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Identification of a 27-kDa High Affinity Phenylalkylamine-Binding Polypeptide as the σ_1 Binding Site by Photoaffinity Labeling and Ligand-Directed Antibodies

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SUMMARY

The verapamil-like arylazide (−)-[^{3}H]azidopamil specifically photoaffinity labeled two low molecular mass polypeptides, with apparent molecular masses of 22 and 27 kDa, in the endoplasmic reticulum of guinea pig liver, kidney, adrenal gland, and lung. It was recently shown that the 22-kDa polypeptide binds the anti-ischemic phenylalkylamine (−)-[^{3}H]emopamil and other anti-ischemic drugs with high affinity. We now provide evidence that the photolabeling of the 27-kDa polypeptide is blocked by nanomolar concentrations of σ ligands [order of potency, haloperidol > pentazocine > 1,3-ditolylguanidine > dextromethorphan > (+)-SKF10,047]. The apparent affinities of these and other drugs closely corresponded to those for 1,3-[^{3}H]ditolylguanidine-labeled σ binding sites. Based on its high affinity for the (+)-enantiomer [but not the (−)-enantiomer] of SKF10,047 (K_i = 51 nM), pentazocine (K_i = 3 nM), and dextromethorphan (K_i = 30 nM), the (−)-[^{3}H]azidopamil-labeled site on the 27-kDa polypeptide was classified as being of the σ_1 subtype. Using antiphenylalkylamine antibodies, we developed a novel immunological detection method that allows the rapid and sensitive staining of the photolabeled 27-kDa polypeptide after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. We conclude that the phenylalkylamines emopamil and azidopamil represent a novel class of σ ligands, highly suitable for the further structural characterization of polypeptides carrying σ_1 binding sites.

We previously identified two polypeptides (molecular masses of 22 and 27 kDa) in the endoplasmic reticulum of liver, kidney, lung, and adrenal gland that bind phenylalkylamine Ca^{2+} antagonists with nanomolar affinity (1). Both could be specifically photoaffinity labeled with the arylazide phenylalkylamine (−)-[^{3}H]azidopamil[1] and are associated in larger oligomeric complexes after solubilization with digitonin (1). Phenylalkylamine binding to these 27- and 22-kDa polypeptides was inhibited by similar concentrations of the anti-ischemic drug emopamil and by tetraethylammmonium, but only (−)-[^{3}H]azidopamil photolabeling of the 22-kDa polypeptide was modulated by low micromolar concentrations of Zn^{2+} and millimolar concentrations of Na^+. The latter polypeptide forms the previously described (2) cation-sensitive, high affinity binding domain for the anti-ischemic phenylalkylamine (−)-[^{3}H]emopamil and other anti-ischemic drugs. Reversible binding of (−)-[^{3}H]emopamil to the 22-kDa polypeptide was blocked by low concentrations of the σ site ligand opipramol. We therefore investigated whether one or both of the two high affinity phenylalkylamine binding domains are related to σ binding sites. Due to the low affinity of the (−)-[^{3}H]emopamil binding site for other σ ligands [haloperidol, (+)-SKF10,047 (*N*-allylnormetazocine), DTG, and (+)-3-PPP], this could be ruled out, in an earlier study, for the phenylalkylamine binding domain on the 22-kDa polypeptide (1).

In this study we characterize in detail the (−)-[^{3}H]azidopamil-labeled phenylalkylamine binding domain of the 27-kDa polypeptide in guinea pig liver. Evidence is presented that it carries a σ_1 binding site (for review, see Refs. 3 and 4). A method for the rapid and sensitive detection of the 27-kDa polypeptide using antiphenylalkylamine antibodies was developed. We show that the verapamil-related phenylalkylamines azidopamil and emopamil are σ ligands with potencies similar to those of DTG and haloperidol. These phenylalkylamines are novel tools for the further pharmacological and structural characterization of polypeptides carrying σ binding sites.

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[1] (−)-[^{3}H]Azidopamil is identical to [*N-methyl*-^{3}H]LU49888, 5-[3-azidophenethyl-[*N-methyl*-^{3}H]methylamino]-2-(3,4,5-trimethoxyphenyl)-2-isopropylvaleronitrile.

ABBREVIATIONS: DTG, 1,3-ditolylguanidine; HSA, human serum albumin; 3-PPP, 3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl flouride; DMSO, dimethylsulfoxide; ELISA, enzyme-linked immunosorbent assay; TBS, Tris-buffered saline; PVDF, polyvinylidene difluoride.

Experimental Procedures

Materials. (−)-[^{3}H]Emopamil (67 Ci/mmol), (−)-[^{3}H]azidopamil (87 Ci/mmol), and the unlabeled phenylalkylamines were kindly provided by Knoll A.G. (Ludwigshafen, Germany). σ ligands were a gift of Dr. J. Traber (Tropon, Cologne, Germany). Other chemicals were obtained from the following sources: [^{3}H]DTG (39.7 Ci/mmol), NEN (Wien, Austria); opipramol, Ciba-Geigy (Wien, Austria); Bradford protein reagent, electrophoresis reagents, and molecular weight markers, Bio-Rad; prestained molecular weight markers, BRL-GIBCO; all other chemicals, Sigma.

