Binding of Sulfonamides to Erythrocytes and Their Components

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Binding of zonisamide, a new antiepileptic sulfonamide derivative, was examined to human erythrocytes, their lysate and their carbonic anhydrase by centrifugation for cells or by ultrafiltration for the others. Scatchard plots revealed that the binding to intact and lysed cells was composed of high- and low-affinity components and that to carbonic anhydrase, of the high-affinity component alone. Parameters for high-affinity binding were similar in all three preparations and those for low-affinity binding were similar in the former two preparations. Dissociation constants for these bindings to erythrocytes were smaller than the dissociation constant for serum albumin. These results may explain the concentration of sulfonamides in red cells, and suggest the participation of cellular protein component(s) in addition to previously known carbonic anhydrase in the binding.

Acetazolamide, sulthiame, zonisamide, hydrochlorothiazide and sulfanilamide inhibited carbonic anhydrase in a non-competitive manner to different extents. The K_i values of these sulfonamides were of the order of 0.1—0.2 of their respective K_d values determined by ultrafiltration, suggesting that under the present conditions, physicochemical interactions between sulfonamides and carbonic anhydrase primarily occur at common sites that affect the activity of the enzyme.

Keywords sulfonamides; erythrocytes; carbonic anhydrase; binding

It is known[1] that sulfonamides with an aromatic substituent exhibit high affinity for carbonic anhydrase and are significantly concentrated in erythrocytes, possibly because red cells contain a large amount of the enzyme. However, it is not established that concentration of those compounds in intact red cells is primarily or exclusively due to their binding to the enzyme. Moreover, the sulfonamides generally inhibit the enzyme activity[1a] but the physicochemical and functional interactions between the compound and enzyme protein are not well understood. The present studies were performed to answer these problems, mainly using zonisamide, a newly developed antiepileptic agent possessing a sulfonamide group,[2] as a model drug.

Experimental

Chemicals Zonisamide (1,2-benzisoxazole-3-methanesulfonamide) and the drug ^{14}C-labeled at the sulfamoylmethyl moiety with a specific radioactivity of 32.4 μCi/mg and a radiochemical purity of >99%[3] were prepared in our laboratories as described. Sulthiame and hydrochlorothiazide were prepared from commercial tablets (Bayer A. G. and Banyu Pharmaceutical Co., Ltd., respectively). Acetazolamide and sulfanilamide were commercial products (Lederle Japan, Ltd. and Nakarai Chemicals, Ltd., respectively). Human serum albumin (fr. V) and human carbonic anhydrase B were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were of reagent grade and were purchased from commercial sources.

Erythrocytes Human blood collected with heparin by venipuncture from healthy volunteers was centrifuged at 3000 rpm for 15 min to separate erythrocytes. Cells were washed with two volumes of 0.9% sodium chloride solution and finally suspended in Krebs–Ringer phosphate (KRP) buffer of pH 7.4. Cells were lysed by addition of three volumes of cold water and centrifuged at 12000 rpm for 20 min for removal of the ghost fraction.

Incubation Intact cells (0.5 ml) were incubated with 0.5 ml of [^{14}C]zonisamide solution in KRP buffer at final concentrations of 0—800 nmol/ml at the hematocrit of 50% at 37 °C. Two milliliters of hemolysate preparation described above, of carbonic anhydrase preparation (13.2 μM) or of human serum albumin (588 μM) was incubated with 2 ml of the sulfonamide solution at final concentrations of 0—800 nmol/ml in 0.1 M phosphate buffer of pH 7.4.

Analysis Binding to the intact red cells was determined by measuring the cellular [^{14}C]zonisamide radioactivity after centrifugation at 3000 rpm for 15 min post incubation.

Protein binding in the hemolysate and the solution of carbonic anhydrase or human serum albumin was determined by the ultrafiltration method using a CF 25 membrane (Amicon Corp., Danvers, MA, U.S.A.) as described previously.[3] Bindings were analyzed by means of a Scatchard plot[4] based on the equation $C_b/C_f = n\ (Pt)/K_d - C_b/K_d$, where C_b is the concentration of bound sulfonamide, C_f is the concentration of unbound sulfonamide, n is the number of binding sites, Pt is the concentration of the protein, and K_d is the dissociation constant. The parameters were determined by the least-squares method using a PC-9801 personal computer (NEC, Tokyo, Japan).

For estimation of zonisamide, the ^{14}C-labeled compound was used throughout the present studies and radioactivity was measured with a Tri-Carb liquid scintillation spectrometer (model 460 CD, Packard Instruments Inc., Downers Grove, IL, U.S.A.) as described previously.[3] Sulthiame was estimated by gas chromatography.[3] Other sulfonamides were estimated by measuring the absorbance at 265 nm for acetazolamide, 270 nm for hydrochlorothiazide and 255 nm for sulfanilamide.

