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Porin Proteins in Mitochondria from Rat Pancreatic Islet Cells and White Adipocytes: Identification and Regulation of Hexokinase Binding by the Sulfonylurea Glimepiride

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The binding of hexo-/glucokinase and glycerol kinase to mitochondria via the channel forming protein, porin, in pancreatic islet β-cells and adipocytes, was recently proposed to participate in nutritional signaling, glucose sensing, and the control of high-energy phosphate distribution and oxidative phosphorylation. In this study we demonstrate that polyclonal antisera against purified rat liver porin recognize unique proteins in rat pancreatic islets, adipocytes, and RINm5F cells, each with an apparent M_r about 2000 smaller than that of liver porin. Immunoblotting of subcellular fractions, the purity of which has been controlled by the distribution of marker proteins, revealed the mitochondrial localization of the cross-reacting proteins. Their enrichment with a method used for the purification of porin proteins, the characteristic behavior during isoelectric focusing, and the specific binding of rat liver hexokinase and glycerol kinase to phospholipid vesicles containing the purified cross-reacting β-cell or adipocyte proteins strongly suggest their identity with mitochondrial porin. The subtle differences in the apparent M_r and charge heterogeneity raise the possibility of the existence of porin isoforms expressed in a tissue-specific manner. Anti-porin antisera coimmunoprecipitated hexo-/glucokinase from rat insulinoma cell (RINm5F) and adipocyte mitochondria as determined by subsequent immunoblotting of the immunoprecipitates with polyclonal antisera against yeast hexokinase and rat liver glucokinase, respectively. This indicates that some rat pancreatic glucokinase (54 kDa) and liver hexokinase (102 kDa), respectively, is bound to mitochondrial porin. The major portion of the bound fraction is released from mitochondria after treatment with glucose 6-phosphate. Incubation of RINm5F and fat cells with the insulin releasing sulfonylurea drug, glimepiride (20 nM and 5 μM, respectively) for 30 min reduces the amount of hexo-/glucokinase associated with mitochondria and porin to about 50–30%. The reduced kinase binding activity of porin is preserved after isolation of porin from glimepiride-treated cells, reconstitution into phospholipid vesicles and assaying for glucose 6-phosphate inhibitable binding of rat liver hexokinase. The sulfonylurea tolbutamide (20 μM and 5 mM) is significantly less effective. The sulfonylurea-induced inhibition of hexo-/glucokinase binding to mitochondrial porin does not require glucose metabolism or Ca^{2+} influx into the cells. These data suggest that the sulfonylurea glimepiride, which is thought to inhibit the ATP-regulated K^+-channel in β-cells, may have, in addition, an intracellular site of action in pancreatic islet and adipocyte cells at the level of regulation of gluco-/hexokinase binding to mitochondrial porin.

The outer mitochondrial membrane is exclusively permeable for polar molecules as large as several thousand Daltons along a channel forming protein, termed porin. The isolated channels formed by these 29- to 32-kDa transmembrane polypeptides have been called VDAC (voltage-dependent anion-selective channel), a name that describes their electrical properties (1). The porin protein specifically binds hexokinase, glucokinase, and glycerol kinase in some cell types (2, 3). The mitochondrial binding of these enzymes may play a significant role in the control of high-energy phosphate distribution and oxidative phosphorylation. Therefore, the functioning of hexo-, gluco-, and glycerol kinase and glucose/glycerol as acceptors for newly synthesized mitochondrial ATP which does not equilibrate with the extramitochondrial pool may ensure maximal efficiency of oxidative phosphorylation in

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nonmuscular tissues (4–9). For instance, it was recently proposed that the mitochondrial binding of glucokinase isoenzymes participates in the fuel-sensor function of the pancreatic islet β-cell (10, 11). First, bound hexokinase is less sensitive than the cytosolic enzyme to inhibition by D-Glc-6-P[2] (9, 10). Second, the postulated binding of hexokinase isoenzymes to mitochondrial porin provides a direct pathway for coupling D-glucose phosphorylation to mitochondrial respiration (12). Third, bound hexokinase preferentially uses ATP generated inside the mitochondria rather than extramitochondrial ATP as a substrate for D-glucose phosphorylation (7, 13, 14). It should be stressed, however, that porin had, so far, not been identified in pancreatic islet cells.

The present study documents the presence of porin in rat pancreatic islets, adipocytes, and an insulinoma cell line by immunological methods and reveals subtle differences in size and charge between these porins. Furthermore, despite the proposed physiological significance of the association between gluco-/hexokinase with mitochondria and porin (10, 11) which may form part of the glucose sensing mechanism in β-cells and adipocytes, these interactions have not been studied in those cells so far. Here we demonstrate that β-cell and adipocyte porin binds gluco- and hexokinase, respectively, under physiological conditions. The binding capacity of the porin proteins is reduced after incubation of the cells with the hypoglycemic sulfonylurea, glimepiride.

MATERIALS AND METHODS

Materials. Polyclonal and affinity-purified ^{125}I-labeled anti-rabbit IgG and ^{125}I-labeled anti-mouse IgG from sheep and Hybond C extra nitrocellulose sheets were bought from Amersham-Buchler, Braunschweig, FRG; hydroxyapatite Bio-Gel HTP was purchased from Bio-Rad, Munich, FRG; celite 535 was from Roth, Osterode, FRG; DEAE-cellulose, Percoll, and Ampholines were provided by Pharmacia/LKB, Freiburg, FRG; visking tubings 18/32 were from Union Carbide, Fresno, Arizona; Genapol X-100 was made available by Hoechst Aktiengesellschaft, Frankfurt, FRG; collagenase type CLS II was bought from Worthington, Freehold, New Jersey; bovine serum albumin (fraction V), asolectin (type II-S from soybean), protease inhibitors, deoxyglucose, and cell culture media were provided by Sigma, Deisenhofen, FRG; Triton X-114 was purchased from Fluka, Buchs, Switzerland; male Wistar rats were bred and delivered by the Hoechst Aktiengesellschaft (Tierzucht Kastengrund, FRG); glimepiride, tolbutamide, and carboxytolbutamide were prepared by the Pharma Synthesis Department of the Hoechst Aktiengesellschaft, Frankfurt, FRG; polyclonal rabbit antibodies against the purified SRP-R α-subunit from dog pancreas microsomes, cytochrome c from mouse liver mitochondria (mouse cytochrome c has the same amino acid sequence as rat cytochrome c), F_1-ATPase α-subunit from rat liver mitochondria and hexokinase from the yeast *Saccharomyces cerevisiae* were kindly donated by T.A. Rapoport, Berlin, FRG, E. Margoliash, Evanston, Illinois, G.C. Shore, Montreal, Quebec, and G. Schatz, Biocenter Basel, respectively.

Isolation and subcellular fractionation of pancreatic islets. Groups of 600 pancreatic islets each were isolated from fed female albino rats by the collagenase procedure (13), collected in an albumin-free Hank's solution (13), and lyophilized. In another series of experiments, groups of 1000 islets each were homogenized in Potter–Elvehjem tubes (12 strokes) with 0.55 ml of a Hepes–NaOH buffer (10 mM, pH 7.4) containing 280 mM sucrose and 2.5 mM EDTA. After a first centrifugation (600*g*, 5 min) for deposition of intact cells, cell debris, and nuclei, the supernatant material (0.5 ml) was centrifuged (5500*g*, 5 min, 4°C) for isolation of a mitochondria-rich pellet. The supernatant of this second centrifugation was again centrifuged (24,000*g*, 10 min, 4°C) for isolation of a pellet enriched in secretory granules. All samples, including the supernatant of the last centrifugation, were lyophilized and finally resuspended in sample buffer (15).

RINm5F culture. RINm5F cells of the insulin-producing cell line derived from a rat islet cell tumor (16, 17) were grown in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal calf serum, 50 IU/ml penicillin, 0.25 μg/ml fungizone, and 50 μg/ml streptomycin at 37°C in an atmosphere of humified air/CO_2 (19:1) according to Praz *et al.* (18) with the following modifications: The cells were seeded at a density of 3.5×10^4 cells/ml in 20 ml of medium (75-cm^2 culture flasks). The medium was replaced four times per week (one passage). Thereafter, the cells were treated (2–5 min, 37°C) with trypsin (0.02% trypsin in 0.9% NaCl/0.2 mM EDTA). The trypsin-treated cells were diluted, reseeded at a density of 2×10^6 cells per 75-cm^2 culture flask, and grown to 70% confluency.

Isolation of RINm5F mitochondria. The cell layer from culture flasks was washed twice with ice-cold 25 mM Hepes/KOH (pH 7.4), 0.1 M sucrose, 0.2 M mannitol, 0.5 mM EDTA, scraped with 5 ml/flask of the same buffer, and homogenized with 10 strokes of a tight fitting Potter–Elvehjem homogenizer followed by sonication (bath sonicator, 4°C, 10 s, maximal power). The homogenate was centrifuged (800*g*, 5 min). The supernatant was saved and the pellet resuspended in 10 ml of 0.2 M mannitol, 0.1 M sucrose, 10 mM Hepes/KOH (pH 7.4), 0.1 mM EDTA and homogenized as above. Centrifugation was repeated and the combined supernatants were centrifuged (20,000*g*, 15 min). The pellet consisted of a loosely packed fluffy top layer and a tightly packed bottom layer. The top layer was carefully removed and recentrifuged (20,000*g*, 15 min). The resulting bottom layer was resuspended with the previous bottom layer and then centrifuged (1000*g*, 20 min). The supernatant was centrifuged (20,000*g*, 20 min). The resulting pellet was carefully rinsed with the medium above to remove the fluffy material on top and then suspended at 0.5–1 mg protein/ml medium. Five milliliters of crude mitochondria was mixed with 5 ml of 20% Percoll, 0.2 M sucrose, 0.1 M mannitol, 0.05 mM EDTA, 0.1 mM PMSF, 20 mM Hepes/KOH (pH 7.4) and layered on top of a 5-ml cushion of the same medium. After centrifugation (13,000 rpm, 30 min, 4°C, Sorvall Hb-4 swing-out rotor), mitochondria were withdrawn with a Pasteur pipet from the lower third of the gradient and diluted with 5 vol of 0.2 M sucrose, 0.1 M mannitol, 10 mM Hepes/KOH (pH 7.5). The purified mitochondria were centrifuged (20,000*g*, 15 min), washed once with 15 ml of medium, and suspended at 0.5 mg of protein/ml of the medium above.

