Sulfonylureas Rapidly Cross Phospholipid Bilayer Membranes by a Free-Diffusion Mechanism

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Because sulfonylureas directly activate the exocytotic machinery, we were interested in the extent to which these compounds penetrate the β-cell plasma membrane and the underlying molecular mechanism(s). We now provide evidence that sulfonylureas cross phospholipid bilayer membranes rapidly and effectively by a free-diffusion mechanism. Two sulfonylurea compounds investigated by ^{1}H nuclear magnetic resonance spectroscopy, glibenclamide and tolbutamide, were found to incorporate into phospholipid bilayers, with the ionizable sulfonamide exposed to the aqueous interface and its apparent dissociation constant (pK_a) increased to ~7.0. Diffusion of weak amphiphilic acids across membranes is associated with a measurable change in pH. Thus, by using a fluorescence-based pH assay, we could investigate the diffusion of sulfonylurea compounds across phospholipid bilayer membranes. A fluorescent pH indicator (pyranin or [2′,7′-bis (2-carboxyethyl)-5(6)-carboxyfluorescein] [BCECF]) was trapped in egg phosphatidylcholine vesicles. Addition of glibenclamide decreased internal pH (pH_{in}), and addition of albumin reversed this drop by 50%. With the same amount of tolbutamide, the decrease in pH_{in} was much smaller, primarily because of the lower partitioning of tolbutamide into phospholipid bilayers. Using similar protocols, we also demonstrated diffusion by the same mechanism across the β-cell plasma membrane. Thus, we now provide a molecular mechanism by which sulfonylureas can penetrate the plasma membrane and reach intracellular sites regulating exocytosis. *Diabetes* 52:2526–2531, 2003

Sulfonylureas are drugs that stimulate secretion of insulin from the pancreatic β-cells (1,2) and are therefore used extensively in the treatment of type 2 diabetes. It is well established that sulfonylureas stimulate insulin release by interacting with the high-affinity 140-kDa SUR1 protein of the ATP-regulated K^+ channel at the cytoplasmic leaflet of the plasma membrane. This interaction closes the channel, causing membrane depolarization, the opening of voltage-gated L-type Ca^{2+} channels, an increase in cytoplasmic-free Ca^{2+} concentration, and the activation of the secretory machinery (3,4). We have also shown that sulfonylureas stimulate insulin exocytosis by directly interacting with the secretory machinery and not through closure of the plasma membrane ATP-regulated K^+ channel (5–7). This effect may constitute part of the therapeutic benefits of sulfonylureas and contribute to their hypoglycemic action in diabetes. Although this direct effect of sulfonylureas on insulin exocytosis is now well established and suggestions for underlying molecular mechanisms have been put forward (8), the physiological relevance of these findings are still in question. This skepticism originates primarily from the difficulty in envisaging how the sulfonylureas bypass the β-cell plasma membrane rapidly and effectively, thereby interacting with intracellular binding sites involved in the regulation of exocytosis. Indeed, earlier studies suggested that sulfonylureas do not penetrate into β-cells and therefore only interact with specific binding sites in the plasma membrane. Nevertheless, some studies have clearly demonstrated that the second-generation sulfonylurea glibenclamide accumulates progressively in the β-cell. Moreover, autoradiography studies have shown that sulfonylureas are internalized by the β-cell and bind to intracellular sites such as secretory granules (5,9–12).

In view of our own findings that sulfonylureas directly activate the exocytotic machinery (5–8), we were interested in clarifying the extent to which these compounds penetrate the β-cell plasma membrane and the underlying molecular mechanism(s). We now provide evidence that sulfonylureas cross phospholipid bilayer membranes rapidly and effectively by a free-diffusion mechanism.

RESEARCH DESIGN AND METHODS

Materials. Egg phosphatidylcholine was purchased from Avanti Polar Lipids (Pelham, AL). Tolbutamide and glibenclamide were purchased from Sigma (St. Louis, MO). Pyranin (8-hydroxy-1,3,6-pyrenetrisulfonate), [2′,7′-bis (2-carboxyethyl)-5(6)-carboxyfluorescein] (BCECF), and its acetoxymethylester (BCECF-AM) were purchased from Molecular Probes (Eugene, OR). D_2O was purchased from Cambridge Isotope Labs (Boston, MA), and G-25 Sephadex gel was from Amersham Pharmacia Biotech (Uppsala, Sweden). Sodium

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BCECF, [2′,7′-bis (2-carboxyethyl)-5(6)-carboxyfluorescein]; BCECF-AM, acetoxymethylester to BCECF; FA, fatty acid; LUV, large unilamellar vesicle; MES, 2-morpholinoethanesulfonic acid; NMR, nuclear magnetic resonance; pH_{in}, internal pH; pK_a, dissociation constant; SUV, small unilamellar vesicle; TSP, sodium 3-(trimethylsilyl)[2,2,3,3-^{2}H4] propionate (trimethylsilyl)[2,2,3,3-^{2}H4] propionate.

