0026-895X/90/030362-08$02.00/0

MOLECULAR PHARMACOLOGY, 38:362-369

Mitochondrial Ca^{2+} Antagonist Binding Sites Are Associated with an Inner Mitochondrial Membrane Anion Channel

GERALD ZERNIG, IVO GRAZIADEI, THOMAS MOSHAMMER, CHRISTINA ZECH, NORBERT REIDER, and HARTMUT GLOSSMANN

Institut für Biochemische Pharmakologie, Universität Innsbruck, A-6020 Innsbruck, Austria

Received March 23, 1990; Accepted June 18, 1990

SUMMARY

The inner mitochondrial membrane contains specific Ca^{2+} antagonist binding sites unrelated to the L-type Ca^{2+} channel. The mitochondrial 1,4-dihydropyridine (DHP) and phenylalkylamine sites are reciprocally allosterically coupled, require anions (e.g., Cl^-, No_3^-) for optimal binding, and are inhibited by purine and pyrimidine nucleotides in a noncompetitive manner. In mitochondrial swelling experiments, a concentration-dependent inhibition of an inner mitochondrial membrane anion channel (IMAC) by Ca^{2+} antagonists from different chemical classes can be demonstrated. Under the conditions of the swelling experiments, affinity of different Ca^{2+} antagonists and amiodarone, a known IMAC inhibitor, for the mitochondrial (±)-[^{3}H]nitrendipine binding site (K_d, 7.2 ± 2.0 μM; B_{max}, 1.03 ± 0.37 nmol/mg of protein) strongly correlated with their inhibitory potency for the IMAC. Linear regression of pIC_{50} values for IMAC-induced swelling versus pIC_{50} values for (±)-[^{3}H]nitrendipine binding inhibition yielded a correlation coefficient of 0.91 for all tested DHPs (n = 12, $p < 0.001$). Amiodarone inhibited (±)-[^{3}H]nitrendipine binding and IMAC-induced swelling with pIC_{50} values of 6.11 and 5.93, respectively. The correlation coefficient between binding and inhibition of IMAC-induced swelling for amiodarone and all tested Ca^{2+} antagonists (including non-DHP compounds) was 0.76 (n = 20, $p < 0.001$), with the slopes approaching unity. These results suggest the association of the mitochondrial Ca^{2+} antagonist binding sites with an IMAC.

In addition to their stereoselective interactions with the α_1 subunit of the L-type Ca^{2+} channels (for recent reviews, see Refs. 1–6), certain Ca^{2+} antagonists from the DHP, PAA, and BTZ classes modulate a variety of cellular systems, causing a multitude of effects that cannot be explained by Ca^{2+} channel inhibition (for review, see Ref. 7). For example, Ca^{2+} antagonist interaction with the multidrug resistance-related P-glycoprotein (8) has already been employed for the therapy of anticancer drug resistance in malignant lymphoma patients (9).

Non-L-type channel DHP binding sites have also been reported in mitochondria (10–12). DHPs have been shown to regulate [^{3}H]Ro5-4864 binding to the peripheral benzodiazepine receptor (10), which is located on the outer mitochondrial membrane (13). However, only the DHP sites located on the inner mitochondrial membrane have been partially purified after solubilization (14) and they contain binding domains for DHPs (12) and PAAs (14, 15), which are coupled to each other in a negative heterotropic allosteric manner and are allosterically inhibited by purine and pyrimidine nucleotides (16). The DHP site characterized by our group is most likely identical to the mitochondrial DHP site described by Brush *et al.* (11), although these authors reported a lack of DHP binding regulation by PAAs.

In animal studies, Ca^{2+} antagonists can prevent mitochondrial Ca^{2+} overload and preserve mitochondrial ultrastructure and function under ischemic stress (for reviews, see Refs. 1, 4, 17, and 18). These effects cannot be explained solely by an inhibition of the plasmalemmal L-type Ca^{2+} channel (19). Although several mitochondrial Ca^{2+}-regulating systems (e.g., energy-driven Ca^{2+} uptake or the mitochondrial Na^+/Ca^{2+} exchange) have been shown to be modulated by Ca^{2+} antagonists (see Ref. 7 for a review), the molecular basis for the "anti-ischemic" effects has not yet been provided.

This investigation was supported in part by the Dr. Legerlotz Foundation (G.Z.) and the Fonds zur Förderung der wissenschaftlichen Forschung in Österreich, Grants P7492-MED (G.Z.) and S45-01 (H.G.).

ABBREVIATIONS: DHP, 1,4-dihydropyridine; B_{max}, maximal density of receptor sites; Bay K 8644, 3-methyl-1,4-dihydro-2,6-dimethyl-5-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate; Bay M5579, 3-ethyl-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine dicarboxylate; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; D600, methoxyverapamil (gallopamil); D619, 2-methyl-3-cyano-3-(3′,4′-dimethoxyphenyl)-6-dimethylaminohexane; D888, desmethoxyverapamil; DMSO, dimethyl sulfoxide; IMAC, inner mitochondrial membrane anion channel; IC_{50}, concentration causing 50% of maximal inhibition; k_{+1}, association rate constant; k_{-1}, dissociation rate constant; K_d, equilibrium dissociation constant; K_i, inhibition constant; (±)-LU47781, (±)-5-[(3-azidophenethyl)methylamino]-2-(3,4,5-trimethoxyphenyl)-2-isopropylvaleronitrile; (±)-LU50106, 1-(3-aminophenyl)-3-methylaza-7-cyano-7-(3,4,5-trimethoxyphenyl)-8-methyl-nonane; PAA, phenylalkylamine; BTZ, benzothiazepine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

We were prompted to search for effects of Ca^{2+} antagonists on anion-related functions because binding of certain DHPs to non-L-type Ca^{2+} channel-linked sites in various tissues was stimulated by anions (20, 21). We later found that inorganic anions stimulated DHP binding to the mitochondrial inner membrane sites (12). With the aid of radioligand binding as well as functional experiments, we provide evidence that the mitochondrial Ca^{2+} antagonist binding site may be associated with an inner mitochondrial membrane anion channel. Preliminary results have been presented at a recent meeting (22).

Experimental Procedures

Materials. Nigericin, A23187, CCCP, and amiodarone were from Sigma (Munich, FRG). (±)-[^{3}H]Nitrendipine [(±)-[5-*methyl*-^{3}H]3,5-ethylmethyl-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine dicarboxylate; specific activity 84–87 Ci/mmol] was obtained from New England Nuclear (Vienna, Austria). DHPs were gifts from Bayer AG (Wuppertal, FRG) and PAAs from Knoll AG (Ludwigshafen, FRG). Ca^{2+} antagonists were from sources given elsewhere (12, 23). Guinea pig liver mitochondrial membranes for binding studies were prepared, as described previously (12), from mitochondria isolated by differential centrifugation (24). Intact mitochondria from guinea pig liver for swelling assays were isolated from nonfasting animals of either sex according to the method of Broekemeier *et al.* (25), with the following modifications. During homogenization and the first 5-min 1000 × *g* centrifugation step, mannitol-sucrose buffer (210 mM mannitol, 70 mM sucrose, 3 mM Tris-HEPES, pH 7.4, 0.2 mM Tris-EDTA) supplemented with 0.4 mg/ml bovine serum albumin (fatty acid-free; Sigma, Munich, FRG) was used. After sedimentation of the mitochondria for 5 min at 8000 × *g*, the pellet was washed in mannitol-sucrose buffer, recentrifuged, and stored in K buffer (55 mM KCl, 5 mM Tris·HCl, pH 7.4, 0.2 mM Tris-EGTA, 0.2 mM Tris-EDTA) at a concentration of 4–8 mg of protein/ml, on ice, up to 4 hr before the swelling assay.

