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Photoaffinity Labeling and Purification of ZG-16p, a High-Affinity Dihydropyridine Binding Protein of Rat Pancreatic Zymogen Granule Membranes that Regulates a K^+-Selective Conductance

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ABSTRACT

In rat pancreatic zymogen granules (ZG), an ATP-sensitive K^+ conductance and a Cl^- conductance have been characterized that are inversely regulated by an ≈65-kDa multidrug resistance P-glycoprotein (*mdr1*) gene product. In search of a label for purification of this protein, we found that the dihydropyridine derivative (−)-[^{3}H]BZDC-DHP, a recently developed high-affinity ligand for Mdr1, binds with similar affinity to ZG membranes (ZGM) (K_d = 6.2 nM). Binding was inhibited by nanomolar concentrations of the L-type Ca^{2+} channel blockers azidopine and verapamil and by micromolar concentrations of the K^+ channel blockers glibenclamide and quinidine. Inhibition by glibenclamide was noncompetitive. The Mdr1 modulators cyclosporin A and vinblastine did not inhibit binding, which is different from Mdr1. In addition, only (±)-BZDC-DHP, azidopine, and verapamil selectively inhibited the K^+ conductance in ZGs, whereas the Cl^- conductance was not affected. In photoaffinity labeling experiments, (−)-[^{3}H]BZDC-DHP surprisingly specifically and selectively labeled a ≈19-kDa protein in ZGM with a pharmacological profile identical with the high-affinity binding site but did not label a 65-kDa protein. The 19-kDa protein was purified by ion exchange chromatography and SDS-polyacrylamide gel electrophoresis and sequenced. The sequence obtained corresponds to ZG-16p, a recently cloned ZG protein with no apparent homology to Mdr1. The identity of the 19-kDa protein was confirmed by immunoprecipitation of (−)-[^{3}H]BZDC-DHP-labeled ZGM with an anti-ZG-16p antibody. Furthermore, it is shown that ZG-16p is associated with the ZGM. We propose that ZG-16p, as part of the submembranous granule matrix, regulates the ATP-sensitive K^+ conductance of ZGs.

In permeabilized pancreatic acini, enzyme secretion evoked by hormones and second messengers depends on the presence of Cl^- and K^+ in the surrounding medium and is abolished by application of Cl^- and K^+ channel blockers (Fuller et al., 1989). This suggests that hormonally regulated Cl^- and K^+ channels present in the membrane of zymogen granules (ZG) promote enzyme secretion elicited by secretagogues.

Subsequently, we have demonstrated the presence of regulated Cl^- (De Lisle and Hopfer, 1986; Thévenod et al., 1990) and K^+ (Thévenod et al., 1992a) conductances in rat pancreatic ZG membranes (ZGM). The Cl^- conductance is activated by ATP and nonhydrolyzable ATP analogs, such as AMP-PCP, and blocked by the Cl^- channel blocker DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid) (De Lisle and Hopfer, 1986; Thévenod et al., 1990). The K^+ conductance is blocked by typical K^+ channel blockers, e.g., quinidine and Ba^{2+}, but is also inhibited by ATP, nonhydrolyzable ATP analogs, and glibenclamide and activated by diazoxide in a manner similar to ATP-sensitive K^+ channels (Thévenod et al., 1992a).

Previously, we have shown that monoclonal antibodies against multidrug resistance P-glycoprotein (Mdr1) inhibit the ZG Cl^- conductance and label a ≈65-kDa protein in rat pancreatic ZGM (Thévenod et al., 1994). Furthermore, using *mdr1a* knockout mice, we have provided evidence that ZG Cl^- and K^+ conductances are regulated by two low-molecular weight *mdr1a* and *mdr1b* gene products in ZGM that are distinct from the known Mdr1 proteins with a molecular mass of ≈180 kDa (Thévenod et al., 1996a). Recently, we presented evidence suggesting that the 65-kDa protein is the receptor for [^{3}H]glibenclamide in ZG (Braun et al., 1997).

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ABBREVIATIONS: ZG, zymogen granule; ZGM, ZG membrane; Mdr1, multidrug resistance P-glycoprotein; IEC, ion exchange chromatography; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; AMP-PCP, adenosine 5′-(β,γ-methylenetriphosphate); PAGE, polyacrylamide gel electrophoresis; BZDC-DHR, 2,6-dimethyl-4-(2-(trifluoromethyl)-phenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid{2-[3-(4-benzoylphenyl)propionylamino] ethyl}ester ethyl ester.

Because the binding affinity of [^{3}H]glibenclamide is ≈6 μM, it did not seem a suitable marker for the purification of the protein. In contrast, we found that a benzoyldihydrocinnamic acid derivative of dihydropyridine, (−)-[^{3}H]BZDC-DHP, a newly developed high-affinity ligand for MDR1 (Boer et al., 1996), binds with similar affinity to ZGM (K_d ≈8 nM), but with a pharmacological profile distinct from MDR1 (Thévenod et al., 1996b).

We therefore attempted to purify the putative ZGM 65-kDa *mdr1* gene product with (−)-[^{3}H]BZDC-DHP as a ligand. To our surprise, (−)-[^{3}H]BZDC-DHP labeled a single ≈19-kDa protein but not the 65-kDa Mdr1-like protein in ZGM. By purification and sequencing, we identified this protein as ZG-16p, a recently cloned ZG protein of unknown function with homology to lectins and no sequence homology to Mdr1 (Cronshagen et al., 1994). In addition, we show that BZDC-DHP specifically blocks the K^+-selective conductance in ZG. We hypothesize that ZG-16p, as part of the submembranous granule matrix, is involved in the regulation of the ZG ATP-sensitive K^+ conductance.

Experimental Procedures

Isolation of ZG and Purification of ZGM. ZGs from rat exocrine pancreas were isolated according to previously published protocols (Thévenod et al., 1992a,b). Briefly, pancreatic tissue of male Wistar rats (180–200 g, fasted overnight) was homogenized by nitrogen pressure cavitation (500 psi for 1 min) and centrifuged on a self-forming Percoll gradient (40% Percoll, 20,000*g* for 20 min). ZGs were collected from the bottom of the centrifuge tube and washed in isotonic buffer containing 50 mM succinate for removal of mitochondrial contaminations before use.

For preparation of ZGM, isolated ZGs were lysed on ice in a hypotonic buffer (10 mM HEPES, 0.1 mM $MgSO_4$, pH 7) containing a protease inhibitor cocktail (10 μM leupeptin, 100 μg/ml trypsin inhibitor, 2 mM benzamidine, 0.2 mM Pefabloc SC) followed by centrifugation for 1 h at 100,000*g* (Thévenod et al., 1992b). The membrane pellet was washed in the same buffer and finally stored at −70°C until use. Protein concentration was determined according to Bradford (1976). Subcellular fractionation of rat pancreatic tissue was performed according to a published protocol (Imamura and Schulz, 1985).

