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HARMALINE INTERACTION WITH SODIUM-BINDING SITES IN INTESTINAL BRUSH BORDER SUCRASE

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Summary

The effect of harmaline on rabbit brush border sucrase has been studied at pH 6.8. An initial analysis in classical kinetic terms revealed harmaline to be a fully competitive inhibitor of the substrate, sucrose. In spite of this result however, the following hypothesis has been tested. Harmaline, which is positively charged in the physiological range of pH, might in fact compete, not directly with the substrate site, but rather with an allosterically-related sodium-binding site which has been postulated to be involved in the activation of sucrase by the alkali-metal ions (Mahmood and Alvarado, Arch. Biochem. Biophys. 168, 585, 1975). Because of its size, harmaline, when bound to the metal site, could at least partially overlap with the substrate site, thereby behaving as if it were an authentic fully competitive inhibitor of the substrate. This hypothesis appears to be confirmed by the fact that the alkali metals can completely reverse the inhibition caused by harmaline.

Introduction

The psychotomimetic alkaloid, harmaline, has recently attrached much interest because of its apparent interference with the binding of the sodium ion to enzymes and to transport systems dependent on this cation (for review see ref. 1). In particular, the detailed studies of Sepúlveda and Robinson [2] strongly indicate that harmaline competes with Na^+ for the sites implicated in the activation of sugar and amino acid transport systems in the small intestine. These workers questioned whether harmaline may be a highly specific ligand for Na^+-binding sites in certain proteins; because of their similarity of function, all such Na^+-binding sites could involve the same reactive groups or at least have

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some common molecular features. On the other hand, harmaline could simply bind non-specifically to cation-binding sites since it possesses a positive charge.

In order to differentiate between these two possibilities, the present study has been undertaken to examine the effect(s) of harmaline on another Na^+-activated system, intestinal brush border sucrase (sucrose α-glucohydrolase, EC 3.2.1.48). This enzyme shares with the above-mentioned transport systems the properties of: (1) location in the same subcellular organelle, the apical or brush border membrane of the enterocyte; and (2) activation by Na^+ according to a similar, allosteric, non-compulsory mechanism [3]. Even though important quantitative differences exist between sucrase and Na^+-linked transport systems [3], sucrase remains an excellent model for Na^+-activation that could possibly clarify the still poorly understood mechanism of solute and Na^+ cotransport. With these differences in mind, we are currently engaged in a study of sucrase, both in its native state (still bound to the membrane matrix) and in the isolated form. The interest of our studies is compounded in view of the old observation that sucrase may be functionally linked to the D-glucose transport system(s) in the intestine [4,5]. An explicit suggestion has even been made that sucrase may act as a carrier for D-glucose residues resulting from the enzymatic hydrolysis of sucrose [6].

Before proceeding, however, it must be mentioned that alkali metal activation of sucrase is strongly dependent on pH. In addition to the metal-binding site involved in alkali metal activation in the pH range 5—7, brush border sucrase ionizes above pH 7 to generate an additional "anionic site" at which level both organic and inorganic cations can bind and cause inhibition [7]. In order to simplify matters, the present report (first in a series) is restricted only to observations made using partially purified brush-border membrane fragments from rabbit jejunum at pH 6.8. The effects of both harmaline and other organic cations at pH values other than neutral will be dealt with in subsequent publications. Preliminary accounts of this work have been given [7,8].

Materials and Methods

Enzyme preparation. This work has been done using relatively pure brush-border membrane fragments obtained from rabbit jejunum according to the method of Schmitz et al. [9]. The preparation used is equivalent to Fraction P_2 of these workers and is essentially free of contamination by other cell constituents, notably DNA, endoplasmic reticulum, mitochondria and basolateral membranes. The key step in the isolation procedure is a treatment with solid $CaCl_2$ to give a final 10 mM Ca^{2+} concentration. All membrane material other than brush-border fragments tends to aggregate under these conditions and can be sedimented at low speed (2000 $\times g$). A subsequent centrifugation at 42 000 $\times g$ yields an essentially pure population of brush-border fragments. Details of the procedure used and evidence for the lack of an effect of Ca^{2+} on the kinetics will be published elsewhere.

