Purification and Amino-terminal Sequencing of the High Affinity Phenylalkylamine Ca<sup>2+</sup> Antagonist Binding Protein from Guinea Pig Liver Endoplasmic Reticulum

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A high affinity phenylalkylamine Ca<sup>2+</sup> antagonist binding polypeptide (Moebius, F. F., Burrows, G. G., Striessnig, J., and Glossmann, H. (1993) Mol. Pharmacol. 43, 128–145) was purified to homogeneity from the endoplasmic reticulum of guinea pig liver with the aid of [3H]lemopamil, an antiischemic agent, and [3H]azidopamil, a photoaffinity label. The purified protein retained its high affinity for the antiischemic drugs emopamil (K<sub>d</sub> = 4 nM), opipramol (IC<sub>50</sub> = 15 nM), trifluoperazine (IC<sub>50</sub> = 2 nM), and for Zn<sup>2+</sup> (IC<sub>50</sub> = 2 μM). Ferguson plots revealed a molecular mass of 27.2 kDa. Partial amino acid sequence information was obtained by Edman degradation and revealed no homology to known protein sequences. Antibodies raised against a synthetic peptide corresponding to the first 25 N-terminal amino acid residues specifically immunoprecipitated the [3H]azidopamil photoaffinity-labeled polypeptide and recognized the protein in Western blots. Cross-linking with a variety of homo- and heterobifunctional agents lead to the formation of dimers. Since in the purified preparation no other subunit could be identified with different protein stains, our results indicate that the [3H]lemopamil binding site is formed by the homodimer of a novel membrane protein.

Organic Ca<sup>2+</sup> channel blockers (like verapamil, nifedipine, and diltiazem) are widely used clinically to treat cardiovascular disorders like angina, hypertension, and certain arrhythmias. Their pharmacological effects are exerted by blocking depolarization-induced Ca<sup>2+</sup> entry into smooth and cardiac muscle through L-type voltage-gated Ca<sup>2+</sup> channels after high affinity interaction with binding domains on the α<sub>1</sub> subunit (1).

In addition to their cardiovascular effects, tissue protective properties have been reported for many Ca<sup>2+</sup> antagonists. In various animal models Ca<sup>2+</sup> antagonists were found to protect from ischemic (2) as well as toxic cell injury (3). Such drugs may therefore be useful to minimize ischemic damage during stroke and myocardial infarction as well as for the prevention of damage after organ transplantation. An extensive review of the literature is given in Ref. 4.

One of the most extensively studied tissue-protective drugs is the phenylalkylamine (PAA)1 emopamil that is structurally closely related to the Ca<sup>2+</sup> antagonist verapamil. Although it is a weaker Ca<sup>2+</sup> channel blocker than verapamil it exerts more potent antiischemic effects in animal models of global and cerebral ischemia (see Ref. 5, for references; Refs. 6–10). We have recently identified with radiolabeled [3H]lemopamil possible molecular targets of this drug that could mediate its antiischemic effects. Indeed we characterized a novel high affinity (K<sub>d</sub> = 10 nM), sodium- and zinc-sensitive binding site for [3H]lemopamil in guinea pig liver, brain, kidney, adrenal gland, and lung (5, 11). Further biochemical studies and photoaffinity labeling with the PAA [3H]azidopamil revealed that the new binding site is located on a polypeptide with an apparent molecular mass of 22.5 kDa (5). This emopamil-binding polypeptide (EBP) is preferentially localized in the endoplasmic reticulum as shown by subcellular fractionation of guinea pig liver membranes (5). More recently, we demonstrated that EBP shares many properties of so-called c-binding sites (SBBSs) (12). SBBSs have been discussed as mediators for a variety of pharmacological actions of so-called σ ligands (for review, see Ref. 13) including antiischemic effects (14). Despite considerable efforts their functional role has, however, not yet been determined. Thus, similar to other high affinity drug-binding polypeptides with still undefined function (i.e. imidazoline-binding sites (15), peripheral benzodiazepine receptors (16)), the assessment of the physiological role of EBP and σ sites may be greatly facilitated by analysis of their molecular structure.

Here we report the purification, partial amino acid sequencing, and analysis of the oligomeric structure of the EBP. Our experiments reveal that this protein is structurally unrelated to the PAA-binding α<sub>1</sub> subunit of L-type Ca<sup>2+</sup> channels or any other known protein. We also rule out that this protein represents any known drug-metabolizing enzyme including cytochrome P450, as has been suggested for SBBSs (17, 18).