Measurement of Ion Conductances. Ion conductances of pancreatic ZG were assayed according to previously reported protocols (Thévenod et al., 1990, 1992a). The assay relies on the measurement of osmotic lysis of ZG resuspended in buffered isotonic salt solutions, which occurs after maximal permeabilization of the ZGM with electrogenic ionophores for counterions and is therefore limited by ion fluxes through the endogenous conductance pathways. Granule lysis causes a decrease in absorbance of the suspension that is measured at a wavelength of 540 nm at 37°C in a Beckman DU-64 spectrophotometer equipped with a Peltier constant-temperature chamber, an automatic six-unit sampler, and a kinetics Soft-Pac module.

For measurement of Cl^- conductance, ZGs were suspended in 150 mM KCl, 5 mM $MgCl_2$, and 50 mM HEPES (adjusted to pH 7 with Tris). Granule lysis was measured after addition of 5 μM valinomycin, which maximally and selectively permeabilizes the granule membranes for K^+. The influx of salt and water causes lysis of ZG and is limited under these conditions by the permeation of Cl^- through endogenous conductances (De Lisle and Hopfer, 1986; Thévenod et al., 1990).

For measurement of K^+ conductance, ZGs were suspended in 150 mM K^+ acetate containing 5 mM $MgCl_2$ and 1 mM EDTA and buffered with 50 mM imidazole (pH 7.0, adjusted with acetic acid). Because the intragranule pH is ~6.5, an inside-to-outside directed H^+ concentration gradient of ~0.5 pH units is generated across the granule membrane. By maximally permeabilizing the granule membrane to H^+ by addition of the electrogenic protonophore CCCP (16 μM), the H^+ concentration gradient is converted into an inside negative H^+ diffusion potential. This in turn drives K^+ influx through endogenous K^+ permeabilities. Anion influx occurs through the uncharged molecule acetic acid, which permeates through the lipid membrane by nonionic diffusion and dissociates, thus continuously providing the intragranule space with protons for protonation of imidazole and for proton efflux from the acidic interior. Under these conditions, K^+ influx through endogenous K^+ permeabilities is rate limiting for bulk salt influx into the intragranule space and for the resulting granule lysis (Thévenod et al., 1992a).

Lysis rates were expressed as inverse half-times of lysis, which were considered proportional to the rate constants of lysis. Half-time of granule lysis was estimated from the slope of the decrease in absorbance with time between addition of ionophore and either experimental half-time or the entire observation period if the half-time was not reached. The slope of the absorbance change with time was calculated by linear regression of the digitized data. Inhibitors were dissolved in dimethyl sulfoxide (DMSO), which was added in the same concentration (0.1%) to the control cuvettes.

Equilibrium Binding Studies. Equilibrium binding studies were performed as described by Boer et al. (1996). Ten to 25 μg of ZGM was incubated with 0.5 to 1 nM (−)-[^{3}H]BZDC-DHP in the absence or presence of inhibitors in 50 mM Tris-HCl buffer (pH 7.4; total volume, 250 μl) for 1 h at 22°C. Stock solutions of inhibitors were prepared in DMSO, the final concentration of which was 1% in the assay (at this concentration, DMSO did not affect equilibrium binding). All determinations were performed in duplicate. Bound and free ligand were separated by filtration through Whatman GF/C glass fiber filters under reduced pressure immediately after addition of 3.5 ml of ice-cold filtration buffer (10 mM Tris-HCl, 10% polyethylene glycol 6000, 10 mM $MgCl_2$, pH 7.4), followed by three washes with 3.5 ml of the same buffer. Filter-bound radioactivity was determined by liquid scintillation counting.

IC_{50} values were obtained from competition experiments by fitting of the data to the general dose-response equation for a single binding component (DeLean et al., 1978). According to the Cheng-Prusoff equation, IC_{50} values were equated with K_i values or, in the case of homologous competition, with K_d values (Tallarida, 1995).

Photoaffinity Labeling. Photoaffinity labeling experiments were performed according to Boer et al. (1996), with minor modifications. One hundred to 200 μg of ZGM was incubated with ≈10 nM (−)-[^{3}H]BZDC-DHP (or 50 nM [^{3}H]azidopine) in the absence or presence of inhibitors in 750 μl of 50 mM Tris-HCl buffer (pH 7.4) for 1 h at room temperature. After irradiation with black light for 1 h (or 20 min for [^{3}H]azidopine) at 4°C, samples were diluted 1:2 in assay buffer and centrifuged at 100,000*g* for 30 min. The membrane pellets were resuspended in sample buffer, heated to 60°C for 5 min, then separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 15% acrylamide minigels and blotted onto nitrocellulose membranes. Note that heating to 60°C or addition of 8 M urea to the sample buffer was necessary for solubilization of specifically labeled protein. The membranes were cut into 3-mm stripes, which were placed into scintillation vials and counted after addition of 2 ml of Ultima Gold (Sunahara et al., 1990).

Protein Purification and Sequencing. ZGMs (600–700 μg) were labeled with (−)-[^{3}H]BZDC-DHP as described above, then diluted in assay buffer and centrifuged at 100,000*g* for 30 min. The pellet was resuspended in 4 M urea, 0.5 M NaCl, and 50 mM HEPES (pH 7.5) and, after incubation at 25°C for 15 min, centrifuged at 100,000*g* for 1 h. The supernatant was diluted in 4 M urea and 50 mM HEPES (pH 8, final NaCl concentration 50 mM) and bound to a Sartobind S15X Membrane Adsorber unit (Sartorius AG, Göttingen, Germany), a cation exchange system in which sulfonic acid groups are attached to a microporous membrane filter, allowing application of samples and buffers with a syringe. The unit was washed with 10 volumes of the same buffer and subsequently adjusted to pH 4 with

buffer containing 4 M urea, 50 mM NaCl, and 50 mM formic acid. Elution was performed at pH 4 with an NaCl step gradient (400 → 420 → 500 mM). The eluate fractions (1-ml volume each) were concentrated by trichloroacetic acid precipitation or with Microcon microconcentrators (Amicon, Inc., Beverly, MA) and separated by SDS-PAGE on 20 × 30-cm 10 to 18% acrylamide gradient gels. After blotting onto nitrocellulose membranes, the protein bands were visualized for documentation by amido black or silver staining, and bound radioactivity was analyzed by cutting the membrane as described above.

For protein sequencing, unlabeled ZGMs were separated by ion exchange chromatography (IEC) and SDS-PAGE as described above and blotted onto polyvinylidene difluoride (PVDF) membranes. After visualization by Coomassie blue staining, the bands of interest were cut out of the membranes and sequenced by Edman degradation.