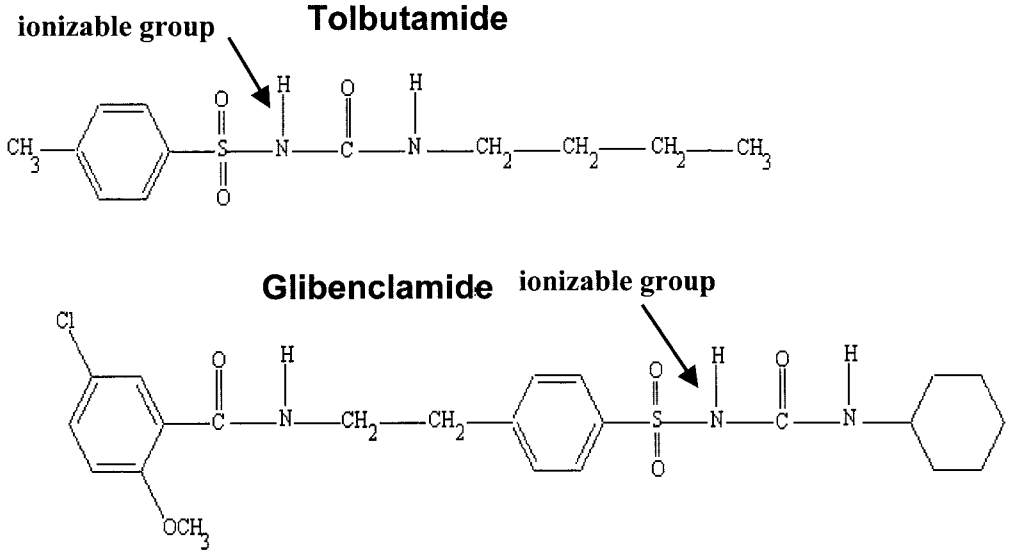

FIG. 1. Structure of tolbutamide and glibenclamide showing the ionizable proton.

3-(trimethylsilyl)[2,2,3,3-$^2H_4$] propionate (TSP) was from Wilmad (Buena, NJ). Fatty acid–free albumin was from Sigma.

Sample preparation for ^{1}H nuclear magnetic resonance experiments. Tolbutamide was dissolved (3.5 mmol/l final concentration) in 10 mmol/l 2-morpholinoethanesulfonic acid (MES) buffer with 50 mmol/l KCl. An equivalent amount of NaOH was added to dissolve all tolbutamide, and D_2O was added for nuclear magnetic resonance (NMR) analysis. After each measurement, the sample was removed from the NMR tube to adjust the pH to the desired value with small (5-μl) aliquots of 0.1 mmol/l HCl or 0.1 mmol/l NaOH. The sample of 20 mol% tolbutamide (0.2 mol tolbutamide/mol phospholipid) incorporated in small unilamellar vesicles (SUVs) was prepared as follows: 45 mg egg phosphatidylcholine in chloroform was mixed with 3.0 mg tolbutamide. The chloroform was evaporated under nitrogen, and the sample was lyophilized for 1 h; 1.62 ml of 0.5% wt/vol of KCl and 0.18 ml D_2O were added to the lyophilized sample, and the sample was allowed to hydrate for 2 h at 4°C. The solution was then sonicated (Branson 350 sonifier) in an ice/water bath for 1 h using a microtip and a 30% duty cycle. The sample of 10 mol% glibenclamide (0.1 mol glibenclamide/mol phospholipid) incorporated into the SUV was prepared by adding a small aliquot of glibenclamide in DMSO to SUV prepared as above except without any sulfonylurea added. Samples were titrated from low to high pH by adding 1- to 2-μl aliquots of 1 mol/l KOH. The pH was measured in the NMR sample with a Beckman pH meter fitted with a glass micro-electrode.

