Effect of Substituted Benzoylglycines (Hippurates) and Phenylacetylglycines on p-Aminohippurate Transport in Dog Renal Membrane Vesicles

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The effect of substituted benzoylglycines (hippurates) and phenylacetylglycines on the transport of p-aminohippurate (PAH) was studied in basolateral (BLMV) and brush border membrane vesicles (BBMV) isolated from dog kidney cortex. The probenecid-sensitive part of 100 μM [^{3}H]PAH uptake into BLMV and BBMV was measured in the presence and absence of 5 mM glycine conjugate. The benzoyl- and phenylacetylglycines studied were substituted in the 2-, 3-, or 4-position with an H, CH_3, OCH_3 or OH group. Benzoylglycines were stronger inhibitors of PAH transport than phenylacetylglycines and the inhibitory potency of the conjugates was in general lower against the transporter in BBMV than BLMV. The specificities of the transporters in both membranes appear to be very similar. The inhibitory potency of the benzoylglycines, expressed as the apparent inhibition constant ($\log K_i$), did not show a linear relationship with their lipophilicity as determined by reversed phase HPLC. Deviation from linearity was caused mainly by the 3-OH and 4-OH analogs, which showed a greater inhibitory potency than expected from their lipophilicity. Phenylacetylglycines only showed a small variation in $\log K_i$ values, indicating that insertion of a CH_2 group between the ring and the carbonyl practically abolishes the influence of the ring and its substituents. In conclusion, both hydrophobic and electronic properties are important determinants of affinity for the PAH transport system. An additional partially negative hydroxyl group in the ring, located preferably at the 3- or 4-position, increases the interaction with the transport system.

KEY WORDS: p-aminohippurate; glycine conjugates; isolated membrane vesicles; organic anion transport; dog kidney.

INTRODUCTION

Glycine conjugation is next to glucuronidation an important metabolic pathway in the elimination of aromatic carboxylic acids (1). It leads to less toxic compounds that are good substrates for the renal organic anion transport system and therefore more readily excreted than the unconjugated molecules (2). Although aromatic carboxylic acids may be actively secreted by the kidney themselves, the efficiency of their renal clearance is generally low because of extensive passive backdiffusion and/or active reabsorption (3,4). A classical example is hippurate (benzoylglycine), the conjugation product of benzoate and glycine. It is excreted in large amounts in normal urine, depending on the daily intake of benzoate precursors in the diet (5). The renal clearance of hippurate and related conjugates like p-aminohippurate (PAH) is so efficient at low concentrations, that they are completely extracted from renal plasma in a single pass through the kidney (2,3). In the past, several studies have been undertaken with the hippurate class of compounds in an attempt to identify the structural properties that are essential for active renal secretion. It was concluded that the carbonyl and carboxylate groups play critical roles in the secretion of hippurates (6). Essig and Taggart (7) studied the influence of ring methylation, halogenation and nitration on the transport properties of hippurate in rabbit kidney cortex slices. Competitive inhibition of PAH transport was observed, and hippurates substituted in the meta-position were better inhibitors than the corresponding para- and ortho-analogs. The capacity to inhibit PAH transport was inversely related to their own transport rates and within the meta- and para-series the inhibitory potency was related to the weight or bulk of the substituent and not to the electron distribution in the aromatic ring. A difficulty in interpreting these results, and of slice studies in general, is that they do not allow the differentiation of luminal and peritubular (basolateral) membrane processes (2). There is now abundant evidence that PAH transport across both membranes is carrier-mediated and susceptible to inhibition by other anions (2,3).

In recent years, the specificity of basolateral organic anion transport has been studied extensively by Ullrich and coworkers in microperfused rat tubules. They evaluated systematically the interaction of up to a thousand compounds, originating from a wide variety of chemical classes, with basolateral PAH transport, and with the related dicarboxylate and sulfate systems (8–13). On the basis of molecular modeling it was concluded that the three systems show overlapping specificities and that determinant factors for inhibitory activity against the PAH system are charge distribution and location and size of hydrophobic domains (14). In contrast with the abundance of information concerning basolateral transport, only a few studies have addressed the specificity of the PAH transporter in the brush border membrane (15–17).

In the present study we used isolated brush border (BBMV) and basolateral membrane vesicles (BLMV) to evaluate and compare the specificity of PAH transport across each membrane separately. The objective was to characterize the relative affinity of mono-substituted benzoylglycines (hippurates) and phenylacetylglycines for the PAH transport system by measuring their inhibitory effect on PAH uptake into BLMV and BBMV and to determine how inhibitory potency is related to the physicochemical properties of these compounds.

