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INHIBITION BY β-PHENYLETHYLAMINE AND SIMILAR COMPOUNDS OF GLYCINE TRANSPORT BY PIGEON RED CELLS*

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SUMMARY

1. β-Phenylethylamine gave first-order inhibition of Na^+-dependent glycine entry ($K_i = 5.35$ mM). The ion rather than the free amine was the inhibitor. Inhibition was reversible and (as a first approximation) noncompetitive to both glycine and Na^+.

2. β-Phenylethylamine also inhibited Na^+-independent glycine entry and $^{22}Na^+$ entry, but less strongly.

3. Specificity was low but present; *e.g.*, *n*-hexylamine and α-phenylethylamine were as effective as β-phenylethylamine as inhibitors of Na^+-dependent glycine entry, benzylamine and tyramine were about half as effective, and *N,N,N*-trimethyl-β-phenylethylammonium ion was ineffective.

INTRODUCTION

Glycine transport by pigeon red cells can be analyzed into Na^+-independent and Na^+-dependent components. The Na^+-independent component shows diffusion-like kinetics. Most of the glycine transport by these cells is Na^+-dependent and obeys Michaelis–Menten kinetics with respect to both the glycine concentration and the square of the Na^+ concentration. It is primarily the K_m term rather than the V term of the kinetic equation that is affected by the Na^+ concentration[1]. The bulk of the Na^+-dependent glycine transport occurs by a route highly specific for glycine[2,3]. Recent work[3] has shown that a minor, but significant, amount of Na^+-dependent glycine transport by pigeon red cells occurs by a second route (the "ASCP" route) and probably depends on the first power of the Na^+ concentration.

Inhibitors, in general, are useful tools. β-Phenylethylamine inhibition of Na^+-dependent glycine transport by pigeon red cells had been briefly reported[1]. The high amino acid specificity of the main glycine transport route[2] made it unlikely that β-phenylethylamine was a competitive inhibitor for glycine, and the high specificity of glycine transport for Na^+ (ref. 1) similarly made competition with Na^+ unlikely. This suggested that some translocation step(s) was preferentially inhibited. This possibility was supported by the present study.

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Since the agent might be useful, a survey was made of some of its properties. It was found that the protonated form is the active species, inhibition is predominantly noncompetitive, and structural specificity is low but not absent. Several different transport processes are inhibited, but not equally. The Na^+-independent route for glycine entry is inhibited less strongly than the main Na^+-dependent route. The inhibition kinetics show that the "ASCP" route for glycine entry is also inhibited.

MATERIALS AND METHODS

Preparation of cells and determination of the rates of Na^+-dependent and Na^+-independent glycine entry with [^{14}C]glycine were done as previously described. Media were the (modified) Krebs–Ringer phosphate–glucose solutions described before[1]. Procedures or treatments not described in ref. 1 are described in the legends or footnotes of the appropriate figures or tables. Chemicals were reagent grade except for those listed below. The following were Eastman products: β-phenylethylamine (EK 2642), *n*-hexylamine (EK 5251), benzylamine (EK 579), β-phenylethyl alcohol (EK 313). Tyramine hydrochloride (AX 921) and DL-α-phenylethylamine (MX 740) were from Matheson, Coleman and Bell. Octanoic acid was from Eldorado Oil Works, San Francisco, Calif. The tyramine hydrochloride was decolorized with charcoal before use. Mono-methyl β-phenylethylamine was prepared and purified by the procedure of CAROTHERS *et al.*[4] and *N*,*N*-dimethyl-β-phenylethylamine by the procedure of ICKE *et al.*[5]. These were further purified by recrystallization of the hydrochlorides from ethanol–ether (m.p., 163–164.5° (ref. 12, 156–157°)) and ethanol (m.p., 163–164°, (ref. 6, 171°; ref. 7, 205°)). The mixed melting range (108–140°) showed these to be different compounds. As the hydrochlorides were poor compounds for identification purposes, picrates were prepared (crystallized from ethanol). The melting points were, monomethyl derivative, 141.4°–142.8° (ref. 8, 141°–143°); dimethyl derivative, 135.4°–136.4° (ref. 9, 135°–136°). (β-Phenylethylamine picrate has a m.p. of 171° (ref. 8).)