The purification of EBP will permit more direct studies on this class of drug-binding proteins and eventually help to reveal their role in mediating responses elicited by antiischemic Ca<sup>2+</sup> antagonists and σ ligands.

EXPERIMENTAL PROCEDURES

Materials—(→)[3H]Emopamil (67 Ci/mmol), (→)[3H]Azidopamil (97 Ci/mmol), and the unlabeled phenylalkylamines were kindly provided by Knoll A. G. (Ludwigshafen, Germany). Sigma ligands were a gift of

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1 The abbreviations were used: PAA, phenylalkylamine; EBP, emopamil-binding polypeptide; IC<sub>50</sub> concentration causing half-maximal inhibition; K<sub>HPO</sub><sub>4</sub>/K<sub>H</sub>PO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>/PAGE, polyacrylamide gel electrophoresis; SAPD, N-succinimidyl(4-azidophenyl)-1,4-diethylopropane; SANPAH, N-succinimidyl(6-azido-2'-nitrophenylamino)-hexanoate; SBS, σ binding site; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; MeSO<sub>2</sub>, dimethyl sulfoxide; RIA, radioimmunoassay; CHAPS, 3-(3-cholamidopropyl)dimethylammonium)-1-propanesulfonic acid.
Purification and NH2-terminal Sequencing of EBP

Dr. Traber (Troppon, Cologne, Germany). Other chemicals were obtained from the following sources: opipramol, Ciba-Geigy (Vienna, Austria); hydroxyapatite, Bradford protein reagent, electrophoresis reagents, and molecular weight markers, Bio-Rad; DEAE-, SP-, and chelating Sepharose, Pharmacia Biotech Inc.; all other chemicals, Sigma (Deisenhofen, Germany).

Binding Assays—[3H]Emopamil binding experiments with membrane-bound and solubilized EBP were carried out as described previously (5). Briefly, 0.8–2.3 nm [3H]Emopamil were incubated with protein in 0.1% (w/v) digitonin, 10 mM Tris-HCl, pH 7.4 (37 °C), 0.1% phenylmethylsulfonyl fluoride in the absence or presence of other drugs for 1 h at 22 °C. Samples were then irradiated for 55 s with an ultraviolet lamp (Sylvania GTE germicide) at 10 cm distance. Photolyzed protein was dialyzed against 0.5% (w/v) SDS, lyophilized, resuspended in sample buffer containing 10 mM N-ethylmaleimide (nonreducing conditions) (5), and separated on SDS-polyacrylamide gels as described (5). For fluorography, Coomasie Blue-stained gels were equilibrated in AmplifyR, dried, and exposed to Kodak X-Omat AR5 films for the indicated times (–80 °C). Ferguson plot analysis of purified EBP was carried out as described in the legend to Fig. 2.

Partial Amino Acid Sequencing of EBP—The Cu2+-chelating Sepharose eluate was dialyzed (molecular weight cutoff of 12,000–14,000) for 48 h against 0.05% (w/v) SDS. The lyophilized sample was resuspended in sample buffer containing 10 mM dithiothreitol (reducing conditions) and separated by preparative SDS-PAGE. After negative staining of the gel with CuCl2 (20), the 22-kDa band was cut out, the gel pieces placed in a Bio-Rad electrophoresis apparatus in 0.01% (w/v) SDS, 25 mM Tris, 192 mM glycine, and electrophoresed for 15 h at 200 V. The electrophoretic protein formed an insoluble precipitate in the electrophorete (0.6 ml). The precipitate was collected by centrifugation at 12,500 × g and washed twice by resuspension and centrifugation with distilled water (1.0 ml).

After photolysis the protein was dissolved in hexafluoroacetone (HFA) and insoluble material was removed by centrifugation. 10% of the supernatant were loaded on Porton protein support and subjected to automated Edman degradation in a Porton Instruments P1290E E4 microsequencer (see also Table III). To obtain internal sequence information, 350 pmol of protein were digested with TPK-Ctrypsin (Pro-mega, sequencing-grade, final concentration 0.4 mg/ml in 0.5% (w/v) CHAPS, 100 mM Tris-HCl, pH 8.7, at 37 °C. Tryptic fragments were isolated from the digest by reversed-phase high performance liquid chromatography on a Vydac C8 column (2.1 × 150 mm, 35°C) eluted with a linear gradient from 2% (v/v) to 50% (v/v) acetonitrile over 70 min at a flow of 0.1 ml/min. The individual peaks were collected directly onto Porton peptone support which were dried and subjected to automated Edman degradation.