Membrane preparation. Microsomal membranes were prepared from guinea pig liver according to a previously published procedure (2). Protein was measured according to the method of Bradford (5), with bovine serum albumin as a standard.

Binding assays. Binding experiments with [^{3}H]DTG were carried out as described previously for (−)-[^{3}H]emopamil binding (2). Briefly, 0.6–1.2 nM [^{3}H]DTG was incubated with 0.04–0.08 mg/ml microsomal membrane protein in 10 mM Tris·HCl, pH 7.4, 0.1 mM PMSF (buffer A), in a final volume of 0.5 ml, at 22° for 1 hr. Nonspecific binding was defined with 1 μM DTG. Drugs were diluted in DMSO and added directly to the incubation mixture. The final DMSO concentration was 1% (v/v), which did not affect radioligand binding. Samples were incubated for 10 min at 2° before separation of bound and free ligand by filtration over GF/C glass fiber filters as described (1).

Photoaffinity labeling and SDS-PAGE. (−)-[^{3}H]Azidopamil (1–127 nM) was incubated in the dark with 0.4–0.5 mg/ml microsomal membrane protein in buffer A, in the absence or presence of other drugs. Serial drug dilutions were made in DMSO and added directly. After incubation for 1 hr at 22°, the samples were irradiated for 55 sec with an UV lamp (Sylvania GTE germicide) at 10-cm distance. Photolyzed membranes were collected by centrifugation, denaturated in sample buffer containing 10 mM *N*-ethylmaleimide (6), and separated on 12 or 13% polyacrylamide gels. For fluorography, Coomassie Blue-stained gels were treated with Amplify (Amersham), dried, and exposed to Kodak X-Omat AR5 films for the indicated times (at −80°). To quantify the amount of photoincorporated ligand, fluorograms were digitized and analyzed using image analysis software (Image-Pro Plus; Media Cybernetics, Silver Spring, MD).

Preparation of (−)-[^{3}H]azidopamil-labeled HSA. One hundred-fifty microliters of a 10 mM stock solution of (±)-azidopamil dissolved in DMSO (1.5 μmol) were added to 15 mg of HSA dissolved in 1.5 ml of 10 mM Tris·HCl, pH 7.4. (−)-[^{3}H]Azidopamil was added to a final concentration of 8 dpm/pmol, to monitor labeling efficiency. The incubation mixture was photolyzed on ice and free ligand was removed by size exclusion high performance liquid chromatography as described previously (7).

Preparation of antiphenylalkylamine antisera. Antiphenylalkylamine antibodies were raised by intracutaneous injections of New Zealand white rabbits every 3–4 weeks with azidopamil-HSA emusified in complete Freund's adjuvant. Rabbits were bled 10 days after injections. Antiphenylalkylamine antibody titers were determined in radioimmunoassays using (−)-[^{3}H]desmethoxyverapamil or (−)-[^{3}H]azidopamil as the radioligand. Radioimmunoassays were carried out as described previously (7), using charcoal to separate antibody-bound from free radioligand. Serum 85/9 was used for all experiments presented in this paper.

Immunoblots and ELISA. After SDS-PAGE, proteins were electrophoretically transferred to a PVDF membrane (Immobilon P; Millipore) using a semi-dry system and Tris-glycine buffer (8). To verify the relative transfer efficiencies for the photolabeled polypeptides, the PVDF membranes were impregnated with Enhance spray (NEN) and fluorographed as described above. Membranes were blocked with 10% (w/v) skim milk in TBS (20 mM Tris·HCl, pH 7.4, 150 mM NaCl) for 2 hr. For immunostaining of phenylalkylamine immunoreactivity, antisera were diluted 2000-fold in 10% (w/v) skim milk, 0.5% (v/v) Triton X-100, 0.1% (v/v) Tween 20, in TBS, pH 7.4 (37°), containing 0.5 mg/ml HSA. Before incubation with the membrane (3 hr), the antisera were preblocked in this solution (3 hr) to saturate anti-HSA antibodies. Bound antibody was detected using a biotin-conjugated second antibody (1/1000 dilution, 1 hr), streptavidin-conjugated horseradish peroxidase (1/500 dilution, 1 hr), and a chemoluminescence detection system (ECL; Amersham). All incubations were carried out at 37°.