The *N*,*N*,*N*-trimethyl-β-phenylethylammonium chloride was prepared by exhaustive methylation of β-phenylethylamine with methyl iodide in methanol (with periodic additions of methanolic KOH) followed by two crystallizations of the iodide from water. The m.p. of the iodide was 232–232.5° (ref. 6, 232°). The iodide was converted to the chloride by passage through Dowex-1 (Cl^-). The I^--free effluent was standardized by titration for Cl^- (ref. 10). All amines were used as their neutral hydrochlorides and octanoate was used as the neutral potassium salt.

RESULTS

In Fig. 1 is shown the effect of 10 mM β-phenylethylamine on the Na^+-dependent component of glycine entry.

Inhibition is due to a large decrease in V (in this experiment, $V_{\text{inhibited}}/V_{\text{uninhibited}} = 0.39$) with little effect on K_m. (Of five experiments, this one showed the least scatter.) The average $V_{\text{inhibited}}/V_{\text{uninhibited}} \pm$ S. E. ($n = 5$) was 0.405 ± 0.052. The average value for the ratio of "inhibited" to uninhibited K_m, $K_{m\,(\text{inhibited})}/K_{m\,(\text{uninhibited})} \pm$ S.E. ($n = 5$), was 1.042 ± 0.099. The K_m here is that for glycine so β-phenylethylamine appears not to compete with glycine. In these experiments the

Na^+ concentration was 50 mM. At this Na^+ concentration the Na^+-dependent and Na^+-independent terms make roughly equal contributions to K_m (uninhibited) so the failure of β-phenylethylamine to increase K_m implies that the inhibitor does not compete with Na^+ either.

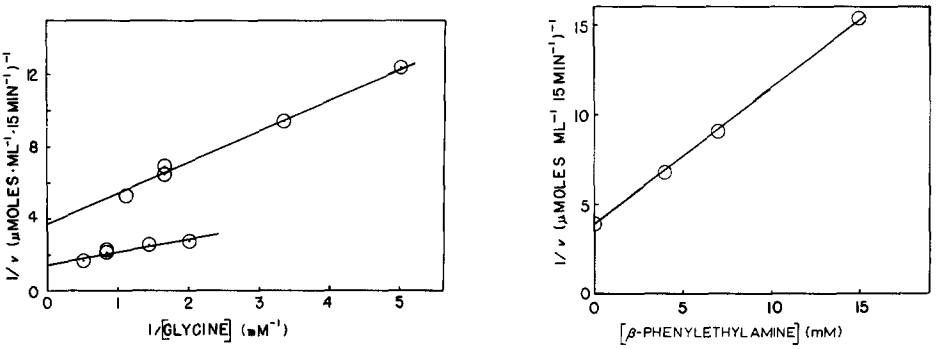

Fig. 1. Effect of 10 mM β-phenylethylamine on V and K_m components of Na^+-dependent glycine entry rate. Cells were prepared and the Na^+-dependent glycine entry rate determined as indicated in MATERIALS AND METHODS. Data are shown as a double reciprocal plot. Entry rate is in μmoles of glycine entering 1 ml pellet water in 15 min at 39°. Upper line, entry in the presence of 10 mM β-phenylethylamine, lower line, entry determined in the same experiment in the absence of β-phenylethylamine The Na^+ concentration was 50 mM. 0 3 g cells were incubated in 2.0 ml medium

Fig 2. Determination of K_i for β-phenylethylamine inhibition of Na^+-dependent glycine entry Procedures and conditions were as for Fig 1 except that β-phenylethylamine was varied as shown and glycine was 0.5 mM for all samples The Na^+ concentration was 50 mM.