^{1}H NMR experiments. NMR experiments were performed on a Bruker DMX 500 and a Varian 400-MHz spectrometer. One-dimensional ^{1}H NMR experiments were performed at 20°C with 500–700 μl of sample containing TSP as an internal chemical-shift standard, using a "zgpr" pulse sequence. The frequency of the water presaturation pulse (O_1) was calibrated for each experiment because its value is pH dependent. The downfield phenyl ring chemical shift of both tolbutamide and glibenclamide protons was measured and plotted against the pH of the solution. All dissociation constant (pK_a) determinations were done by plotting chemical shift values versus bulk pH (13) and curve fitting to a sigmoidal curve. The pH value corresponding to the midpoint of this curve is the apparent pK_a of the sample.

Preparation of samples for fluorimetric assays. SUVs were prepared as above except with pyranin or BCECF present; large unilamellar vesicles (LUVs) were prepared by extrusion as described previously (14). Untrapped pH dye was removed by gel filtration (Sephadex G25), and the phospholipid concentration was determined as described previously (14,15). Clonal pancreatic β-cells (HIT) were cultured and prepared for experiments as before (16). Cells were incubated with 1 μmol/l BCECF-AM for 30 min. Untrapped BCECF and external albumin were removed by three wash steps with Krebs buffer without albumin (16).

Fluorimetric assays. The fluorometric assays were performed on a FluoroMax 2 fluorometer as described in detail before (14,15).

RESULTS

NMR titrations. Sulfonylureas (Fig. 1) are weak acids, with pK_a values of 5.43 (tolbutamide) and 5.3 (glibenclamide) in aqueous solution (17). The pK_a of the sulfonamide group by itself is 10.1 in solution but is lowered to between 5 and 6.5 when the group is next to a carbonyl. Because ionization is difficult to monitor by ^{1}H NMR of the ionizing hydrogen but can be reflected in the chemical shifts of nearby groups in weak acids (13), we measured the downfield chemical shift of a phenyl-ring hydrogen of both tolbutamide and glibenclamide that are ortho to the sulfonamide group (Fig. 1).

The pK_a of tolbutamide was determined both in solution and incorporated in the phospholipid bilayer at a ratio of 20 mol% (0.2 mol tolbutamide/mol phospholipid). The determinations were made as comparable as possible by dissolving similar amounts of tolbutamide in solution and in the vesicle suspension. We found that the pK_a of tolbutamide in solution was 5.41 and was shifted up to 6.64 in the phospholipid bilayer (Fig. 2). This upward shift is similar to that found for fatty acids (FAs) and other weak

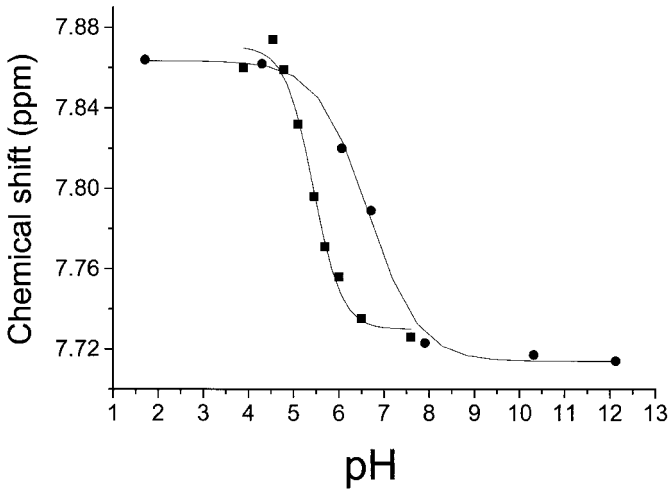

FIG. 2. NMR titrations of tolbutamide. ■, 2.75 mmol/l tolbutamide in water; ●, 5.8 mmol/l tolbutamide in SUVs (0.2 mol tolbutamide/mol phospholipid). The chemical shift of the phenyl ring ortho to the sulfonamide group was monitored as a function of pH. The data were fit to a sigmoidal curve, and the midpoint was taken as the pK_a.