Assay of anion transport by osmotic swelling studies. The net salt transport associated with anion permeation leads to swelling of the mitochondria (26, 27), allowing quantitation of ion intake by measurement of the degree of swelling (28, 29). The degree of swelling was followed by photometric measurement of the associated decrease in absorbance of the mitochondrial suspension after activation of (a) the IMAC or (b) the phosphate and dicarboxylate transporters, according to the method of Garlid and Beavis (26, 30) (see also Fig. 2 for details).

Unless indicated otherwise, mitochondria [equivalent to 80–362 μg of protein, determined according to the method Lowry *et al.* (31)] were incubated in 1.35 ml of K buffer, supplemented with 0.8 μg/ml rotenone, either alone (control blank) or in presence of various drug concentrations for 7 min at 25°. After this suspension was transferred into a stirred six-position photometric cell kept at 25°, absorbance measurement at 520 nm was started at 0 sec and repeated every 20 sec with a Hitachi U2000 photometer. Fifty microliters of 4 μM nigericin at 20 sec and 50 μl of 80 μM CCCP at 80 sec were added to neutralize the charge movement through the IMAC (all additions in K buffer). Addition of 50 μl of 80 μM A23187 at 140 sec (final assay volume, 1.5 ml) together with the chelators present in the incubation medium led to a removal of matrix divalent cations, thus activating the IMAC (for a schematic representation, see Ref. 26). Blank values were determined by adding the respective volumes (i.e., 3 times 50 μl) of K buffer only (instead of nigericine, CCCP, and A23187). For each 20-sec measurement cycle, the decrease in $A_{520\ nm}$/20 sec was determined and expressed as a percentage of control, according to the following formula:

$$\%\ \text{swelling} = ([\Delta A_{520\ nm}/20\ \text{sec}]_{drug} - [\Delta A_{520\ nm}/20\ \text{sec}]_{blank})/([\Delta A_{520\ nm}/20\ \text{sec}]_{control} - [\Delta A_{520\ nm}/20\ \text{sec}_{blank}) \times 100$$

Because of the distorting artifacts due to the opening of the chamber, values of the first cycle were discarded and only data from the second to the fourth cycles were used. Drugs were prepared from 1–100 mM stock solutions in DMSO. Because DMSO affected mitochondrial swelling at concentrations greater than 1% (v/v; not shown), the final DMSO concentration was never allowed to exceed 0.5%.

Radioligand binding assays. Reversible binding of (±)-[^{3}H]nitrendipine to mitochondrial membranes was measured essentially as described (12, 23), with the following modifications. To simulate the conditions of the mitochondrial swelling experiments, mitochondrial membranes (80–318 μg of protein/ml) were incubated with 0.9–4.3 nM (±)-[^{3}H]nitrendipine, in 250 or 500 μl of the K buffer (as used in the swelling experiments; see Materials), at 25° for the times indicated (kinetic experiments). A steady state for saturable binding of (±)-[^{3}H]nitrendipine was reached after 30 min and binding remained stable for at least another 120 min (not shown). Consequently, equilibrium binding was measured after 120-min incubation time. Under the above conditions, total (±)-[^{3}H]nitrendipine binding to mitochondrial membranes ranged from 0.04 to 0.6 pmol/mg of protein, depending on radioligand and membrane concentration. Nonspecific binding was determined in the presence of either 100 μM unlabeled nitrendipine (57% of total binding; Fig. 3 and Table 2) or 30 μM nicardipine (58% of total binding). For equilibrium saturation analysis, the specific activity of (±)-[^{3}H]nitrendipine was varied from 84 to 0.02 Ci/mmol by addition of unlabeled (±)-nitrendipine. In kinetic experiments, dissociation of the (±)-[^{3}H]nitrendipine-receptor complex was initiated either by addition of 10 or 100 μM unlabeled (±)-nitrendipine or, yielding the same result, by 25- or 50-fold dilution of the assay in 25° K buffer. Specific (i.e., saturable) binding for equilibrium saturation analysis and kinetic experiments was calculated by subtraction of nonspecific from total binding. To determine (±)-[^{3}H]nitrendipine binding under more favorable conditions (12), radioligand and membranes (for concentrations, see above) were incubated for 120 min at 37° in NO_3 buffer containing 300 mM $NaNo_3$, 5 mM $MgCl_2$, and 5 mM Tris·HCl (pH 7.0). Under these conditions, equilibrium binding with 1.8–4.3 nM (±)-[^{3}H]nitrendipine ranged from 0.6 to 1.5 pmol/mg of protein, with nonspecific binding amounting to 15% of total binding in the presence of 30 μM unlabeled nitrendipine (Fig. 3, Table 3) or 17% of total binding in the presence of 30 μM nicardipine. For (±)-[^{3}H]nitrendipine binding inhibition experiments, drugs were prepared from 10 or 100 mM stock solutions in DMSO. Because DMSO was inhibitory at concentration greater than 1% (v/v; not shown), the DMSO concentration was never allowed to exceed this value.

To determine the "free" drug concentration in the aqueous medium in the swelling experiment or the binding assay, 0.1–10 μM (±)-[^{3}H]nitrendipine, (+)-[^{3}H]isradipine or (±)-[^{3}H]Bay K 8644 (specific activity, 0.005–0.012 Ci/mmol) were incubated with mitochondrial membranes under the conditions of either the swelling experiment (470 μg of protein, 1.5-ml assay volume, 10-min incubation at 25°) or the binding assay (39.9 μg of protein, 250-μl assay volume, 120-min incubation at 25°). After the incubation, the mitochondrial membrane suspensions were put on ice and centrifuged, and aliquots of the supernatants were counted for radioactivity.

For binding inhibition experiments and calculation of IC_{50} values, nonspecific binding was not subtracted. Instead, using the algorithm of Ref. 32, the data were computer fitted to yield the best parameters for the IC_{50} values, maximal inhibition, and pseudo-Hill slope. Data are given as means ± standard errors of *n* determinations.

Results

The traces contained in Fig. 1 exemplify that the DHP Ca^{2+} antagonist niludipine inhibits the mitochondrial swelling induced by the activation of an IMAC by about 70% at a concentration of 30 μM. Table 1 summarizes the IC_{50} values and pseudo-Hill slopes for IMAC inhibition by a variety of DHP, PAA, and BTZ Ca^{2+} antagonists and compares them with amiodarone, a known IMAC blocker (26, 33). The Ca^{2+} channel activator Bay K 8644 also inhibited the IMAC, albeit at high concentrations (Table 1). The IC_{50} value and pseudo-Hill slope for amiodarone under our experimental conditions (1.2 μM and

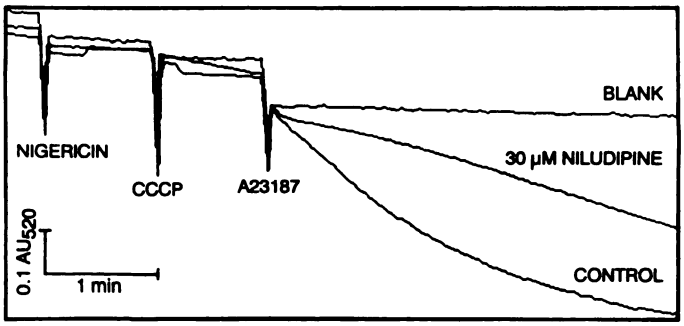

Fig. 1. Inhibition of IMAC-induced swelling of mitochondria by 30 μM niludipine. Guinea pig mitochondria (341–378 μg of protein) were assayed for IMAC-induced swelling, as described in Experimental Procedures. Tracings show the effect on absorbance (27) caused by mitochondrial swelling after addition of nigericin, CCCP, and A23187 alone (control) or in the presence of 30 μM niludipine. The blank sample received only equivalent volumes of K buffer instead of nigericin, CCCP, and A23187 additions.