The specifity of the antiphenylalkylamine antiserum was verified by competitive ELISA (see legend to Fig. 2C). Microtiter plates (96-well) were coated overnight at 4° with 0.1 ml of azidopamil-HSA in 0.1 M $NaHCO_3/Na_2CO_3$, pH 9.6 (0.15 μg of protein/well). Unbound antigen was removed by rinsing the wells three times with TBS containing 0.05% (v/v) Tween 20. Additional protein binding sites were blocked by incubation (1 hr, 22°) with 0.15 ml of TBS containing 0.2% (w/v) gelatin, followed by washing with TBS-Tween. Antiphenylalkylamine antiserum (1/3000 dilution in TBS-gelatin) was preincubated (1 hr, 37°) with 0.5 mg/ml HSA to saturate antibodies directed against HSA. Blocked antisera were incubated (1 hr, 37°) with drugs diluted in DMSO, in a final volume of 0.5 ml (final DMSO concentration, 1%). Aliquots (0.1 ml) of the assay mixture were then added to the antigen-coated microtiter plates and incubated for 2 hr (22°). Unbound antibodies were removed by three washes with TBS-Tween. Antibody binding to the solid phase-bound antigen was quantified using alkaline phosphatase-conjugated anti-rabbit IgG antibody (1/1000 dilution) and *p*-nitrophenyl phosphate as the chromogenic substrate. Absorption was measured at 405 nm. After substraction of background absorbance (<0.1), data were normalized with respect to control absorbance at 405 nm (0.9–1.1 arbitrary units).

Statistics. Binding parameters (IC_{50}, slope factor, K_d, and B_{max} values) were calculated by nonlinear curve-fitting of individual binding (or photolabeling) experiments to the general dose-response equation (9) (binding inhibition data) or a rectangular hyperbola (equilibrium saturation data). Constants are given as means ± standard deviations.

Results

σ ligands block specific (−)-[^{3}H]azidopamil photolabeling of the 27-kDa polypeptide. To investigate whether the high affinity phenylalkylamine binding domain on the 27-kDa polypeptide is pharmacologically related to σ binding sites, the modulation of (−)-[^{3}H]azidopamil photolabeling of this polypeptide by a variety of σ ligands was studied. Fig. 1A shows that the labeling of the 27-kDa polypeptide was completely blocked by nanomolar concentrations of the σ ligands DTG (150 nM) (Fig. 1A, *lane 3*), haloperidol (50 nM) (Fig. 1A, *lane 5*), 3-PPP (200 nM) (Fig. 1A, *lane 6*), and 10 μM (+)-SKF10,047 (Fig. 1A, *lane 7*). In contrast, the 22-kDa polypeptide labeling was almost unaffected. Opipramol (100 nM), amiodarone (500 nM), and emopamil (500 nM) blocked labeling of both polypeptides. BMY-14802 (150 nM) (Fig. 1A, *lane 8*) showed slightly higher selectivity for the 27-kDa polypeptide. Cocaine was a weak inhibitor of the labeling of both polypeptides. The pharmacological profile of the photolabeling of the 27-kDa polypeptide (Fig. 1A) was in excellent agreement with the modulation of reversible [^{3}H]DTG binding by σ ligands (Table 1). The affinities for the σ ligands haloperidol and DTG were studied in detail using different ligand concentrations. Their apparent K_i values (haloperidol, 0.8 ± 0.2 nM; DTG, 19 ± 1 nM; two experiments) (see Table 1) for inhibition of (−)-[^{3}H]azidopamil photolabeling closely correspond to the apparent K_i values (haloperidol, 0.6 ± 0.2 nM; DTG, 8.4 ± 2.8 nM; three experiments) for the [^{3}H]DTG-labeled binding site. We therefore conclude that this polypeptide forms a high affinity haloperidol-sensitive σ binding site.

In contrast to the 27-kDa polypeptide, σ ligands blocked azidopamil photolabeling of the 22-kDa polypeptide (Fig. 1A) in accordance with their potency for (−)-[^{3}H]emopamil binding