MATERIALS AND METHODS

Isolation of Membrane Vesicles. Kidneys from Beagle dogs that became available from other, mainly surgical, experiments were used as starting material. Brush border (BBMV) and basolateral membrane vesicles (BLMV) were prepared from cortical homogenate by $CaCl_2$ precipitation and differential Percoll density gradient centrifugation as described previously (18). The isolated membrane vesicles were suspended in uptake buffer, consisting of 100 mM KCl,

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100 mM mannitol and 5 mM HEPES-Tris pH 7.4, at a protein concentration of 8–12 mg/ml.

The purity of the membrane preparations was evaluated by marker enzyme analysis, according to previously described methods (18). Compared to the initial homogenate, BLMV showed a 8- to 10-fold enrichment in (Na^+-K^+)-ATPase, 0.8-fold in maltase, 2.2-fold in alkaline phosphatase, 1.3-fold in acid phosphatase, and <0.9-fold in succinate dehydrogenase and NADPH-cytochrome-c reductase. BBMV were enriched for maltase and alkaline phosphatase 12- to 15-fold, while the enrichment factors for the other marker enzymes were all <0.9.

The membranes were rapidly frozen in liquid nitrogen and stored at −80°C in small aliquots until used. In preliminary experiments, we found no difference in transport activity between frozen and freshly prepared membrane vesicles.

Transport Studies. The uptake of [^{3}H]PAH in BBMV and BLMV was measured at 37°C by rapid filtration. Transport was initiated by the addition of 40 μl solution to 10 μl of membrane suspension. The experimental conditions were based on the previously determined characteristics of PAH transport in dog renal membrane vesicles (15,18). The initial composition of the extravesicular medium for BLMV was 100 mM NaCl, 20 mM KCl, 100 mM mannitol, 5 mM HEPES-Tris pH 7.4 and for BBMV 100 mM KCl, 100 mM mannitol, 5 mM 2-(N-morpholino)-ethanesulfonic acid (MES)-Tris pH 6.0. The final concentration of [^{3}H]PAH was 100 μM, and in interaction experiments probenecid or glycine conjugate were present in a final concentration of 5 mM. Uptake of PAH was terminated after 15 s by diluting the incubation mixture with 3 ml ice-cold transport buffer. This sample was immediately filtered under vacuum through a prewetted 0.45 μm cellulose nitrate filter (Schleicher and Schüll, Dassel, Germany) and washed twice with 3 ml of ice-cold buffer. The radioactivity remaining on the filters was counted using standard liquid scintillation techniques after dissolution in 10 ml Aqualuma plus (Lumac, Schaesberg, The Netherlands). Corrections were made for the radioactivity bound to the filters in the absence of vesicles. To make sure that the glycine conjugates had no indirect effect on PAH uptake by generating a transmembrane diffusion potential, controls were performed under voltage clamp conditions. In these experiments inhibition of PAH uptake was evaluated by using vesicles with equal internal and external K^+ concentrations (100 mM) in the presence of the K^+-selective ionophore valinomycin (5–8 μg per mg protein) to short-circuit anion diffusion potentials and maintain the membrane potential at zero voltage. Both in BLMV and BBMV the inhibitory effects were not different from those observed without a voltage clamp, indicating that there was no indirect effect of an anion diffusion potential on transport.

Determination of $\log k_w$. In order to study the relative lipophilicity of the substituted glycine conjugates, the capacity factor $\log k_w$ was determined by reversed phase HPLC as described by Yamana *et al.* (19). A Hewlett-Packard 1084B chromatograph equipped with a UV detector (254 nm), autosampler and terminal (HP7850LC) was used. The stainless-steel column (250 × 4.6 mm I.D.) was packed with CP™Spher C18, particle size 10 μm (Chrompack, Middelburg, The Netherlands). The mobile phase consisted of a mixture of methanol and double-distilled water containing 0.01 M phosphate buffer of pH 2.9. The column temperature was 35°C and the eluent was delivered at a flow rate of 2.0 ml/min. The compounds were dissolved in methanol (5 mM) and 5 μl was injected into the column. Retention is quantitatively described by the capacity factor, $k' = (t_R - t_0)/t_0$ where t_R is the retention time of the compound and t_0 is the retention time of an unretained reference compound, for which potassium dichromate was used. For each compound k' was determined at at least four methanol concentrations ranging from 5–50% v/v and differing 5% or more, such that peaks with a reasonable retention time (1.5–25 min) were obtained. A linear relationship was observed between the $\log k'$ and the methanol concentration of the mobile phase and extrapolation to 100% water (0% methanol) gave the $\log k_w$ value.