TABLE 1

Inhibition of the IMAC-induced swelling of mitochondria by various Ca^{2+} antagonists and amiodarone

Assay conditions are described in Experimental Procedures. Data are means ± estimated standard errors of pooled data from *n* determinations. The numbers in parentheses (see also Tables 2 and 3) refer to Fig. 4. *h*, pseudo-Hill coefficient. Values in parenthesis refer to the highest concentrations employed.

Compound	IC_{50}	Maximal inhibition	*h*	*n*
	μM	%		
DHPs				
1 (±)-Nicardipine	8.4 ± 0.4	86	1.55 ± 0.10	10
2 (±)-Nitrendipine	20.2 ± 5.9	80	1.75 ± 0.53	8
3 (+)-Isradipine	38.5 ± 3.8	62 (100 μM)	1.36 ± 0.19	8
4 (−)-Isradipine	107 ± 27	61 (100 μM)	1.57 ± 0.83	4
5 (±)-Bay K 8644	181 ± 172	61	1.38 ± 1.93	6
6 Niludipine	8.7 ± 0.8	89	2.32 ± 0.52	4
7 (±)-Bay M 5579	207 ± 47	33 (100 μM)	0.65 ± 0.11	6
8 (±)-Nisoldipine	12.0 ± 2.7	87	1.13 ± 0.34	4
9 (±)-Nimodipine	19.3 ± 2.1	80	3.16 ± 1.02	4
10 Nifedipine	64.8 ± 18.1	26 (50 μM)	2.91 ± 2.71	6
11 (±)-Niguldipine	3.5 ± 0.2	93	1.85 ± 0.15	13
12 (±)-Amlodipine	2.0 ± 0.2	92	2.29 ± 0.48	9
PAAs				
13 (±)-LU47781	6.1 ± 1.0	92	2.02 ± 0.67	6
14 (±)-LU50106	60.3 ± 8.9	96	0.88 ± 0.13	10
15 (±)-D888	17.6 ± 3.1	96	1.07 ± 0.23	8
16 (±)-Verapamil	129 ± 37	96	1.61 ± 0.60	4
17 (±)-D600	69.5 ± 4.9	88	2.36 ± 0.37	8
(±)-D619	339 ± 132	53 (500 μM)	0.49 ± 0.18	4
BTZs				
18 (+)-(*cis*)-Diltiazem	61.3 ± 24.0	85	1.16 ± 0.33	6
16 (−)-(*cis*)-Diltiazem	58.2 ± 2.3	85	0.86 ± 0.04	4
Anion channel inhibitor				
17 Amiodarone	1.2 ± 0.3	86	1.58 ± 0.82	8

1.58, respectively) closely corresponded to those reported by Beavis [0.6 μM and 1.51, respectively (33)]. Ca^{2+} antagonists inhibited swelling only if the anion permeated through the anion channel; if anion permeation involved the phosphate and dicarboxylate transporters, the same drugs were without effect (Fig. 2).

Under conditions simulating the mitochondrial swelling experiment (i.e., low ionic strength of K buffer and an incubation temperature of 25°), (±)-[^{3}H]nitrendipine saturably bound to hepatic mitochondrial membranes with a K_d of 7.2 ± 2.0 μM

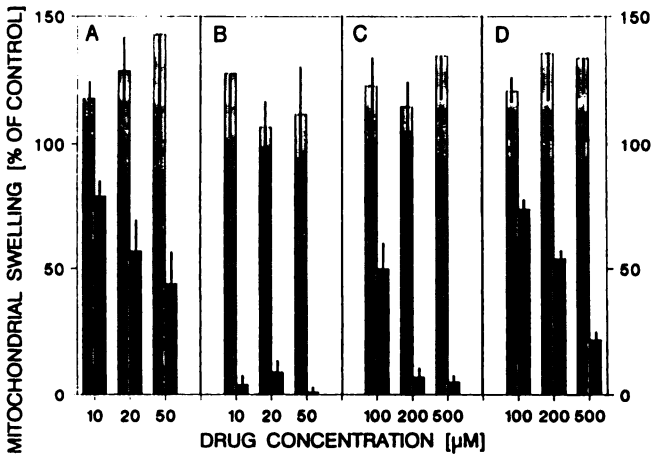

Fig. 2. Ca^{2+} antagonists inhibit mitochondrial swelling induced by the IMAC but not by activation of the phosphate and dicarboxylate transporters. Guinea pig liver mitochondria were preincubated for 7 min at 25° either without or in presence of (±)-nitrendipine (A), (±)-nicardipine (B), (±)-verapamil (C), or (+)-(*cis*)-diltiazem (D), in 1.4 ml of buffer containing 37 mM malic acid-KOH (pH 7.4), 20 mM KCl, 1.8 mM Tris·HCl (pH 7.4), 0.07 mM Tris-EDTA, and 0.07 mM Tris-EGTA, before absorbance measurements were started, as described in Experimental Procedures. Drug concentrations are given in μM. Malate was used as the permeant anion for either the dicarboxylate transporter (under concomitant activation of the phosphate transporter, see Ref. 27 for a schematic description) or the IMAC (see Experimental Procedures for details). To test the dicarboxylate and phosphate transporters, to intact mitochondria (equivalent to 82–242 μg of protein), 50 μl of 4 μM nigericin were added after 20 sec and 50 μl of 150 mM KH_2PO_4/K_2HPO_4 (pH 7.4) after 80 sec (1.5 ml final assay volume). To determine IMAC activity, intact mitochondria (80–242 μg of protein) were assayed as described in Experimental Procedures. Shown are means ± standard errors of three to eight determinations from three different mitochondrial preparations. □, Mitochondrial swelling induced by the dicarboxylate and the phosphate transporters; ■, swelling induced by activation of the IMAC.