Immunoprecipitation. (−)-[^{3}H]BZDC-DHP-labeled ZGMs (300 μg) were solubilized by heating to 95°C for 5 min in 50 μl of buffer containing 50 mM Tris-HCl (pH 7.5) and 1% SDS. The samples were then diluted 10 times in radioimmune precipitation buffer (1% Nonidet P-40, 0.5% deoxycholate, 150 mM NaCl, and 50 mM Tris-HCl (pH 8); final SDS concentration, 0.1%) and centrifuged at 100,000*g* for 30 min. Next, 7.5 μl of preimmune serum or anti-ZG-16p antibody was added to the supernatants, followed by incubation at 4°C overnight. The antigen-antibody complexes were precipitated by incubation with 6.5 mg of protein A-agarose for 1 h. The precipitates were washed once with radioimmune precipitation buffer, with 150 mM NaCl plus 50 mM Tris-HCl (pH 8) and with 10 mM Tris-HCl (pH 8), respectively. The beads were resuspended in sample buffer and heated to 95°C for 5 min, and the supernatant was separated by SDS-PAGE on 15% acrylamide minigels. After blotting onto nitrocellulose membranes, protein bands were visualized by amido black staining, and bound radioactivity was analyzed as described above.

Immunoblotting. Samples were heated to 95°C for 5 min in sample buffer, separated by SDS-PAGE on 15% acrylamide minigels, and blotted onto PVDF membranes. After blocking with Tris-buffered saline containing 0.1% Tween 20 and 3% milk, the membranes were incubated with anti-ZG-16p antibody (1:2000) or anti-amylase antibody (1:1,000) overnight followed by horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:5000) for 1 h. The blots were developed with enhanced chemiluminescence reagent (Amersham, Braunschweig, Germany).

Statistics. Unless indicated otherwise, experiments were repeated at least three times, and results are given as means ± S.D. Statistical analysis of the data was performed with paired Student's *t* test. Results with levels of $P < .05$ were considered significant.

Materials. (−)-[^{3}H]BZDC-DHP (~40 Ci/mmol) was synthesized according to a previously published protocol (Boer et al., 1996). Unlabeled (±)-BZDC-DHP was a gift from Prof. Dr. J. Striessnig (Department of Biochemical Pharmacology, Innsbruck, Austria). [^{3}H]Azidopine (50 Ci/mmol) was purchased from Amersham. Wistar rats were obtained from Charles River Wiga GmbH (Sulzfeld, Germany). Ultima Gold was purchased from Packard (Groningen, the Netherlands). PVDF membranes were obtained from NEN-Dupont (Bad Homburg, Germany), nitrocellulose membranes from Schleicher & Schuell (Dassel, Germany) and Whatman filters from Herolab (Wiesloch, Germany). Carbonyl-cyanide *m*-chlorophenylhydrazone (CCCP), valinomycin, and protein A-agarose were purchased from Sigma Chemical Co. (Deisenhofen, Germany). All other chemicals were of analytical grade. Anti-ZG-16p antibody and anti-amylase antibody were kindly provided by Prof. Dr. H.-F. Kern (Department of Cell Biology, Marburg, Germany) and Prof. Dr. R. Zimmermann (Department of Medical Biochemistry, Homburg, Germany), respectively. Prof. Dr. V. Flockerzi (Department of Pharmacology, Homburg, Germany) donated (+)- and (−)-desmethoxyverapamil.

Results

ZGM Contain a High-Affinity Binding Site for (−)-[^{3}H]BZDC-DHP. (−)-[^{3}H]BZDC-DHP, a recently developed high-affinity ligand for MDR1 (Boer et al., 1996), binds specifically and with high affinity to rat pancreatic ZGM in a rapid filtration assay (Thévenod et al., 1996b). This binding site was now further characterized. In Fig. 1, the results of homologous competition experiments with increasing concentrations of (±)-BZDC-DHP are shown. By fitting of pooled data from five experiments to the general dose-response equation, a K_d for (−)-[^{3}H]BZDC-DHP binding of 6.22 nM and a B_{max} of 20.2 pmol/mg were obtained. A Hill plot of the data yielded a K_d of 5.4 nM and a slope factor of 0.97, indicating a lack of cooperativity of binding. Specific binding was also detected with [^{3}H]azidopine, another dihydropyridine that photolabels MDR1 (Safa et al., 1987). From competition experiments with unlabeled azidopine, a K_d of ≈85 nM was calculated (data not shown).

A pharmacological characterization of the (−)-[^{3}H]BZDC-DHP binding site revealed clear differences to known high-affinity binding proteins for (−)-[^{3}H]BZDC-DHP, i.e., MDR1 and the L-type Ca^{2+} channel (Fig. 2A). Isradipine, which binds with high affinity to L-type Ca^{2+} channels (Ferry et al., 1983), reduced (−)-[^{3}H]BZDC-DHP (1 nM) binding to ZGM only by 7% at a concentration of 100 nM ($n = 2$). Similarly, cyclosporin A and vinblastine, which both inhibit (−)-[^{3}H]BZDC-DHP binding to MDR1 with IC_{50} values in the nanomolar range (Boer et al., 1996), had only a small inhibitory effect on (−)-[^{3}H]BZDC-DHP binding to ZGM (15 and 3% at 1 μM, respectively; $n = 2$) (Fig. 2A). The most potent inhibitor of (−)-[^{3}H]BZDC-DHP binding to ZGM tested was verapamil, with an IC_{50} of 30 nM ($n = 2$; data not shown). The verapamil derivative desmethoxyverapamil inhibited (−)-[^{3}H]BZDC-DHP binding with an IC_{50} similar to the parent compound and was mildly stereoselective: IC_{50} values were 31 ± 15 nM for the (+)-enantiomer and 54 ± 17 nM for

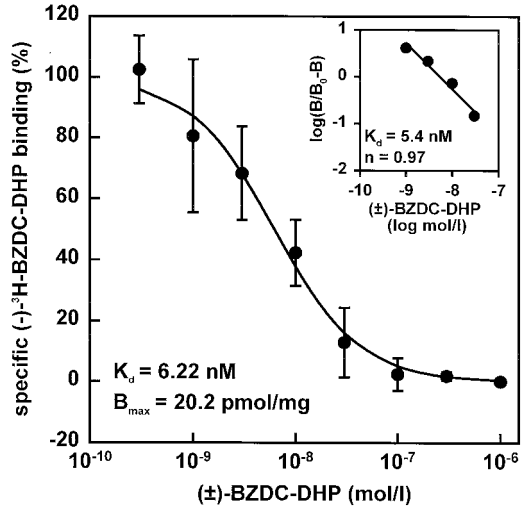

Fig. 1. Binding inhibition of (−)-[^{3}H]BZDC-DHP by (±)-BZDC-DHP in ZGM and Hill plot of the data (inset). Binding of (−)-[^{3}H]BZDC-DHP (0.5–1 nM) to ZGM was determined in the absence and presence of increasing concentrations of unlabeled (±)-BZDC-DHP. Nonspecific binding was defined at 1 μM (±)-BZDC-DHP and accounted for 19.9 ± 9.6% of total binding. Specific binding in the absence of unlabeled drug amounted to 827 ± 348 cpm. The curves show pooled data from five experiments (means ± S.D.).

the (−)-enantiomer, respectively ($n = 4$; data not shown). This suggests that the binding site in ZGM is stereoselective, but it differs from that of L-type Ca^{2+} channels, where (−)-desmethoxyverapamil binds with 30-fold higher affinity than the (+)-enantiomer (Ruth et al., 1985). Also effective at micromolar concentrations were the MDR1 inhibitor tamoxifen and the K^+ channel blockers quinidine and glibenclamide (Fig. 2A), which have been shown to inhibit the K^+ conductance of ZG (Thévenod et al., 1992a) and bind to the 65-kDa *mdr1* gene product (Braun et al., 1997).