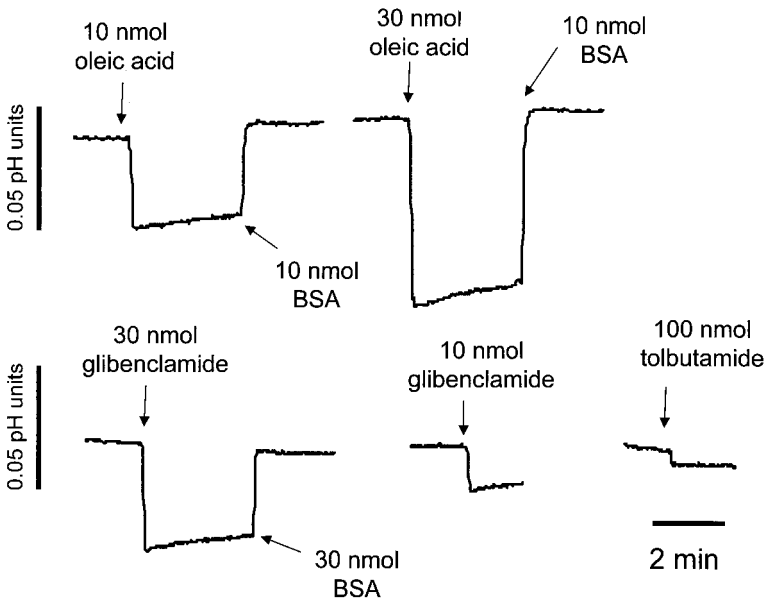

FIG. 3. Flip-flop of sulfonylureas in phospholipid bilayers. Additions of different amounts of oleic acid (upper traces) and sulfonylureas (lower traces) to a suspension of LUVs with trapped pyranin. The changes in pH_{in} as monitored by the pyranin fluorescence reflect flip-flop of the non-ionized form of the sulfonylureas. Relative to oleic acid, larger amounts of sulfonylureas had to be added to yield similar pH changes because of the weaker partitioning of sulfonylureas into the bilayer. Addition of BSA reversed the pH effects as a result of extraction of oleic acid or glibenclamide from the bilayer. The traces shown are individual runs in the fluorimeter; these experiments were done at least twice on the same day and subsequently repeated with three different vesicle preparations.

lipophilic acids (18). Sulfonylurea derivatives are amphiphilic molecules and have limited solubility in pH 7.4 HEPES buffer (19) and, in the presence of SUVs, become incorporated into the phospholipid bilayer structure. Additional evidence for the incorporation of tolbutamide in the phospholipid bilayer comes from the differences in chemical-shift values of the phenyl-ring hydrogens at most pH values (Fig. 2) and line-width changes (not shown).

We were unable to obtain spectra for aqueous glibenclamide because of its very low solubility in water. However, glibenclamide could be incorporated into phospholipid vesicles, and we attempted to measure its pK_a in SUVs by the same method we used for tolbutamide. Beginning at a high pH (10.63) with a translucent solution and titrating to a lower pH, we visually observed increasing turbidity and progressive line-broadening of the signals for the phenyl ring next to the sulfonylurea moiety (not shown). The peaks were broadened beyond detection at pH 5.44, so that only a rough estimate of the pK_a (~7.0) was possible.

Fluorescence measurements. Previously, we developed a simple fluorescent pH assay to monitor the diffusion of FAs and other weak lipophilic acids, such as bile acids, across a protein-free phospholipid bilayer (14,15). Because of the increased pK_a in the phospholipid interface, sulfonylureas also exist almost equally in non-ionized and ionized forms at neutral pH.

To test whether the same mechanism (flip-flop) is feasible for sulfonylureas, we added small aliquots (1 mmol/l dissolved in DMSO or ethanol) of glibenclamide or tolbutamide to a suspension of vesicles with trapped pyranin. The observed drop in fluorescence intensity (Fig. 3) is due to a drop in pH inside the vesicles. As with FAs, the decrease in internal pH (pH_{in}) began immediately upon the addition of the sulfonylurea and was complete within 1 s—the time resolution of the measurement (15). Addition of FA-free BSA to the external buffer of SUV with oleic acid, glibenclamide (Fig. 3), or tolbutamide (not shown) resulted in a rapid return to the initial fluorescence intensity. BSA reportedly has three binding sites for tolbutamide, with a K_d of 21 μmol/l (20), and the rise in pH reflects extraction of sulfonylurea from the SUVs (accompanied by flip-flop). The observed drop in fluorescence intensity for tolbutamide was much smaller than that for the same amount of added glibenclamide (Fig. 3), indicating decreased transport of tolbutamide, most likely due to the lower partitioning of tolbutamide into the membrane.

The fluorescence results for both sulfonylureas were reproduced in numerous additional experiments and were independent of whether the trapped pH dye was pyranin