and a B_{max} of 1.03 ± 0.37 nmol/mg of protein ($n = 5$). Kinetic experiments revealed a k_{+1} of 1.84 ± 0.68 × 10^4 M^{-1} × min^{-1} ($n = 4$) and a k_{-1} of 0.102 ± 0.024 min^{-1} ($n = 4$), regardless of whether receptor-ligand complex dissociation was initiated by addition of high concentrations of unlabeled nitrendipine or by dilution (not shown). The kinetically derived K_d (k_{-1}/k_{+1}) of 5.5 μM corresponded well to the K_d determined by equilibrium binding experiments (i.e., 7.2 μM). Under the swelling experiment conditions, DHP and PAA Ca^{2+} antagonists as well as the BTZs (+)- and (−)-(*cis*)-diltiazem inhibited (±)-[^{3}H]nitrendipine binding (Table 2, Fig. 3). Amiodarone also partially inhibited (±)-[^{3}H]nitrendipine binding with a very steep pseudo-Hill slope (Table 2), both characteristics indicative of noncompetitive interaction with the mitochondrial DHP binding site. Fig. 4 shows that (±)-[^{3}H]nitrendipine binding inhibition potency for DHP, PAA, and BTZ Ca^{2+} antagonists as well as amiodarone correlates well with their inhibitory potency for the IMAC when the conditions of the binding assay imitate the swelling experiments. For all tested DHPs (drugs 1–12 of Tables 1 and 2), the correlation coefficient was 0.91 ($n = 12$; $p < 0.001$), the overall relationship being:

$$pIC_{50_{IMAC\text{-}induced\ swelling}} = -0.39 + 0.96 \times pIC_{50_{binding}}$$

Thus, on average, the DHPs displayed a 2.5-fold lower affinity for the IMAC than for the mitochondrial binding site, the difference being larger for DHPs of higher affinity (e.g., niludipine, nitrendipine) and lower for weakly acting DHPs (e.g.,

TABLE 2

Inhibition of mitochondrial (±)-[^{3}H]nitrendipine binding under swelling conditions by DHPs, PAAs, BTZs, and amiodarone

(±)-[^{3}H]Nitrendipine binding to mitochondrial membranes was measured under the conditions of the swelling experiment, as described in Experimental Procedures. Shown are means ± estimated standard errors of pooled data from *n* determinations. Maximal inhibition is given as percentage of total (±)-[^{3}H]nitrendipine binding (no subtraction of nonspecific binding). *h*, pseudo-Hill coefficient. Values in parenthesis refer to the highest concentrations employed.

Compound	IC_{50}	Maximal inhibition	*h*	*n*
	μM	%		
DHPs				
1 (±)-Nicardipine	2.1 ± 1.0	42	1.04 ± 0.52	22
2 (±)-Nitrendipine	2.2 ± 0.6	43	0.99 ± 0.21	16
3 (+)-Isradipine	6.6 ± 1.5	53 (100 μM)	0.82 ± 0.16	6
4 (−)-Isradipine	10.3 ± 1.3	43 (100 μM)	0.54 ± 0.05	8
5 (±)-Bay K 8644	28.1 ± 8.0	46 (100 μM)	1.25 ± 0.28	10
6 Niludipine	1.3 ± 0.6	37	1.70 ± 1.32	6
7 (±)-Bay M 5579	121 ± 33	26 (100 μM)	0.76 ± 0.17	8
8 (±)-Nisoldipine	4.7 ± 0.7	41 (20 μM)	0.87 ± 0.12	4
9 (±)-Nimodipine	7.6 ± 3.9	24	1.90 ± 1.97	4
10 Nifedipine	12.9 ± 11.1	41	0.67 ± 0.36	3
11 (±)-Niguldipine	0.9 ± 0.1	43	0.68 ± 0.08	16
12 (±)-Amlodipine	1.5 ± 0.2	23	1.98 ± 0.46	10
PAAs				
13 (±)-LU47781	22.6 ± 7.3	45	1.34 ± 0.45	8
14 (±)-LU50106	157 ± 50	55	1.27 ± 0.29	12
15 (±)-D888	43.6 ± 38.1	34	1.03 ± 0.84	10
16 (±)-Verapamil	14.7 ± 5.9	23	1.43 ± 0.69	10
17 (±)-D600	36.5 ± 17.0	41	0.82 ± 0.28	10
(±)-D619	NE* (100 μM)			10
BTZs				
18 (+)-(*cis*)-Diltiazem	60.0 ± 49.0	38	0.84 ± 0.60	7
19 (−)-(*cis*)-Diltiazem	66.3 ± 17.8	38	0.58 ± 0.15	9
Anion channel inhibitor				
20 Amiodarone	0.8 ± 0.2	18	4.40 ± 3.42	6

* NE, no effect at the highest drug concentration employed.

Bay K 8644, Bay M 5579). The correlation between (±)-[^{3}H]nitrendipine binding inhibition and functional potency was excellent for the BTZ Ca^{2+} antagonists but poor for the PAA Ca^{2+} antagonists ($r = 0.07$, $n = 5$; $p > 0.1$; drugs 13–17 of Tables 1 and 2), although they proved to be effective inhibitors of the IMAC-induced swelling as well as (with the exception of D619) mitochondrial (±)-[^{3}H]nitrendipine binding. The overall correlation coefficient for all DHP, PAA, and BTZ Ca^{2+} antagonists and amiodarone (with the exception of D619) was 0.76 ($n = 20$; $p < .001$; drugs 1–20 of Tables 1 and 2; relationship: $pIC_{50_{IMAC\text{-}induced\ swelling}} = 1.20 + 0.69 \times pIC_{50_{binding}}$).

We also tested (±)-[^{3}H]nitrendipine binding to mitochondrial membranes in the presence of high concentrations of nitrate, an anion that has been reported to be an excellent permeator of the IMAC (34) and a potent stimulator of (±)-[^{3}H]nitrendipine binding to mitochondrial sites (12). The dissociation constant under these favorable experimental conditions was 0.296 ± 0.054 μM and the B_{max} value was 0.09 ± 0.01 nmol/mg of protein ($n = 4$). Thus, the K_d for the radioligand under swelling experiment conditions was 24-fold higher than that in presence of nitrate but the number of sites accessible to (±)-[^{3}H]nitrendipine was increased by a factor of 10. Thus, overall saturable binding of the radioligand was approximately 3-fold higher under nitrate buffer conditions, compared with K buffer. High concentrations of $NaNO_3$ shifted the (±)-[^{3}H]nitrendipine binding inhibition potency for all DHP and PAA Ca^{2+} antagonists to a variable degree (Fig. 3); the correlation coefficient for $pIC_{50_{NO_3\ buffer}}$ versus $pIC_{50_{K\ buffer}}$ was 0.55 for 11 drugs tested ($p > 0.05$; all drugs listed in Table 3). Notably, amiodarone failed to affect mitochondrial (±)-[^{3}H]nitrendipine binding in the presence of nitrate, whereas niludipine and nicardipine increased their binding inhibition potency 26- and 20-fold, respectively (compare data in Table 2 with Table 3). However, the correlation between (±)-[^{3}H]nitrendipine binding inhibition in nitrate buffer and the inhibition potency for IMAC-induced swelling (tested in K buffer) was very low ($r = 0.12$; $n = 10$; $p \gg 0.1$; drugs 1, 2, 6, 7, 11, 13, 15–17 and 20 of Tables 1 and 3).

Compared with the binding parameters obtained with cardiac mitochondrial membranes in a 500 mM NaCl-containing buffer at an incubation temperature of 37° [where cardiac and hepatic mitochondrial membranes displayed essentially the same (±)-[^{3}H]nitrendipine binding characteristics (12)], K buffer and an incubation temperature of 25° slowed down association of (±)-[^{3}H]nitrendipine to its hepatic mitochondrial binding site by a factor of 2 but increased the dissociation rate 6-fold. Accordingly, the equilibrium binding dissociation constant was increased 12-fold for hepatic mitochondrial membranes.