The inhibition of (−)-[^{3}H]BZDC-DHP binding by glibenclamide was further characterized by performing inhibition experiments at two different concentrations of the label and plotting the data according to Dixon (1953) (Fig. 3). The interception point of the curves indicates a noncompetitive inhibition, and a K_i of 14.6 ± 8.5 μM ($n = 8$) was calculated, which is similar to the K_d of [^{3}H]glibenclamide binding to ZGM.

Next, the binding of (−)-[^{3}H]BZDC-DHP (0.6–1.2 nM) to subcellular fractions of rat pancreatic tissue (12.5–50 μg of protein) was examined. As shown in Fig. 4, specific binding was enriched 4.7 ± 0.9-fold in the ZG fraction and 19.8 ± 7.3-fold in ZGM compared with homogenate. A 2.2 ± 0.8-fold enrichment was detected in plasma membranes. No enrichment was found in the mitochondrial and endoplasmic reticulum fractions (0.3 ± 0.04- and 0.2 ± 0.01-fold, respectively). These data indicate that the binding site is predominantly localized to ZGM.

(±)-BZDC-DHP Inhibits the K^+ Conductance of Rat Pancreatic ZG. ATP and various pharmacological agents, including glibenclamide and quinidine, have been shown to inhibit the K^+ conductance and stimulate the Cl^- conductance of ZG (Thévenod et al., 1994). This inverse regulation is at least partially mediated by a 65-kDa *mdr1* gene product in ZGM (Thévenod et al., 1994, 1996a). We therefore investigated whether (±)-BZDC-DHP, by binding to the 65-kDa *mdr1* gene product, might also inhibit the K^+ conductance and stimulate the Cl^- conductance of ZG. An indirect assay based on the measurement of ionophore-induced lysis of ZG suspended in buffered isoosmotic potassium salts was used. (±)-BZDC-DHP inhibited the K^+ conductance of ZG dose dependently (Fig. 5A and Table 1). At 10 μM, which was the maximally effective dose tested, ZG lysis was inhibited by 41.7 ± 7.3% of control ($n = 4$, $P < .01$). Higher doses could not be tested because of the limited solubility of the substance.

In Fig. 5B, the effects of the Ca^{2+} channel blockers tested in the binding assay are shown on the ZG K^+ conductance compared with (±)-BZDC-DHP. (±)-BZDC-DHP was the most potent inhibitor, and azidopine was similarly effective (42.0 ± 7.1% inhibition at 10 μM, $n = 5$, $P < .01$), whereas isradipine had no effect at the concentrations tested (8.5 ± 7.1% at 10 μM, $n = 5$, NS). This sequence corresponds to the relative binding affinities of these substances to ZGM. For verapamil, however, higher concentrations were needed for inhibition of the ZG K^+ conductance compared with the dihydropyridines (61.5 ± 5.3% inhibition at 100 μM, $n = 7$, $P < .01$). In contrast, the Cl^- conductance of ZG remained unaffected by any of the Ca^{2+} channel blockers investigated at concentrations between 10 and 100 μM (Table 1), suggesting that (±)-BZDC-DHP, azidopine, and verapamil selectively inhibit ZG K^+ conductance by binding to the (−)-[^{3}H]BZDC-DHP binding site (see Fig. 2). Similar to isradipine, vinblastine (10 μM), a substrate of Mdr1 that does not prevent (−)-[^{3}H]BZDC-DHP binding, did not affect ZG K^+ and Cl^- conductances (Table 1). In contrast, cyclosporin A, another Mdr1 substrate that also does not inhibit (−)-[^{3}H]BZDC-DHP binding to ZG (Fig. 2), selectively stimulated K^+ conductance (260.2 ± 29.6% above control at 5 μM, $n = 6$, $P < .01$) (Table 1). Glibenclamide and quinidine, which are known to bind to the 65-kDa *mdr1* gene product (Braun et al., 1997), both effectively blocked ZG K^+ conductance (46.5 ± 1.5 and 48.4 ± 5.2% inhibition, respectively, at 100 μM; $n = 3–5$, $P < .01$) and stimulated Cl^- conductance (42.4 ± 14.8 and 36.2 ± 15.1% above control, respectively, at 100 μM, $n = 4$, $P < .05$). By selectively inhibiting the ZG K^+ conductance without affecting the Cl^- conductance, (±)-BZDC-DHP, azidopine, and verapamil differ from the dual effects of glibenclamide and quinidine on K^+ and Cl^- conductances, thus pointing to a different mechanism of action. Tamoxifen could not be tested on ZG ion conductances because it buffers intragranular pH (Chen et al., 1999) and thereby interferes with conductance assay (see *Experimental Procedures*).

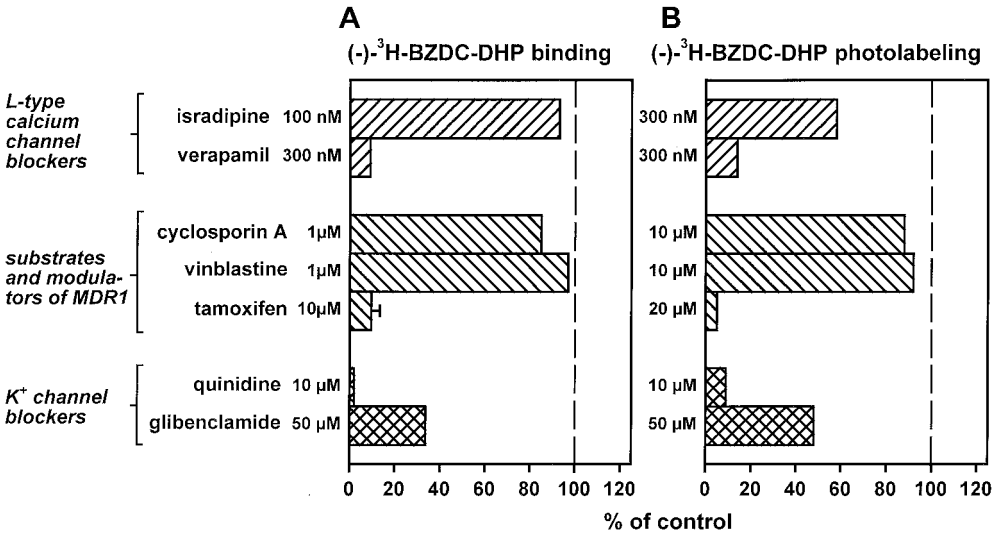

Fig. 2. Effect of inhibitors on high-affinity binding of (−)-[^{3}H]BZDC-DHP (1 nM) to ZGM (A) and (−)-[^{3}H]BZDC-DHP photolabeling of the ≈19-kDa protein (B). For binding experiments, 12.5 to 25 μg of ZGM were incubated with 1 nM (−)-[^{3}H]BZDC-DHP in the absence or presence of the indicated concentrations of inhibitors. For photoaffinity labeling experiments, 100 to 200 μg of ZGM were incubated with 7.5 to 10 nM (−)-[^{3}H]BZDC-DHP in the absence or presence of inhibitors followed by photoaffinity labeling. The bars represent specific binding/photolabeling in the presence of the indicated concentrations of inhibitors as percentage of specific binding/photolabeling without inhibitors (100%). Binding data represent means ± S.D. ($n = 5$) for tamoxifen and means of two experiments for all other inhibitors. Photoaffinity labeling data represent means of two experiments for glibenclamide and single experiments for all other inhibitors.