Immunological Techniques—A polyclonal antiserum was raised in New Zealand White rabbits against a synthetic peptide corresponding to the first 25 NH2-terminal amino acid residues of EBP with an additional NH2-terminal lysine residue (peptide EBP1-25). Peptide synthesis, coupling to bovine serum albumin, immunization, enzyme-linked immunosorbent assay, and affinity purification of antisera were performed as described (21).

For immunoprecipitation of [3H]Emopamil photolabeled EBP microsomal membranes were solubilized in 4% (w/v) SDS for 30 min. Solubilized EBP was diluted 40-fold in RIA buffer (1% (w/v) Triton X-100, 50 mM NaCl, 20 mM Tris-HCl, pH 7.4 (22 °C), 0.5% (w/v) bovine serum albumin) and insoluble material was removed by centrifugation. 10 μl of 55% (w/v) NaCl, 20 mM Tris-HCl, pH 7.4 (22 °C)) were added to an additional concentration of 4% (w/v) SDS, 10% (v/v) glycerol, 62.5 mM Tris-HCl, pH 6.8 (22 °C), 10 mM N-ethylmaleimide.

Table I

| Fraction            | Protein | Recovery % | [3H]Emopamil binding | Recovery % | Density | Purification |
|---------------------|---------|------------|----------------------|------------|---------|-------------|
| Microsomal extract  | 163.7 ± 10.4 | 100 | 1070.1 ± 82.0 | 100 | 6 ± 1 | 1 |
| DEAE-Sepharose      | 66.4 ± 4.4 | 41 | 357.0 ± 92.0 | 33 | 5 ± 2 | 0.8 |
| Hydroxylapatite     | 6.3 ± 1.3  | 3.3 | 203.7 ± 56.7 | 19 | 41 ± 18 | 6 |
| SP-Sepharose        | 0.27 ± 0.05 | 1.6 | 206.0 ± 35.6 | 20 | 9 ± 1 | 12 |
| Green-galactose     | 0.92 ± 0.34 | 0.6 | 147.3 ± 46.9 | 14 | 169 ± 31 | 26 |
| Cu2+-chelating      | 0.035 ± 0.008 | 0.02 | 62.4 ± 18.6 | 6 | 1750 ± 77 | 267 |
| Sepharose           | 0.23 ± 0.15 | 0.15 | 90.8 ± 21.2 | 90 | 1260 ± 43 | 26 |

The binding protein was purified as described under "Experimental Procedures." Data from three independent purifications are given (means ± S.D.). [3H]Emopamil binding activity was measured in duplicate at two protein concentrations at a single ligand concentration (2.0–2.3 mM). Non-specific binding was measured in the presence of 1 μM (1-epi)mephalan. Protein concentrations were determined as described by Bradford (19) using BSA as a standard.
Purification of the EBP from Guinea Pig Liver Microsomes—