In conclusion, binding affinity for the mitochondrial Ca^{2+} antagonist binding sites depended on the experimental conditions (ionic envionment and/or temperature) of the binding assay, with large interindividual differences in affinity shifts for the various drugs tested.

To determine the partitioning of the (hydrophobic) DHP Ca^{2+} antagonists into the mitochondrial membranes, 0.1–10 μM (±)-[^{3}H]nitrendipine, (+)-[^{3}H]isradipine and (±)-[^{3}H]Bay K 8644 were incubated with mitochondrial membranes under conditions simulating the binding or the swelling experiments, and the actual free (i.e., not membrane-associated, available) drug concentrations were measured (see Experimental Procedures). Under the conditions of the binding assays, these free DHP concentrations were always higher than under the conditions of the swelling experiments (Table 4).

Discussion

An electrogenic uptake of anions across the inner mitochondrial membrane has been described by several groups (26, 27, 35), using indirect swelling (27, 28) or $^{36}Cl^-$ uptake experiments (36), and was tentatively assigned to a pore-forming structure in the inner mitochondrial membrane (26, 37). Recently, with the aid of patch-clamp studies, the existence of an anion channel in the inner mitochondrial membrane was demonstrated in giant mitochondria from cuprizone-fed mice (38).

The correlation between the inhibitory potency of DHP, PAA, and BTZ Ca^{2+} antagonists as well as amiodarone for the mitochondrial swelling induced by the activation of the IMAC and their affinity for the mitochondrial (±)-[^{3}H]nitrendipine binding site, simulating the ionic and temperature conditions of the swelling experiments, suggests that the two structures are closely associated. "Nonspecific" effects of the Ca^{2+} antagonists on mitochondrial swelling in general can be excluded. The antagonists proved to be inhibitory only when swelling was induced by activation of the IMAC and not when it was induced by activation of the dicarboxylate and phosphate transporters (Fig. 2). The inability of DHP, PAA, or BTZ Ca^{2+} antagonists to inhibit mitochondrial swelling induced by the addition of 5 mM phosphate under our experimental conditions

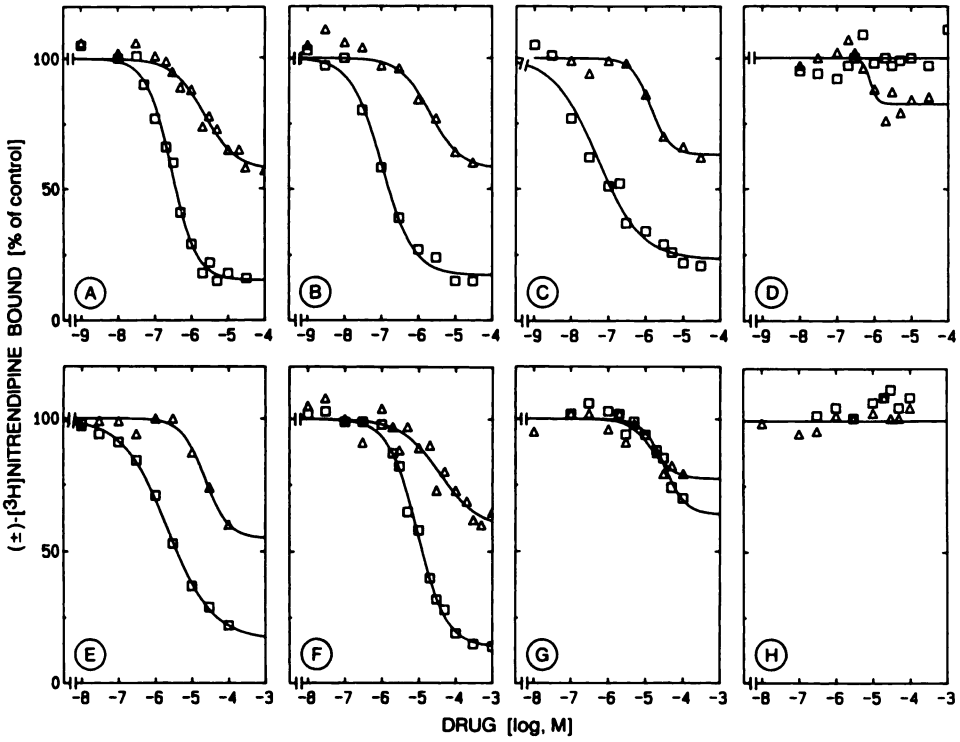

Fig. 3. Mitochondrial (±)-[^{3}H]nitrendipine binding inhibition by DHPs, PAAs, and amiodarone under different ionic conditions. Inhibition of reversible (±)-[^{3}H]nitrendipine binding to mitochondrial membranes was measured by incubating various concentrations of drugs (as indicated) with (±)-[^{3}H]nitrendipine, as described in Experimental Procedures, either in the buffer used for swelling experiments (K buffer at 25°) (Δ) or in a $NaNO_3$-containing buffer (NO_3 buffer at 37°) (□). The plotted data are normalized with respect to total control (±)-[^{3}H]nitrendipine binding (in the absence of added unlabeled drugs) and are expressed as percentages. Control binding ranged from 0.04 to 0.6 pmol/mg of protein under swelling experiment conditions and varied between 0.6 and 1.5 pmol/mg of protein in NO_3 buffer. Data are means of 4–22 determinations. A, nitrendipine; B, nicardipine; C, niludipine; D, amiodarone; E, LU 47781; F, gallopamil; G, verapamil; H, D619.

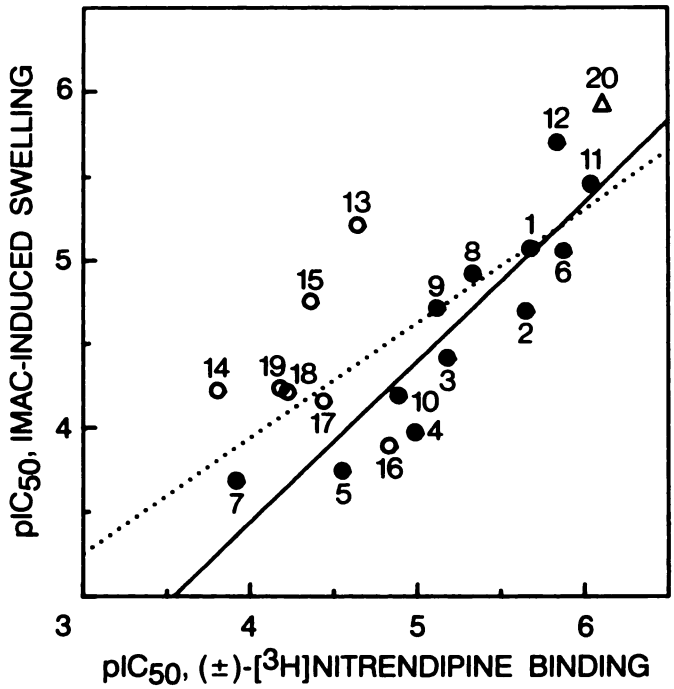

Fig. 4. Correlation between affinity for the mitochondrial (±)-[^{3}H]nitrendipine binding site and inhibitory potency for the IMAC. Plotted are pIC_{50} values for (±)-[^{3}H]nitrendipine binding inhibition under swelling experiment conditions (*abscissa*) versus pIC_{50} values for inhibition of mitochondrial swelling induced by the IMAC (*ordinate*). *Numbers* refer to the substances listed in Tables 1 and 2. Linear regression for the DHPs (substances 1–12 of Tables 1 and 2) yielded a correlation coefficient of 0.91 ($p < 0.001$) with the following relationship (——): $pIC_{50_{swelling}} = -0.39 + 0.96 \times pIC_{50_{binding}}$. The overall correlation coefficient (· · · ·) (DHPs, PAAs, BTZs, and amiodarone; substances 1–20) was 0.76 ($p < 0.001$), the relationship being: $pIC_{50_{swelling}} = 1.20 + 0.69 \times pIC_{50_{binding}}$. ●, DHPs; ○, PAAs and BTZs; △, amiodarone.