Isolation and subcellular fractionation of rat adipocytes. Adipocytes were isolated from epididymal fat pads of 160- to 180-g male Wistar rats as described (19). For subcellular fractionation (according to Refs. 20, 21 with modifications) cells from 10 g tissue (approx. 5×10^7 cells) were washed three times with homogenization buffer (20 mM Tris/HCl, pH 7.4, 1 mM EDTA, 0.25 M sucrose), supplemented with protease inhibitors (200 μM PMSF, 100 μg/ml aprotinin, 2 μg/ml each of leupeptin, pepstatin, and antipain), resuspended in 30 ml of homogenization buffer, homogenized with 10 strokes in a Teflon-in-glass homogenizer, and centrifuged (1500*g*, 5 min, 20°C). The supernatant (homogenate) was removed carefully from the fat cake and pellet fraction (cell debris) by suction with a needle and centrifuged (16,000*g*, 15 min, 4°C). The supernatant was saved for preparation of microsomes and cytoplasm. The pellet (mitochondria/nuclei) was resuspended, recentrifuged, resus-

[2] Abbreviations used: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SRP-R, signal recognition particle receptor; Glut, glucose transporter isoforms; Glc-6-P, glucose 6-phosphate; ATP-K^+-channel, ATP-regulated K^+-channel; PEG, polyethylene glycol; PMSF, phenylmethylsulfonyl fluoride; Mops, morpholinepropanesulfonic acid; BSA, bovine serum albumin; TCA, trichloroacetic acid; TBS, Tris-buffered saline; GPI-PLC, glycosyl-phosphatidylinositol-specific phospholipase C; PIG, phosphoinositol-glycans.

pended in 10 ml of homogenization buffer, and layered onto a 5-ml cushion of 20 mM Tris/HCl (pH 7.2), 1 mM EDTA, 1.12 M sucrose. After centrifugation (100,000*g*, 60 min, 4°C) the pellet (crude mitochondria) was washed once and suspended in 30 ml of 20 mM Tris/HCl (pH 7.2), 0.5 mM EDTA, 0.25 M sucrose, 24% Percoll. After centrifugation (40,000*g*, 30 min, 4°C) the purified mitochondria were withdrawn from the lower third of the gradient with a Pasteur pipet, diluted with 10 vol of SEM buffer (0.25 M sucrose, 1 mM EDTA, 20 mM Mops/KOH, pH 7.4), and collected by centrifugation (13,000*g*, 10 min, 4°C). The pellet was washed once, recentrifuged, and finally dissolved in sample buffer. The initial supernatant was centrifuged (245,000*g*, 90 min, 4°C). The pellet (microsomes) was suspended in SEM buffer, recentrifuged, and finally dissolved in sample buffer. The supernatant (cytoplasm) was supplemented with twofold concentrated sample buffer.

Incubation of cells with sulfonylureas. RINm5F cells seeded in culture flasks at a density of 2×10^6 cells and grown to 70% confluency were treated with trypsin/EDTA (see above), washed with 3×10 ml Hepes-based Krebs-Henseleit buffer containing 20 mM Hepes/KOH (pH 7.4), 125 mM NaCl, 5 mM KCl, 7.5 mM $NaHCO_3$, 2 mM $CaCl_2$ (as indicated), 0.8 mM $MgSO_4$ suspended at a density of 2×10^7 cells/ml in the same buffer, and then incubated (60 min, 25°C) with glimepiride (20 nM), tolbutamide (20 μM), or carboxytolbutamide (20 μM). Subsequently, the cells were washed two times with the same buffer. Isolated adipocytes were washed three times with Dulbecco's MEM lacking glucose and containing 20 mM Hepes, 2% fetal calf serum, 1% BSA, 50 units/ml penicillin, 10 mg/ml streptomycin by flotation onto the top of the medium after centrifugation (1000*g*, 1 min). The packed cell suspension was finally adjusted to 2×10^5 cells/ml medium. After incubation (30 min, 37°C) under slight shaking with glimepiride (5 μM), tolbutamide (5 mM), or carboxytolbutamide (5 mM), the cells were washed two times with Krebs-Ringer bicarbonate buffer, 25 mM Hepes/KOH (pH 7.4), 0.1% BSA.

Preparation of rat liver homogenate and mitochondria (according to Ref. 22 with modifications). Briefly, the liver removed from one male 250-g Wistar rat in 150 ml of ice-cold buffer I (25 mM Hepes/KOH pH 7.4, 250 mM sucrose, 1 mM EDTA) containing antipain, leupeptin, pepstatin (each at 5 μg/ml), PMSF (100 μM), and aprotinin (200 μg/ml) was minced, homogenized with 15 strokes in a tight-fitting motor-driven (1500 rpm) Teflon-in-glass homogenizer, and then filtered through two layers of nylon mesh and centrifuged (1000*g*, 15 min, 4°C). The upper three quarters of the supernatant (homogenate) were removed and further centrifuged (10,000*g*, 10 min). The resulting crude mitochondrial pellet was suspended in 2 ml of 10 mM Hepes/KOH, pH 7.5, 0.3 M mannitol, 70 mM sucrose, 1 mM EGTA and layered onto a continuous linear urographin gradient (25–55% in 10 mM Hepes/KOH, pH 7.5, 0.5 mM EDTA). After centrifugation (250,000*g*, 12 h, 4°C) the resulting band was recovered, diluted 10-fold with 10 mM Hepes/KOH, 0.5 mM EDTA, 0.6 M mannitol, and centrifuged (12,000*g*, 10 min). The pellet was washed once for removal of urographin and recentrifuged (12,000*g*, 10 min) to collect purified mitochondria which were finally dissolved in sample buffer.

Treatment of mitochondria with Glc-6-P. Mitochondria (0.5 mg protein/ml) were incubated (20 min, 30°C) without or with 2 mM Glc-6-P and then centrifuged (20,000*g*, 15 min, 4°C). The pellet was rinsed once with the initial volume of buffer, carefully resuspended in buffer, and recentrifuged as above. The final pellet was suspended in the desired buffer.

Purification of porin. A mitochondrial pellet was dissolved in 1 ml ice-cold Triton X-114 (2%) in 25 mM Tris/HCl (pH 7.4), 144 mM NaCl per 10 mg of protein. Phase separation was performed as described (23, with modifications according to Ref. 24). Proteins recovered from the aqueous phase were precipitated with PEG-6000 (12% final concn.), washed two times with 4% PEG-6000, and dissolved in 0.5 ml of buffer A (2.5% Genapol X-100, 50 mM KCl, 25 mM Tris/HCl, pH 7.2, 10 mM KP_i, 1 mM EDTA). The supernatant of a centrifugation (50,000*g*, 15 min) was applied to a small column of hydroxyapatite (1 g dry wt of Bio-Gel HTP). The column was eluted with buffer A. A 0.5-ml sample of the eluate was collected and applied to a small column of hydroxyapatite and celite (0.5 g dry wt each). The column was eluted with buffer A. A 0.5-ml sample of the eluate was collected and applied onto a second column of hydroxyapatite and celite. A 0.5-ml aliquot of the last eluate was dialysed in visking tubing, 18/32, against 10 vol of 0.5% Genapol X-100, 10 mM Tris/HCl (pH 7.5) (two times, 12 h), subjected to chromatography on DEAE-cellulose, equilibrated with 0.5% Genapol X-100, 10 mM Tris/HCl (pH 7.5), and eluted with the same buffer. The protein peak which appeared at the buffer front was collected. For SDS-PAGE, the protein was precipitated with 5% TCA and 30% (v/v) methanol. After 30 min on ice the samples were centrifuged, washed successively with 0.5% TCA and two times with acetone, dried, and dissolved in sample buffer.

Binding of hexokinase and glycerol kinase. Soybean asolectin was extracted with acetone and dissolved in chloroform and a portion of the solution (1.4 mg of lipid) was dried as a film at the bottom of a conical centrifuge tube. Porin protein fractions (0.5 ml) collected from the DEAE chromatography were added to the tube. The mixture was dispersed by ultrasonic oscillation (Bransonic 12 bath sonicator, maximal power, 1 min), diluted with 25 ml of 20 mM Hepes/KOH (pH 7.4), 0.5 mM EDTA, and centrifuged (300,000*g*, 1 h, 4°C). The vesicle pellet was washed once and resuspended in the same buffer at 0.1 mg protein/ml. Aliquots (50 μl) of the reconstituted vesicles were incubated (30 min, 25°C) with 200 μl of liver cytosol (300,000*g* supernatant of a liver homogenate in 0.25 M sucrose, 20 mM Hepes/KOH, pH 7.4, 100 μM PMSF) in the presence of 10 mM $MgCl_2$ without or with 10 mM Glc-6-P (as indicated). Subsequently, the sample was diluted with 25 ml of ice-cold sucrose/Hepes buffer and centrifuged (300,000*g*, 1 h, 4°C). The pellet was rinsed with 10 ml of the same buffer, suspended in 25 ml of ice-cold buffer, recentrifuged as above, and suspended in buffer containing 0.2% Lubrol WX. Aliquots were tested for gluco-/hexokinase or glycerol kinase activity and protein by enzymatic assay and immunoblotting, respectively.

Preparation of antisera. Antibodies against Glut isoforms were raised in rabbits by injection of synthetic peptides coupled to the carrier protein keyhole-limpet hemocyanin using published procedures (25). Anti-Glut2 antibodies were raised against the rat liver Glut2 C-terminal peptide sequence CRKATVQMEFLGSSETV (26). Anti-Glut4 antibodies were raised against the 24 C-terminal amino acids of rat Glut4 (27). Antibodies against rat liver porin were raised in rabbits (antiserum I) and mice (antiserum II) by using purified porin (see above) and against rat liver glucokinase by using isolated glucokinase (purified by a published procedure according to Ref. 28 and estimated to be more than 90% pure by SDS-PAGE). Both proteins were subjected to SDS-PAGE and stained with Coomassie blue, and the protein band was excised and reelectrophoresed on a second SDS-polyacrylamide gel slab (8%). The single band (200 μg protein) obtained was excised, and the protein was electroeluted, dialyzed, TCA-precipitated, dissolved in 4% SDS, 120 mM Tris/HCl (pH 6.8), mixed with an equal volume of complete Freund's adjuvant, and injected. After two further injections at 3 and 5 weeks, the animals were bled. This procedure yielded strong and monospecific antisera. Preimmune rabbit and mouse sera were used in control experiments and did not generate signals in immunoblotting.

Immunoblotting. Proteins separated by SDS-PAGE were transferred electrophoretically onto nitrocellulose filter (29) using a semidry blotting apparatus. The transfer efficiency was controlled by staining of the gel with Coomassie blue and of the filter sheet with Ponceau-S in each case. The sheets were blocked (1 h, 25°C) with Tris-buffered saline (TBS; 25 mM Tris/HCl, pH 7.4, 125 mM NaCl) containing 5% (w/v) defatted milk powder and 0.2% (w/v) Tween 20. After addition of antibodies diluted in blocking buffer (anti-porin I, 1:8000; anti-porin II, 1:2500; anti-cytochrome c, 1:800; anti-F_1-ATPase, 1:500; anti-Glut4, 1:1000; anti-Glut2, 1:500; anti-SRP-R, 1:2000; anti-hexokinase, 1:50; anti-glucokinase, 1:600), the incubation was continued (16 h, 4°C). Subsequently, the sheets were washed successively (30 min each) with TBS containing 0.25 M NaCl, TBS containing 0.2% Tween 20 (two times), and TBS. Bound antibodies were detected by incubation (1 h, 4°C) with ^{125}I-labeled sheep anti-rabbit/mouse IgG diluted 1:1000 in blocking buffer (2 μCi/ml). After washing (see above) the sheets were air-dried and subjected to autoradiography (Kodak XAR-5 films). The films were developed after 36 to 240 h of exposure at −80°C. For sequential immunodecoration of the

same blot using different antibodies, the nitrocellulose sheet was incubated (1 h, 25°C each) after each autoradiography successively with TBS containing 2 M NaCl plus 0.5% SDS, TBS containing 2 M NaCl plus 0.2% Tween 20, TBS containing 0.2% Tween 20 (three times), and blocking buffer for removal of bound antibodies and then incubated with the next primary antiserum.