(−)-[^{3}H]BZDC-DHP Labels a 19-kDa Protein in ZGM. To identify the receptor for (−)-[^{3}H]BZDC-DHP in ZGM, photoaffinity labeling experiments were performed. (−)-[^{3}H]BZDC-DHP contains a photoreactive benzophenone group and thus can be covalently linked to its receptors by irradiation with ultraviolet light (Boer et al., 1996). In ZGM, a ≈19-kDa protein was efficiently and exclusively labeled by 10 nM (−)-[^{3}H]BZDC-DHP (Fig. 6), whereas no labeling of a ≈65-kDa protein was detected. Labeling could be inhibited by 300 nM unlabeled (±)-BZDC-DHP, indicating a high-affinity site. Moreover, photoaffinity labeling of the 19-kDa protein was inhibited by the same substances that inhibited (−)-[^{3}H]BZDC-DHP binding to the high-affinity site (Fig. 2). These results indicate that the 19-kDa protein carries the high-affinity binding site for (−)-[^{3}H]BZDC-DHP characterized in the reversible binding assay. A specific peak of radioactivity of the same molecular weight was also obtained in photoaffinity labeling experiments with [^{3}H]azidopine (data not shown).

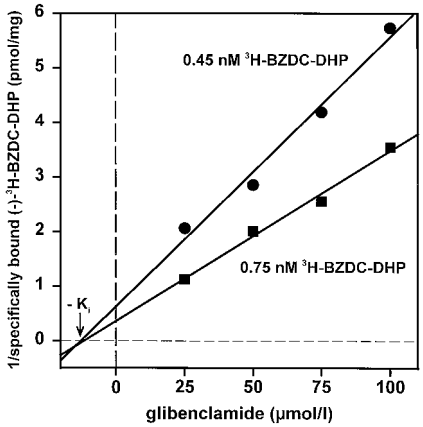

Fig. 3. Noncompetitive inhibition of (−)-[^{3}H]BZDC-DHP binding to ZGM by glibenclamide. Data from binding inhibition at two different concentrations of (−)-[^{3}H]BZDC-DHP by glibenclamide were plotted according to Dixon (1953). An interception point of the two resulting curves on the *x*-axis indicates a noncompetitive inhibition, and the interception point also gives the K_i of the inhibitor. K_i values of 12.7 and 11.5 μM were calculated at the higher and lower concentrations of label, respectively. One of four similar experiments is shown.

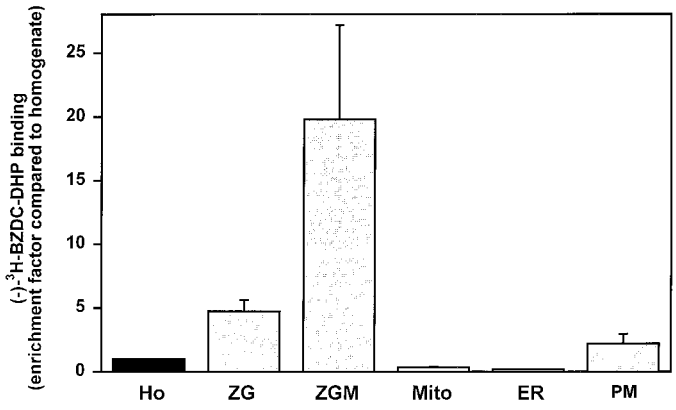

Fig. 4. Binding of (−)-[^{3}H]BZDC-DHP to subcellular membrane fractions from rat pancreatic tissue. Binding experiments with (−)-[^{3}H]BZDC-DHP (0.6–1.2 nM) were performed as described in *Experimental Procedures*. Specific binding to the different membrane fractions was compared with that of homogenate. Data show enrichment compared with homogenate (two to five measurements). Ho, homogenate; Mito, mitochondria; ER, endoplasmic reticulum; PM, plasma membranes.

Identification of the (−)-[^{3}H]BZDC-DHP Receptor of ZGM as ZG-16p. To identify the (−)-[^{3}H]BZDC-DHP receptor of ZGM at the molecular level, the (−)-[^{3}H]BZDC-DHP-labeled 19-kDa protein was purified. The purification protocol consisted of IEC followed by SDS-PAGE. The receptor could be solubilized by procedures known to solubilize peripheral membrane proteins (4 M urea + 0.5 M NaCl or 1 M guanidinium-HCl). For purification experiments, ZGMs were labeled with ≈10 nM (−)-[^{3}H]BZDC-DHP or ≈50 nM [^{3}H]azidopine and then solubilized with 4 M urea and 0.5 M NaCl. NaCl was diluted to 50 mM after solubilization of the membranes, which did not influence the solubility of the labeled proteins. Significant binding of the specifically radioactively labeled protein to an anion exchange matrix was only seen at pH ≥ 10. Subsequently, cation exchange chromatography was chosen to enable work at a more gentle, neutral pH. Initially, a protein band was identified that behaved similarly to the specifically bound radioactivity during both IEC and SDS-PAGE. In further experiments, this band turned