Guinea pig liver microsomes possess the highest density of EBP as previously shown by subcellular fractionation experiments (5). In guinea pig liver microsomes its apparent molecular mass as well as its highly characteristic reversible (H)emopamil binding properties (e.g., high affinity for Zn²⁺ and Na⁺) are indistinguishable from its properties in various other tissues investigated (5). Guinea pig liver microsomes were therefore selected as a starting material for purification. From the specific binding activity (about 30 pmol/mg of membrane protein (11)) we estimated that EBP represents less than 0.07% of the total membrane protein, requiring about 1500-fold purification (to a maximal specific activity of 45 nmol/mg protein assuming a 1:1 stoichiometry of drug binding). 70–90% (range, n > 8) of the binding activity was extracted from the membranes after solubilization in 1% (w/v) digitonin. As binding activity time dependently became associated with insoluble protein fractions, low binding recoveries were obtained from most resins to which crude binding activity was adsorbed. This problem was minimized by passing the solubilized material through DEAE-Sepharose under conditions (pH = 9.1, 0.5% (w/v) digitonin, 5 mM NaCl) where most of the contaminating protein adsorbed to the resin but about 40% of the binding activity were recovered in the flow-through. No further activity could be eluted from the column with NaCl up to 1 M, indicating that the low recovery was most likely due to protein aggregation. After the DEAE step no further tendency of the binding activity to aggregate was observed and thus facilitated further purification with reasonable recoveries (see Table I). Despite the lack of enrichment of specific binding activity DEAE-Sepharose chromatography allowed removal of several prominent contaminating proteins as revealed by silver staining (Fig. 1A). The flow-through of the DEAE column was then adsorbed to hydroxyapatite and eluted with 0.9–1.5 M NaH₂PO₄. In the eluate binding activity was reversibly inhibited by K_2HPO₄ but was recovered after removal of salt by overnight dialysis. Major contaminating proteins (42, 32, and 18 kDa) were then removed by passing the eluate through SP-Sepharose and green-agarose resins (Fig. 1A). The resulting material was enriched about 26-fold with respect to reversible (H)emopamil binding activity as compared to the solubilized membranes (Table I) and contained a prominent 22-kDa polypeptide (Fig. 1C) indicating an underestimation of its molecular mass. The EBP was more tightly bound to this resin than other proteins and could be eluted at high purity (Fig. 1A, lane 7) with 15 mM imidazol. The purified fraction contained 6% of the starting binding activity and less than 0.02% of the total protein indicating an at least 270-fold overall purification from the solubilized material. Obviously this represents a lower estimate because we did not attempt to correct the specific activity for irreversible losses of (H)emopamil binding activity during the 72-h purification process.

To obtain a more precise estimate of the molecular mass of EBP by SDS-PAGE, the purified protein was subjected to Ferguson analysis as illustrated in Fig. 1B. From these experiments an apparent molecular mass of 27.2 kDa was calculated (Fig. 1C), indicating an underestimation of its molecular mass.
when analyzed at a single polyacrylamide concentration. The migration in SDS-PAGE was not affected by the previous reduction of disulfide bonds (not shown).

To confirm the identity of the purified binding activity with the previously characterized microsomal \( ^3 \text{H} \)emopamil binding site the pharmacological properties of the Cu\(^{2+}\)-chelating Sepharose affinity were studied in more detail. Saturation analysis with \( ^3 \text{H} \)emopamil revealed a dissociation constant of \( 4.4 \) ± 1.5 \( \text{nm} \) \((n = 3) \) and a \( B_{\text{max}} \) of \( 8.5 \) ± 3.2 \( \text{nmol/mg protein} \) \((n = 3) \). Binding activity was stable upon storage in 15% (v/v) glycerol at 5 °C (half-life 4-6 weeks, range, \( n = 2 \) ) but rapidly deteriorated at 37 °C (half-life 6 ± 1 min, \( n = 5 \) ).

The binding inhibition profile of reversible \( ^3 \text{H} \)emopamil interaction by a variety of drugs and cations was essentially unchanged after purification as compared to the membrane-bound state (Table II). From all substances tested only the inhibition of reversible \( ^3 \text{H} \)emopamil binding (complete block by 0.15 \( \mu \text{M} \) \(+\)-emopamil and 50 \( \mu \text{M} \) Zn\(^{2+}\), half-maximal block by 1 \( \mu \text{M} \) \(+\)-verapamil and incomplete block by 150 \( \mu \text{M} \) Na\(^{+}\)) in Fig. 2A). Together with the fact that the photolabeled band strictly comigrated with the silver-stained polypeptide in SDS-PAGE, electroeluted after negative staining with CuCl\(_2\), and directly subjected to gas-phase sequencing. Amino acid sequence was obtained from five individual peaks: two corresponding to the NH\(_2\)-terminal amino acid sequence (not shown), two peaks gave other single sequences (peptides B and C, Table III), and one peak contained two peptide sequences running at about the same level (peptide D/E, Table III). Like the NH\(_2\)-terminus, the internal amino acid sequence obtained for peptide B revealed also no homology to known protein sequences.

To unequivocally demonstrate that the sequenced protein corresponds to the EBP we raised polyclonal antibodies against a synthetic peptide corresponding to the first 25 NH\(_2\)-terminal amino acid residues (EBP\(_{1-25}\)). As shown in Fig. 2B, 25 \( \mu \text{l} \) of the anti-EBP\(_{1-25}\) antiserum specifically immunoprecipitated the \( ^3 \text{H} \)azidopamil-photolabeled EBP from solubilized membranes. Immunoprecipitation was specific because it was completely blocked in the presence of 1 \( \mu \text{M} \) antigenic peptide and was not observed with preimmune serum. In Western blots affinity purified antibodies specifically recognized EBP in membranes (Fig. 3A, lane 1) as well as purified preparations (not shown). Taken together these results clearly demonstrate that the NH\(_2\)-terminal sequence information was derived from EBP.