Immunoprecipitation. Mitochondrial protein (0.1 mg in up to 0.2 ml) was diluted with 0.8 ml of 20 mM Hepes/KOH (pH 7.4), 150 mM NaCl, 1% TX-100, 0.1 mM PMSF, and centrifuged (12,000*g*, 5 min). The supernatant was incubated with 50 μl of anti-porin antiserum II (30 min, 4°C). After addition of 50 μl of protein A-Sepharose beads (100 mg/ml buffer), the incubation was continued under constant shaking (2 h, 4°C). The beads were collected by centrifugation (12,000*g*, 1 min) and washed sequentially with 1 ml each of buffer containing 1% TX-100/100 mM NaCl, (omitted during coimmunoprecipitation), 0.1% TX-100/50 mM NaCl, and 0.1% TX-100. The final pellet with the immunocomplex-containing beads was solubilized in 50 μl of sample buffer (5 min, 95°C) and centrifuged. The supernatant was analyzed by SDS-PAGE and immunoblotting.

Miscellaneous procedures. Published procedures were used for determination of soluble (30) and membrane protein content (31) and activity of hexokinase and glycerol kinase (32), SDS-PAGE on vertical slab gels (17.5%, 1 mm thick) in the presence of urea (33), and isoelectric focusing (34) using 6% polyacrylamide gels, 15% *N,N'*-diallyltartardiamide crosslinker and ampholines (pH range of 3.5–10.0; 26) in the presence of 6 M urea.

RESULTS

Polyclonal Antisera against Rat Liver Porin Recognize Rat Islet and Adipocyte 28-kDa Proteins

Rat liver porin was isolated from total mitochondrial proteins which had been depleted from the majority of "typical" transmembrane proteins by TX-114 partitioning (23, 24). Based on its relative hydrophilic character (which agrees well with its secondary structure consisting of a number of β-sheets, but lacking extended hydrophobic α-helical transmembrane domains), most of the porin was recovered from the detergent-depleted (aqueous) phase. After PEG precipitation of the proteins partitioned into the aqueous phase, porin was further enriched using a method adapted from the purification of porin from *Neurospora crassa* (35). The PEG-precipitated proteins were solubilized in the nonionic detergent Genapol X-100 and passed through a column of dry hydroxyapatite. The eluate was passed two times through a mixture of hydroxyapatite and celite. This "neurospora" procedure resulted in ~150-fold enrichment of rat liver porin toward total mitochondrial protein with ~60% yield and is thus slightly more efficient than the original purification scheme developed for rat liver porin (36). After the final DEAE-chromatography step SDS-PAGE revealed a single band (see Fig. 4, lane 11) with the apparent M_r of 30,000–31,000, as has been described for rat liver porin (36). Polyclonal antisera were raised against this protein in rabbits (antiserum I) and mice (antiserum II). In immunoblots both antisera reacted with a single protein (apparent M_r = 31,000) in liver homogenates (Fig. 1, lanes 2–4) and mitochondria (lane 1) and with porin isolated by the "neurospora" method (lane 5), depending on the amount of protein applied to the gel. Control experiments demonstrated the dependence of signal height calculated from densitometric scanning of the autoradiograms on the concentration of porin transferred onto the blots. The antisera also recognized rat liver porin isolated by the original method (kindly provided by B.D. Nelson, Stockholm; data not shown). These data (and the competition of the immunodecoration of total mitochondrial protein and isolated porin by excess of purified porin, which has been incubated with submaximal amounts of antisera I and II prior to addition to the filter; see Fig. 3, lanes 1–6) demonstrated the specificity of both antisera against rat liver porin. The antisera also yielded strong signals (depending on the amount of protein) with homogenates from rat islet cells (Fig. 1, lanes 6–11) and adipocytes (lanes 12–15) migrating with an apparent M_r of 28,000.

The Cross-Reacting Islet and Adipocyte 28-kDa Proteins Are Associated with Mitochondria

To assess the intracellular localization of the cross-reacting proteins, adipocytes and islet cells were fractionated into cytoplasm, mitochondria, and microsomes or secretory granules, respectively, which were tested for immunoreactivity with anti-porin antisera I and II (Fig. 2). The purity of the fractions was analyzed by immunoblotting with antibodies against marker proteins of known topology. Figure 2 and the corresponding quantitative evaluation of the immunoblot data (Table I) shows that the intracellular distribution of the 28-kDa protein from both adipocytes and islets recognized by the anti-porin antibodies (2a and 2b, lanes 2–5) closely resembled that of the mitochondrial proteins, F_1-ATPase α-subunit (inner membrane protein; 2a, lanes 10–13) and cyto-

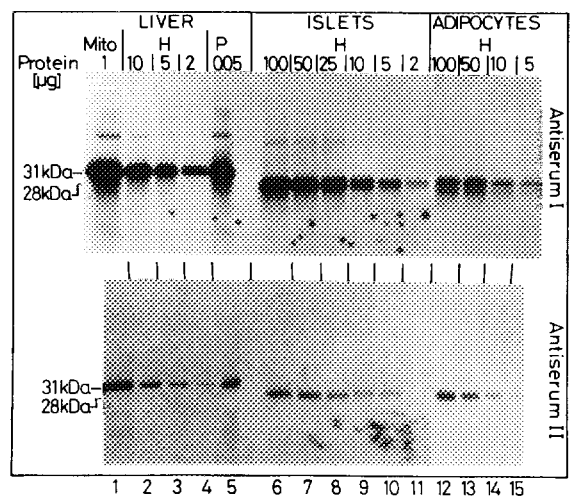

FIG. 1. Cross-reactivity of islet and adipocyte proteins with antisera against liver porin. Various amounts of protein of purified rat liver porin (P), rat liver mitochondria (Mito), and homogenate (H) from rat liver, islet cells, and adipocytes were separated by SDS-PAGE, immunoblotted with two different polyclonal antisera against rat liver porin, and autoradiographed. The molecular masses indicated on the left margin were derived from marker proteins run in parallel.

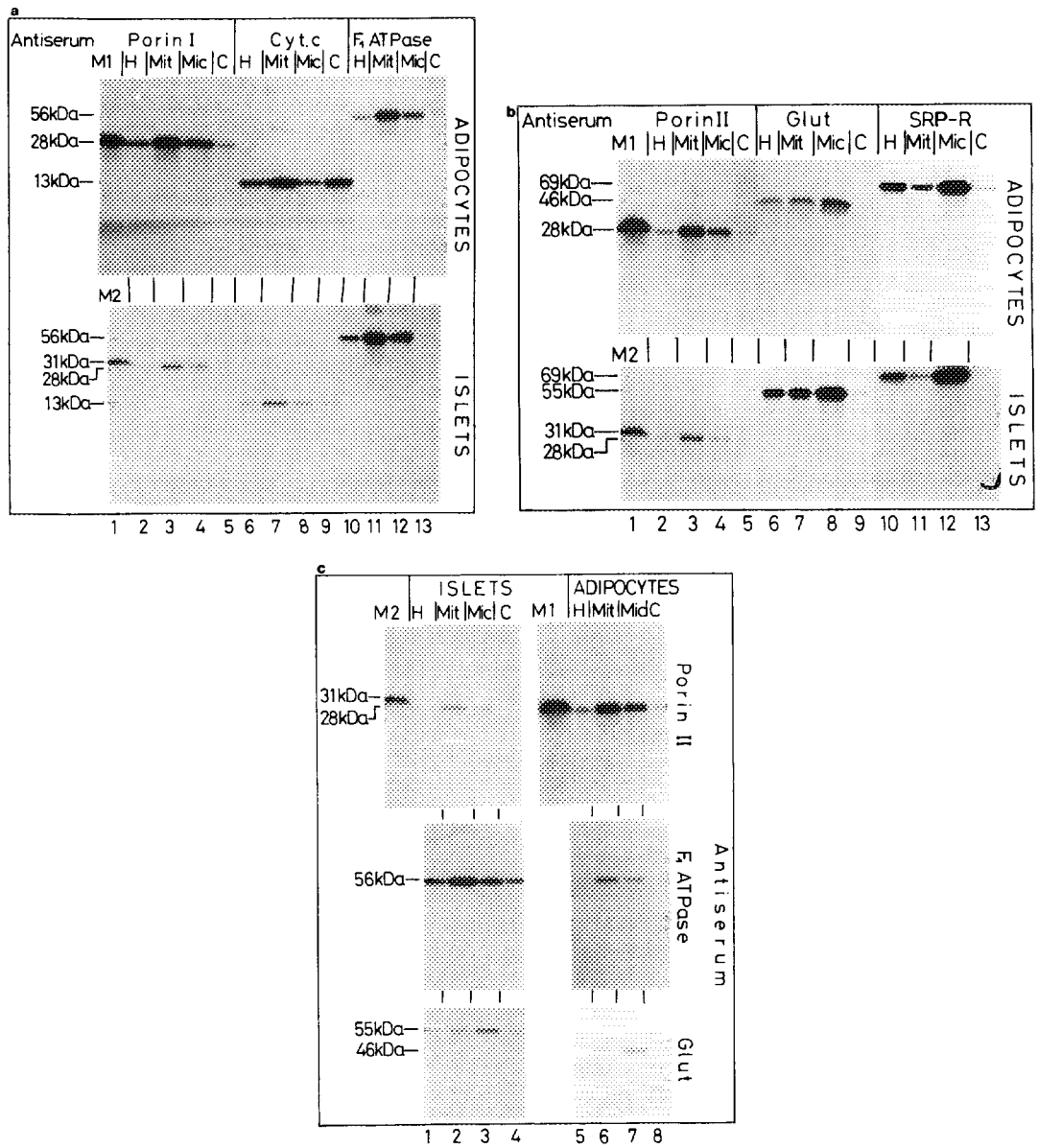

FIG. 2. Subcellular localization of the cross-reacting islet and adipocyte proteins. 150 μg of protein from rat adipocyte and islet homogenate (H), mitochondria (Mit), microsomes (Mic), and cytoplasm (C) was separated by SDS–PAGE, immunoblotted separately (a and b) or sequentially (c, see Materials and Methods) with polyclonal antibodies against rat liver porin (Porin I, II), cytochrome c (Cyt. c), rat F_1-ATPase α-subunit, Glut 4 or Glut 2 (adipocyte- or islet-specific, respectively), and dog pancreas SRP-R α-subunit, and autoradiographed. Mitochondrial proteins from islets (M1) and liver (M2) were run in parallel as controls. The molecular masses indicated on the left margin were derived from marker proteins run in parallel.