The activity of carbonic anhydrase was determined from the rate of hydrolysis of *p*-nitrophenyl acetate at the enzyme concentration of 2 μM at 20 °C in 0.1 M Tris–sulfate buffer of pH 7.6 by measuring the absorbance at 400 nm as described previously.[5]

Results

Bindings of Zonisamide to Erythrocytes, Their Components and Albumin Bindings of zonisamide to intact human erythrocytes, their lysate, purified carbonic anhy-

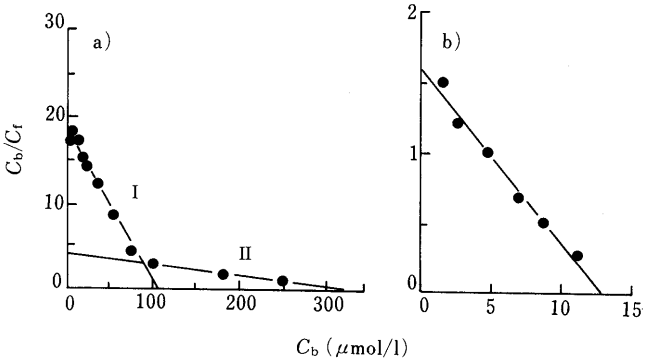

Fig. 1. Scatchard Plots of Zonisamide Binding to Intact Human Erythrocytes (a) and Carbonic Anhydrase (b)

Points are means of duplicate determinations. C_b, concentration of bound zonisamide (μmol/l); C_f, concentration of unbound zonisamide (μmol/l). a) I, $C_b/C_f = 18.7 - 1.76 \times 10^5 \cdot (C_b \times 10^{-6})$ ($r = 0.996$); II, $C_b/C_f = 4.17 - 1.29 \times 10^4 \cdot (C_b \times 10^{-6})$ ($r = 0.999$). b) $C_b/C_f = 1.59 - 1.22 \times 10^5 \cdot (C_b \times 10^{-6})$ ($r = 1.000$). The concentration of carbonic anhydrase was 13.2 μM.

TABLE I. Binding Parameters of Zonisamide to Components of Human Erythrocytes Determined from Scatchard Plots

Component	Parameters for binding			
	K_{d1} (μM)	B_{max1} (μmol/l)	K_{d2} (μM)	B_{max2} (μmol/l)
Intact erythrocytes	5.7	106	77.5	323
Erythrocyte hemolysate	6.7	115	92.6	362
Carbonic anhydrase	8.2	124	—	—

B_{max} for carbonic anhydrase was calculated from n (0.99, *cf.* Table II) $\times$ 125 μmol/l (content of carbonic anhydrase B in red cells[7]).

TABLE II. Binding Parameters of Sulfonamides to Carbonic Anhydrase Determined by Physicochemical Means (K_d) and by Enzyme Inhibition (K_i)

Agent	K_d (μM)	n	K_i (μM)	K_i/K_d
Acetazolamide	2.6	2.7	0.34	0.13
Sulthiame	4.5	0.93	0.63	0.14
Zonisamide	8.2	0.99	1.5	0.18
Hydrochlorothiazide	23.4	1.9	4.5	0.19
Sulfanilamide	36.0	2.8	7.6	0.21

n, number of binding sites.

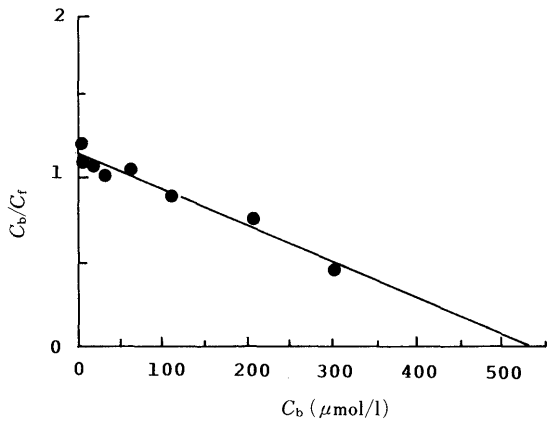

Fig. 2. Scatchard Plots of Zonisamide Binding to Human Serum Albumin

Points are means of duplicated determinations. $C_b/C_f = 1.14 - 2.12 \times 10^3$ ($C_b \times 10^{-6}$) ($r = 0.975$). The concentration of human serum albumin was 588 μM. See also the legend to Fig. 1.

drase of human erythrocyte and human serum albumin were analyzed by means of Scatchard plots.[4] In intact cells, the plot (Fig. 1a) revealed two components: high-affinity and low-affinity components. Their dissociation constants (K_d) and the maximal binding capacities (B_{max}) are listed in Table I. Zonisamide was also found to bind to the hemolysate with two binding components with parameters (Table I) similar to those for intact cells.

In contrast, binding of zonisamide to purified carbonic anhydrase was homogenous (Fig. 1b). Calculated parameters (Table I) were similar to those for high affinity binding to intact and lysed cells.