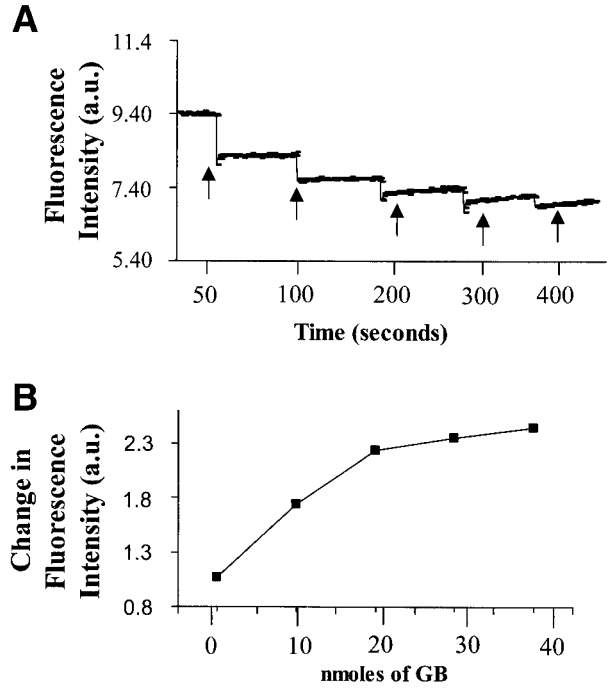

FIG. 4. Dose-dependent changes in fluorescence after addition of glibenclamide. A: Fluorescence intensity responses of pyranin entrapped in SUVs to glibenclamide added to the external buffer; 10 nmol of glibenclamide was added to the same sample at the points indicated by each arrow. *B*: Maximum change in pyranin fluorescence estimated from the data in *A*, subsequent to the addition of the indicated amounts of glibenclamide. Traces shown are representative of three trials. a.u., arbitrary units.

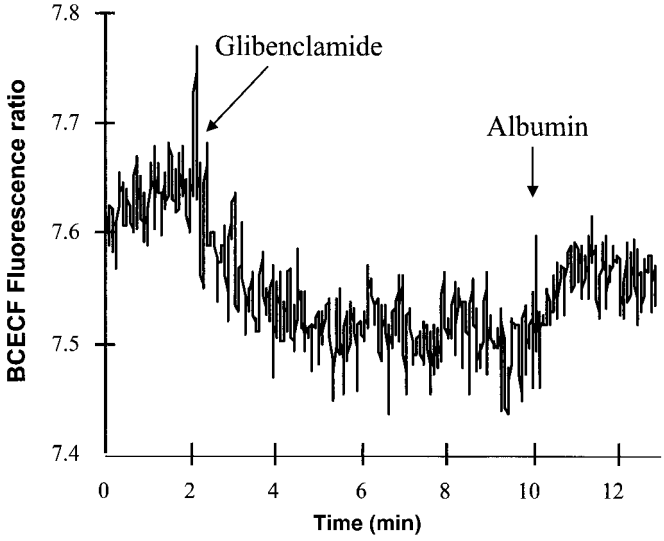

FIG. 5. Diffusion of glibenclamide to the cytosolic side of insulin-secreting HIT cells. Glibenclamide (150 nmol) was added to HIT cells with trapped BCECF (10^6 cells/ml in a stirred cuvette; 2.2 ml total volume). At 10 min, 50 nmol of BSA was added to the suspension. The ratio of the pH-dependent and pH-independent fluorescence was sampled every 2 s. The experiment was repeated twice with three different HIT cell preparations. Other doses (50 and 100 nmol) showed the same pattern.

or BCECF and whether the model membranes were SUV or LUV. The change in fluorescence was consistently nonlinear and progressively smaller with increasing concentration of added sulfonylurea, as illustrated for glibenclamide in Fig. 4. This pattern is due to the build-up of a pH-gradient across the phospholipid bilayer, as previously demonstrated for FAs in protein-free phospholipid vesicles, and provides further evidence for the flip-flop mechanism (14).

To assess the physiological relevance of the flip-flop of sulfonylurea observed in model membranes, we monitored the intracellular pH of insulin-secreting (HIT) cells. When glibenclamide was added to a suspension of 2 ml of cells preloaded with BCECF, the pH_{in} dropped ~0.1 pH units in a time course of 3 min (Fig. 5). On addition of albumin, the pH_{in} partially recovered. As with FAs (16), the pH_{in} changes after exposure of HIT cells to glibenclamide were slower than those observed in SUVs (Fig. 2).