Fig. 2 shows the reciprocal of the Na^+-dependent glycine entry rate plotted against the β-phenylethylamine concentration. For pure noncompetitive inhibition[11], K_i would be the concentration of β-phenylethylamine giving 50 % inhibition. The K_i values so obtained from two experiments (Fig. 2 shows one) were 5.2 and 5.5 mM. Inhibition depends on the first power of the β-phenylethylamine concentration, so a single molecule of β-phenylethylamine combining at a critical site inactivates. It also indicates that the "ASCP" route[3] is also inhibited, otherwise curvature would be expected. This can be seen from the following. Suppose the "ASCP" route for glycine entry (V_{ASCP}) is not inhibited by β-phenylethylamine, and suppose this were 10 % of the total (which is less than the percentage indicated by Fig. 5 of ref. 3). Let the glycine entry rate by both routes be $v = V\{K_i/(K_i + [I])\}\{[Gly]/(K_m + [Gly])\} + v_{ASCP}$, where [Gly] = glycine concentration and $[I]$ = β-phenylethylamine concentration. Then if $v_{ASCP} = 0.1$ in the absence of β-phenylethylamine, the percentage of entry at 15 mM β-phenylethylamine due to entry through the main, β-phenylethylamine inhibitable route would be 16 %. Then K_i would be 2.85 mM and the points at 4 and 7 mM β-phenylethylamine in Fig. 2 should be at 8.9 and 11.9 instead of 6.9 and 9.2, respectively, producing a grossly convex curve.

Pure noncompetitive kinetics might arise if β-phenylethylamine irreversibly inactivated the transport mechanism. The data in Table I show that the β-phenylethylamine effect is largely reversible, although some damage to the transport mechanism may occur when cells are exposed to the agent at 39° in the absence of both transport substrates, Na^+ and glycine.

TABLE I

REVERSIBILITY OF β-PHENYLETHYLAMINE INHIBITION OF TOTAL GLYCINE ENTRY

Cell pellets (about 0.3 g) were prepared and glycine-uptake rate determined as indicated in MATERIALS AND METHODS except that total uptake was not corrected for the Na^+-independent component which is minor. (At 50 mM Na^+ and 0.5 mM glycine, Na^+-independent glycine entry is generally 20% or less of the total entry.) As indicated in the table, some samples were preincubated with or without β-phenylethylamine and with or without Na^+ *plus* glycine Controls not previously incubated were simply held at 4° as 15% (w/v) suspensions in Na^+- and glycine-free medium For preincubation, about 0.3 g of cells and 2.0 ml of the usual medium were used Preincubated samples were chilled, centrifuged once and resuspended in 2.0 ml medium containing 50 mM Na^+ and 0.5 mM [^{14}C]glycine and β-phenylethylamine as indicated. The table shows glycine uptake from these media.

Preincubated 15 min at 39° with			*β-Phenylethylamine present in medium during uptake of [^{14}C]glycine (mM)*	*Total glycine taken up in 15 min at 38° (μmoles/ml pellet water)*		*Glycine uptake as % of corresponding β-phenylethylamine free control sample*		*Av. % inhibition*
β-Phenylethylamine (mM)	*Na^+ (mM)*	*Glycine (mM)*		*Expt. 1*	*Expt. 2*	*Expt. 1*	*Expt. 2*	
20	50	0 5	00	0.216	0.214	76.2	96.2	13 8
0	50	0.5	0	0.284	0 223	(100)	(100)	
20	0	0	0	0.170	0 202	57.0	76.4	33 3
0	0	0	0	0 297	0 264	(100)	(100)	
—	—	—	10	0 127	0 137	38.4	51 3	54 3.
—	—	—	0	0.331	0 257	(100)	(100)	

Cell samples were exposed to β-phenylethylamine (in the presence and absence of substrates) for 15 min at double (20 mM) the usual β-phenylethylamine test concentration, and the β-phenylethylamine then removed. The capacity of the various cell samples to take up glycine was then measured and compared (Table I). The inhibition remaining after exposure to, and removal of 20 mM β-phenylethylamine (14% with substrates present during exposure, 33% with substrates absent) is less than that caused by 10 mM β-phenylethylamine present only during glycine uptake (54%) and much less than that (Fig. 2) excepted from the presence of 20 mM β-phenylethylamine (74%). Since only one wash was used, removal of β-phenylethylamine may not have been complete.