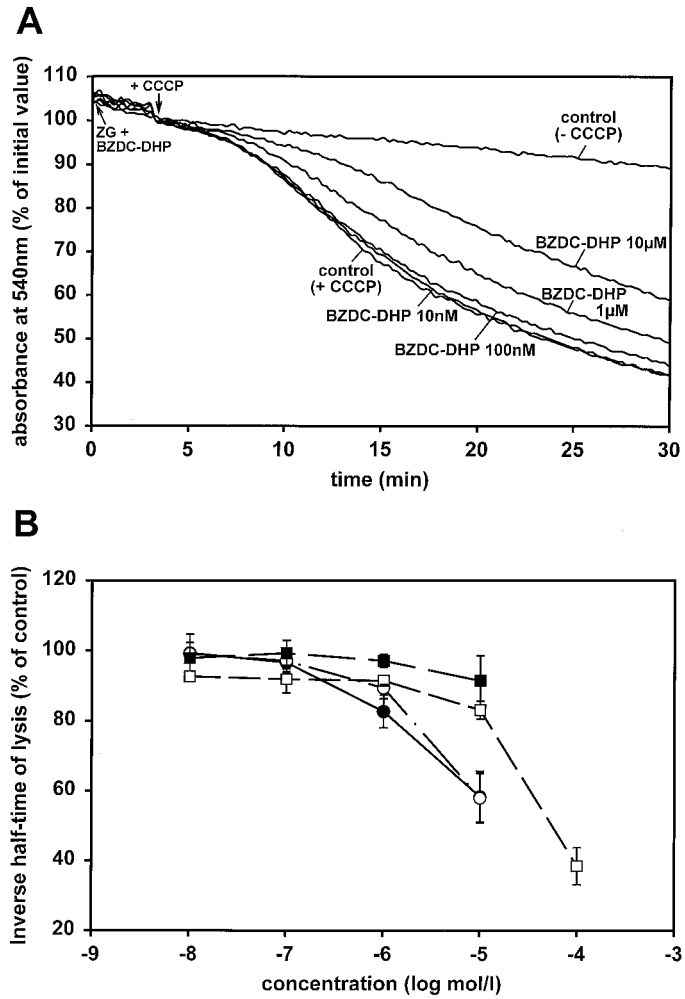

Fig. 5. Inhibition of ZG K^+ conductance by (±)-BZDC-DHP and related compounds. A, a representative experiment showing the effect of (±)-BZDC-DHP on ZG K^+ conductance. ZGs were incubated without (control) or with increasing concentrations of (±)-BZDC-DHP at 37°C. Lysis was induced by addition of the protonophore CCCP (16 μM; at the arrow). Lysis rates were calculated from the slope of the absorbance change. B, summary of the effect of the dihydropyridines (±)-BZDC-DHP (●), azidopine (○), and isradipine (■) and the phenylalkylamine verapamil (□) on ZG K^+ conductance. The curves show means ± S.D. of four or five experiments for each drug concentration.

out to consist of two proteins, only one of which correlated with specifically bound radioactivity. The best separation of these two proteins was achieved by binding the samples to the IEC unit at pH 8 and elution at pH 4 with an NaCl step gradient, followed by separation on 10 to 18% acrylamide gradient gels (Fig. 7). For protein sequencing, the same purification procedure was performed with unlabeled ZGM. The sequence obtained by Edman degradation of the protein band of interest corresponded to the N-terminal 17 amino acids of ZG-16p, a recently cloned ZG protein with homology to a lectin (Cronshagen et al., 1994), beginning with the last two amino acids of the secretory signal peptide. To confirm this result, the purified protein was digested with 0.05 μg of V8-protease according to the method of Cleveland et al. (1977). An ≈8-kDa band was produced that comigrated with specifically bound radioactivity. N-terminal sequencing of the peptide yielded amino acids 85 to 95 of ZG-16p.

For verification of the purification results, (−)-[^{3}H]BZDC-DHP (12.5 nM)-labeled ZGMs were immunoprecipitated with a polyclonal antibody directed against ZG-16p (Cronshagen et al., 1994). As shown in Fig. 8, anti-ZG-16p immunoprecipitated a peak of bound radioactivity of ≈19 kDa. This peak could be displaced by unlabeled (±)-BZDC-DHP with the same efficiency as the peak characterized in photoaffinity labeling experiments. The 19-kDa (−)-[^{3}H]BZDC-DHP receptor was precipitated by anti-ZG-16p with efficiency (≈10%) similar to that of unlabeled ZG-16p (as determined by immunoblot experiments; data not shown), indicating specific binding of the antibody. In Coomassie stains of gels or amido black stains of blots from immunoprecipitated material, only the protein band corresponding to ZG-16p could be detected in addition to the Ig bands (data not shown). No specifically bound radioactivity could be immunoprecipitated by preimmune serum (Fig. 8). This confirms that ZG-16p is the (−)-[^{3}H]BZDC-DHP receptor in ZGM.

ZG-16p Is Associated with the ZGM. ZG-16p contains no transmembrane domain (Cronshagen et al., 1994), but binding and photoaffinity labeling experiments suggested an association of the ZG (−)-[^{3}H]BZDC-DHP receptor with the granule membrane. We therefore determined the distribu-

TABLE 1

Effect of L-type Ca^{2+} channel blockers, modulators of Mdr1, and inhibitors of K^+ channels on ZG K^+ and Cl^- conductances

Conductances are expressed as inverse half-times of lysis of ZG as described in the *Experimental Procedures* section. Control K^+ and Cl^- conductances were 4.26 ± 1.01 and 1.13 ± 0.22 h^{-1}, respectively. Data are means ± S.D. of three to seven different experiments.

Drug	Concentration	K^+ conductance	Cl^- conductance
	M	*% Difference*	
(±)-BZDC-DHP	10^{-5}	−41.7 ± 7.3	−6.2 ± 7.9
Azidopine	10^{-5}	−42.0 ± 7.1	−6.9 ± 6.2
Verapamil	10^{-5}	−16.8 ± 2.6	7.0 ± 1.8
Verapamil	10^{-4}	−61.5 ± 5.3	18.8 ± 11.2
Isradipine	10^{-5}	−8.5 ± 7.1	−15.2 ± 9.9
Vinblastine	10^{-5}	−1.6 ± 1.1	−0.7 ± 4.2
Cyclosporin A	10^{-6}	30.3 ± 7.2	−1.7 ± 5
Cyclosporin A	5×10^{-6}	260.2 ± 29.6	−12.5 ± 5.1
Quinidine	10^{-5}	−4.1 ± 6.3	18.8 ± 22.9
Quinidine	10^{-4}	−48.4 ± 5.2	36.2 ± 15.1
Glibenclamide	10^{-5}	−13.8 ± 2.9	1.8 ± 6.6
Glibenclamide	10^{-4}	−46.5 ± 1.5	42.4 ± 14.8

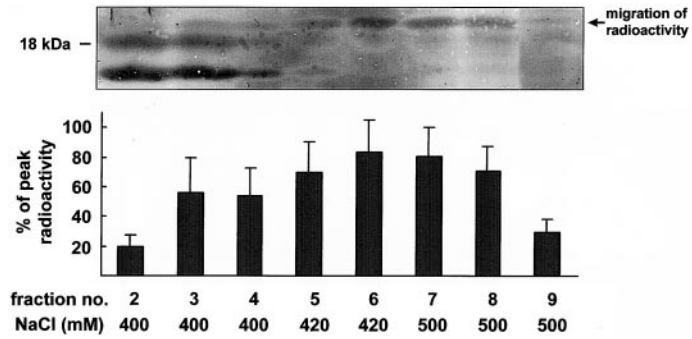

Fig. 7. Purification of the ZGM (−)-[^{3}H]BZDC-DHP receptor. ZGMs were photolabeled with 10 nM (−)-[^{3}H]BZDC-DHP, solubilized, and bound to a cation exchange unit, and the fractions obtained by elution with the indicated concentrations of NaCl were separated by SDS-PAGE. Bottom, the distribution of specifically bound radioactivity in the gel is plotted as percentage of peak radioactivity (means ± S.D. from four or five experiments). Top, a silver stain of the gel of a representative experiment in the molecular weight range of interest. The protein band that is enriched in fractions 6 to 8 and comigrates with radioactivity was sequenced.