Determination of sucrase activity. Sucrase activity was assayed by measuring D-glucose formation with glucose oxidase and peroxidase [10,11], as previously described [3]. In experiments involving harmaline, it was necessary to remove first this alkaloid, which interferes with the glucose analysis. The 0.5 ml incuba-

tion mixtures were treated with an equal volume of 0.5 M Tris, pH 7.2 (to stop the sucrase reaction) and subsequently extracted with isobutanol [12]. Two extractions, first with 2 ml, then with 1 ml, were found to be sufficient: glucose was then assayed in 0.5-ml aliquots of the aqueous extracts. The isobutanol treatment did not interfere with the glucose analysis: on the contrary, it was found that the colour yield was enhanced by about 40%. This increase is attributed to a stabilizing effect on the chromogen, *o*-dianisidine (which is known to be poorly soluble in water) by traces of isobutanol in the aqueous extracts. It has also been verified that essentially identical results can be obtained whether isobutanol extraction is involved or not (see, for instance, the results shown in Table II). Protein was determined according to the method of Lowry et al. [13].

Reagents and buffers. All reagents were of A.R. grade. All solutions were prepared in distilled, then deionized, water. Sucrose, harmaline and Tris were obtained from Sigma. The glucostat reagent came from Worthington and contained 1% Triton X-100 [11]. No truly inert buffer has yet been found for use with brush border sucrase: all cations so far used, either inorganic or organic, have some effect on the enzyme. Nevertheless, and for the purposes of the present study, carried out only at pH 6.8, a maleate buffer containing 5—10 mM Li^+ has been used and can be assumed to be 'inert' for all practical purposes. We are beginning to use metal-free buffers such as that utilized for the experiments in Table II, where the only cation other than H^+ is the *t*-butylammonium ion. Even this organic cation cannot be considered as truly inert, but its use seems justified for our present purposes, similar to the case of Li^+ just mentioned. All cations were added in the form of their chlorides. For the reasons stated above, no inert cation was available to maintain the ionic strength of the buffers constant.

Calculation of the results. Velocities are expressed as units per mg of protein (1 unit = 1 μmol/min under standard conditions [3]). V and K_m were calculated using the $[S]/v = f[S]$ transformation [14]. Straight lines were calculated by the method of least squares, using directly the raw experimental data, without 'weighting'. Six values of [S] were used for each curve (range: 8—40 mM): at least 2—3 determinations were made for each experimental point.

Results and Discussion

Rationale. At neutral or slightly acidic pH, brush border sucrase may be envisaged as having two distinct, allosterically related binding sites: one for the substrate, sucrose, and another for the metal activator [3]. If harmaline were a general, specific ligand for Na^+-binding sites, then it could be expected to interact only with the second of these sites.

In initial experiments, therefore, we established first that harmaline indeed inhibits intestinal sucrase from hamster, guinea pig, rat and rabbit, both in the presence and in the absence of sodium, and in the pH range 5.2—9. The obvious questions followed: (1) Does harmaline inhibit sucrase through a direct interaction with the Na^+-binding site? (2) Is this the only action of harmaline? (3) If harmaline indeed competes for the metal site, is its binding to this site 'specific'

or is it simply due to the fact that harmaline is a cation? The harmala alkaloids have a β-carboline nucleus possessing a lone pair of electrons. Because of its high pK_a (about 9.8), harmaline would be protonated and bear a positive charge in the pH range mentioned.