### Purification and NH\(_2\)-terminal Sequencing of EBP

#### Table II

Inhibition of \( ^3 \text{H} \)emopamil binding to purified EBP by different drugs and cations

| Compound       | Microsomes IC\(_{50}\) | Purified IC\(_{50}\) | Slope | Ratio purified/microsomes |
|----------------|------------------------|---------------------|-------|--------------------------|
| \(+\)-Emopamil | 0.006 ± 0.001          | 0.009 ± 0.003       | 1.03 ± 0.12 | 1.5                      |
| Trifluoperazine| 0.008 ± 0.002          | 0.002 ± 0.0004      | 1.14 ± 0.11 | 0.3                      |
| Opipramine\(^b\)| 0.015 ± 0.004          | 0.015 ± 0.011       | 0.85 ± 0.04 | 12                       |
| Ifenprodil\(^b\)| 0.005 ± 0.0004         | 0.90 ± 0.150        | 0.87 ± 0.95 | 70                       |
| \(+\)-Verapamil| 0.690 ± 0.09           | 0.340 ± 0.140       | 0.94 ± 0.16 | 0.5                      |
| \(-\)-Verapamil| 1.120 ± 0.160          | 0.680 ± 0.060       | 0.91 ± 0.10 | 0.6                      |
| Haloperidol    | 0.190 ± 0.030          | 0.190 ± 0.050       | 0.90 ± 0.17 | 1                        |
| Pentetrazine   | 0.500 ± 0.140          | 0.590 ± 0.210       | 0.92 ± 0.18 | 1.2                      |
| Ditolylguanidine| 6.740 ± 2.4            | 5.760 ± 1.800       | 0.82 ± 0.12 | 0.9                      |
| ZnCl\(_2\)     | 1.180 ± 0.180          | 2.320 ± 0.740       | 1.41 ± 0.71 | 2.0                      |
| NaCl           | 14,000 ± 1,000         | 180,000 ± 80,000    | 1.28 ± 0.50 | 13                       |

\(^a\)Tissue protective effects reported (6–10, 22–25).

\(^b\)Data taken from Ref. 11.

\(^c\)Data taken from Ref. 5.

\(^d\)Data taken from Ref. 13.

\(^e\)Data taken from Ref. 31.
Fig. 2. (−)-[3H]Azidopamil photoaffinity labeling and immunoprecipitation of EBP. A, photoaffinity labeling was carried out by incubating the Cu2+-chelating Sepharose eauate in 0.75 ml of 0.1% (w/v) digitonin, 10 mM Tris-HCl, pH 7.4 (37°C), with 18.3 nM (−)-[3H]azidopamil at a protein concentration of 0.2 µg/ml for 1 h at 22°C in the presence (lane 1) or absence (lane 2) of (+)-emopamil (150 nM, lane 2). NaCl (150 mM, lane 3), NaN3 (50 mM, lane 4), and (+)-verapamil (1 µM, lane 5). After photolysis (5) samples were dialyzed against 0.05% (w/v) SDS overnight or processed (5) as described (5). One of three experiments giving almost identical results is shown. The arrow indicates the migration of EBP monomer and dimer, respectively. B, specific immunoprecipitation of the (−)-[3H]azidopamil photoaffinity labeled EBP with anti-EBP (lanes 1 and 4) or preimmune serum (lanes 2 and 3) to which (+)-emopamil (150 nM, lane 2) or (-)-verapamil (1 µM, lane 3) was added. After incubation for 12 h at 4°C the protein A-Sepharose was washed five times with 1.25 ml of RIA buffer and then with 1.25 ml of TBS. 10 µl of sample buffer were added to a final concentration of 4% (w/v) SDS, 10% glycerol, 62.5 mM Tris-HCl, pH 6.8 (22°C), 10 mM N-ethylmaleimide. 15 µg of photolabeled microsomes were separated in parallel (lane 1) on a 10% (w/v) polyacrylamide SDS gel. The fluorogram after 15 days of exposure is shown.