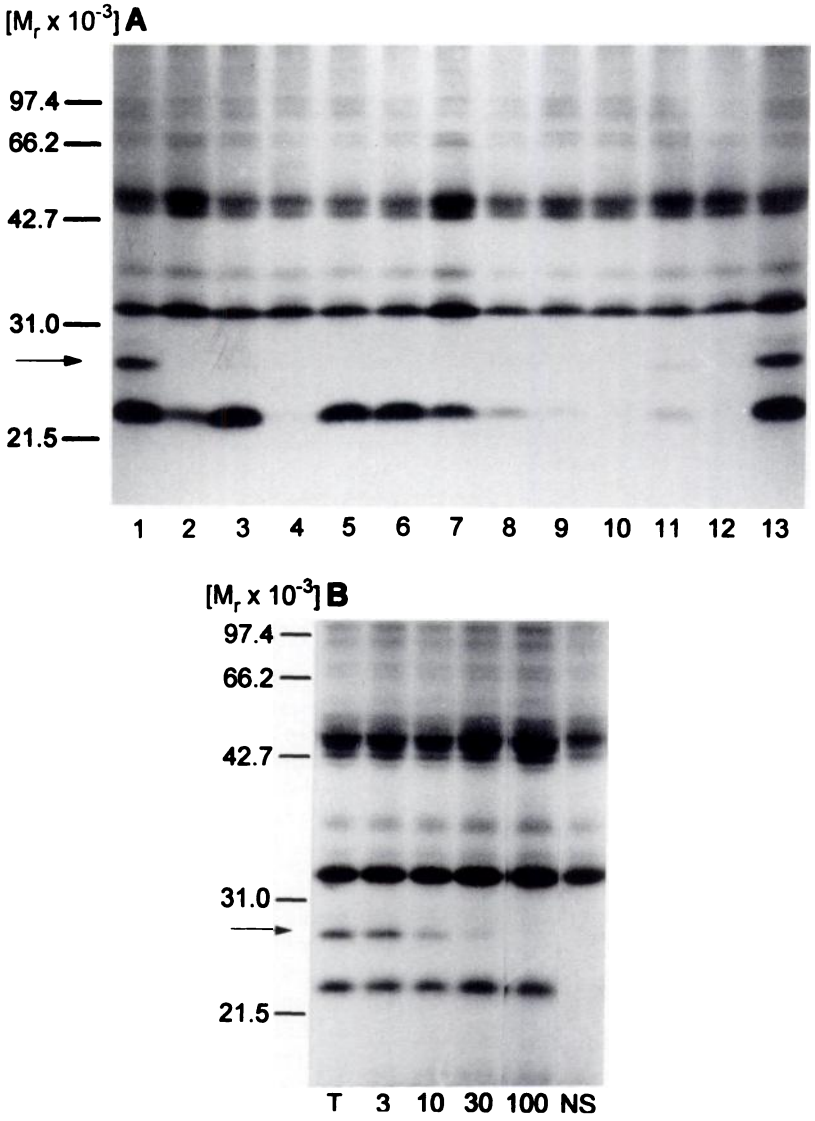

Fig. 1. A, Photoaffinity labeling of guinea pig liver microsomes with (−)-[^{3}H]azidopamil in the presence of various σ ligands. Microsomal protein (0.35–0.50 mg/ml) was incubated for 1 hr at 22° with 9.3–24.4 nM (−)-[^{3}H]azidopamil in the absence (*lanes 1* and *13*) or presence of 10 μM (*lane 2*) or 0.15 μM (*lane 3*) DTG, 5 μM (*lane 4*) or 0.05 μM (*lane 5*) haloperidol, 0.2 μM (+)-3-PPP (*lane 6*), 10 μM (+)-SKF10,047 (*lane 7*), 0.15 μM BMY-14802 (*lane 8*), 0.1 μM opipramol (*lane 9*), 0.5 μM amiodarone (*lane 10*), 10 μM cocaine (*lane 11*), or 0.5 μM (−)-emopamil (*lane 12*). After photolysis, samples were centrifuged and 0.2 mg of protein was separated on a 12% polyacrylamide gel. The gel was impregnated with Amplify, dried, and exposed to film for 20 days at −80°. One of three independent experiments giving almost identical results is shown. B, Pentazocine inhibition of (−)-[^{3}H]azidopamil photolabeling. Microsomal liver protein (0.50 mg/ml) was incubated for 1 hr at 22° with 23.6 nM (−)-[^{3}H]azidopamil in the absence (*T*) or presence of 3–100 nM pentazocine in 10 mM Tris·HCl, pH 7.4, 0.1 mM PMSF. Nonspecific labeling (*NS*) was defined with 1 μM (+)-emopamil. Photolyzed microsomal protein (0.25 mg) was subjected to SDS-PAGE. The gel was treated with Amplify, dried, and exposed to X-ray film for 20 days at −80°. One of two independent experiments giving almost identical results is shown. To quantify the amount of photoincorporated ligand, fluorograms were digitized and analyzed using image analysis software (Image-Pro Plus; Media Cybernetics). To compensate for different sample loads, densities were normalized with respect to a nonspecifically labeled 35-kDa band. Because of the high receptor and ligand concentrations used for photoaffinity labeling, the K_i for (±)-pentazocine was calculated according to the method of Linden (20). A K_i of 2.6 ± 0.5 nM (two experiments) was calculated using the following parameters: K_d for (−)-[^{3}H]azidopamil, 18 nM; ligand concentration, 20–24 nM; receptor concentration, 5 nM; measured IC_{50} value, 8.4 ± 1.0 nM. *Arrow*, migration of the 27-kDa polypeptide.

inhibition (1). These data confirm our previous report (1) that reversible (−)-[^{3}H]emopamil binding occurs to the 22-kDa polypeptide.