To determine whether the charged or uncharged species was responsible for inhibition, inhibition was measured at different pH values (Table II). Cells were pre-equilibrated at 0° with media at the nominal pH shown and incubated in [^{14}C]-glycine and β-phenylethylamine-containing media at the same nominal pH. Although the actual pH values of the cell suspensions during incubation are uncertain, since the measured pH values of supernatants and (stored) cell suspensions are lower than the nominal ones, it is clear that a several-fold change in $[H^+]$ had no significant effect on the extent of inhibition. Since the pK of β-phenylethylamine is 9.83 (ref. 4), the ion concentration is essentially the same at the different pH values, while the free amine concentration varies inversely with the $[H^+]$. Using the data of Fig. 2 as a dose–response curve, it can be seen that a pH difference of 0.1 corresponds to (approximately) a 20% difference in glycine entry rate if unionized β-phenylethylamine is the active form; the pH difference of 0.45 between nominal 6.8 and 7.7 corresponds to a 3-fold change in unionized β-phenylethylamine concentration and a 2-fold change in entry rate. The ratio of membrane-bound uncharged amine to membrane-bound ion

TABLE II

THE pH INDEPENDENCE OF β-PHENYLETHYLAMINE INHIBITION OF Na^+-DEPENDENT GLYCINE ENTRY

Cells were washed twice with cold buffered Na^+- and glycine-free medium (20 ml per 3.4 g cells) to adjust their pH values. Buffer species in the nominal pH 6 8 media and washes were 3 mM $H_2PO_4^-$ and 3 mM HPO_4^{2-}; in the nominal pH 7.1, 3 mM $H_2PO_4^-$ and 6 mM HPO_4^{2-}, and in the nominal pH 7.7, 3 mM $H_2PO_4^-$ and 3.8 mM Tris base. Glycine uptake was measured as usual from media buffered as the corresponding wash media and containing either 9 mM β-phenylethylamine or none. Percent inhibition is 100 (1 − glycine entry (β-phenylethylamine present)/glycine entry (β-phenylethylamine absent)) In one of the two otherwise identical experiments, supernatants were warmed to room temperature and their pH values measured shortly after obtaining them Unincubated aliquots of cells suspensions from the same experiment were capped with parafilm and stored at 4° for 24 h and then warmed to room temperature and their pH values measured. Two different samples at each nominal pH were measured. As agreement was reasonable, the averages are listed In the other (the first) experiment pH values of the supernatants were measured after storage 24 h at 4°; these pH values were 6.81 (6.83, 6.79), 6.97 (range of 10, 6.92–7 07), and 6 98 for the nominal pH values 6.8, 7 1 and 7.7, respectively

Nominal pH	*Measured pH supernatant*	*Cell suspension*	*% Inhibition of Na^+-dependent glycine entry*	
			Expt. 1	*Expt 2*
6 8	6.72	6.71	57 0	55.8
7.1	6.89	6.91	63.9	63.5
7.7	6.92	7.16	53.1	54.5

might be much higher than the ratio of these species in solution. Since inhibition is reversible, the membrane-bound forms are presumably in equilibrium with the dissolved forms, and the membrane-bound free amine must also change with pH. As pH does not affect inhibition, the charged species is responsible for the observed inhibition. Free amine would probably also inhibit, since the isosteric β-phenethyl alcohol does (Table III), but free amine concentration is much lower than the alcohol concentration required to produce an effect.

The data of Table III show that specificity for inhibitor structure is low but not absent. These data are grouped according to inhibitor strength. The differences in strength among the first group of five compounds are not statistically significant ($P > 0.05$ relative to β-phenylethylamine by Students "t" test) although mono- and dimethyl-β-phenylethylamine seem slightly stronger than β-phenylethylamine itself. The compounds of the second group, which includes the nonionic β-phenethyl alcohol, are significantly weaker than β-phenylethylamine ($P < 0.05$ relative to β-phenylethylamine). The *N,N,N*-trimethyl-β-phenylethylammonium ion, the sole member of the third group, does not inhibit. Its apparent stimulatory effect is not statistically significant, although possibly real. Octanoate, and octanoate *plus* β-phenylethylamine are put into a fourth group.

Octanoate at 10 mM (38°) lyses the cells. It is not clear whether inhibition by 5 mM octanoate properly belongs in the same class as the other effects. (β-Phenylethylamine at 20 mM and 39° caused no lysis at all.)