chrome c (peripherally associated with the outer face of the inner membrane; 2a, lanes 6–9). In contrast, its distribution clearly differed from that of the plasma membrane and *trans*-Golgi network-associated Glut proteins (isoform 4 in adipocytes, Ref. 27, and isoform 2 in islet cells, Ref. 26; 2b, lanes 6–9) and of the endoplasmic reticulum-associated protein, SRP-R α-subunit (37; 2b, lanes 10–13). The major portion of these proteins was recovered in the microsomal fraction (2b, lanes 8 and 12). The minor amounts of 28-kDa protein recognized in the microsomes and cytoplasm (lanes 4 and 5) are due to mitochondria contaminating these fractions. This became evident from the presence of cytochrome c and F_1-ATPase α-subunit (2a, lanes 8, 9, 12, and 13). On the other hand, adipocyte and islet mitochondria were strongly deprived of plasma membranes, Golgi vesicles, and endoplasmic reticulum membranes as demonstrated by the small amounts of Glut4/2 and SRP-R recovered in mitochondria (2b, lanes 7 and 11). The enrichment of the cross-reacting 28-kDa adipocyte and islet proteins in the mi-

TABLE I

Quantitative Evaluation of Fig. 2a and 2b

Antiserum	Subcellular fractions of adipocytes: Homogenate	Mitochondria	Microsomes	Cytosol
Porin I	2,298	16,122 (7.0)	7,592 (3.3)	477 (0.2)
Porin II	613	7,929 (12.9)	2,345 (3.8)	285 (0.5)
Cytochrome c	3,140	12,977 (4.1)	1,142 (0.4)	4013 (1.3)
F_1-ATPase	390	5,828 (14.9)	2,142 (5.5)	172 (0.4)
Glut	877	1,719 (2.0)	8,268 (9.4)	388 (0.4)
SRP-R	18,294	6,821 (0.4)	54,120 (3.0)	844 (>0.1)
	Subcellular fractions of islets			
Antiserum	Homogenate	Mitochondria	Microsomes	Cytosol
Porin I	119	1,385 (11.6)	582 (4.9)	53 (0.4)
Porin II	291	3,651 (12.5)	872 (3.0)	94 (0.3)
Cytochrome c	455	1,972 (4.3)	694 (1.5)	302 (0.7)
F_1-ATPase	5117	29,720 (5.8)	10,421 (2.0)	432 (<0.1)
Glut	4244	5,817 (1.4)	19,247 (4.5)	558 (0.1)
SRP-R	2812	437 (0.2)	17,025 (6.1)	161 (<0.1)

Note. Immunoreactivity was quantitated by cutting rectangular strips, corresponding to the locations of the blotted proteins on the developed films, from the nitrocellulose filters and counting the filter strips in a gamma-counter. Specific binding of ^{125}I-labeled secondary antibody (dpm) was determined after subtracting half of the sum of the background radioactivities present on size-matched strips of the filter cut from areas above and below each immunoreactive band. The results of one typical fractionation experiment repeated three times are shown. The relative enrichment factors of the marker proteins toward the homogenate (set at 1) are given in the brackets.

tochondrial fraction and their low levels in the homogenate of other membrane fractions and the cytoplasm (2a and 2b, lanes 2, 6, and 10) strongly support their mitochondrial localization. These results were confirmed by sequential immunodecoration of subcellular fractions of both islet cells and adipocytes using the same gel for the incubations with antibodies against porin, F_1-ATPase α-subunit, and Glut2/4 (used in that order; Fig. 2c). This procedure excludes varying transfer efficiencies in different blotting experiments.

The specificity of the cross-reaction of antisera I and II raised against rat liver porin with the 28-kDa proteins from adipocyte and islet mitochondria was confirmed by using submaximal amounts of antibodies which had been preincubated with excess purified rat liver porin (Fig. 3). The signal of the immunoreactive adipocyte and islet 28-kDa band decreased with increasing amounts of porin added (lanes 7–12). As a control for the specificity of the antisera used, competition of the homologous immunoreaction with total mitochondrial protein (lanes 4–6) and purified rat liver porin (lanes 1–3) was demonstrated. In addition, the subcellular fractionation of isolated rat adipocytes was followed by measuring the enzymatic activities of the marker proteins, lipoprotein lipase (microsomal fraction), succinate:cytochrome c oxidoreductase (mitochondrial fraction), and lactate dehydrogenase (cytoplasmic fraction) as has been shown previously for cultured 3T3 adipocytes (38). Comparable enrichment factors for microsomes, mitochondria, and cytoplasm, were calculated from the immunoblot data and distribution of marker enzyme activities (data not shown).

The Cross-Reacting Adipocyte and Islet 28-kDa Proteins Can Be Purified Like Porin

To corroborate the identity of the cross-reacting 28-kDa adipocyte and islet proteins with mitochondrial porin, total mitochondrial proteins from islet, RINm5F cells, and adipocytes were subjected to the purification scheme used for liver porin in this study. As can be seen from the SDS–PAGE of the resulting fractions (Fig. 4, upper panel) and from the corresponding purification scheme (Table

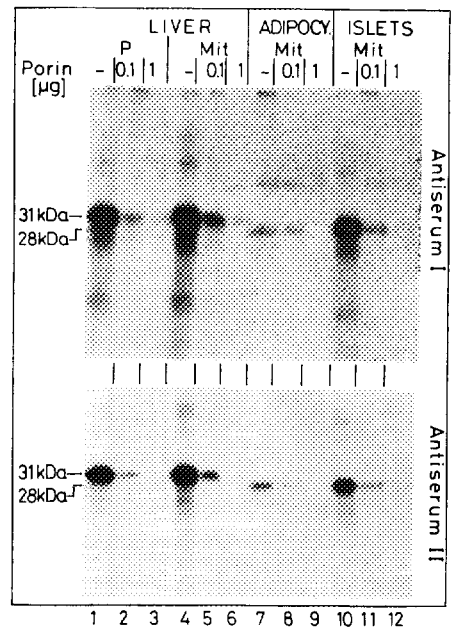

FIG. 3. Competition of the cross-reaction by isolated rat liver porin. 20 μg of mitochondrial protein (Mit) from rat liver, adipocytes, and islet cells and 0.2 μg of purified rat liver porin (P) were separated by SDS–PAGE and transferred onto nitrocellulose filters. The filters were cut into strips according to each lane of the SDS–PAGE. Each strip was incubated (1 h, 4°C) with blotting buffer containing anti-porin antibodies which had been preincubated with purified porin or BSA (as a control) according to the following protocol: 200 μl of 10 m*M* Tris/HCl (pH 7.5), 2.5% Genapol X-100 containing 1 or 10 μg of purified porin, or 10 μg of BSA was added to 800 μl of blocking buffer containing anti-porin I (1:16,000) or II (1:5000) antibodies and incubated for 16 h at 4°C (final detergent concn., 0.5%). For incubation of a single strip, 0.9 ml of blocking buffer containing 0.1% Genapol X-100 was supplemented with 100 μl of anti-porin antibody/porin complexes (corresponding to 0.1 μg [lanes 2, 5, 8, 11] or 1 μg [lanes 3, 6, 9, 12], respectively, final amount of porin present during the immunoblot) or anti-porin antibody/BSA mixture (control in lanes 1, 4, 7, 10). The final detergent concentration during the immunoblotting was 0.15%. Subsequent steps of the immunoblotting were carried out as described under Materials and Methods. An autoradiogram of the blot (after having pasted the lanes together) is shown. The molecular masses indicated on the left margin were derived from marker proteins run in parallel.

II), the majority of the mitochondrial proteins from islet cells and adipocytes which partitioned into the TX-114-depleted phase (Fig. 4, lanes 1 and 6) were adsorbed by hydroxyapatite. The proteins recovered from the eluate (lanes 2 and 7) were further separated by two successive chromatographic runs on a mixture of hydroxyapatite and celite. This procedure removed most of the ADP/ATP-carrier which comigrates with porin in SDS–PAGE during the preceding purification steps (see lanes 2 and 7) from the eluate of the columns (lanes 3, 4, 8, and 9). The remaining minor contaminants in the eluate, which are in the apparent M_r range of 18,000–66,000, were eliminated by chromatography on DEAE-cellulose (lanes 5 and 10). Coomassie blue staining after SDS–PAGE revealed a single 28-kDa band that comprised ~0.15–0.30 of the total mitochondrial protein. This is somewhat less than has been reported for the mitochondrial porins of *Neurospora crassa* (35) and rat liver (36). The enrichment factors (~100- to 200-fold) and yields (~50%) of porin proteins from total mitochondria of rat islets, RINm5F cells, and adipocytes were comparable (Table II). A unique islet and adipocyte protein reacted with anti-porin antibodies in the immunoblot at each purification step (Fig. 4, lower panel). They comigrated on SDS–PAGE with the 28-kDa cross-reacting protein of total mitochondria from islets and adipocytes (lanes 1–10) but not with the 31-kDa protein from liver (lane 11).

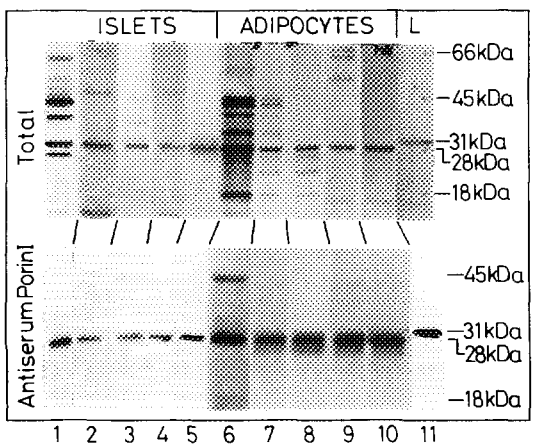

FIG. 4. Purification of the cross-reacting proteins from islet cells and adipocytes. Islet and adipocyte mitochondria were solubilized and subjected to the purification protocol as described under Materials and Methods and analyzed after each step of the isolation. Proteins recovered from the detergent-enriched phase after TX-114 partitioning (lanes 1 and 6), eluted from the HTP column (lanes 2 and 7), eluted from the first HTP/celite column (lanes 3 and 8), eluted from the second HTP/celite column (lanes 4 and 9), and eluted from DEAE-cellulose (lanes 5 and 10) were precipitated with TCA, redissolved in sample buffer, and separated by SDS–PAGE. As a control, proteins from liver mitochondria processed as above and finally eluted from DEAE-cellulose were run in parallel (lane 11). One-half of the samples were analyzed by staining the gel directly with Coomassie blue (Total, upper panel), and the gel of the other half of the samples was immunoblotted with antiserum I against rat liver porin and autoradiographed (lower panel). The molecular masses indicated on the left margin were derived from marker proteins run in parallel. The yields and relative enrichment factors for each purification step are given in Table I.