Data Analysis and Presentation. All experiments were performed on at least three different membrane preparations. Under all given conditions, the initial uptake rate of PAH measured at 15 s was linear with respect to time and concentration. Total PAH uptake can be described as uptake via a Michaelis-Menten process in parallel with passive diffusion (9). The carrier-mediated or specific component of PAH uptake was defined as the difference between uptake in the absence and presence of 5 mM probenecid. Inhibition was expressed relative to the respective control uptakes as $\%inh. = 100(v - v')/v\ \%$, where v = probenecid sensitive part of PAH uptake (control) and v' = probenecid sensitive part of PAH uptake in presence of benzoate. Assuming that mediated PAH uptake follows Michaelis-Menten kinetics and that inhibition is competitive for a single binding site, the apparent inhibition constant K_i was calculated as:

$$K_i = \frac{I[v'/(v - v')]}{(1 + S/K_m)}$$

where I = concentration inhibitor (5 mM), S = PAH concentration (100 μM), and K_m = apparent Michaelis-Menten constant of PAH transport. The values for K_m were identical to previously determined values, *viz.* 0.8 mM for uptake into BLMV and 5.7 mM for BBMV (15). Data are expressed as means ± SEM. Curve fitting was done by least-squares regression analysis of the unweighted data using the computer program PCNONLIN (Version 3.0, SCI Software, Lexington, KY). Student's t-test and an F-test were used to evaluate statistical significance in the correlation studies.

Preparation of the Glycine Conjugates. Benzoylglycine and 2-OH-benzoylglycine were commercially available, and the other conjugates were prepared by chemical synthesis. Benzoylglycines and phenylacetylglycines with a H, CH_3 or OCH_3 substituent were prepared according to the Schotten-Baumann method from the corresponding benzoyl and phenylacetyl chlorides by reaction with glycine in the presence of a slight excess of sodium hydroxide (20). Acyl chlorides were obtained commercially from Aldrich (Brussel, Belgium) or prepared by reaction of the substituted acid with a slight molar excess of thionyl chloride (21). The Schotten-Baumann method is not suitable for the preparation of hydroxy-substituted conjugates because the phenolic groups can react with the acid chlorides. For these compounds an activated ester was prepared by reaction of the acid with

N-hydroxysuccinimide in presence of N,N'-dicyclohexylcarbodiimide in dry dioxan (22). The activated ester was separated and subsequently added to a slight alkaline sodium bicarbonate solution to yield the hydroxy-substituted glycine conjugate. The substituted benzoyl- and phenylacetylglycines were purified by recrystallization from boiling water. The identity of the compounds was checked by comparing melting points with available literature values (20,22–27) and incidently by IR, mass spectrometry and NMR analysis. Purity was checked by TLC and HPLC. TLC was performed on silicagel GF_{254} plates (Merck, Darmstadt, Germany) with a mobile phase of chloroform:methanol:acetic acid (80:20:3 or 85:15:3). Spots were detected under UV light and by immersing the plates in a saturated iodine solution in carbon tetrachloride. The HPLC method was the same as used for the determination of the $\log k_w$ values.

Chemicals. p-Amino[^{3}H]hippuric acid (440 mCi/mmol) was obtained from Amersham (Buckinghamshire, UK). Unlabeled PAH, benzoylglycine (hippuric acid) and 2-OH-benzoylglycine (salicyluric acid), glycine and thionylchloride were from Merck (Darmstadt, Germany), and N-hydroxysuccinimide and N,N'-dicyclohexylcarbodiimide from Janssen Chimica (Beerse, Belgium). All other chemicals were purchased either from Sigma (St. Louis, MO) or Merck.

RESULTS

Physicochemical Properties. The capacity factors ($\log k_w$) of the substituted benzoylglycines and phenylacetylglycines, determined by reversed phase HPLC at pH 2.9 are given in Table I. $\log k_w$ was obtained by extrapolation of retention data from methanol-water eluents to 100% water and was used to characterize the relative lipophilicity of the glycine conjugates. The pK_a of most compounds is not known, but on theoretical grounds it is reasonable to assume that the values are not much different from the pK_a of benzoylglycine (3.80). This means that $\log k_w$ values determined at pH 2.9 provide a measure of the lipophilicity of the unionized molecules. The lipophilicity of a compound is usually expressed by the partition coefficient, log*P*, derived from distribution studies between *n*-octanol and water. Because of the practical advantages of the chromatographic system over conventional shake-flask measurements we favored the use of $\log k_w$. However, to have a comparison with log*P*, shake-flask values were determined for eight glycine conjugates ($\log k_w$ ranging from 0.72–1.89) with the same buffer as used in HPLC. A linear relationship was found, given by the equation $\log P = 0.852 \log k_w - 0.089$ ($r = 0.967$, $n = 8$), indicating that $\log k_w$ is a reliable measure of lipophilicity of the glycine conjugates.