Octanoate does not affect β-phenylethylamine inhibition. The assumption that both octanoate and β-phenylethylamine inhibit noncompetitively and independently allows calculation of the inhibition when both are present together. The calculated

TABLE III

INHIBITION OF GLYCINE ENTRY BY β-PHENYLETHYLAMINE ANALOGUES

Cells were prepared and glycine entry measured as indicated in MATERIALS AND METHODS Na^+-containing media had 50 mM Na^+ and all media had 0.5 mM [^{14}C]glycine. Average percent inhibition is the percentage, ± S.E., by which the analogue at the concentration listed reduced total glycine entry (*n*-hexylamine, β-phenethyl alcohol) or the Na^+-dependent component of glycine entry (all others). (Na^+-dependent entry is generally 80% or more of the total entry at these Na^+ and glycine concentrations.) In the final column are the ratios, ± S.E., of percent inhibition by analogues to percent inhibition by β-phenylethylamine in the same experiment and at the same concentration.

Agent	*Concn. (mM)*	*n*	*Av % inhibition ± S.E.*	*Av. % inhibition relative to β-phenylethylamine at the same concn. ± S.E.*
N-Methyl-β-phenylethylamine hydrochloride	10	2	70.0 ± 5.4	1.27 ± 0.06
N,N-Dimethyl-β-phenylethylamine hydrochloride	10	2	65.1 ± 5.5	1.18 ± 0.04
n-Hexylamine hydrochloride	12	2	72.1 ± 0*	1.04 ± 0.01*
α-Phenylethylamine hydrochloride	10	2	58.6 ± 2.6	1.03 ± 0.06
β-Phenylethylamine hydrochloride	10	12**	61.2 ± 2.1	1.00
Tyramine hydrochloride	10	3	36.5 ± 8.6	0.585 ± 0.155
Benzylamine hydrochloride	12	2	37.2 ± 3.6	0.546 ± 0.042
β-Phenethyl alcohol	12	2	32.2 ± 2.6*	0.465 ± 0.033*
N,N,N-Trimethyl-β-phenylethylammonium chloride	10	3	—15.3 ± 9.3	
Potassium octanoate	5	2	35.9 ± 11.9	1.04 ± 0.04
Potassium octanoate *plus* β-phenylethylamine hydrochloride	5 and 10	2	71.3 ± 2.9	—

* Total entry; see legend of this table.

** This average includes values from experiments not otherwise entered in this table, *e.g.*, those of Figs. 1 and 2. The average β-phenylethylamine inhibition from experiments involving analogues is 61.9 ± 2.2 ($n=7$).

TABLE IV

INHIBITION OF NET Na^+ ENTRY BY β-PHENYLETHYLAMINE

Cells, prepared as usual were preincubated 16 min at 38° in media lacking K^+ (Na^+ = 146 mM) and containing 5 μg/ml strophanthin *k*. After centrifuging, the cell pellets were resuspended in $^{22}Na^+$-containing (122 μC/ml, 146 mM in Na^+) glycine-free medium which also had 2.5 μg/ml strophanthin *k*. Uptake of Na^+ was calculated from uptake of $^{22}Na^+$ as for glycine uptake. Incubation was for 15 min at 38–39°.

Expt. No.	*β-Phenylethylamine concn. (mM)*	*Na^+ entry (μmoles/ml pellet H_2O in 15 min, 38°)*	*Inhibition (%)*
1	0	1.20	
1	10	1.12	6.5
2a	0	2.17	
2a	20	0.97	55.0
2b	0	2.23	
2b	20	1.78	21.0

inhibition is 70.8%, in good agreement with the measured 71.3%. Octanoate antagonism of β-phenylethylamine inhibition was looked for because it was known that lowering the pH decreased glycine entry[1,3]. If this pH effect were due to an increase in the net positive charge distributed over (or in) the membrane, β-phenylethylamine might inhibit transport by likewise merely increasing the positive charge of the membrane. Then octanoate might add anions to the membrane and so antagonize (or augment) the β-phenylethylamine effect. The lack of octanoate effect makes this mode of action for β-phenylethylamine less probable.