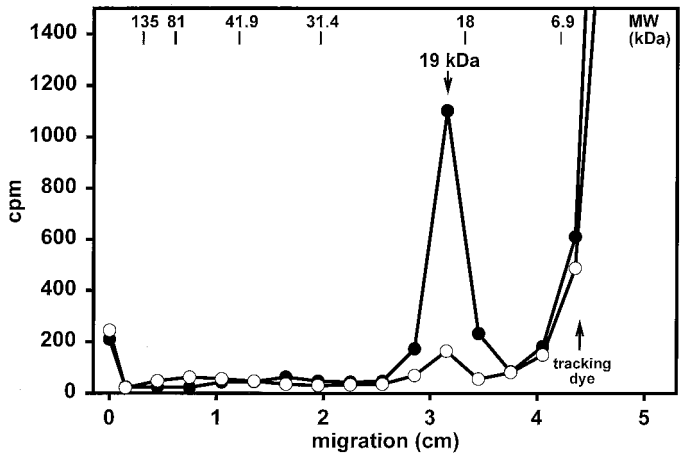

Fig. 6. Photoaffinity labeling of ZGM with (−)-[^{3}H]BZDC-DHP. Photoaffinity labeling was performed with 10 nM (−)-[^{3}H]BZDC-DHP in the absence (●) or presence (○) of 300 nM unlabeled (±)-BZDC-DHP. A single peak of covalently incorporated radioactivity was detected at 19.6 ± 1 kDa (n = 14; six preparations) that could be displaced by unlabeled drug. Each point represents a 3-mm slice of nitrocellulose membrane; the positions of the molecular weight markers are shown on top of the graph.

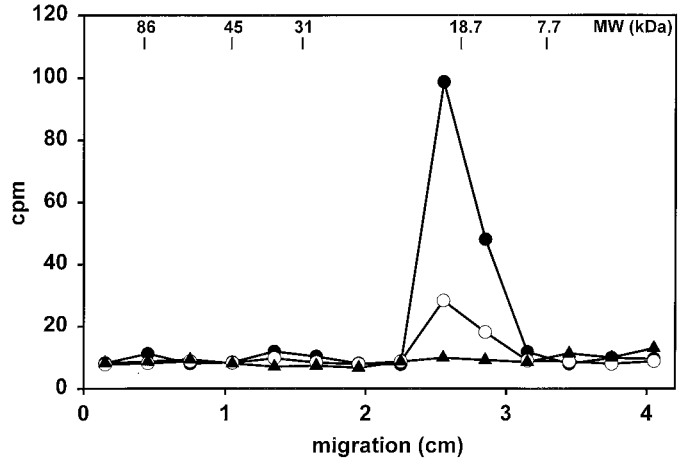

Fig. 8. Immunoprecipitation of (−)-[^{3}H]BZDC-DHP-labeled ZGM. ZGMs were labeled with 12.5 nM (−)-[^{3}H]BZDC-DHP in the absence (●, ▲) or presence (○) of 500 nM unlabeled (±)-BZDC-DHP and immunoprecipitated with equal amounts of anti-ZG-16p antibody (●, ○) or preimmune serum (▲). The precipitates were separated by SDS-PAGE and analyzed for bound radioactivity. The molecular weight markers are shown on the top.

tion of ZG-16p by immunoblotting. As shown in Fig. 9, ZG-16p was mainly associated with the granule membrane, whereas amylase was mainly found in the ZG content. The distribution of ZG-16p correlated with (−)-[^{3}H]BZDC-DHP (15 nM) photoaffinity labeling.

Discussion

As previously reported, rat pancreatic ZGMs contain a high-affinity binding site for (−)-[^{3}H]BZDC-DHP (Thévenod et al., 1996b). By purification and immunoprecipitation, we now identified ZG-16p as the ZGM (−)-[^{3}H]BZDC-DHP receptor. ZG-16p shows homologies to two secretory proteins from other exocrine glands (common salivary protein 1 and prostatic spermine binding protein) and to the plant lectin jacalin but not to known enzymes (Cronshagen et al., 1994). ZG-16p does not contain a transmembrane domain, but as we and others (Kleene et al., 1999) show, it is associated with the granule membrane, indicating that it is part of the submembranous granule matrix. Recently, ZG-16p has been implicated with protein sorting in the *trans*-Golgi network (Kleene et al., 1999). Our data propose a novel function of the protein, namely, the regulation of a K^+ conductance in ZG.

We found that (±)-BZDC-DHP and the other drugs tested inhibit the ATP-sensitive K^+ conductance of ZG with a rank of potencies similar to that of inhibition of (−)-[^{3}H]BZDC-DHP binding to ZGM (Figs. 2A and 5 and Table 1). However, whereas the dihydropyridines (±)-BZDC-DHP and azidopine and the phenylalkylamine verapamil selectively inhibited ZG K^+ conductance, the K^+ channel blockers glibenclamide and quinidine inhibited K^+ conductance but increased Cl^- conductance (Table 1), pointing to a different mode of action of these two groups of compounds. We propose that the dihydropyridines (±)-BZDC-DHP and azidopine and verapamil may specifically bind to ZG-16p to mediate inhibition of K^+ conductance. In contrast, the inhibition of (−)-[^{3}H]BZDC-DHP binding by glibenclamide suggests an interaction of ZG-16p with a ≈65-kDa *mdr1* gene product that serves as a receptor for glibenclamide and quinidine in ZG (Braun et al., 1997) and inversely regulates ZG Cl^- and K^+ conductances (Thévenod et al., 1994, 1996a). This is supported by the observation that [^{3}H]glibenclamide binding to ZGM and inhibition of (−)-[^{3}H]BZDC-DHP binding by glibenclamide occurred with a similar affinity, but the inhibition curves indicated a noncompetitive mechanism for the latter (Fig. 3). A noncompetitive inhibition mode is also compatible with the fact that [^{3}H]glibenclamide (10 nM) binding to ZGM was only weakly inhibited by 1 μM (±)-BZDC-DHP (30%, $n = 2$; data not shown). A possible physical interaction of ZG-16p with a K^+ channel protein or the 65-kDa *mdr1* gene product therefore remains to be investigated.