Even if we restrict the ensuing discussion only to experiments performed at pH 6.8, when the simplified situation mentioned above can be assumed to hold, it would be impossible to find a simple, direct answer to the first of the above-listed questions. In hamster, guinea pig and rat sucrase, Na^+ is a pure affinity-type or *K*-activator [3,15,16]. This means that, if a classical 'competitive' effect were found using harmaline (increase in the apparent K_m without significant effect on *V*), it could be interpreted either as a direct competition for the substrate site, or as a reversal of the metal activation. In principle, the use of rabbit sucrase could simplify the interpretation if it were true that, in this species, Na^+ is essentially a pure capacity-type or *V*-activator having no significant effect on K_m [15]. However, we have not been able to confirm this finding since, in our hands, the effect of Na^+ on rabbit sucrase is mixed: both an increase in *V* and a reduction in K_m are induced by this cation (these results, which exceed the limits imposed upon this paper, will be presented in detail elsewhere). Nevertheless, and in spite of the above reservations, we have attempted an analysis of the effect of harmaline on rabbit sucrase, in classical kinetic terms, as follows.

Harmaline behaves as a fully competitive inhibitor with regard to the substrate, sucrose. A kinetic analysis at constant [I] and variable [S] revealed that harmaline inhibits sucrase by increasing the apparent K_m, with no significant effect on *V* (Tables I and II). This is the criterion for competitive inhibition, and application of the appropriate equation gave a value of $K_{i(harmaline)}$ = 2.5 mM (range: 1.5—3.5 in several similar experiments). Essentially identical results were found using either a Li^+ buffer (Table I) or a metal-free buffer (Table II).

TABLE I

RABBIT BRUSH BORDER SUCRASE: INHIBITION BY HARMALINE AND ITS REVERSAL BY THE SODIUM ION

Kinetic analysis at constant [I] and variable [S]. Buffer: 8 mM lithium maleate, pH 6.8. (*r*) is the correlation coefficient for the plot $[S]/v = f[S]$. For the calculation of K_i in the last series, the average control value in expts. 1 and 2 was used as the reference K_m (9.5 mM).

Expt.	[Harmaline] (mM)	[Na^+] (mM)	(*r*)	*V* (units/mg protein)	K_m (mM)	K_i (mM)
1	—	—	(0.997)	1.39	8.35	—
	3.2	—	(0.995)	1.67	22.5	1.89
	9.6	—	(0.961)	1.40	51.0	1.88
2	—	—	(0.998)	1.66	10.6	—
	3.2	—	(0.999)	1.63	22.1	2.97
	9.6	—	(0.946)	1.67	64.6	1.89
3	6.4	—	(0.977	1.33	27.8	3.30
	6.4	2.	(0.986)	2.18	20.7	—
	6.4	20.	(0.997	2.16	11.5	—
	6.4	100.	(0.995)	2.16	9.8	—

TABLE II

RABBIT BRUSH BORDER SUCRASE: INHIBITION BY HARMALINE AND ITS REVERSAL BY THE POTASSIUM ION

Conditions similar to those in Table I, except for the use of a metal-free buffer (succinic acid/phosphoric acid/glycine/*t*-butylamine = 10/10/10/22.4 mM: pH adjusted to 6.8 with HCl). As in Table I, the incubation mixtures were extracted with isobutanol, except for the case of expts. 1 and 2.

Expt.	[Harmaline] (mM)	[K^+] (mM)	(r)	V (Units/mg protein)	K_m (mM)	K_i (mM)
1	—	—	(0.999)	1.08	13.2	—
2	—	—	(0.997)	1.16	17.2	—
(1 + 2) *	—	—	(0.995)	1.12	15.2	—
3	—	—	(0.992)	1.02	14.5	—
4	—	—	(0.997)	0.93	12.1	—
(3 + 4) *	—	—	(0.993)	0.97	13.2	—
3	3.2	—	(0.985)	1.16	34.9	2.27
4	3.2	—	(0.990)	0.83	26.3	2.73
(3 + 4) *	3.2	—	(0.914)	0.97	30.0	2.51
4	3.2	1.0	(0.996)	0.93	14.4	—

* For this calculation, all the available [S] and v values (n = 12) were utilized.