Competition Experiments. The transport of 100 μM PAH into BLMV and BBMV was measured with or without the presence of 5 mM probenecid, in order to distinguish carrier-mediated PAH transport from unmediated processes. Uptake at 15 s was proportional to incubation time and was used to measure the initial rate of transport. The probenecid-sensitive part of uptake accounted for 72% of total uptake into BLMV (108 ± 4 *vs* 30 ± 3 pmol/mg protein, n = 8) and for 80% of uptake into BBMV (150 ± 10 *vs* 30 ± 5 pmol/mg protein, n = 8). The inhibitory effects of the substituted benzoylglycines and phenylacetylglycines on probenecid-sensitive PAH uptake into BLMV and BBMV are shown in Table I. Control uptakes were measured in presence of 5 mM gluconate and were not different from the uptake values without gluconate. Since the glycine conjugates are weak acids, they might interfere indirectly with pH-dependent PAH uptake into BBMV by accelerating the dissipation of the transmembrane pH gradient. To test this we measured uptake in presence of acetate, an anion that accumulates rapidly into the vesicles by nonionic diffusion (16). With acetate concentrations up to 10 mM no inhibition was observed, indicating that the intravesicular buffering was sufficient. The inhibitory potency of the glycine conjugates was in general lower against transport into BBMV than BLMV. The strongest inhibitory effects were found with 2-OCH_3- and 3-CH_3-benzoylglycine. In both BLMV and BBMV, phenylacetylglycines showed a lower and much less differentiated inhibitory effect, as compared to the corresponding benzoylglycines.

The logarithm of the apparent inhibition constant ($\log K_i$) was used to look for a relationship between the inhibitory potency of the glycine conjugates and their capacity factors listed in Table I. We could not elicit a consistent pattern relating $\log K_i$ to $\log k_w$ values. Linear regression analyses revealed poor correlations for both the benzoylglycines and phenylacetylglycines. In particular 3-OH- and 4-OH-benzoylglycine disturbed a linear relationship by showing

Table I. Capacity Factors ($\log k_w$) and Effect on PAH Uptake of Substituted Glycine Conjugates[a]

		BLMV		BBMV	
Substituent	$\log k_w$	*%inh.*	K_i (mM)	*%inh.*	K_i (mM)
benzoylglycines					
H	1.01	50 ± 5	4.8	30 ± 5	11.6
2-CH_3	1.35	39 ± 6	6.9	26 ± 6	14.0
3-CH_3	1.75	74 ± 6	1.6	54 ± 7	4.2
4-CH_3	1.74	56 ± 6	3.5	43 ± 3	6.5
2-OCH_3	1.76	76 ± 5	1.4	56 ± 4	3.9
3-OCH_3	1.44	63 ± 6	2.6	47 ± 7	5.5
4-OCH_3	1.47	51 ± 7	4.3	37 ± 3	8.4
2-OH	1.54	57 ± 9	3.3	52 ± 7	4.5
3-OH	0.86	46 ± 6	5.2	43 ± 2	6.5
4-OH	0.72	56 ± 6	3.5	50 ± 3	4.9
phenylacetylglycines					
H	1.29	47 ± 4	5.0	25 ± 3	14.7
2-CH_3	1.66	47 ± 4	5.0	33 ± 3	10.0
3-CH_3	1.89	45 ± 4	5.4	34 ± 4	9.5
4-CH_3	1.91	44 ± 4	5.6	40 ± 5	7.4
2-OCH_3	1.68	51 ± 5	4.3	29 ± 2	12.0
3-OCH_3	1.55	49 ± 5	4.6	29 ± 1	12.0
4-OCH_3	1.43	56 ± 3	3.5	30 ± 1	11.5
2-OH	1.08	61 ± 4	2.8	20 ± 6	19.7
3-OH	0.83	38 ± 5	7.2	30 ± 4	11.5
4-OH	0.65	36 ± 4	7.9	26 ± 2	14.0

[a] The probenecid-sensitive part of 100 μM PAH uptake at 15 s was measured into basolateral (BLMV) and brush border membrane vesicles (BBMV) in presence of 5.0 mM glycine conjugate. Control uptakes were measured in presence of 5 mM gluconate. Inhibition (*%inh.*) is expressed relative to the respective control uptakes as means ± SEM of three to five experiments.

inhibitory potencies comparable to conjugates with $\log k_w$ values almost two times greater. Since values of the independent variable, $\log K_i$, did not vary more than one order of magnitude, data are inadequate to search for a quantitative structure-activity relationship. However, it seems clear that lipophilicity of the glycine conjugates alone cannot explain their affinity for the PAH transport system.