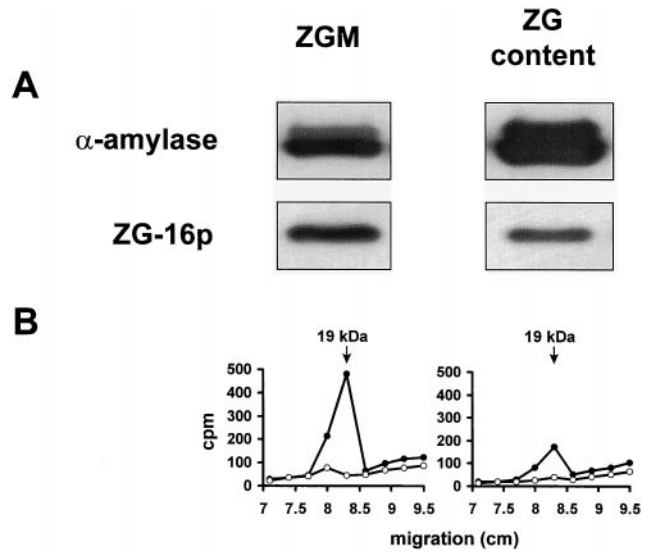

Fig. 9. Distribution of ZG-16p between ZGM and ZG content. Isolated ZGs were lysed in a hypotonic buffer as described in *Experimental Procedures* and centrifuged at 100,000*g* for 1 h. Aliquots of supernatant and pellet containing equal amounts of protein were immunoblotted with anti-ZG-16p (1:2000) and anti-α-amylase (1:1000) antisera, respectively (A), or photoaffinity labeled with (−)-[^{3}H]BZDC-DHP (B) [●, 15 nM (−)-[^{3}H]BZDC-DHP; ○, 15 nM (−)-[^{3}H]BZDC-DHP + 1 μM verapamil].

Whereas verapamil inhibited (−)-[^{3}H]BZDC-DHP binding to ZGM with an affinity similar to (±)-BZDC-DHP and azidopine, higher concentrations of verapamil than of dihydropyridines were needed for inhibition of the ZG K^+ conductance (Figs. 2 and 5B and Table 1). A similar observation has been made in multidrug-resistant cell lines with dihydropyridines and phenylalkylamines, where a strict quantitative correlation between functional effects of MDR1 modulators (i.e., restoration of intracellular doxorubicin accumulation) and their ability to inhibit [^{3}H]azidopine binding to membrane extracts was observed only within a chemical family of modulators but not between the different classes of drugs (Hu et al., 1996).

Higher concentrations of the dihydropyridines and verapamil were needed for inhibition of K^+ conductance than for inhibition of (−)-[^{3}H]BZDC-DHP binding (Figs. 2 and 5B). A similar discrepancy between the affinities of binding and of the functional effect of pharmacological agents, especially of dihydropyridines, has also been observed in other cases. For instance, the EC_{50} of (±)-BZDC-DHP for inhibition of MDR1-mediated rhodamine transport is ≈100-fold higher than its IC_{50} for inhibition of (−)-[^{3}H]BZDC-DHP binding to MDR1 (Boer et al., 1996). Similarly, the EC_{50} values of dihydropyridines for negative inotropy in the heart muscle are 77- to 3450-fold higher than their K_I values for binding to heart muscle membranes (Goll et al., 1986). One possible explanation for the discrepancy observed in ZG is that ZGMs are used for binding experiments, whereas the ZG K^+ conductance is measured in intact granules where the accessibility of the binding site inside the granules might be restricted. Another reason might be a yet unidentified cofactor that influences binding and/or functional effects. In addition, binding studies were carried out under equilibrium conditions, whereas inhibitors were added 3 to 5 min before measurement of K^+ conductance in the functional assay. Therefore, we cannot exclude that BZDC-DHP exerts its functional effect through an additional, low-affinity binding site, but neither the binding studies nor the photoaffinity labeling experiments yielded evidence for such a low-affinity site.

The Mdr1 modulator cyclosporin A, which does not bind to ZG-16p (Fig. 2), strongly stimulated ZG K^+ conductance (Table 1). Currently, its mode of activation is unclear. We have excluded effects on multidrug transporters (e.g., Mdr1 or multidrug resistance-related protein) or on calcineurin phosphatase. First, equimolar concentrations of the cyclosporin A analog PSC 833 (5 μM), which has a higher affinity to these transporters than cyclosporin A and no inhibitory effect on calcineurin phosphatase (Twentyman and Bleehen, 1991),

were much less stimulatory than cyclosporin A (55% above control; $n = 2$; data not shown). In addition, FK506, an inhibitor of calcineurin phosphatase and Mdr1-mediated transport (Liu et al., 1991; Naito et al., 1992) had no stimulatory effect on ZG K^+ conductance (8.3% inhibition at 10 μM; $n = 2$; data not shown). The observation that cyclosporin A selectively activates ZG K^+ conductance is intriguing and could indicate that it directly binds to the channel protein.

ZG-16p and MDR1 have similar affinities to dihydropyridines (BZDC-DHP, azidopine), but apart from this, their pharmacological profiles are quite different. Verapamil binds with considerably higher affinity to ZG-16p than to MDR1 ($K_d \approx 30$ nM and 15 μM, respectively) (Boer et al., 1996), whereas the cytotoxic drug vinblastine and the chemosensitizer cyclosporin A display a substantially lower affinity for ZG-16p (Fig. 2) than for MDR1 (Boer et al., 1996; Thévenod et al., 1996b). In that context, it would also have been useful to compare the stereoselectivity of binding inhibition of (−)-BZDC-DHP by the pure enantiomers of BZDC-DHP with that of MDR1 (Boer et al., 1996); however, only the racemate was available. The binding sites for [^{3}H]azidopine in MDR1 have been localized to transmembrane domains 6 and 11 and 12 (Greenberger et al., 1991; Loo and Clarke, 1993). However, ZG-16p does not contain a transmembrane region, and accordingly, no substantial sequence homology could be detected between ZG-16p and the above-mentioned regions of MDR1. In analogy, no homology was found between ZG-16p and a region critical for dihydropyridine binding in L-type Ca^{2+} channels, transmembrane segment IVS6 (Grabner et al., 1996). The precise localization of the (−)-[^{3}H]BZDC-DHP binding site in ZG-16p remains to be performed.

In exocrine and endocrine glands, evidence has been found for a role of anion and cation channels of secretory granules in exocytosis (Fuller et al., 1989; Anderie and Thévenod, 1996; Barg et al., 1999; Jensen et al., 1999). In pancreatic acinar cells and intestinal goblet cells, both of which express ZG-16p (Cronshagen et al., 1994), it has been proposed that these channels promote a "flushing out" of the granule content (i.e., enzymes or mucins) during exocytosis (De Lisle and Hopfer, 1986; Guo et al., 1997). In line with this idea, it has been shown that monovalent cations inhibit the aggregation of the ZG content (Dartsch et al., 1998). A direct coupling of granule fusion to the plasma membrane to activation of channels in the granule membrane would promote this process. In neurons, coupling of synaptic vesicle fusion and activation of channels in the vesicles is supposed to occur via the membrane potential (Rahamimoff and Fernandez, 1997). We speculate that a similar coupling could be mediated after an initial, fusion pore-mediated granule swelling by the granule matrix, including ZG-16p.

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