The 27-kDa polypeptide forms the σ_1 binding site. As shown in Table 1, [^{3}H]DTG labeled a homogeneous population of high affinity binding sites in guinea pig liver microsomes (K_d = 7.9 ± 0.5 nM, B_{max} = 30 ± 3 pmol/mg of protein; linear Scatchard plot, $r > 0.97$; means ± standard deviations, three experiments). The pharmacological profile is consistent with the reversible labeling of σ sites (see, for example, Refs. 3 and 10). The anti-ischemic phenylalkylamine emopamil was also a potent inhibitor, whereas the structurally related Ca^{2+} antagonists devapamil and verapamil displayed 20–50-fold lower affinity. Previous studies showed that [^{3}H]DTG labels at least two different subtypes of σ binding sites, termed σ_1 and σ_2, with indistinguishable affinity (11, 12). These subtypes are discriminated by benzomorphans (e.g., pentazocine and SKF10,047) and morphinans (e.g., dextromethorphan). In accordance with their selectivity for σ_1 binding sites (10, 12), these drugs inhibited [^{3}H]DTG binding with pseudo-Hill slopes smaller than

TABLE 1

Inhibition by phenylalkyamines and σ ligands of [^{3}H]DTG binding to liver microsomes

Guinea pig liver microsomes (0.04–0.08 mg/ml protein) were incubated for 1 hr with [^{3}H]DTG (0.6–1.2 nM) in the absence or presence of drugs. Nonspecific binding was defined with 1 μM DTG. The inhibition by σ ligands of (−)-[^{3}H]azidopamil photolabeling was determined by incubation of 0.5 mg/ml microsomal protein and 9–17 nM (−)-[^{3}H]azidopamil with six serial drug dilutions, over a 300-fold concentration range, for 1 hr. Nonspecific binding was defined with 1 μM (±)-emopamil. After photolysis, protein (0.06 mg/lane) was separated by SDS-PAGE. The gel was treated with Amplify, dried, and exposed to X-ray film for 15 days at −80°. Fluorograms were analyzed as described in the legend to Fig. 1B. IC_{50} values and slope factors (n_H) were determined by nonlinear curve fitting of the inhibition data. Data are given as mean ± standard deviation from two to six separate experiments.

Drug	[^{3}H]DTG binding			(−)-[^{3}H]Azidopamil photolabeling, apparent K_i[b]
	IC_{50}	n_H	Apparent K_i[a]	
	nM		nM	
Phenylalkylamines				
(+)-Emopamil	12 ± 4	0.88 ± 0.17	8.6 ± 1.1	ND[c]
(−)-Emopamil	12 ± 2	1.02 ± 0.07	8.5 ± 3.0	7.1 ± 0.7
(+)-Devapamil	300 ± 120	0.76 ± 0.07	230 ± 90	ND
(−)-Devapamil	200 ± 10	0.75 ± 0.05	150 ± 6	ND
(+)-Verapamil	700 ± 130	0.80 ± 0.10	540 ± 100	ND
(−)-Verapamil	820 ± 170	0.90 ± 0.07	630 ± 130	ND
Azidopamil	140 ± 120	0.73 ± 0.08[d]	13 ± 4	26 ± 5
σ ligands				
Haloperidol	0.9 ± 0.3	1.31 ± 0.15	0.6 ± 0.2	0.8 ± 0.2
Opipramol	11 ± 6	1.21 ± 0.06	6.1 ± 22.6	ND
DTG	13 ± 5	1.04 ± 0.14	8.4 ± 2.8	19 ± 1
Pentazocine	22 ± 4	0.64 ± 0.05[d]	0.9 ± 0.3	2.6 ± 0.5
(+)-3-PPP	26 ± 11	0.84 ± 0.10	18 ± 9	ND
Dextromethorphan	159 ± 49	0.62 ± 0.03[d]	18 ± 3	30 ± 10
(+)-SKF10,047	770 ± 170	0.45 ± 0.03[d]	43 ± 18	51 ± 23
(−)-SKF10,047	2790 ± 290	0.88 ± 0.02	2130 ± 220	ND
Cocaine	8390 ± 950	0.82 ± 0.06	5080 ± 1420	ND
Naloxone	>10 μM[e]	ND	ND	ND

[a] Apparent K_i values for σ sites were calculated according to the method of Linden (20), assuming a K_d for [^{3}H]DTG of 7.9 nM and a B_{max} of 30 pmol/mg of protein. For σ_1-selective drugs, the apparent K_i was calculated for the high affinity (σ_1) binding component assuming a B_{max} of 10 pmol/mg of protein (see footnote *d*).

[b] Apparent K_i values for inhibition of photoaffinity labeling were calculated according to the method of Linden (20), assuming a K_d for (−)-[^{3}H]azidopamil of 18 nM and a B_{max} for σ_1 binding sites of 10 pmol/mg of protein (see above), from the following IC_{50} values: (−)-emopamil, 14 ± 1 (two experiments); azidopamil, 44 ± 8 (two experiments); haloperidol, 1.4 ± 0.4 nM (two experiments); pentazocine, 8.4 ± 1.0 nM (two experiments); DTG, 40 ± 1 nM (two experiments); dextromethorphan, 63 ± 22 nM (three experiments); (+)-SKF10,047, 97 ± 42 nM (six experiments); (−)-SKF10,047, >1 μM (two experiments).

[c] ND, not determined.