β-Phenylethylamine inhibited Na^+-independent entry less than Na^+-dependent entry. 10 mM β-phenylethylamine inhibited Na^+-independent glycine entry by 26.4 ± 6.5 % (inhibition ± S.D., $n = 8$). The data for this average were from different experiments, and percent inhibition was estimated in different ways and with different glycine concentrations. The average is therefore reported with S.D. rather than S.E. in order not to understate the error. The different methods of estimation gave similar values: maximal and minimal group estimates, 30 and 22 %; maximal and minimal single values, all groups, 37 and 16 %.

The effect of β-phenylethylamine on $^{22}Na^+$ entry was also briefly examined (Table IV). Inhibition was seen in all trials, but the effect was small and erratic.

Thus β-phenylethylamine inhibits at least four different transport processes (main-route Na^+-dependent glycine entry, "ACSP"-route glycine entry, Na^+-independent glycine entry, and Na^+ entry) but not all equally.

DISCUSSION

As a first approximation, β-phenylethylamine acts as a reversible, first-power, noncompetitive inhibitor of Na^+-dependent glycine transport. With respect to the noncompetitive character, $\{1 - V_{\text{inhibited}}/V_{\text{uninhibited}} + 2 \times \text{S.E.}\} = 0.491$, while $\{1 - (K_{m(\text{uninhibited})}/K_{m(\text{inhibited})}) + 2 \times \text{S.E.}\} = 0.238$, so the effect on K_m is unlikely to be as much as half as great as that on V. From kinetic studies with hemolyzed and restored cells[12], Na^+-dependent glycine transport into Na^+-poor cells involves an inside-to-outside transition of empty transport mechanism ($E_i \rightarrow E_0$) and an outside-to-inside transition of a fully loaded complex ($E\text{–}Na_2Gly_0 \rightarrow E\text{–}Na_2Gly_1$). These two transitions appear to be jointly rate-limiting. If β-phenylethylamine equally inhibits both, the effects of β-phenylethylamine on the K_m terms would cancel, and inhibition would be purely noncompetitive. If inhibition of the steps were markedly unequal, an effect of β-phenylethylamine on K_m should have been observed. If β-phenylethylamine affected the glycine, Na^+, (or Cl^-) combination reactions, an effect on K_m would again be expected. The simplest interpretation of the β-phenylethylamine inhibition is that the transition reactions are (roughly) equally impeded. The combining site for β-phenylethylamine may not, however, be in (or on) the transport mechanism itself, but in its immediate surroundings.

Specificity for β-phenylethylamine is low. Several amines were about equally effective, with *n*-hexylamine being least like β-phenylethylamine. The approximate equivalence of *n*-hexylamine, β-phenylethylamine, and *N*,*N*-dimethyl-β-phenylethylamine suggests that the chief requirements for inhibitory action are a positive charge and a medium-sized hydrophobic region, although substituents (*c.f.* tyramine) may modify activity. The failure of *N*,*N*,*N*-trimethyl-β-phenylethylammonium ion to

inhibit invites explanation. The capacity to hydrogen bond might be critical, but this is unlikely since β-phenylethylamine and its mono- and di-*N*-methyl derivatives were all about equally effective. A more attractive hypothesis is that a cation permeability barrier lies between the medium and the inhibitor combining site. The other amines might cross this in their unionized forms, followed by either H^+, or OH^- *plus* H_2O a path not open to the quaternary ion.

Some specificity for transport systems was observed in that Na^+-dependent glycine transport was more sensitive to β-phenylethylamine than Na^+ transport or Na^+-independent glycine transport. The effects on the latter two were not resolved. The Na^+-independent glycine transport shows diffusion-like kinetics[1,3] but could be, or include, mediated transport by a high K_m route(s). Na^+ entry can occur by several routes, *e.g.*, reversal of the strophanthin-sensitive Na^+–K^+ pump, possibly reversal of the ethacrynic acid-sensitive[13] Na^+ pump, pure diffusion leak (if any), and amino acid routes.

Interpretation of the Na^+-transport effect is complicated by the probability that Na^+ exit is also inhibited by β-phenylethylamine. Strophanthin-poisoned cells were used to reduce exit by one of the major routes. However, inhibition by β-phenylethylamine of other routes, including, for Na^+ exit, the main glycine route, may be responsible for the variability of inhibition of $^{22}Na^+$ uptake.

From the properties found, β-phenylethylamine should be a useful experimental tool.

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