The identity of the purified proteins with porin was further examined by isoelectric focusing of the islet, adipocyte, and liver mitochondrial protein fractions at each step of the purification. Figure 5 shows that the majority of the islet, adipocyte, and liver mitochondrial proteins had p*I* values lower than p*I* 7.5 (upper panel; lanes 10, 11, and 12). In contrast, the enriched proteins were separated into two major polypeptides with p*I* values of 7.9 and 7.7, irrespective of their origin and purity (lanes 1–9). Both components were recognized by the anti-porin antibodies (lower panel, lanes 1–9). This behavior during isoelectric focusing is typical for mitochondrial porin proteins studied so far (35, 36) and indicates that the porin polypeptides exhibit charge heterogeneity. Thus, the findings of Fig. 5 support the identity of the cross-reacting and purified 28-kDa mitochondrial proteins and their relationship to porins. Interestingly, the relative abundance of the two components characterized by different p*I* values was very similar with the islet and adipocyte proteins (Fig. 5, lower panel, lanes 1–6). It differed, however, between the islet/adipocyte and liver proteins by a significant shift to the basic component consistently observed with the liver porin (lanes 7–9).

The Cross-Reacting Islet and Adipocyte 28-kDa Proteins Specifically Bind Hexokinase and Glycerol Kinase

Porin from rat liver or brain has been reported previously (4, 39) to function as hexokinase and glycerol kinase binding protein. Consequently, we studied whether the purified cross-reacting rat islet and adipocyte proteins have the ability to specifically bind hexokinase and glycerol kinase from rat liver cytosol when incorporated into asolectin vesicles. The data in Table III demonstrate that in the absence of Glc-6-P significant hexokinase activity was recovered with asolectin vesicles containing the purified liver, adipocyte, and islet 28-kDa proteins. The amount of vesicle-associated hexokinase was reduced by Glc-6-P. Glycerol kinase sedimented with the three types of reconstituted asolectin vesicles to an extent comparable to that of hexokinase. However, Glc-6-P must be present for glycerol kinase binding to occur. The opposite effects of Glc-6-P on hexokinase and glycerol kinase binding from liver cytosol, where both enzymes are present, indicate that glycerol kinase competes with hexokinase for the same binding site. Thus, binding of glycerol kinase became more prominent when binding of hexokinase was hindered by Glc-6-P. This characteristic behavior is well documented for rat liver porin (4). The specificity of the Glc-6-P-regulated binding of hexokinase/glycerol kinase to reconstituted proteoliposomes containing the islet or adipocyte 28-kDa proteins was confirmed by inhibition of binding when antibodies against purified rat liver porin had been included in the binding assay (Table IV). In contrast, preimmune serum did not affect the binding reaction. Thus, the combined experimental data strongly

TABLE II

Purification of Mitochondrial Porin from Pancreatic Islet Cells, RINm5F Cells, and Fat Cells

Step	Total protein (mg)			% of total mitochondrial protein			% of total immunoreactivity			Purification factor		
	Islets	RINm5F cells	Fat cells	Islets	RINm5F cells	Fat cells	Islets	RINm5F cells	Fat cells	Islets	RINm5F cells	Fat cells
Mitochondria solubilized with TX-114	125	34	68	100	100	100	100	100	100	1	1	1
Aqueous phase after TCA Precipitation	42	14	23	33.6	41.2	33.8	88.6	91.2	83.7	2.6	2.2	2.5
Eluate from HTP	2.52	0.85	1.54	2.02	2.50	2.26	80.2	83.9	76.1	39.7	33.6	33.7
Eluate from HTP/celite I	0.72	0.44	0.61	0.82	1.29	0.90	72.1	69.2	65.1	87.9	53.6	72.3
Eluate from HTP/celite II	0.45	0.24	0.25	0.44	0.71	0.37	60.3	54.2	56.8	137.0	76.3	153.5
Eluate from DEAE cellulose	0.32	0.15	0.19	0.26	0.44	0.28	53.9	49.2	51.2	207.3	111.9	182.9

Note. The purification steps were carried out as described under Materials and Methods. Specific immunoreactivity was determined by densitometric scanning of the autoradiograms after immunoblotting of each fraction with anti-porin I antiserum (see Materials and Methods) and calculated as arbitrary units/mg of protein applied onto the blot. The amount of immunoreactive material contained in each fraction of the purification procedure is given as a percentage of the total immunoreactivity measured for TX-114-solubilized mitochondria.

support the identity of the cross-reacting islet and adipocyte 28-kDa proteins with mitochondrial porin.

Sulfonylurea Drugs Reduce the Binding of Hexo-/Glucokinase to Porin of RINm5F Cell and Adipocyte Mitochondria

We next studied whether a specific interaction between adipocyte and islet porin proteins and hexokinase, which has been demonstrated in the reconstituted system (see above), can be observed *in vivo*. For this mitochondria were isolated from rat adipocytes and cultured β-cells which were derived from a rat insulinoma cell line (16, 17) and used instead of isolated rat pancreatic islets. The porin proteins were immunoprecipitated with anti-porin antiserum II. The immunoprecipitates were separated by SDS–PAGE and tested for the presence of porin, glucokinase, and hexokinase by immunoblotting (Fig. 6). It can be seen that the anti-porin immunoprecipitates from both RINm5F cells and adipocytes contain porin. The apparent M_r of porin protein from RINm5F cells (as identified with anti-porin antiserum I) is identical to that of islet and adipocyte porin (28,000) but lower than that of liver porin (31,000). In addition, proteins that were recognized by polyclonal antisera directed against rat liver glucokinase (in RINm5F cells, 54,000, lane 1) and yeast hexokinase (in adipocytes, 102,000, lane 8) coimmunoprecipitated. Their molecular masses are very similar to the size of rat β-cell glucokinase (40, 41) and rat brain hexokinase (42), respectively. In mammalian tissues, there are three isozymes of low K_m hexokinases, commonly referred to as Types I, II, and III (for a review see Ref. 43). These isozymes have several properties in common, including a K_m for glucose in the submillimolar range and a marked sensitivity to inhibition by the product Glc-6-P. Isozymes I–III are also similar in that they consist of a single polypeptide chain of ~100 kDa (43). These properties contrast with those of a fourth type of hexokinase found in mammalian liver and endocrine pancreas, called Type IV or "glucokinase," exhibiting lower affinity for glucose and sensitivity for inhibition by Glc-6-P (for a review see Ref. 44). The latter enzyme is similar to the hexokinase found in yeast, consisting of a single polypeptide chain of ~55 kDa (45). It has been speculated that the mammalian isozymes I–III might have evolved by a process involving duplication and fusion of a gene encoding an ancestral hexokinase similar to present-day glucokinase and yeast hexokinase (42, 46, 47). Therefore, it is not surprising that a polyclonal antiserum raised against purified yeast hexokinase cross-reacted with rat adipocyte hexokinase and shows (very weak) cross-reactivity with rat pancreatic glucokinase (data not shown). However, the latter reactivity did not interfere with our studies since rat adipocytes do not contain glucokinase to any detectable extent (40, 48). The antibody against rat liver glucokinase strongly cross-reacts with glucokinase from RINm5F cells due to the known homology between both proteins (40, 48). RINm5F cells possess a considerable amount of hexokinases I–III (49). However, as can be seen from Fig. 6, the antibody did not recognize any of these isoforms. The identity of the cross-reacting proteins with hexo- and glucokinase, respectively, was substantiated by their dramatically reduced coimmuno-

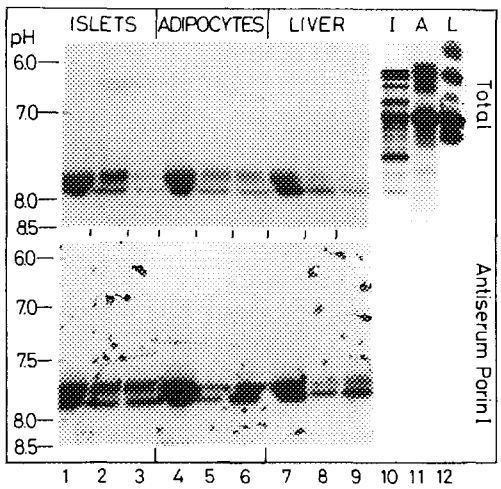

FIG. 5. Isoelectric focusing of the cross-reacting islet and adipocyte proteins and of liver porin. Mitochondrial proteins from rat islets (I), adipocytes (A), and liver (L) recovered from the detergent-enriched phase after TX-114 partitioning (lanes 10, 11, and 12), eluted from the HTP column (lanes 1, 4, and 7), eluted from the HTP/celite column (lanes 2, 5, and 8), and eluted from DEAE-cellulose (lanes 3, 6, and 9) were precipitated with TCA, dissolved in upper reservoir electrolyte, and subjected to isoelectric focusing. One-half of the samples were analyzed by staining the gel directly with Coomassie blue (Total, upper panel), and the gel of the other half of the samples was immunoblotted with antiserum I against rat liver porin and autoradiographed (lower panel). The pH values indicated on the left margin were determined using a semiautomatic pH gradient measuring apparatus.

precipitation with anti-porin antibodies after incubation of isolated mitochondria with Glc-6-P (lanes 2 and 9). Thus a portion of hexo-/glucokinase is bound to mitochondrial porin in rat adipocytes and β-cells.

The interaction of the glucose-phosphorylating enzymes with mitochondrial porin under physiological conditions is important for the control of the cytoplasmic ATP pool which is critical for the regulation of insulin secretion in β-cells and for glucose utilization in adipocytes (see Discussion). Since sulfonylurea drugs which are widely used as hypoglycemic agents for the therapy of NIDDM are known to have pancreatic (reflected in depolarization and stimulation of insulin secretion in cultured β-cells) and extrapancreatic sites of action (reflected in stimulation of glucose utilization in isolated adipocytes), we studied whether these drugs affect the interaction of hexo-/glucokinase with mitochondrial porins from RINm5F cells and rat adipocytes. Consequently, the coimmunoprecipitation experiment described above was carried out with RINm5F cells and rat adipocytes incubated with the novel efficient sulfonylurea, glimepiride (EC_{50} = 0.31 nM for depolarization of mouse β-cells; 50), the "first generation" sulfonylurea, tolbutamide ($EC_{50} \approx$ 7 μM; 51), and the pharmacologically inactive sulfonylurea derivative carboxytolbutamide (Fig. 6). Interestingly, glimepiride reduced the amount of gluco-/hexokinase coimmunoprecipitated with anti-porin antibodies from RINm5F cells (lane 3) and rat adipocytes (lane 9) to ~50–

TABLE III

Binding of Hexokinase and Glycerol Kinase to Porin Proteins from Liver, Adipocyte, and β-Cell Mitochondria

Asolectin vesicles reconstituted with	Glc-6-P	Bound to proteoliposomes	
		Hexokinase (mU/mg)	Glycerol kinase (mU/mg)
No protein	−	0.0	0.2
	+	0.1	0.1
Liver porin	−	157.4 ± 32.1	2.7 ± 0.9
	+	1.3	35.7 ± 8.9
β-Cell porin	−	24.7 ± 10.1	2.8 ± 1.3
	+	0.9	109.4 ± 51.3
Adipocyte porin	−	71.4 ± 13.2	0.7
	+	1.4	12.6 ± 3.3

Note. Purified liver, β-cell, and adipocyte porin was incorporated into asolectin vesicles. Portions of the reconstituted vesicles containing identical amounts of protein and of control vesicles lacking protein were incubated with liver cytosol in the absence or presence of Glc-6-P. The specific activity of hexokinase and glycerol kinase associated with the vesicles after centrifugation was determined. The values were obtained from four different vesicle preparations with double measurements each (±SD).