TABLE 3

Inhibition of (±)-[^{3}H]nitrendipine binding by DHPs, PAAs, BTZs and amiodarone in the presence of 300 mM $NaNO_3$ and 5 mM $MgCl_2$

Assay of conditions are given in Experimental Procedures. Shown are means ± estimated standard errors of pooled data from *n* determinations. Maximal inhibition is given as percentage of total (±)-[^{3}H]nitrendipine binding (no subtraction of nonspecific binding). *h*, pseudo-Hill coefficient. Values in parenthesis refer to the highest concentrations employed.

Compound	IC_{50}	Maximal inhibition	*h*	*n*
	μM	%		
DHPs				
1 Nicardipine	0.107 ± 0.015	83	1.02 ± 0.13	14
2 Nitrendipine	0.281 ± 0.025	85	1.28 ± 0.14	8
6 Niludipine	0.050 ± 0.013	77	0.74 ± 0.14	4
7 Bay M 5579	NE* (20 μM)			2
11 Niguldipine	3.630 ± 0.690	64	1.75 ± 0.50	4
PAAs				
13 (±)-LU47781	2.21 ± 0.26	84	0.70 ± 0.04	4
15 (±)-D888	37.6 ± 28.4	35	0.48 ± 0.27	4
16 (±)-Verapamil	32.2 ± 12.3	36	1.51 ± 0.61	4
17 (±)-D600	9.4 ± 0.8	86	1.10 ± 0.08	4
(±)-D619	NE (100 μM)			4
Anion channel inhibitor				
20 Amiodarone	NE (100 μM)			4

* NE, no effect at the highest drug concentration employed.

is in contrast to the findings of Vaghy *et al.* (39) and Matlib *et al.* (40), who found an inhibitory effect of verapamil, diltiazem, and several DHPs at similar concentrations. These researchers measured phosphate-induced swelling in actively respiring mitochondria [ADP and pyruvate added (39)]. However, in our experiments mitochondrial respiration was inhibited by addition of rotenone (see Experimental Procedures). We suggest that the different Ca^{2+} antagonist effects are accounted for by the different experimental conditions.

The close correlation between binding and effect could justify the term *drug receptor* for the mitochondrial Ca^{2+} antagonist binding site. It fulfills the following criteria: (a) chemical

TABLE 4

Partitioning of (±)-[^{3}H]nitrendipine, (+)-[^{3}H]isradipine, and (±)-[^{3}H] Bay K 8644 into the mitochondrial membrane compartment under the conditions of the swelling and the binding experiments

Assay conditions are given in Experimental Procedures. The actual free (i.e., not membrane-associated) drug concentration is expressed as a percentage of the total drug concentration employed. Shown are means ± standard errors of three determinations.

Compound (total concentration)	Actual free concentration under the conditions of the	
	Swelling experiment	Binding experiment
μM	% of total	
(±)-[^{3}H]Nitrendipine		
0.1	47 ± 1	65 ± 2
1	51 ± 1	64 ± 2
10	57 ± 1	72 ± 1
(+)-[^{3}H]Isradipine		
0.1	66 ± 4	80 ± 2
1	65 ± 1	76 ± 1
10	69 ± 1	75 ± 1
(±)-[^{3}H]Bay K 8644		
0.1	72 ± 1	87 ± 2
1	73 ± 1	85 ± 2
10	76 ± 4	81 ± 12

selectivity (12, 14), (b) reversible ligand binding (11, 12, 14, 15, 41), (c) allosteric regulation by endogeneous ligands [e.g., ATP (16)], (d) correlation of binding parameters with phenomenological parameters under certain experimental conditions (this study), and (e) localization on a complex of polypeptides that can be solubilized and partially purified from mitochondrial membranes (14).

There are two major arguments against proposing the association of the mitochondrial Ca^{2+} antagonist sites with the IMAC, based on our experimental evidence, which can be answered as follows.

First, there is a discrepancy between binding inhibition and functional potency, which is on average 2.5-fold. The discrepancy is higher when overall potency is high and decreases when overall potency is low (Tables 1 and 2). We offer the following explanations. (a) Mitochondrial swelling had to be initiated after preincubation times not allowing for equilibrium binding; in the swelling experiments, at most 10 min passed between addition of the drugs and the A23187-induced activation of the IMAC. It is reasonable to expect that high inhibition potency is correlated with slower off kinetics of a drug. The expected percentage of occupied sites at times far from equilibrium is, therefore, inversely correlated with affinity and could explain the larger difference between binding affinity and functional potency for Ca^{2+} antagonists with high overall potency and *vice versa* (Tables 1 and 2). (b) In the case of α_{1A}-adrenoceptors and L-type Ca^{2+} channel radioligand binding experiments, partitioning of hydrophobic DHP molecules (e.g., niguldipine) into membrane compartments can often cause dramatic shifts in binding inhibition parameters (42, 43). Table 4 shows that, indeed, a consistently greater percentage of ligand partitioned into the mitochondria under the conditions of the swelling experiments than under the conditions of the binding experiments. In our investigations, binding experiments with intact mitochondria yielded unsatisfactory results (not shown) due to a very large nonspecific uptake of (±)-[^{3}H]nitrendipine. Thus, partitioning of drugs into the mitochondrial matrix and unavailability for IMAC inhibition may also account for the observed differences in affinity, depending on the hydrophobicity of the drug.