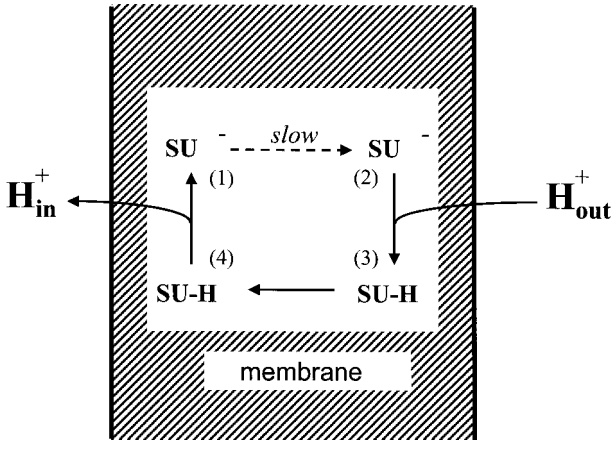

FIG. 6. Model of flip-flop of sulfonylureas across a phospholipid bilayer. It is postulated that sulfonylureas can be in four states: with the ionizable group either at the inside (states 1 and 4) of the membrane or at the outside (states 2 and 3). When sulfonylureas are added at the outside, they rapidly redistribute among the four states. Because flip-flop of the ionized form is slow, this leads to transport of about one H^+ per four sulfonylurea molecules added. SU, sulfonylurea; SU-H, protonated SU.

DISCUSSION

In recent years, many studies have proposed a role for transport proteins in the delivery of FAs to cells, and the importance of free diffusion for the transport of FAs across the phospholipid bilayer has been a topic of debate (21–25). Sulfonylureas are good candidates for the investigation of the free-diffusion model, because these compounds are known to be hydrophobic and are also weak acids. These derivatives are the kinds of molecules for which the Overton rule was formulated more than 100 years ago (26,27). According to this rule, the entry of a molecule into a cell depends on the solubility of that molecule in the phospholipid membrane surrounding the cell. Provided that the molecule can assume an uncharged form in the lipid bilayer, it also can translocate through the bilayer. The major findings of our study are that *1*) upon binding to phospholipid bilayers, the apparent pK_a values for tolbutamide and glibenclamide increase to neutral pH values; *2*) the uncharged form of the sulfonylurea rapidly diffuses across a protein-free phospholipid bilayer; and *3*) glibenclamide diffuses through the plasma membrane of insulin-secreting HIT cells.

To study molecular interactions between sulfonylureas and phospholipid bilayers and the transport of sulfonylureas through bilayers, we used ^{1}H NMR spectroscopy to demonstrate sulfonylurea binding to vesicles and to measure the pK_a of the ionizable sulfonamide group. Direct interactions of the sulfonylurea with phospholipids were shown by changes in chemical shifts and line-widths of selected ^{1}H NMR peaks of the sulfonylurea in the presence of phospholipid bilayers relative to those of the sulfonylurea in aqueous buffer. The pK_a of tolbutamide increased in accord with predictions from previous studies of weak amphiphilic acids such as FAs and bile acids (27–29). Although the pK_a could only be estimated for glibenclamide, the estimate was good enough to permit us to conclude that the interfacial pK_a values for the two sulfonylureas are not significantly different. The titration of the sulfonylurea in vesicles indicated that the sulfonamide group is readily accessible to the H^+/OH^- added to the external buffer and therefore positioned at the aqueous surface of the phospholipid bilayer. Exposure of the titratable group (Fig. 1) would most likely be accomplished by immersion of the more hydrophobic groups into the bilayer; such a bent conformation also would enhance diffusion of the molecule through the bilayer by reducing its cross-sectional area. A study of 53 compounds that have a clinically established ability or lack of ability to cross the blood-brain barrier by diffusion (30) revealed three main requirements for diffusion. These are *1*) partitioning into the lipid bilayer, *2*) a pK_a between 4 and 10 if the molecule is charged, and *3*) a small cross-sectional area (<80 Å) in the interface. The NMR results show directly that both glibenclamide and tolbutamide fulfill the first and third requirements and show indirectly that the second requirement is also fulfilled.

To study the transmembrane movement of sulfonyl-

ureas, we performed fluorescence measurements of pH_{in} of entrapped pH dye after addition of the sulfonylurea to suspensions of SUV or LUV. Both sulfonylureas caused a dose-dependent decrease in pH_{in} and a progressively smaller decrease with increasing equivalent doses. These results are in accord both with theoretical predictions for the flip-flop mechanism and experimental results for FAs and other weak lipophilic acids (14). The pH changes observed with tolbutamide were much smaller than those observed with glibenclamide (Fig. 3). Because the interfacial pK_a values were similar, this is likely a result of the lower partitioning into the lipid phase of the more water-soluble tolbutamide. Figure 3 provides a graphic illustration that lower partitioning results in decreased diffusion through the membrane and that higher doses of tolbutamide would be required to achieve the same cellular concentrations as those for glibenclamide.