[d] Drugs with pseudo-Hill slopes of <0.75 were fitted according to a two-site model. The following IC_{50} values were obtained for the high and low affinity components: azidopamil, 15 ± 4 nM (22 ± 4% of sites) and 290 ± 60 nM; (±)-pentazocine, 1.4 ± 0.3 nM (29 ± 7% of sites) and 60 ± 5 nM; (+)-SKF10,047, 51 ± 22 nM (45 ± 6% of sites) and 8500 ± 4400 nM; dextromethorphan, 21 ± 4 nM (44 ± 12% of sites) and 680 ± 210 nM (three experiments). From these data we estimated that 34 ± 13% (12 experiments) of [^{3}H]DTG binding sites are related to the σ_1 binding site. This corresponds to a B_{max} of 10 pmol/mg of protein.

[e] Inhibition of [^{3}H]DTG binding of 16 ± 8% (four experiments) at 10 μM concentration.

unity (<0.75) (see Table 1). Computer fitting of the inhibition curves revealed that a two-site model can adequately describe our data; a high affinity site [(±)-pentazocine, $IC_{50(high)}$ = 1.4 ± 0.3 nM, 29 ± 7% of sites; (+)-SKF10,047, $IC_{50(high)}$ = 51 ± 22 nM, 45 ± 6% of sites; dextromethorphan, $IC_{50(high)}$ = 21 ± 4 nM, 44 ± 12% of sites; three experiments] and a low affinity site [(±)-pentazocine, $IC_{50(low)}$ = 60 ± 5 nM; (+)-SKF10,047, $IC_{50(low)}$ = 8500 ± 4400 nM; dextromethorphan, $IC_{50(low)}$ = 680 ± 210 nM; three experiments] were discriminated, suggesting that the high affinity site corresponds to the σ_1 subtype. To clarify which σ subtype exists on the 27-kDa polypeptide, we photoaffinity labeled the 27-kDa polypeptide with (−)-[^{3}H]azidopamil in the presence of different concentrations of the σ_1-selective ligands (±)-pentazocine, (+)-SKF10,047, and dextromethorphan (see Fig. 1B and Table 1). These drugs inhibited specific (−)-[^{3}H]azidopamil photolabeling with IC_{50} values of 8.4 ± 1.0 nM (two experiments), 97 ± 42 nM (six experiments), and 63 ± 22 nM (three experiments), respectively. A comparison of the apparent K_i values calculated from the IC_{50} values listed above (see legend to Table 1) shows that all drugs tested [(−)-emopamil, azidopamil, haloperidol, DTG, pentazocine, dextromethorphan, and (+)-SKF10,047] displayed almost identical affinities for the inhibition of (−)-[^{3}H]azidopamil photolabeling and the [^{3}H]DTG-labeled σ_1 binding site (Table 1). We therefore conclude that this high affinity phenylalkylamine binding domain on the 27-kDa polypeptide is identical to the σ_1 binding site. This is further supported by the observed stereoselectivity for SKF10,047 [low affinity for the (−)-enantiomer, IC_{50} > 1 μM; two experiments] (see Table 1).

Estimates for the affinity of (−)-[^{3}H]azidopamil for the σ_1 binding site were obtained by photoaffinity labeling experiments carried out in the presence of increasing concentrations of (−)-[^{3}H]azidopamil (Fig. 2A). Half-maximal photolabeling of the 27-kDa polypeptide as determined by gel slicing (Fig. 2B) occurred at a ligand concentration of 18 nM. This high affinity interaction of (−)-[^{3}H]azidopamil with the σ_1 binding site was also evident from the shallow azidopamil inhibition curve of [^{3}H]DTG binding (see Table 1); fitting of the data to a two-site model revealed a high affinity component with an apparent K_i value of 13 ± 4 nM (three experiments) (see legend to Table 1). Our results demonstrate that σ_1 binding sites bind verapamil-like phenylalkylamines with high affinity and can be specifically photoaffinity labeled with (−)-[^{3}H]azidopamil.

Ligand-directed antisera differentiate the azidopamil-photolabeled polypeptides. Ligand-directed antibodies with high affinity and specificity for azidopamil-related phenylalkylamines (see legend to Fig. 2C) were raised for the rapid detection of azidopamil-labeled polypeptides in immunoblots after SDS-PAGE. A comparison of Fig. 2, A (labeling visualized by fluorography) and C (labeling visualized by immunostain-