In order to establish whether fully or partially competitive inhibition [17] is taking place, a second kinetic analysis at constant [S] and variable [I] was performed. A typical result, plotted in the form of $1/v$ = f [I] is shown in Fig. 1. The fact that straight lines are obtained indicates that the inhibition is not partial. The fact that several such straight lines, each obtained at a different value of [S], all intercept at a common point above the x-axis indicates that the inhibition is fully competitive (type Ia of Dixon and Webb [17]). The point at which the three lines join gives the value of $K_{i(harmaline)}$ = 2 mM (range: 1.8—2.5 in several other similar experiments).

A strict interpretation of the above results would have been that harmaline competes with sucrose directly for the substrate site, an interpretation exactly the opposite of our working hypothesis. Such an explanation, however, seems hardly plausible in view of the lack of structural similarity between harmaline and sucrose. Therefore, we chose to investigate the following alternative interpretation. Even if harmaline binds selectively to the metal site, it seems conceivable that this bulky organic cation could at least partially overlap with the substrate site, which by definition must not be very far away. Such an overlapping could then result in an interference with the binding of sucrose to its own site, e.g. by steric hindrance. This situation would yield kinetics of fully competitive inhibition even when harmaline may have no significant affinity for the substrate site proper. Situations similar to this one have been proposed to explain, for instance, the fully competitive inhibition by phloretin of the D-glucose transport system in erythrocytes [18].

Harmaline inhibition is fully reversed by alkali metal ions. The statement that harmaline is a fully competitive inhibitor of sucrase is equivalent to saying that the inhibition is readily reversible. We nevertheless verified this reversibility in experiments involving extreme conditions, such as preincubation of the

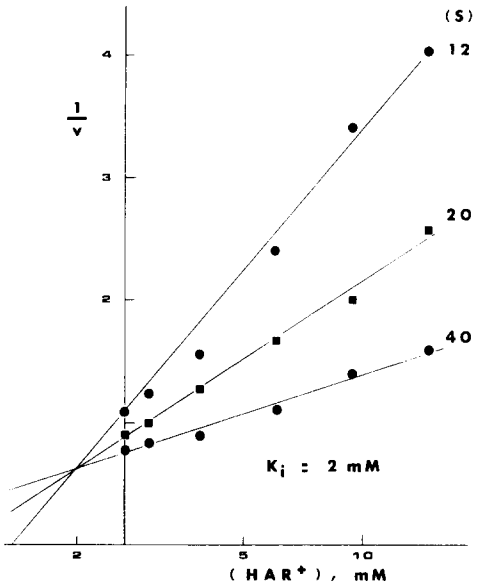

Fig. 1. Fully competitive inhibition caused by harmaline. Results plotted and calculated according to Dixon's method [17]. [S] indicates the substrate concentrations used to prepare each series of points. Maleate buffer, pH 6.8, containing 5 mM Li^+.

enzyme with 12.8 mM harmaline for 1 h, either at pH 5.2 or 9.0, at 4° C or at 37° C. Subsequent dialysis or simple dilution of the concentrated enzyme resulted in the recovery of essentially all the enzymatic activity (results not shown).

Another kind of reversibility, however, seems considerably more relevant to our enquiry. If it were true that the primary bond of harmaline occurs at the metal- rather than at the substrate-binding site, then the inhibition should be fully reversed by the alkali-metal ions. The concentration-dependent reversal of harmaline inhibition by Na^+ is illustrated in Fig. 2. First, a control curve demonstrates the activation of sucrase by Na^+. A plateau is reached at 40% activation, a result in agreement with data in the literature [15,16]. Second, the lower curve in Fig. 2 shows the inhibition caused by 12.8 mM harmaline and its complete reversal by Na^+. In the absence of this cation, inhibition is 65%; at 15 mM Na^+ the control level is reached; and at 100 mM Na^+ the plateau of maximal activation is attained.

