up to 1.1 nm. Even 1,2,3-benzenetricarboxylate^{3-}, with its rigid center, its three adjacent carboxyl groups spanning 0.8–0.9 nm, and its dimensions of roughly 0.9 nm × 0.7 nm × 0.3 nm, can still be accommodated by the binding domain. All group VIIa anions with unhydrated diameters up to 0.43 nm (I^-) (Table 2) stimulated [^{3}H]NTR binding. $PO_4^{1.5-}$ and SO_4^{2-}, roughly spherical molecules with diameters around 0.6 nm, affected [^{3}H]NTR binding (Table 3). On the other hand, gluconate$^-$, with its bulky hexose heterocycle (dimensions of the chair form, roughly 0.8 nm × 0.7 nm × 0.4 nm), could not reach the anion-binding domain of the partially purified mitochondrial DHP receptor complex (Table 3; see Results). Thus, the anion-binding domain can accommodate either spherical ligands with diameters up to 0.6 nm or molecules with a flat backbone with dimensions of approximately 0.9 nm × 0.7 nm × 0.3 nm.

Comparison of the effects on [^{3}H]NTR binding of the anions that have been reported to permeate the IMAC (9) gave the following picture. Whereas Cl^-, SCN^-, and NO_3^- stimulated [^{3}H]NTR binding, butylmalonate^{2-}, 1,2,3-benzenetricarboxylate^{3-}, $PO_4^{1.5-}$, and SO_4^{2-} were clearly inhibitory (Tables 2 and 3). Of the anions reported not to pass through the IMAC (9), gluconate qualitatively behaved as a partial agonist with respect to the full agonist chloride (Tables 2 and 3), being slightly stimulatory when given alone but inhibiting NaCl-stimulated [^{3}H]NTR binding in the same preparations. On the other hand, glucuronate seemed not to affect [^{3}H]NTR binding to the partially purified receptor at all (Tables 2 and 3).

Changing the ionic environment also affected the affinity of PAA Ca^{2+} antagonists for the partially purified mitochondrial Ca^{2+} antagonist receptors, as well as the degree of their allosteric coupling to the DHP-binding domain, as assessed with the PAA Ca^{2+} antagonist gallopamil (see Results). A detailed description of these effects, however, would exceed the scope of the present study.

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