Our fluorescence assay also showed that glibenclamide diffused through the lipid bilayer plasma membrane of HIT cells, although not as rapidly as in model membranes. The bi-directional movement of sulfonylureas was demonstrated by the reversal of pH_{in} by the incomplete recovery reflecting weaker binding of glibenclamide to albumin compared with the binding of long-chain FAs. Similarly slow kinetics of pH_{in} changes such as those obtained for glibenclamide diffusion across the plasma membrane of HIT cells have been observed for the entry of FAs into HIT cells (16). Entry of FAs into adipocytes is significantly faster (31,32). In interpreting the kinetics of the pH_{in} changes, one must keep in mind that the pH assay measures the arrival of protons in the cytosol after adsorption of glibenclamide to the outer leaflet of the plasma membrane and its translocation through the membrane. Passive diffusion through the plasma membrane of the HIT cell might be slower than diffusion through protein-free model membranes because of the diffusion through the unstirred water layer (glycocalyx) adjacent to the plasma membrane or slower diffusion through the plasma membrane. This is not a unique characteristic of the sulfonylurea: the same considerations apply to the relatively slow pH changes after the addition of FAs to HIT cells (16).

We have shown that both tolbutamide and glibenclamide can cross the phospholipid bilayer rapidly by a free-diffusion mechanism (Fig. 6). Although our assay does not provide information about the rate of dissociation of sulfonylureas from the bilayer, it is likely that these molecules dissociate rapidly (or at least at a time scale significant for cell physiology), as in the case of FAs (33), and become available for binding sites in cytosol after they have diffused through the plasma membrane. These findings are especially interesting in that we have shown previously that these compounds can directly stimulate exocytosis of insulin (5–8). The discovery of a transport mechanism for sulfonylurea compounds across the phospholipid bilayer offers an explanation at the molecular level for how the sulfonylurea reaches intracellular targets. Furthermore, knowledge that sulfonylureas effectively permeate the plasma membrane and understanding of an underlying molecular mechanism may be of fundamental clinical importance in identifying new pharmacological principles and developing drugs for the treatment of type 2 diabetes.

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REFERENCES

1. Otto H, Mikosch M, Otto-Bendfeldt E: Indications for combined use of insulin and sulfonylureas in the treatment of diabetes. *Med Welt* 3:1864–1966, 1966
2. Krall LP. Glyburide (DiaBeta): a new second-generation hypoglycemic agent. *Clin Ther* 6:764–762, 1984
3. Ashcroft FM, Rorsman P: Electrophysiology of the pancreatic beta-cell. *Prog Biophys Mol Biol* 54:87–143, 1989
4. Aguilar-Bryan L, Nichols CG, Wechsler SW, Clement JP 4th, Boyd AE 3rd, Gonzalez G, Herrera-Sosa H, Nguy K, Bryan J, Nelson DA: Cloning of the beta cell high-affinity sulfonylurea receptor: a regulator of insulin secretion. *Science* 268:423–426, 1995
5. Flatt PR, Shibier O, Szecowka J, Berggren P-O: New perspectives on the actions of sulphonylureas and hyperglycaemic sulphonamides on the pancreatic β-cell. *Diabete Metab* 20:157–162, 1994
6. Eliasson L, Renström E, Ämmälä C, Berggren P-O, Bertorello AM, Bokvist K, Chibalin A, Deeney JT, Flatt PR, Gäbel J, Gromada J, Larsson O, Lindström P, Rhodes CJ, Rorsman P: PKC-dependent stimulation of exocytosis by sulfonylureas in pancreatic β cells. *Science* 271:813–815, 1996
7. Eliasson L, Ma X, Renström E, Barg S, Berggren PO, Galvanovskis J, Gromada J, Jing X, Lundquist I, Salehi A, Sewing S, Rorsman P: SUR1 regulates PKA-independent cAMP-induced granule priming in mouse pancreatic β-cells. *J Gen Physiol* 121:181–197, 2003
8. Barg S, Renström E, Berggren P-O, Bertorello A, Bokvist K, Braun M, Eliasson L, Holmes WE, Köhler M, Rorsman P, Thevenod F: The stimulatory action of tolbutamide on Ca^{2+}-dependent exocytosis in pancreatic β cells is mediated by a 65-kDa MDR-like P-glycoprotein. *Proc Natl Acad Sci U S A* 96:5539–5544, 1999
9. Hellman B, Sehlin J, Taljedal I-B: Glibenclamide is exceptional among hypoglycaemic sulphonylureas in accumulating progressively in B-cell-rich pancreatic islets. *Acta Endocrinol* 105:385–390, 1984
10. Carpentier J-L, Sawano F, Ravazzola M, Malaisse WJ: Internalization of ^{3}H-glibenclamide in pancreatic islets. *Diabetologia* 29:259–261, 1986
11. Marynissen G, Smets G, Kloppel L, Gerlache L, Malaisse WJ: Internalisation of glimepridine and glibenclamide in the pancreatic B-cell. *Acta Diabetol* 29:113–114, 1992
12. Geng X, Li L, Watkins S, Robbins PD, Drain P: The insulin secretory granule is the major site of K_{ATP} channels of the endocrine pancreas. *Diabetes* 52:767–776, 2003
13. Hamilton JA, Small DM: Solubilization and localization of triolein in phosphatidylcholine bilayers: a ^{13}C study. *Proc Natl Acad Sci U S A* 78:6878–6882, 1981
14. Kamp F, Westerhoff HV, Hamilton JA: Movement of fatty acids, fatty acid analogues, and bile acids across phospholipid bilayers. *Proc Natl Acad Sci U S A* 32:11074–11086, 1993
15. Kamp F, Hamilton JA: pH gradients across phospholipid membranes caused by fast flip-flop of un-ionized fatty acids. *Proc Natl Acad Sci U S A* 89:11367–11370, 1992
16. Hamilton JA, Civelek VN, Kamp F, Tornheim K, Corkey BE: Changes in internal pH caused by movement of fatty acids into and out of clonal pancreatic beta-cells (HIT). *J Biol Chem* 269:20852–20856, 1994
17. Newton DW, Kluza RB: PK_a values of medicinal compounds in pharmacy practice. *Drug Intell Clin Pharm* 12:546–554, 1978
18. Small DM, Cabral DJ, Cistola DP, Parks JS, Hamilton JA: The ionization behavior of fatty acids and bile acids in micelles and membranes. *Hepatology* 4 (Suppl. 5):77S–79S, 1984
19. Vorum H, Brodersen R, Kragh-Hansen U, Pedersen AO: Solubility of long-chain fatty acids in phosphate buffer at pH 7.4. *Biochim Biophys Acta* 1126:135–142, 1992
20. Jakoby MG 4th, Covey DF, Cistola DP: Localization of tolbutamide binding sites on human serum albumin using titration calorimetry and hetero-