In Tables I and II it is demonstrated that Na^+ and K^+ both have kinetic effects opposite to that of harmaline, i.e., both metals reverse the increase in K_m caused by the inhibitor, without any concomitant effect on V. A 1 mM concentration of K^+ is practically sufficient to counteract fully the inhibition caused by 3.2 mM harmaline. This result agrees with the observation that K^+ has greater affinity than Na^+ for the metal site [3,7].

Conclusions. Taken together, the experiments just described strongly support our working hypothesis that the primary site of action of harmaline is at the metal-binding site of sucrase. Furthermore, since this site is not strictly specific for Na^+ (both K^+ and Li^+ can activate the enzyme [7]), we conclude

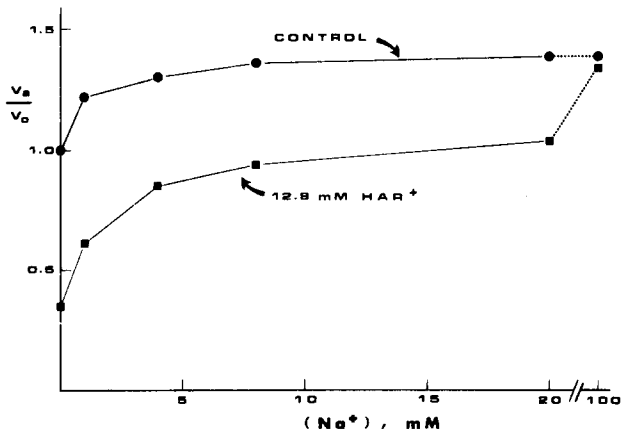

Fig. 2. Inhibition by 12.8 mM harmaline and its reversal by Na^+. Results expressed as v_a/v_0 ratios, where v_0 is the reference velocity in the absence of both harmaline and Na^+. Same buffer as in Fig. 2. Sucrose concentration: 20 mM.

that harmaline competes with the alkali metals for their binding site primarily because of its positive charge. We also have noted the inhibition of sucrase by other organic cations, e.g. some protonated amines (unpublished observations). What appears to be peculiar about harmaline, however, is that this relatively bulky molecule seems to overlap and block the substrate site, thereby giving the kinetics of fully competitive inhibition which smaller organic cations cannot produce.

Before closing we would like to compare the effects of harmaline on sucrase and on the organic solute transport systems in intestine. In both cases, it seems that harmaline competes with Na^+ for the same sites involved in Na^+-activation. * Furthermore, the apparent K_i for harmaline in each case is of the same order of magnitude: 2.5 mM for sucrase according to the present work and 1.6 mM for phenylalanine transport according to Sepúlveda and Robinson [2]. However, in our experiments this constant was calculated in terms of an apparent competition between harmaline and sucrose for the substrate site, whereas in the transport work, K_i was determined in experiments involving a direct competition between harmaline and Na^+ [2].

There is, therefore, a clear difference between the action of harmaline on sucrase and its behaviour towards phenylalanine transport. According to our model, harmaline bound to the metal site of sucrase can at least partially overlap with the substrate site: This implies that the two sites are very close to one another. In contrast, harmaline appears to have no direct effect on the substrate site of the transport system: In the absence of Na^+, no inhibition of phenylalanine transport could be induced by harmaline [2]. This difference could be interpreted in either of two ways. On one hand, the substrate and

* One referee has suggested that we consider the possibility that the effect of harmaline on intestinal transport systems is not due to a direct action on the Na^+-coupled carrier, but rather on the (Na,K)-ATPase. This interpretation can be rejected in view of the evidence published by Sepúlveda and colleagues [1,19]. These workers have shown quite clearly that, although harmaline is an inhibitor of the ATPase, its effect on non-electrolyte influx into the enterocyte is not secondary to its action on this enzyme.

metal sites of the transport systems could be separated from each other by a distance longer than that postulated for sucrase; in this way no overlap of harmaline would be possible. On the other hand, when bound to the metal site of the transport carrier, harmaline could be oriented away from the substrate site, also resulting in lack of overlap with this site. Further work will be needed, however, in order to choose between these two possibilities.

Acknowledgements

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