Binding of other sulfonamides, acetazolamide, sulthiame, hydrochlorothiazide and sulfanilamide, to carbonic anhydrase was then determined by ultrafiltration. Their binding was found to be of homogenous, like that of zonisamide, though the K_d values were different from each other (Table II).

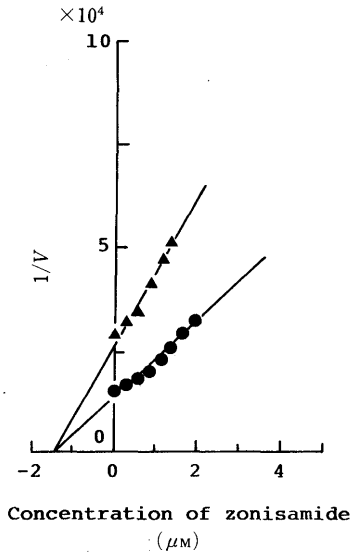

Fig. 3. Dixon Plots of Carbonic Anhydrase Inhibition by Zonisamide

Points are means of duplicated determinations. Substrate concentration, 125 μM (▲) or 250 μM (●).

The K_d and the number of binding sites (n) of zonisamide for binding to human serum albumin, determined by ultrafiltration, were 472 μM and 0.91, respectively, as shown in Fig. 2. The n value can be used to calculate the plasma maximum binding capacity (B_{max}) for zonisamide of 0.91×606 μM (physiological level of albumin) = 551 μmol/l.

Inhibition of Carbonic Anhydrase by Sulfonamides The K_m and V_{max} of carbonic anhydrase for hydrolysis of *p*-nitrophenyl acetate were found to be 3.5 mM and 50 molecules per enzyme molecule per min, respectively, which are consistent with those reported previously.[5] Inhibition of carbonic anhydrase by sulfonamides was analyzed by means of a Dixon plot[6] (*e.g.*, Fig. 3) and was found to be noncompetitive. The K_i values are also listed in Table II and were smaller than the K_d values for all sulfonamides examined.

Discussion

In the present studies, we examined the binding of sulfonamides to carbonic anhydrase B. It was reported[1b,7] that human erythrocytes also contain carbonic anhydrase C at about 1/5 the content of carbonic anhydrase B, and the former isozyme also binds sulfonamides. If this is taken into consideration with regard to the binding of zonisamide to intact erythrocytes, their lysate and their carbonic anhydrase B, the high-affinity dissociation constants listed in Table I can be regarded as virtually identical, around 6.8 μM sulfonamide on average. It can be concluded that this dissociation constant is that of the physicochemical interaction between the sulfonamide and carbonic anhydrase.

In the present analysis, another protein component(s) besides carbonic anhydrase in red cells was found to bind zonisamide with low affinity since the binding was detected to the intact and lysed cells but not to carbonic anhydrase. The K_{d2} of this protein (Table I), around 100 μM zonisamide, is small relative to the dissociation constant to extracellular albumin, 472 μM. Despite the smaller maximal binding capacities of those red cell components in Table I than the B_{max} of albumin, their higher affinities may account for the concentration of the sulfonamide in red

cells, since the ordinary plasma levels of zonisamide are $< 100\,\mu M$ at the therapeutic dose in man[8] and animals.[9] Thus, the present studies revealed that, in addition to carbonic anhydrase, another protein(s) present in red cells operates in the concentration of sulfonamides in the cells.

Since the hemolysate preparation used for the present experiment did not contain the ghost fraction, the other protein(s) in question was not associated with the red cell membrane. In a separate experiment, hemoglobin was found not to be the binding protein.

The K_i values, functionally determined dissociation constants between sulfonamides and carbonic anhydrase, were lower than the K_d values, the physicochemically determined dissociation constants (Table II). The mode of inhibition of carbonic anhydrase by sulfonamides was noncompetitve. These findings suggest that the interaction between sulfonamides and carbonic anhydrase predominantly occurs at a site(s) affecting the catalytic function of the enzyme.

Under the present conditions, the K_i/K_d ratios in Table II were in the range of 10—20%, indicating that the physicochemical interaction occurred with affinity lower than but dependent on the affinity of the more specific interaction influencing enzyme activity. In other words, the physicochemical and functional interactions occur at common sites on the enzyme protein. Thus, the interaction between sulfonamides and carbonic anhydrase is essentially similar to other previously documented cases such as the interaction with serum albumin[10] of certain drugs with inhibitory effect on its esterase like-activity, or that of adenosine triphosphatase[11] with the local anesthetic tetracaine.

The present studies provide direct evidence that concentration of sulfonamides in erythrocytes is due to their binding to cellular carbonic anhydrase and another protein with higher affinities than to extracellular albumin. Furthermore, the physicochemical interaction between sulfonamides and carbonic anhydrase is related to the more specific interaction affecting the activity of the enzyme.

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