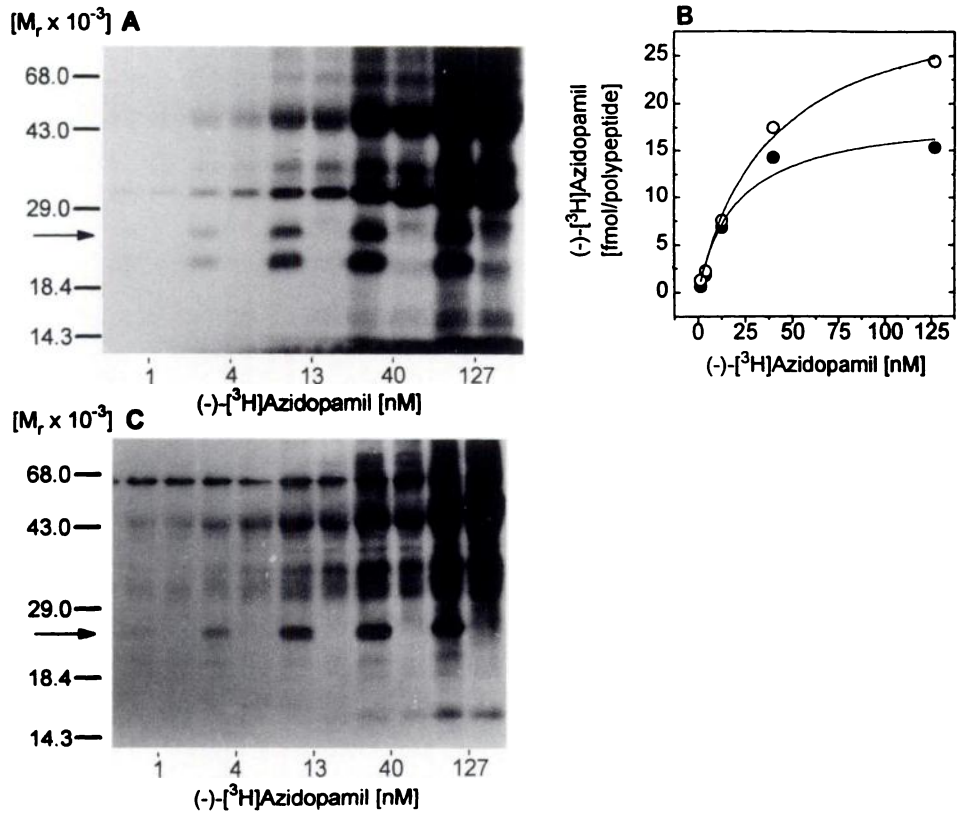

Fig. 2. Detection of saturable azidopamil photolabeling of the 27-kDa polypeptide with fluorography, gel slicing, and antiphenylalkylamine antisera. A, Fluorography. Microsomal liver protein (0.5 mg/ml) was incubated (1 hr at 22°) with increasing concentrations of (−)-[^{3}H]azidopamil (1–127 nM) in 10 mM Tris·HCl, pH 7.4, 0.1 mM PMSF, in the absence (*left lanes*) or presence (*right lanes*) of 1 μM (±)-emopamil. After photolysis, microsomes (0.06 mg of protein/lane) were separated by SDS-PAGE on four 13% minigels (Bio-Rad). One gel was treated with Amplify and processed for fluorography (15-day exposure). *Arrow*, migration of the 27-kDa polypeptide. B, Gel slicing. Specific (−)-[^{3}H]azidopamil photoincorporation into the 27-kDa (●) and 22-kDa (○) polypeptides was quantified by liquid scintillation counting after incubation of gel slices with 1 ml of 30% H_2O_2, 1.2% NH_3, 12 mM dithiothreitol, at 56° for 4 hr. By fitting these data to a rectangular hyberbola (assuming that the concentration of free ligand equals the concentration of total ligand), concentrations giving half-maximal photolabeling of 18 nM for the 27-kDa polypeptide and of 39 nM for the 22-kDa polypeptide were determined. Data given are means of duplicate determinations. C, Detection of (−)-[^{3}H]azidopamil photolabeling by immunostaining. For immunostaining with the antiphenylalkylamine antiserum, proteins separated on the fourth gel were electrophoretically transferred to a PVDF membrane. *Arrow*, migration of the 27-kDa polypeptide. The (−)-[^{3}H]azidopamil-photolabeled 22- and 27-kDa polypeptides were electroblotted with similar efficiencies, as verified by fluorography of the PVDF membrane (see Experimental Procedures). The specificity of the antiphenylalkylamine antiserum was tested by competitive ELISA (see Experimental Procedures). The following binding parameters for inhibition of antibody binding to solid phase-bound photolyzed azidopamil were obtained by nonlinear curve fitting (means ± standard deviations from three experiments): (±)-azidopamil, IC_{50} = 0.5 ± 0.5 nM, n_H = 1.61 ± 0.73; (±)-gallopamil, IC_{50} = 0.5 ± 0.1 nM, n_H = 1.81 ± 0.34; (±)-verapamil, IC_{50} = 28 ± 20 nM, n_H = 0.56 ± 0.05. The structurally unrelated calcium antagonists (+)-(*cis*)-diltiazem and (±)-isradipine did not inhibit binding at 10 μM concentrations. Thus, (±)-azidopamil and the structurally most closely related compound (±)-gallopamil (21) displayed the highest affinities for this antiserum. Steep inhibition curves, as displayed by these drugs, have been observed for other antidrug antisera (22, 23) and may be explained by, for example, cooperative binding mechanisms.