nuclear 2-D NMR. *Biochemistry* 34:8780–8787, 1995
21. Abrumrad N, Harmon C, Ibrahimi A: Membrane transport of long-chain fatty acids: evidence for a facilitated process (Review). *J Lipid Res* 39: 2309:2318, 1998
22. Berk PD, Stump DD: Mechanisms of cellular uptake of long chain free fatty acids. *Mol Cell Biochem* 192:17–31, 1999
23. Hamilton JA, Kamp F: How are free fatty acids transported in membranes? Is it by proteins or by free diffusion. *Diabetes* 48:2255–2269, 1999
24. Zakim D: Thermodynamics of fatty acid transfer (Review). *J Membr Biol* 176:101–109, 2000
25. Kleinfeld AM: Lipid phase fatty acid flip-flop: is it fast enough for cellular transport? *J Membr Biol* 175:79–86, 2000
26. Overton E: On the general osmotic properties of the cell, their probable origin, and their significance for physiology. *Vierteljahrsschr Naturforsch Ges Zurich* 44:88–135, 1899
27. Al-Awqati Q: One hundred years of membrane permeability: does Overton still rule? *Nat Cell Biol* 1:E201–E202, 1999
28. Hamilton JA, Cistola DP: Transfer of oleic acid between albumin and phospholipid vesicles. *Proc Natl Acad Sci U S A* 83:82–86, 1986
29. Cabral DJ, Hamilton JA, Small DM: The ionization behavior of bile acids in different aqueous environments. *J Lipid Res* 27:334–343, 1986
30. Beschiaschvili G, Seelig J: Peptide binding to lipid bilayers: nonclassical hydrophobic effect and membrane-induced pK shifts. *Biochemistry* 31: 10044–10053, 1992
31. Civelek VN, Hamilton JA, Tornheim K, Kelly KL, Corkey BE: Intracellular pH regulation in adipocytes: effects of free fatty acids, lipolytic agents and insulin. *Proc Natl Acad Sci U S A* 93:10139–10144, 1996
32. Kamp F, Guo W, Souto R, Pilch PF, Corkey BE, Hamilton JA: Rapid flip-flop of oleic acid across the plasma membrane of adipocytes. *J Biol Chem* 278:7988–7995, 2003
33. Zhang F, Kamp F, Hamilton JA: Dissociation of long and very long chain fatty acids from phospholipid bilayers. *Biochemistry* 35:16055–16060, 1997