Second, although they are interesting, we do not understand the affinity changes for different DHPs (compare Tables 2 and 3) upon changes in the experimental conditions for binding. Amiodarone, a known inhibitor of the IMAC (26, 33), affects (±)-[^{3}H]nitrendipine binding only in the presence of low concentrations of the permeant anion Cl^-. The incomplete inhibition [18% of total (±)-[^{3}H]nitrendipine binding, which corresponds to 43% of nicardipine- or 42% nitrendipine-displaceable (i.e., specific) (±)-[^{3}H]nitrendipine binding] and the steep pseudo-Hill slope (Table 2) suggest a negative heterotropic allosteric interaction with the mitochondrial DHP binding site. On the other hand, amiodarone was without effect on (±)-[^{3}H] nitrendipine binding in the presence of high concentrations of NO_3^-, another permeant anion of the IMAC (34). Apparently nitrate, by occupation of an anion site, increases the affinity of various Ca^{2+} antagonists for the DHP sites but abolishes allosteric inhibition by amiodarone. Alternatively, all these changes might be caused by the nonspecific chaotropic effect of $NaNO_3$ (44). In contrast, interaction of (+)- or (−)-(*cis*)-diltiazem with the mitochondrial DHP binding site becomes measurable only under the conditions of the swelling experiments, whereas these BTZs had no effect in a Tris-based buffer containing 500 mM NaCl (12). Thus, there was only a weak correlation between the binding parameters obtained in K buffer and those obtained in NO_3 buffer; in addition, no correlation between the binding parameters obtained with NO_3 buffer and the IMAC inhibition data (assessed in K buffer) was observed. One explanation could be heterogeneity of the binding sites. One class, which is absolutely dependent on certain anions for high affinity DHP binding, has no relationship to the IMAC and the other class is associated with the IMAC but cannot be differentiated from the former with our binding methodology in NO_3 buffer; we have no evidence to support or reject this argument. However, we tend to favor an alternative view, because an apparent lack of correlation between binding constants and functional data for Ca^{2+} antagonists is not unprecedented. For example, K_i values for Ca^{2+} antagonist binding in depolarized membrane preparations do not correlate with, for example, negative inotropic effects on heart papillary muscle. The discrepancy is, however, more apparent than real because there is "state-dependent" binding of the drugs to the L-type Ca^{2+} channel (see Ref. 6 for a detailed discussion). Thus, by analogy, a single class of interchangeable sites on the inner mitochondrial membrane could be in different states, stabilized, for example, by anions and/or temperature, and only the state induced under the K buffer conditions reveals the association with the IMAC. It is hoped that this question, as well as others, can be resolved by functional reconstitution of the solubilized and partially purified mitochondrial Ca^{2+} antagonist binding sites. As reported elsewhere, these preparations exhibit allosteric coupling of PAA and DHP sites, ATP regulation, and anion stimulation (14). With respect to the poor correlation of (±)-[^{3}H]nitrendipine (DHP) binding inhibition and IMAC-induced swelling inhibition by the PAA Ca^{2+} antagonists, it should be noted that allosteric inhibition constants of the PAAs at the mitochondrial DHP binding site are compared with the PAA effects on IMAC activity. Thus, poor correlation could be explained by loose allosteric coupling of the two distinct Ca^{2+} antagonist binding domains, each, however, being tightly associated with the

IMAC. Perhaps, direct labeling of the mitochondrial PAA sites (see Ref. 14) will demonstrate correlations as good as shown by us now for the DHP site.

Although the existence of an anion channel in the inner mitochondrial membrane has been demonstrated by a variety of functional assays (see above), neither has its (patho)physiological role been established yet nor have there been any reports of drugs that selectively interact with the IMAC. Garlid and Beavis (26) discuss the IMAC as a possible "physiological uncoupler" as well as a structure that can be used by mitochondria upon recovery from (ischemic) stress to free themselves from the accumulated ionic load and, consequently, shrink to normal size. On the other hand, for significant Ca^{2+} accumulation to occur, overall electroneutrality (by proton extrusion) must be maintained across the inner mitochondrial membrane (for recent reviews, see Refs. 45 and 46). The accompanying proton extrusion imposes limits on the amount of Ca^{2+} that can be accumulated. Concomitant permeation of anions into the mitochondrial matrix, however, removes any (theoretical) restraint to Ca^{2+} accumulation (45, 46). Inhibition by Ca^{2+} antagonists of a structure allowing the influx of anions (i.e., the IMAC) could inhibit excessive Ca^{2+} uptake by mitochondria and prevent the resulting structural and functional impairment. The latter are well documented Ca^{2+} antagonist effects in a variety of animal studies (for reviews, see Refs. 17 and 18). If Ca^{2+} antagonists, as suggested by our studies, bind to this hitherto only functionally characterized channel, they may be exploited for structural characterization, as exemplified for the L-type Ca^{2+} channel.

Acknowledgments

Doris Kandler is thanked for technical assistance and Christian Trawöger for doing some of the art work. We also want to thank Prof. W. G. Hanstein, Bochum, for fruitful discussions, the Institut für Biostatistik und Dokumentation (Prof. A. Neiss), University of Innsbruck, for statistical consulting, and our colleagues from Bayer and Knoll AG for drugs.