40% and 35–25%, respectively, compared to untreated cells. The residual binding of both kinases was specific since it was competed for by Glc-6-P (lanes 4 and 10). The inhibition of kinase binding to mitochondrial porin seems to be correlated with the insulin releasing potency of sulfonylureas since it was significantly less pronounced with the less active sulfonylurea tolbutamide (lanes 5 and

TABLE IV

Inhibition of Hexokinase and Glycerol Kinase Binding to Porin Proteins by Anti-porin Antibodies

Asolectin vesicles reconstituted with	Serum	% Bound to proteoliposomes	
		Hexokinase	Glycerol kinase
Liver porin	Anti-porin	1.9 ± 0.5	3.9 ± 2.5
	Control	105.3	93.8
β-Cell porin	Anti-porin	7.9 ± 4.8	9.6 ± 3.7
	Control	89.7	103.6
Adipocyte porin	Anti-porin	16.8 ± 6.7	19.7 ± 5.6
	Control	98.5	94.6

Note. Purified liver, β-cell, and adipocyte porin was incorporated into asolectin vesicles. Portions of the reconstituted vesicles containing identical amounts of porin were incubated (30 min, 4°C) in the absence or presence of anti-porin antiserum I (1:8000) or preimmune serum (control) and subsequently with rat liver cytosol in the absence (for binding of hexokinase) or presence of Glc-6-P (for binding of glycerol kinase). The specific activity of hexokinase and glycerol kinase associated with the vesicles after centrifugation was determined. The specific activity obtained in the absence of serum was set at 100%. The values are means from four different vesicle preparations with double measurements each (±SD).

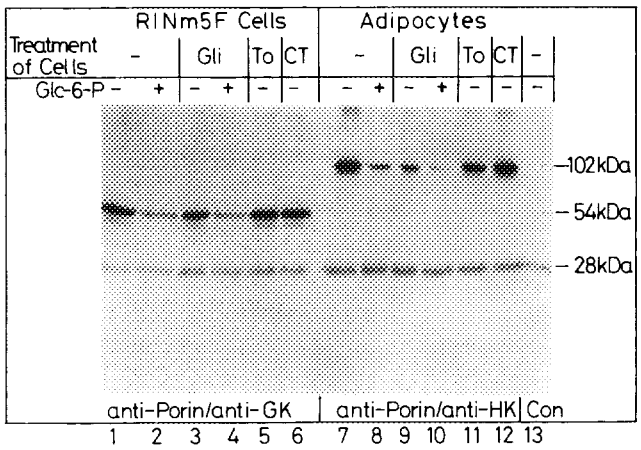

FIG. 6. Coimmunoprecipitation of gluco-/hexokinase with anti-porin antibodies from mitochondria of untreated and sulfonylurea-treated RINm5F cells and adipocytes. RINm5F cells and adipocytes in medium containing 5 mM glucose were incubated in the absence or presence of glimepiride (Gli), tolbutamide (To), or carboxytolbutamide (CT). Mitochondria were isolated, incubated without or with Glc-6-P, and centrifuged. Total SDS-solubilized mitochondrial protein was immunoprecipitated with anti-porin antiserum II, separated by SDS–PAGE, and analyzed by immunoblotting with anti-porin antiserum I alone (as a control in lane 13) or in combination with anti-glucokinase antiserum (anti-GK; lanes 1–6) and anti-hexokinase antiserum (anti-HK; lanes 7–12). An autoradiogram of the immunoblot is shown. The molecular masses indicated on the right margin were derived from marker proteins run in parallel on the same gel. The use of preimmune serum instead of anti-porin antiserum for the immunoprecipitation did not lead to any signal during the subsequent immunoblotting (not shown).

11) and absent entirely with the structurally related but pharmacologically inactive carboxytolbutamide (lanes 6 and 12). The amount of porin recovered by sequential immunoprecipitation and immunoblotting with antisera I and II against rat liver porin did not vary significantly between drug-treated and untreated cells. These data provide the first evidence that sulfonylureas interfere with the interaction between porin and glucose phosphorylating enzymes.

The sulfonylurea-induced inhibition of kinase binding to mitochondrial porin should also be reflected in a reduced recovery of hexo-/glucokinase with mitochondria isolated from sulfonylurea-treated cells. For this, mitochondria were isolated from RINm5F cells (Fig. 7a) or adipocytes (7b) which had been incubated in the absence or presence of glimepiride under various conditions, purified from loosely bound cytosolic proteins by centrifugation through a cushion of Percoll (see Materials and Methods) and then analyzed for the presence of porin and hexo-/glucokinase by immunoblotting. Figure 7 demonstrates that RINm5F and adipocyte porin which has an apparent M_r (28,000; lane 2) slightly lower than that of liver porin (31,000; lane 1) but identical to that of rat pancreatic islet porin (28,000; lane 3). In addition, the experiment proves that the antiserum directed against rat liver porin recognized identical amounts of RINm5F cell and adipocyte porin under each incubation condition (lanes 4–13). Immunoblotting of total RINm5F cell and adipocyte mitochondrial protein with antibodies against rat glucokinase/yeast hexokinase (lanes 6–13) revealed the presence of cross-reacting rat glucokinase (54 kDa) and hexokinase (102 kDa). These findings substantiate the mitochondrial association of both enzymes. The amount of mitochondria-bound kinases in glimepiride-treated RINm5F cells (Fig. 7a) and adipocytes (7b), respectively, was reduced to 55–40% and 30–20%, respectively, compared to untreated cells (compare lanes 6 and 7). This portion was further diminished by incubation of the isolated mitochondria with Glc-6-P prior to immunoblotting (7b, lanes 8 and 9). Since it has been hypothesized that sulfonylureas exert their effects on target cells primarily by causing Ca^{2+} influx into β-cells (see Discussion) and possibly also in extrapancreatic cells (52), we asked whether their inhibitory effect on kinase binding to mitochondria depends on extracellular Ca^{2+}. For this, cells were treated with glimepiride in the presence and absence of Ca^{2+} or EGTA in the incubation medium. These conditions had no effect on the amounts of gluco-/hexokinase which were recovered with mitochondria from glimepiride-treated (7a, lanes 9 and 13; 7B, lane 13) and untreated cells (7a, lanes 8 and 12; 7b, lane 12). Thus, the glimepiride effect on kinase binding is not mediated via Ca^{2+} influx. In addition, glimepiride (and carboxytolbutamide) did not diminish kinase binding if incubated with isolated mitochondria from untreated RINm5F cells and adipocytes (lanes 10 and 11).

Glimepiride Treatment of Cells Reduces the Kinase Binding Capacity of Porin after Reconstitution into Liposomes

Finally, we asked whether the sulfonylurea-induced inhibition of kinase binding to mitochondrial porin depends on some cytosolic factor different from Ca^{2+} and/or whether the drug modifies the kinase and/or porin proteins in a way leading to diminished binding affinities for one another. To discriminate between these possibilities, we tried to restore the sulfonylurea effect in a reconstituted system. If the porin protein becomes modified in response to the drug, it may be possible to observe reduced interaction between exogenously added hexokinase and isolated porin after its reconstitution into liposomes in the absence of cytosolic factors. This was tested by purification of porin from mitochondria of glimepiride-, tolbutamide-, and carboxytolbutamide-treated RINm5F cells and rat adipocytes and subsequent reconstitution into phospholipid vesicles (Fig. 8). The proteoliposomes were incubated with rat liver cytosol, then separated from soluble proteins by centrifugation and analyzed for bound rat liver hexokinase and for the presence of identical amounts of reconstituted liposomes per assay by immunoblotting with anti-hexokinase and anti-porin antibodies, respectively. Figure 8 shows that liposomes with porin purified from glimepiride- and tolbutamide-treated cells

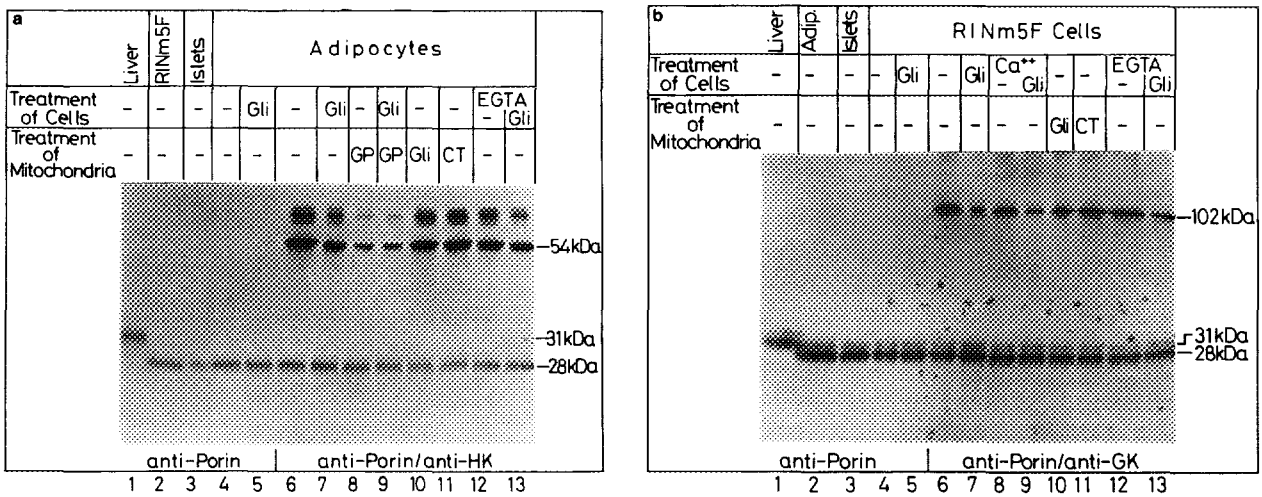

FIG. 7. Immunodetection of gluco-/hexokinase with mitochondria of untreated and sulfonylurea-treated RINm5F cells and adipocytes. RINm5F cells (a) and adipocytes (b) in medium containing 5 mM glucose and supplemented with 2 mM Ca^{2+} or 1 mM EGTA (as indicated) were incubated without or with glimepiride (lanes 4–13). Mitochondria were isolated, incubated in the absence or presence of Glc-6-P (GP; as indicated), glimepiride (Gli; lane 10), and carboxytolbutamide (CT; lane 11), and centrifuged. Total SDS-solubilized mitochondrial proteins were separated by SDS-PAGE and analyzed by immunoblotting with anti-porin antiserum I alone (lanes 4 and 5) or in combination with anti-glucokinase (anti-GK; a, lanes 6–13) and anti-hexokinase antiserum (anti-HK; b, lanes 6–13). As controls, isolated mitochondrial porin from rat liver (lane 1), rat adipocytes (a, lane 2) or RINm5F cells (b, lane 2) and pancreatic islets (lane 3) were run in parallel on the same gel and analyzed for porin by immunoblotting with anti-porin antiserum II (lanes 1–3). An autoradiogram of the immunoblot is shown.