ing), demonstrates the sensitive immunolabeling of azidopamil incorporated into the 27-kDa σ_1 polypeptide. In contrast, azidopamil in the 22-kDa polypeptide is not available for high affinity antibody binding. The sensitivity of antibody staining was similar to that of fluorograms exposed for 2 weeks (Fig. 2A, *lanes* with 1 and 4 nM (−)-[^{3}H]azidopamil). One to 3 fmol of azidopamil photoincorporated into the σ_1 polypeptide [Fig. 2, B and C, *lanes* with 1 and 4 nM (−)-[^{3}H]azidopamil] were detected within <1 day. Immunostaining also allowed detection of the 27-kDa polypeptide in SDS-polyacrylamide gels after photoaffinity labeling with saturating concentrations of unlabeled (±)-azidopamil. About 100 fmol of 27-kDa polypeptide could be easily detected by this nonradioactive immunological method (data not shown).

Discussion

Phenylalkylamines label σ binding sites with high affinity. (−)-[^{3}H]azidopamil is an arylazide phenylalkylamine that has been developed for specific photolabeling of the phenylalkylamine Ca^{2+} antagonist binding domain of the α_1 subunit of L-type Ca^{2+} channels (13, 14). Here we report that this ligand photolabels a 27-kDa polypeptide that carries a σ binding site. This was proven by an extensive analysis of the pharmacological profile of (−)-[^{3}H]azidopamil photolabeling of this polypeptide with a variety of σ ligands (Fig. 1A; Table 1). (−)-[^{3}H]azidopamil (K_d estimated by photolabeling, 18 nM) (Fig. 2B) and emopamil (apparent K_i for [^{3}H]DTG binding inhibition, 8.5 ± 3.0 nM; three experiments) are the phenylalkylamines with the highest affinity for this σ binding site. Devapamil bound to the σ site with only moderate affinity. Verapamil was a weak inhibitor, in accord with earlier reports (12, 15). σ binding sites recognized all phenylalkylamines tested in a nonstereoselective manner (see Table 1). This is in contrast to the stereoselective phenylalkylamine binding to Ca^{2+} channels [higher affinity for (−)-enantiomers] (16) and to the 22-kDa polypeptide [higher affinity for (+)-enantiomers] (2).

The 27-kDa polypeptide is most likely identical to 26- and 29-kDa high affinity haloperidol-binding proteins photolabeled with [^{3}H]azido-DTG (17) and [^{125}I]iodoazidococaine (18), respectively, as shown by its high affinity for haloperidol and DTG. None of these earlier studies (17, 18) attempted to clarify which σ binding site subtype was labeled by these compounds. Our experiments with the σ_1-selective benzomorphan derivative pentazocine, the enantiomers of SKF10,047, and the morphinan dextromethorphan revealed that the σ binding site labeled by (−)-[^{3}H]azidopamil on the 27-kDa polypeptide corresponds to the σ_1 subtype.

The 27-kDa polypeptide shares many biochemical (similar molecular mass, identical subcellular localization, and formation of higher molecular mass complexes) and pharmacological (almost identical affinities for emopamil, azidopamil, opipramol, and cocaine) properties with the 22-kDa polypeptide previously identified as a high affinity acceptor for anti-ischemic drugs (1). Therefore, the phenylalkylamine binding domain on the 22-kDa polypeptide reversibly labeled by (−)-[^{3}H]emopamil (1) could be structurally related to the 27-kDa polypeptide carrying the σ_1 binding site. Additional biochemical characterization will be necessary to confirm this hypothesis.

The photolabeled σ_1 polypeptide can be selectively detected with antiphenylalkylamine antibodies. Ligand-directed antibodies were raised to detect azidopamil-photolabeled polypeptides. The sensitivity of this immunological method critically depends on the accessibility to the antibodies of the incorporated photolabel. The phenylalkylamine binding domains on both polypeptides could be photolabeled with azidopamil to similar extents (Fig. 2B). However, only azidopamil immunoreactivity in the 27-kDa polypeptide was efficiently recognized by the antiserum (Fig. 2C). This can be used to track the photolabeled σ_1 site during purification without the uncertainties of reversible binding stability. The differential immunoreactivity of the two photolabeled polypeptides indicates differences in the molecular architecture of their phenylalkylamine binding domains. Identification of the amino acid residues participating in the formation of these domains should be easily accomplished with the help of sequence-directed antibodies (19) as soon as the primary structures of the two polypeptides become available. This could eventually allow definition of common molecular properties of high affinity phenylalkylamine binding domains (19).

We conclude that the verapamil-like phenylalkylamines azidopamil and emopamil are high affinity ligands for the further characterization of σ_1 binding sites. Together with a phenylalkylamine-directed antiserum, azidopamil is an important tool for the purification of σ_1 sites, to elucidate their primary structure and function. This should also help to clarify the role of σ ligand-binding polypeptides (4) in the pathophysiology of ischemia (1).

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