References

1. Triggle, D. J., D. A. Langs, and R. A. Janis. Ca^{2+} channel ligands: structure-function relationships of the 1,4-dihydropyridines. *Med. Res. Rev.* **9**:123–180 (1989).
2. Catterall, W. A., M. J. Seagar, and M. Takahashi. Molecular properties of dihydropyridine-sensitive calcium channels in skeletal muscle. *J. Biol. Chem.* **263**:3535–3538 (1988).
3. Vaghy, P. L., J. S. Williams, and A. Schwartz. Receptor pharmacology of calcium entry blocking agents. *Am. J. Cardiol.* **59**:9A–17A (1987).
4. Godfraind, T., R. Miller, and M. Wibo. Calcium antagonism and calcium entry blockage. *Pharmacol. Rev.* **38**:321–416 (1986).
5. Glossmann, H., and J. Striessnig. Calcium channels. *Vitam. Horm.* **44**:155–328 (1988).
6. Janis, R. A., P. J. Silver, and D. J. Triggle. Drug action and cellular calcium regulation. *Adv. Drug Res.* **16**:309–591 (1987).
7. Zernig, G. Widening potential for Ca^{2+} antagonists: non-L-type Ca^{2+} channel interaction. *Trends Pharamcol. Sci.* **11**:38–44 (1990).
8. Gottesman, M. M., and I. Pastan. Resistance to multiple chemotherapeutic agents in human cancer cells. *Trends Pharamcol. Sci.* **9**:54–58 (1988).
9. Dalton, W. S., T. M. Grogan, P. S. Meltzer, R. J. Scheper, B. G. M. Durie, and C. W. Taylor. Drug-resistance in multiple myeloma and non-Hodgkin's lymphoma: detection of P-glycoprotein and potential circumvention by addition of verapamil to chemotherapy. *J. Clin. Oncol.* **7**:415–424 (1989).
10. Cantor, E. H., A. Kenessey, G. Semenuk, and S. Spector. interaction of calcium channel blockers with non-neuronal benzodiazepine binding sites. *Proc. Natl. Acad. Sci. USA* **81**:1549–1552 (1984).
11. Brush, K. L., M. Perez, M. J. Hawkes, D. R. Pratt, and S. L. Hamilton. Low-affinity binding sites for 1,4-dihydropyridines in mitochondria and in guinea-pig ventricular membranes. *Biochem. Pharmacol.* **36**:4153–4161 (1987).
12. Zernig, G., and H. Glossmann. A novel 1,4-dihydropyridine-binding site on mitochondrial membranes from guinea-pig heart, liver and kidney. *Biochem. J.* **253**:49–58 (1988).
13. Anholt, R. H., P. L. Pedersen, E. B. De Souza, and S. H. Snyder. The peripheral-type benzodiazepine receptor: localization to the mitochondrial outer membrane. *J. Biol. Chem.* **261**:576–583 (1986).
14. Glossmann, H., G. Zernig, I. Graziadei, and T. Moshammer. Non L-type Ca^{2+} channel linked receptors for 1,4-dihydropyridines and phenylalkylamines, in *Nimodipine and Central Nervous System Function* (W. H. Gispen and J. Traber, eds.), Schattauer Verlag, Stuttgart, 51–67 (1989).
15. Zernig, G., and T. Moshammer. Characterization of a phenylalkylamine binding site allosterically coupled to the high-capacity low-affinity 1,4-dihydropyridine binding site in mitochondria. *Naunyn-Schmiedeberg's Arch. Pharmacol* **339**(suppl.):R45 (1989).
16. Zernig, G., T. Moshammer, I. Graziadei, and H. Glossmann. The mitochondrial high-capacity low-affinity (±)-[^{3}H]nitrendipine binding site is regulated by nucleotides. *Eur. J. Pharmacol.* **157**:67–73 (1988).
17. Fleckenstein, A. History of calcium antagonists. *Circ. Res.* **52**:I3–I16 (1983).
18. Kloner, R. A., and E. Braunwald. Effects of calcium antagonists on infarcting myocardium. *Am. J. Cardiol.* **59**:848–948 (1987).
19. Zsoter, T. T., and J. G. Church. Calcium antagonists: pharmacodynamic effects and mechanism of action. *Drugs* **25**:93–112 (1983).
20. Bellemann, P., D. Ferry, F. Luebbecke, and H. Glossmann. [^{3}H]-Nimodipine and [^{3}H]-nitrendipine as tools to identify directly the sites of action of 1,4-dihydropyridine calcium antagonists in guinea-pig tissues. *Drug Res.* **32(I)4**:361–363 (1982).
21. Glossmann, H., and D. R. Ferry. Molecular approach to the calcium channel. *Drug Dev.* **9**:63–98 (1983).
22. Zernig, G., I. Graziadei, T. Moshammer, D. Kandler, W. Peschina, N. Reider, and H. Glossmann. Association of the mitochondrial Ca^{2+} antagonist binding sites with an inner mitochondrial membrane anion channel. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **341**(suppl.):R43.
23. Glossmann, H., and D. R. Ferry. Assay for calcium channels. *Methods Enzymol.* **109**:513–550 (1985).
24. Matlib, M. A., W. Rouslin, P. L. Vaghy, and A. Schwartz. Isolation of cardiac muscle mitochondria: an update. *Methods Pharmacol.* **5**:25–37 (1985).
25. Broekemeier, K. M., P. M. Schmid, H. H. O. Schmid, and D. R. Pfeiffer. Effects of phospholipase A_2 inhibitors on ruthenium red-induced Ca^{2+} release from mitochondria. *J. Biol. Chem.* **260**:105–113 (1985).
26. Garlid, K. D., and A. D. Beavis. Evidence for the existence of an inner membrane anion channel in mitochondria. *Biochim. Biophys. Acta* **853**:187–204 (1986).
27. Selwyn, M. J., A. P. Dawson, and D. V. Fulton. An anion-conducting pore in the mitochondrial inner membrane. *Trans. Biochem. Soc.* **7**:216–219 (1979).
28. Beavis, A. D., R. D. Brannan, and K. D. Garlid. Swelling and contraction of the mitochondrial matrix. I. Structural intepretation of the relationship between light scattering and matrix volume. *J. Biol. Chem.* **260**:13424–13433 (1985).
29. Garlid, K. D., and A. D. Beavis. Swelling and contraction of the mitochondrial matrix. II. Quantitative application of the light scattering technique to solute transport across the inner membrane. *J. Biol. Chem.* **260**:13434–13441 (1985).
30. Beavis, A. D., and K. D. Garlid. Inhibition of the mitochondrial inner membrane anion channel by dicyclohexylcarbodiimide: evidence for a specific transport pathway. *J. Biol. Chem.* **263**:7574–7580 (1988).
31. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
32. De Lean, A., P. J. Munson, and D. Rodbard. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay and physiological dose-response curves. *Am. J. Physiol.* **4**:E97–E102 (1978).
33. Beavis, A. D. On the inhibition of the mitochondrial inner membrane anion uniporter by cationic amphiphiles and other drugs. *J. Biol. Chem.* **264**:1508–1515 (1989).
34. Beavis, A. D., and K. D. Garlid. The mitochondrial inner membrane anion channel: regulation by divalent cations and protons. *J. Biol. Chem.* **262**:15085–15093 (1987).
35. Azzone, G. F., S. Massari, and T. Pozzan. Mechanism of active shrinkage in mitochondria. II. Coupling between strong electrolyte fluxes. *Biochim. Biophys. Acta* **423**:27–41 (1976).
36. Comerford, J. G., A. P. Dawson, and M. J. Selwyn. Anion transport in sub-mitochondrial particles prepared from rat liver mitochondria. *Trans. Biochem. Soc.* **14**:1044–1045 (1986).
37. Selwyn, M. J. Holes in the mitochondrial inner membrane. *Nature (Lond.)* **330**:424–425 (1987).
38. Sorgato, M. C., B. U. Keller, and W. Stuehmer. Patch-clamping of the inner mitochondrial membrane reveals a voltage-dependent ion channel. *Nature (Lond.)* **330**:498–500 (1987).
39. Vaghy, P. L., M. A. Matlib, L. Szekeres, and A. Schwartz. Protective effects of verapamil and diltiazem against inorganic phosphate induced impairment of oxidative phosphorylation of isolated heart mitochondria. *Biochem. Pharmacol.* **30**:2603–2610 (1981).
40. Matlib, M. A., P. L. Vaghy, D. E. Epps, and A. Schwartz. Actions of certain calcium channel blockers and calmodulin antagonists on inorganic phsophate-induced swelling and inhibition of oxidative phosphorylation of heart mitochondria. *Biochem. Pharmacol.* **32**:2622–2625 (1983).
41. Vaghy, P. L., G. P. Dube, I. L. Grupp, G. Grupp, J. L. Williams, Y. H. Baik, and A. Schwartz. A proposed pharmacological role for dihydropyridine binding sites in heart and coronary smooth muscle, in *Cardiovascular Effects of Dihydropyridine-Type Calcium Antagonists and Agonists. Bayer Symposium*

IX (A. Fleckenstein, C. van Breemen, R. Gross, and F. Hoffmeister, eds.), Springer Verlag, Heidelberg, 156–184 (1985).
42. Boer, R. A., A. Grassegger, C. Schudt, and H. Glossmann. (+)-Niguldipine binds with very high affinity to Ca^{2+} channels and to subtypes of *alpha*$_1$-adrenoceptors. *Eur. J. Pharmacol.* **172**:131–146 (1989).
43. Graziadei, I., G. Zernig, R. Boer, and H. Glossmann. Stereoselective binding of niguldipine enantiomers to *alpha*$_{1A}$-adrenoceptors labeled with [^{3}H]-5-methyl-urapidil. *Eur. J. Pharmacol.* **172**:329–337 (1989).
44. Hatefi, Y., and W. G. Hanstein. Solubilization of particulate proteins and nonelectrolytes by chaotropic agents. *Proc. Natl. Acad. Sci. USA* **62**:1129–1136 (1969).
45. Becker, G. L., G. Fiskum, and A. L. Lehninger. Regulation of free Ca^{2+} by liver mitochondria and endoplasmic reticulum. *J. Biol. Chem.* **255**:9009–9012 (1980).
46. Akerman, K. E. O., and D. G. Nicholls. Physiological and bioenergetic aspects of mitochondrial calcium transport. *Rev. Physiol. Biochem. Pharmacol.* **95**:149–201 (1983).

Send reprint requests to: Gerald Zernig, Institut für Biochemische Pharmakologie, Peter-Mayr-Straße 1, A-6020 Innsbruck, Austria.