bound 50–75% and 25–40%, respectively, less rat liver hexokinase (52 kDa) than liposomes with porin from untreated cells (compare lanes 1 with 3, and 5, 9 with 11, 13). There was no significant difference between RINm5F cells and adipocytes. Porin from carboxytolbutamide-treated cells was not impaired in kinase binding (lanes 6 and 14). The interaction of hexokinase with the proteoliposomes seems to be specific since it was competed for by excess of Glc-6-P prior to immunoblotting in untreated (lanes 2 and 10) as well as glimepiride-treated cells (lanes 4 and 12). Furthermore, only a very small amount of hexokinase was recovered from liposomes lacking porin (lane 8). Glimepiride did not affect the association of hexokinase with porin-containing liposomes when present during the binding reaction (lane 7) as has already been observed for isolated mitochondria (see Fig. 7). This excludes a direct interference of the drug with the association reaction between porin and kinase.

The results from the reconstitution experiments using immunoblotting for the detection of liposome-bound hexokinase were confirmed and extended by determination of the hexokinase and glycerol kinase activity associated with liposomes after incubation with rat liver cytosol. The liposomes have been reconstituted with porin from sulfonylurea-treated and untreated cells (Table V). Glimepiride and significantly less tolbutamide, but not carboxytolbutamide, diminished the liposome-associated hexokinase activity and, to similar degree, also the glycerol kinase activity (detected only in the presence of Glc-6-P) when present during incubation of the cells. They were ineffective when present during the binding reaction. The inhibition of glucose metabolism by the presence of non-metabolizable glucose analogs (2-deoxyglucose, 3-*O*-methylglucose), the presence of pyruvate (instead of glucose), or the absence of extracellular Ca^{2+} during drug treatment of the cells did not antagonize the reduced hexokinase binding affinity of reconstituted porin to a significant extent (Table VI). The latter data corroborate the results from immunoblotting of hexo-/glucokinase using mitochondria from Ca^{2+}/EGTA-treated cells (Fig. 7). The relative efficiencies of inhibition of kinase binding to reconstituted proteoliposomes by glimepiride were in good agreement between the immunological and the activity assays. This correlation provides additional evidence for the identity of the 102-kDa adipocyte and the 54-kDa RINm5F cell proteins with rat adipocyte hexokinase and pancreatic glucokinase, respectively. The reconstitution experiments demonstrate that the inhibitory effect of glimepiride on the interaction between mitochondrial porin and glucose phosphorylating enzymes relies on a drug-induced modified state of the porin molecule itself.

DISCUSSION

Porin proteins in mitochondria have been identified and characterized from lower (yeast, see Ref. 53; *N. crassa,* see Ref. 54) and higher eucaryotes (rat liver, see Refs. 3, 36, 55) and plants (mung bean seedlings, see Ref. 56). They are nuclear-coded, synthesized in the cytoplasm, and post-translationally inserted into the outer membrane of mitochondria (57–59). Their capability of spontaneous insertion into artificial lipid bilayers (60, 61) is accom-

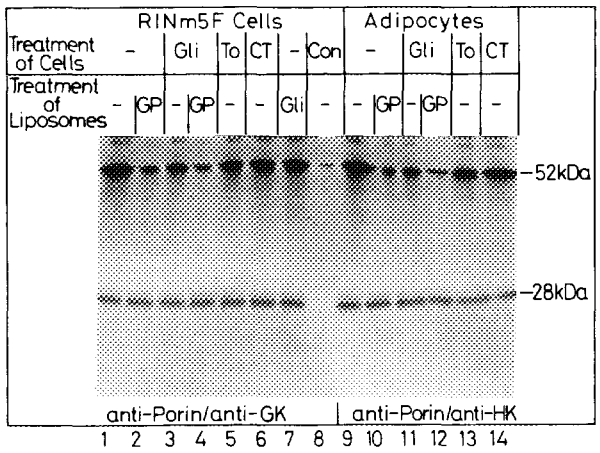

FIG. 8. Binding of hexokinase to proteoliposomes reconstituted with porin proteins from untreated and sulfonylurea-treated RINm5F cells and adipocytes. RINm5F cells (lanes 1–8) and rat adipocytes (lanes 9–14) in medium containing 5 mM glucose were incubated in the absence or presence of glimepiride (Gli), tolbutamide (To), or carboxytolbutamide (CT). The porin proteins were isolated from the cells and reconstituted into phospholipid vesicles as described under Materials and Methods. The proteoliposomes were incubated with rat liver cytosol in the absence or presence of Glc-6-P (GP; lanes 2, 4, 10, and 12) and glimepiride (Gli; lane 7) and centrifuged. The liposome pellets were solubilized in sample buffer. The proteins were separated by SDS–PAGE and analyzed by immunoblotting with anti-porin antiserum I in combination with anti-glucokinase (anti-GK; lanes 1–8) or anti-hexokinase antiserum (anti-HK; lanes 9–14). As a control, liposomes lacking porin were incubated with rat liver cytosol (lane 8) prior to immunoblotting. An autoradiogram of the immunoblot is shown. Molecular masses indicated were derived from marker proteins run in parallel.

panied by the formation of voltage-dependent anion-selective channels (62) which possibly exhibit an asymmetric gating response with respect to voltage. Porin proteins are characterized by an apparent M_r in the range of 30,000 and charge heterogeneity during isoelectric focusing (35, 36).

The present study demonstrates the existence of unique proteins in the mitochondria of RINm5F cells, rat islets, and adipocytes which cross-react with two different antibodies directed against rat liver porin. This cross-reactivity, the subtle differences in the apparent M_r and pI value between the rat β-cell/adipocyte and liver porins (28,000 vs 31,000; relative abundance of the pI 7.7 vs 7.9 component), and the inability to differentiate between the islet and the adipocyte porins by SDS–PAGE and isoelectric focusing suggest that the islet and adipocyte proteins are very similar or even identical to one another and represent isoforms of liver mitochondrial porin. The following points render it unlikely that the 28-kDa β-cell/adipocyte porins are proteolytic degradation products of a 31-kDa "liver type" porin:

(i) Protease inhibitors were included in the homogenization buffer and present during all stages of mitochondria preparation and porin isolation.

(ii) The 28-kDa porins were identified in islet, RINm5F cell, and adipocyte homogenates prepared by the addition of SDS-containing sample buffer and boiling. During cell fractionation and purification of porin no immunoreactive degradation products of the 28-kDa porins were detected. The relative abundance of porin in mitochondria from rat islets and adipocytes calculated from the purification protocol agrees well with reported values for other sources (35, 36, 55) and thus argues against the appearance of significant amounts of non-cross-reacting degradation products.

(iii) Under the same conditions for fractionation of liver cells and purification of liver porin, no degradation was observed. Proteolysis leading to apparently identical 28-kDa degradation products in islets and adipocytes and restricted to these tissues seems unlikely.

(iv) Porin embedded into the outer mitochondrial membrane is very resistant to proteolytic attack (63, 64).

Recent studies from several laboratories revealed tissue-specific porin isoforms which differ both in pI value and apparent M_r (65–67). In contrast to the 28-kDa β-cell/

TABLE V

Effect of Sulfonylureas on the Binding of Hexokinase and Glycerol Kinase to Reconstituted Porin Proteins from RINm5F Cells and Adipocytes

Pretreatment of cells	Reconstituted porin	Additions during binding	Bound to proteoliposomes: Hexokinase	Bound to proteoliposomes: Glycerol kinase
None	RINm5F cells	—	13.9 ± 3.9	1.5 ± 0.9
		Glc-6-P	0.2 ± 0.1	45.7 ± 15.1
		Gli	15.2 ± 6.3	1.9 ± 1.1
None	Adipocytes	—	65.6 ± 10.5	0.4 ± 0.2
		Glc-6-P	2.2 ± 0.5	19.8 ± 4.4
		Gli	78.3 ± 26.4	2.4 ± 0.2
Gli	RINm5F cells	—	7.3 ± 2.2	1.1 ± 0.7
		Glc-6-P	0.4 ± 0.2	25.5 ± 19.8
	Adipocytes	—	22.5 ± 5.7	0.7 ± 0.4
		Glc-6-P	1.6 ± 0.8	9.4 ± 5.9
To	RINm5F cells	—	10.5 ± 2.5	1.9 ± 0.9
		Glc-6-P	0.7 ± 0.4	32.2 ± 22.3
	Adipocytes	—	44.7 ± 7.7	0.9 ± 0.5
		Glc-6-P	2.5 ± 1.1	11.4 ± 4.9
CT	RINm5F cells	—	17.7 ± 5.1	1.9 ± 1.2
		Glc-6-P	0.7 ± 0.2	53.7 ± 19.3
	Adipocytes	—	75.6 ± 21.3	0.9 ± 0.4
		Glc-6-P	3.4 ± 1.4	24.7 ± 6.2

Note. RINm5F cells and adipocytes were incubated in medium containing 5 mM glucose in the absence or presence of glimepiride (Gli), tolbutamide (To), or carboxytolbutamide (CT). The porin proteins were isolated from these cell and reconstituted into asolectin vesicles as described under Materials and Methods. The vesicles were incubated with rat liver cytosol in the absence or presence of Glc-6-P or glimepiride (Gli) and then centrifuged. The specific activity of hexokinase and glycerol kinase associated with the vesicles was determined. The values are means from four different vesicle preparations with double measurements each (±SD).