that is disrupted by high ionic strength buffers or alkaline pH employed upon purification. This is unlikely because indistinguishable binding coefficients were measured in sucrose density gradients for EBP in crude digitonin extracts (s20,w = 12.5 ± 0.1 S, n = 3) and in purified fractions (s20,w = 12.5 ± 0.2 S, n = 3). In both cases (results not shown) the EBP immunoreactivity strictly comigrated with the [3H]hemopamil binding activity. Instead the relatively large s20,w value of 12.5 S must be explained by the formation of larger homo-oligomeric complexes. To investigate this possibility we carried out cross-linking experiments employing various homo- and heterobifunctional cross-linking reagents. The formation of covalently cross-linked EBP oligomers in membranes (Fig. 3A) and purified preparations (results not shown) was then monitored by immuno blotting. 0.02 M disuccinimidyl suberate, N-hydroxysuccinimidyl-4-azidobenzoate, SANPAH, SADP, and p-azidophenylglyoxal incompletely cross-linked EBP resulting mainly in the formation of dimers in digitonin solubilized microsomes. 0.01 M glutaraldehyde lead to complete dimerization (Fig. 3A). The dimer possessed a molecular mass of 39 ± 2 kDa (n = 5) in SDS gels. Sucrose density gradient centrifugation of glutaraldehyde cross-linked microsomes revealed indistinguishable sedimentation coefficients for the dimer (12.4 ± 0.2 S, n = 3) and the monomer (s20,w = 12.5 ± 0.1 S, n = 3, see above). The dimer was still able to bind [3H]emopamil with high affinity as 50% of the binding activity were detectable despite complete dimerization (see Fig. 3B). Moreover the dimer could be specifically photoaffinity labeled with [3H]azidopamil (not shown). This provides strong evidence that a homodimer represents the functional form of EBP.

TABLE III

Purification and NH2-terminal Sequencing of EBP

Amino acid sequences of purified EBP

Amino acid sequences are given using the one-letter abbreviation. Microsequencing was performed as described under "Experimental Procedures." Peptide A: from 330 pmol of EBP the amino-terminal acid sequence was obtained up to the 33rd residue. Alanine in positions 1 and 25 were read at levels of 113 and 43 pmol, respectively. Separate runs confirmed the amino acid sequence from residues 1 to 11 (n = 2) and 1-21 (n = 1). Peptide D/E: two sequences were obtained in parallel at similar levels. —

Peptide A: ATTTSTGPLHPYWPRLRHLRLD

Peptide B: AIILGDOAFLSRLK

Peptide C: ASXXEPLT

Peptide D/E: Q/V A/F Y/T E/V A/S Q/L N/P A X D T

DISCUSSION

We have recently characterized EBP as an intrinsic membrane protein in various tissues that binds several verapamil-like Ca2+ antagonists with affinities comparable to the ¿, subunit of L-type Ca2+ channels (11). However, this binding site is not associated with L-type Ca2+ channels. It is located in the endoplasmic reticulum membrane (5) and clearly differs in its pharmacological binding profile from the Ca2+ channel. It has high affinity not only for the antiischemic PAA emopamil and the PAA photoaffinity ligand azidopamil but also binds a variety of structurally unrelated compounds, like amiodarone, opipramol, ifenprodil, trifluoperazine, and chlorpromazine with low nanomolar dissociation constants (Table II (5)). EBP could therefore mediate effects of these drugs that cannot be attributed to their already known pharmacological targets, like L-type Ca2+ channels (for opipramol), D2-receptors (for trifluoperazine and chlorpromazine), or glutamate receptors (for ifenprodil). Moreover, tissue-protective effects have been reported for each of the above drugs (22–25). Therefore the possibility that EBP could play a pathophysiological role in ischemic or toxic cell death cannot be ruled out.