DISCUSSION

A considerable effort has been made to identify the structural properties that determine whether an anion is secreted by the renal organic anion transport system. Sophisticated models have been proposed to rationalize the vast structural diversity in compounds transported (2,6). Ullrich *et al.* (8–13) showed in an impressive series of in vivo microperfusion experiments in the rat that a negative ionic or partially negative charge in combination with a hydrophobic moiety are in fact sufficient to meet the requirements for interaction with the PAH transporter in the basolateral membrane. The most effective inhibitors had two ionic (e.g., a carboxyl group) or partially negative (e.g., a carbonyl group) charges, separated optimally by 6–7 Å, and a hydrophobic region (e.g., a phenyl group), which does not lie on a direct line between the two charged groups. For substituted benzoates a linear relationship was observed between inhibitory potency and both lipophilicity and acidity (9). These findings correlated well with results obtained in isolated membrane vesicles (15). With the in vivo microperfusion technique it is impossible to measure the specificity of brush border transport. For that purpose, isolated membrane vesicles are most appropriate.

In the present study the substrate specificity of PAH transport across both basolateral and brush border membranes was evaluated with a series of closely related hippurate derivatives. The lipophilicities of the glycine conjugates were determined by reversed phase HPLC. Chromatographic retention parameters, in particular the extrapolated capacity factor for an aqueous eluent ($\log k_w$), are very useful for the assessment of the lipophilic nature of drugs.

Although a quantitative analysis of structure-activity relationship was not possible with our results, it is clear that there exists no simple linear relationship between lipophilicity and $\log K_i$. For the benzoylglycines, deviation from linearity is caused mainly by the 3-OH and 4-OH analogs, which show a greater inhibitory potency than expected on basis of their $\log k_w$ value (Table I). Apparently, the extra partial negative charge of a ring hydroxyl group, in addition to the carbonyl and carboxyl groups, increases the interaction with the transporter and compensates for the reduced lipophilicity. This was also observed for 2-OH-benzoylglycine in BBMV, but to a much lesser extent in BLMV. The unique ability of this compound to form an intramolecular hydrogen bond between the hydroxy and carbonyl group is likely to be responsible for the observed difference between both membranes. The lower pH in the BBMV experiments favors intramolecular hydrogen binding.

Benzoylglycines were better inhibitors than phenylacetylglycines, which only showed a small variation in $\log K_i$ values. Insertion of a CH_2 group between the ring and the carbonyl practically abolishes the influence of the ring and its substituent on the inhibitory potency. In terms of the model proposed by Ullrich and coworkers (9,14), it seems that the distance between the hydrophobic phenyl residue and the negative charges is too long for an effective interaction. Indeed, renal clearance studies in the dog in vivo show that the excretory profile of the substituted phenylacetylglycines are practically identical and characterized by a relatively low capacity of tubular secretion (Russel *et al.*, unpublished results), especially as compared with the corresponding benzoylglycines (28,29).

Overall, the inhibitory potency of the glycine conjugates against PAH transport into BBMV was lower, but the pattern of inhibition was the same as in BLMV, indicating similar transporters. The lower potency against PAH uptake into BBMV suggests that the BLMV transporter has in general a higher apparent affinity for these substrates than does the process in BBMV. This is in accordance with the difference between K_m values for PAH transport across both membranes (15).

It can be concluded that hydrophobic as well as electronic properties are decisive for affinity of glycine conjugates for the PAH transporter. The specificities of the transporters in the basolateral and brush border membrane are very similar. The overall results of our study fit well in the criteria of substrate specificity for the basolateral PAH transporter as recently postulated by Ullrich and coworkers. However, a refinement of this model seems necessary on the basis of our findings. In addition to the ionic carboxyl group and partially negative carbonyl group, a third partially negative charge, located preferably at the 3- or 4-position of the hydrophobic phenyl residue, increases the interaction with the transporter.

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