TABLE VI

Effect of Ca^{2+} and Glucose Metabolism on the Sulfonylurea-Induced Inhibition of Hexokinase Binding to Porin

Pretreatment of cells	Additions during pretreatment	Reconstituted porin	Glc-6-P during binding	Hexokinase bound to proteoliposomes	
				Total	% Inhibition
None	Glc + Ca^{2+}	RINm5F cells		14.6 ± 2.7	
	Glc + EGTA			13.2 ± 3.5	
	Pyr + Ca^{2+}			12.5 ± 2.5	
	Deoxyglc			15.7 ± 3.9	
	Glc + Ca^{2+}	Adipocytes		71.0 ± 13.9	
	Glc + EGTA			63.9 ± 17.5	
	Pyr + Ca^{2+}			78.9 ± 19.8	
	Deoxyglc			65.4 ± 20.3	
Gli	Glc + Ca^{2+}	RINm5F cells	−	7.9 ± 2.5	45.9
	Glc + EGTA		−	6.8 ± 1.9	48.5
	Pyr + Ca^{2+}		−	5.9 ± 4.4	47.2
			+	2.6 ± 0.9	
	Deoxyglc		−	8.4 ± 2.8	46.5
			+	5.3 ± 3.1	
	Methylglc		−	9.1 ± 2.2	42.1
			+	1.7 ± 0.5	
	Glc + Ca^{2+}	Adipocytes	−	24.7 ± 6.6	65.2
	Glc + EGTA		−	20.3 ± 5.1	68.3
	Pyr + Ca^{2+}		−	28.5 ± 18.5	56.1
			+	10.1 ± 3.5	
	Deoxyglc		−	27.4 ± 5.2	60.7
			+	3.9 ± 1.1	
	Methylglc		−	16.7 ± 7.8	74.4
			+	6.8 ± 1.6	

Note. RINm5F cells and adipocytes were incubated in medium lacking a carbon source (2 h, 30°C) and then in medium containing 5 mM glucose (Glc), 20 mM pyruvate (Pyr), (10 mM 2-deoxyglucose (Deoxyglc), or 10 mM 3-*O*-methylglucose (Methylglc) and 2 mM Ca^{2+} or 1 mM EGTA in the absence or presence of glimepiride (Gli). The porin proteins were isolated from these cells and reconstituted into asolectin vesicles as described under Materials and Methods. The liposomes were incubated with rat liver cytosol in the absence or presence of Glc-6-P and then centrifuged. The specific activity of hexokinase associated with the liposomes was determined. The values are means from four different vesicle preparations with double measurements each (±SD). The percentage inhibition of hexokinase binding induced by glimepiride under each condition was calculated. The specific activity of bound hexokinase under each condition in the absence of glimepiride was set at 100%. The hexokinase activity recovered with vesicles lacking porin was subtracted from each value.

adipocyte mitochondrial porin (whose major components show p*I* = 7.7 and 7.9), the human porin (Porin 31HL) purified from B-lymphocytes and identified by its primary structure exhibits a relative molecular mass of 30,641 Da and charge heterogeneity with five different isoelectric points (the major components showing p*I* = 7.2 and 7.5) and is located predominantly at the plasma membrane as shown by indirect immunofluorescence (65, 66). The relationship between the two human porin isoforms (HVDAC1 and 2) identified recently by cDNA cloning and expression in yeast (67) and the rat porin isoforms described in the present report needs to be clarified by determination of their primary structure. However, only HVDAC1 has the capability of specific hexokinase binding arguing against a close similarity of structure and physiological role between HVDAC2 and the rat isoforms.

The functional significance of the putative β-cell/adipocyte and liver isoforms of mitochondrial porin remains to be elucidated. In this respect, it should be underlined that the portion of total cellular hexo-/glucokinase associated with mitochondria strongly depends on the type of tissue studied. Subfractionation experiments performed on rat kidney, liver, and brain revealed that the particulate form of hexokinase distributes with microsomal rather than mitochondrial marker proteins (68–70). These results on normal tissues differ from reports on highly glycolytic tumor cells, the AS-30D and Novikoff hepatoma cell lines, where the particulate location of the Type I isoform is usually regarded as exclusively mitochondrial (8, 9, 69). In these mitochondria it is specifically associated with the outer membrane via porin (71). Recent studies presented evidence that, in pancreatic islets, mitochondrial binding of kinases is not restricted to hexokinase (6, 10) and glycerol kinase (72), but was also observed for glucokinase, in sharp contrast to the situation found in liver extracts (10, 11). The present subfractionation data

extend the list of mitochondrially bound glucose phosphorylating enzymes to glucokinase in a rat insulinoma cell line (RINm5F) and to hexokinase in rat adipocytes. Furthermore, they demonstrate by immunological methods that under physiological conditions the receptor for both kinases may involve, at least in part, the porin molecule. This is in agreement with the hexokinase-binding capacity of purified β-cell/adipocyte porin after reconstitution into liposomes. But obviously, these findings alone cannot be taken as evidence for an interaction between hexokinase and porin in its tissue of origin as is exemplified by rat liver hexokinase/porin. They interact in a reconstitution assay (4) but fail to do so *in vivo* (69). Thus, different isoforms of mitochondrial porin in mammalian tissues may have the ability to bind different enzymes which are involved in high-energy phosphate transfer and couple oxidative phosphorylation to the nutritional situation of the cell (73). The underlying tissue specificity may be determined in part by the growth behavior and glycolytic rate of the cells. These parameters differ considerably between rat liver, RINm5F cells, and rat adipocytes and may be reflected in part in the different portions of porin-associated hexo-/glucokinase. This point merits exact quantitation under more defined experimental conditions.

Previous studies indicated that in skeletal muscle (14), the highly glycolytic AS-30D hepatoma cell line (7), and pancreatic β-cells (13) hexokinase bound to the outer mitochondrial membrane has preferred access to ATP generated in the inner compartment. That is, hexokinase bound to the outer membrane of mitochondria when given a choice prefers to phosphorylate glucose with ATP provided by oxidative phosphorylation rather than ATP provided externally (i.e., cytosolic ATP). Since it is generally believed that most small metabolites gain entry to and leave mitochondria via porin (60), the interaction of glucose phosphorylating enzymes with porin may provide the molecular basis for their preferred access to ATP synthesized via oxidative phosphorylation.

The interaction of hexokinase with mitochondria, *in vitro*, has been shown to be influenced by various cellular constituents. Glc-6-P and ATP promote the release of bound hexokinase, whereas phosphate and magnesium antagonize this liberation process (74, 75). Evidence that changes in the intracellular hexokinase distribution take place *in vivo* is available in tumor cells incubated with an anesthetic (76). Accordingly, this phenomenon may well play a decisive role in energy metabolism of cancer cells as has been proposed in the brain (77). Our findings that incubation of RINm5F cells and rat adipocytes with sulfonylureas interferes with the association between gluco-/hexokinase and porin implicate that this interaction has physiological significance, may underlie regulation, and represents a target for certain drugs in these cells.

It is now unanimously accepted that the entry of glucose into pancreatic β-cells is followed by an acceleration of the glucose metabolism (78, 79) that generates one (ATP) or several signals which close ATP-K^+-channels (for a review see Ref. 80). This in turn activates via a series of events an effector system responsible for exocytosis of insulin granules and thus stimulates insulin secretion (for a review see Ref. 81). The hypoglycemic sulfonylurea drugs bind to receptors at the surface of β-cells (82, for a review see Ref. 83), which causes closure of ATP-K^+-channels and ultimately insulin release (for a review see Ref. 84). However, sulfonylurea drugs are hydrophobic molecules, in general, which are able to cross the plasma membrane and slowly accumulate in the cytoplasm (85–87). They may therefore affect exocytosis of insulin granules by an additional mechanism independent of direct inhibition of the plasma membrane K^+-ATP-channels. In fact, there is some preliminary experimental evidence for this possibility (88, 89). Our findings indeed suggest that glimepiride, by causing dissociation of glucokinase from mitochondria, may affect, at least transiently, the intracellular ATP distribution, as ruled by the consumption of either cytosolic or mitochondrial ATP in the glucokinase-catalyzed phosphorylation of D-glucose. It should be underlined, however, that glimepiride fails to affect the metabolism of D-glucose in intact islets over prolonged incubation (90).

In contrast to the pancreatic β-cell, the primary site of sulfonylurea action, these drugs stimulate glucose transport and metabolism in isolated rat adipocytes (for a review see Ref. 91). This contributes to the so-called extrapancreatic effects which may be involved in their long-lasting hypoglycemic potency in the absence of adequately elevated plasma insulin levels in diverse animal models and humans (92). Specific binding sites for glibenclamide have been identified in rat adipocyte plasma membranes (93), but they are of low affinity and the fat cell plasma membrane lacks an ATP-K^+-channel. Thus fat cells, like β-cells, may possess an intracellular target for sulfonylurea action, which relies on the dissociation of hexokinase from mitochondrial porin. The mechanistic linkage between this glimepiride action and its ability to stimulate glucose transport, Glut translocation from intracellular stores to the plasma membrane (94), and glucose metabolism (G. Müller and S. Wied, in preparation) remains to be elucidated.

The molecular mechanism by which glimepiride reduces gluco-/hexokinase binding to porin is unclear. For β-cells, participation of the ATP-K^+-channel and of Ca^{2+} influx seems to be unlikely since Ca^{2+} in the incubation medium was not required. A rise in cytosolic Glc-6-P, which antagonizes gluco-/hexokinase binding to mitochondria, can be excluded since glucose metabolism is not a prerequisite for the glimepiride effect. Moreover, the participation of soluble factors seems unlikely, since the reduced binding of gluco-/hexokinase to porin from glimepiride-treated cells is preserved after its reconstitution into liposomes. This provides evidence that glimepiride impairs the ability of the porin molecule itself to bind the kinases. Obviously,

the state of reduced affinity can be preserved during the isolation and purification procedure, arguing for a post-translational modification of porin induced by glimepiride. However, so far there is no evidence that porin represents a target for covalent modification. For instance, we did not observe phosphorylation of porin in RINm5F cells and rat adipocytes in the absence or presence of glimepiride under various experimental conditions (data not shown). In this context it is worth mentioning that glimepiride stimulates a glycosyl-phosphatidylinositol-specific phospholipase C (GPI-PLC) in 3T3 adipocytes within the same concentration range shown here to be effective in causing hexokinase release from rat adipocyte mitochondria (38). The cleavage products of the GPI-PLC, phosphoinositol-glycans (PIG), or phosphoinositol-glycan-peptides have been shown to activate certain key enzymes of glucose metabolism, like mitochondrial pyruvate dehydrogenase and pyruvate dehydrogenase phosphatase (95, 96), if incubated with isolated rat adipocytes or adipocyte mitochondria. This mechanism requires transport of PIG structures across the mitochondrial membranes into the matrix compartment.

Our present working hypothesis for the molecular action of glimepiride on porin in extrapancreatic cells implies that soluble, negatively charged PIG structures are transported from the cytoplasm into mitochondria via the outer membrane anion channel, porin. During passage, some of the PIG molecules become associated with porin in a manner which resists the (nondenaturing) conditions of our porin isolation procedure. The binding of the PIG structures to porin reduces its affinity for hexokinase. We are now trying to find experimental support for this speculation.

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