We have recently shown that ¿-binding sites also represent high affinity PAA-binding polypeptides (12). EBP cannot be classified as a typical SBS due to its low affinity for ¿ ligands like ditolylguanidine and pentazocine (Table II). However, EBP shares many pharmacological and biochemical properties with SBSSs: it possesses a similar molecular mass (about 27 kDa) and sedimentation coefficient in sucrose gradients (5), is also localized in the endoplasmic reticulum membrane (5, 26), and displays indistinguishable high affinity for a wide variety of drugs (including emopamil itself, as well as azidopamil, opipramol, ifenprodil, verapamil, and amiodarone). Therefore it seems justified to classify ¿ receptors and EBP as members of a superfamily of small high affinity drug-binding proteins with...
membranes were solubilized in 1% (w/v) digitonin, 50 mM NaCl, 20 mM K$_2$HPO$_4$, pH 7.5, and incubated in the absence (lane 1) or presence of disuccinimidyl suberate (lane 2), N-hydroxy succinimidy1-4-azidobenzolate (lane 3), SADP (lane 4), SANPAH (lane 5), p-azidophenylglyoxal (lane 6) at a final concentration of 0.02 M for 2 h at 22°C. 0.4 M stock solutions were prepared in Me$_2$SO. The final Me$_2$SO concentration was described under "Experimental Procedures." The 5% (lane 6) concentration of glutaraldehyde were separated on sucrose density gradients cross-linked in the presence of glutaraldehyde at 4°C for 5 h, and dialyzed overnight at 4°C against 50 mM NaCl, 20 mM K$_2$HPO$_4$, pH 7.5. Microsomes incubated in the absence (1) or presence (9) of glutaraldehyde were separated on a 5--30% (v/v) sucrose density gradient in 0.1% (w/v) digitonin, 50 mM NaCl, 20 mM Tris-HCl, pH 7.4 (37°C), 4% (v/v) glycerol at 210,000 x g for 5.5 h (4°C), β-Glucosidase (1, 15.9 S), catalase (2, 11.3 S), lactate dehydrogenase (3, 7.3 S), and cytochrome c (4, 1.7 S) were included as markers. Fractions were collected from the bottom of the gradients and analyzed for specific $[^3]$Hmepam binding activity and enzyme activity. C, immunoblotting of sucrose density gradient separated glutaraldehyde cross-linked microsomes. 0.012 ml of each sucrose density gradient fraction was subjected to Western blotting and immunostaining with affinity purified anti-EBP$_1$,$_2$. The lane numbers refer to the fraction numbers shown in B. One of three experiments giving almost identical results is shown. The distribution of the immunoreactivity in the non-cross-linked fractions was identical with the cross-linked fractions (results not shown). The arrow indicates the migration of EBP monomer and dimer, respectively.

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FIG. 3. Crosslinking of EBP and separation of glutaraldehyde cross-linked EBP on sucrose density gradients. A, microsomal membranes were solubilized in 1% (w/v) digitonin, 50 mM NaCl, 20 mM K$_2$HPO$_4$, pH 7.5, and incubated in the absence (lane 1) or presence of disuccinimidyl suberate (lane 2), N-hydroxy succinimidyl-4-azidobenzolate (lane 3), SADP (lane 4), SANPAH (lane 5), p-azidophenylglyoxal (lane 6) at a final concentration of 0.02 M for 2 h at 22°C. 0.4 M stock solutions were prepared in Me$_2$SO. The final Me$_2$SO concentration was described under "Experimental Procedures." The 5% (lane 6) concentration of glutaraldehyde were separated on sucrose density gradients cross-linked in the presence of glutaraldehyde at 4°C for 5 h, and dialyzed overnight at 4°C against 50 mM NaCl, 20 mM K$_2$HPO$_4$, pH 7.5. Microsomes incubated in the absence (1) or presence (9) of glutaraldehyde were separated on a 5--30% (v/v) sucrose density gradient in 0.1% (w/v) digitonin, 50 mM NaCl, 20 mM Tris-HCl, pH 7.4 (37°C), 4% (v/v) glycerol at 210,000 x g for 5.5 h (4°C), β-Glucosidase (1, 15.9 S), catalase (2, 11.3 S), lactate dehydrogenase (3, 7.3 S), and cytochrome c (4, 1.7 S) were included as markers. Fractions were collected from the bottom of the gradients and analyzed for specific $[^3]$Hmepam binding activity and enzyme activity. C, immunoblotting of sucrose density gradient separated glutaraldehyde cross-linked microsomes. 0.012 ml of each sucrose density gradient fraction was subjected to Western blotting and immunostaining with affinity purified anti-EBP$_1$,$_2$. The lane numbers refer to the fraction numbers shown in B. One of three experiments giving almost identical results is shown. The distribution of the immunoreactivity in the non-cross-linked fractions was identical with the cross-linked fractions (results not shown). The arrow indicates the migration of EBP monomer and dimer, respectively.
Purification and NH₂-terminal Sequencing of EBP

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