Biochimica et Biophysica Acta, 323 (1973) 408–414

BBA 76468

PHLORIZIN RECEPTORS IN ISOLATED KIDNEY BRUSH BORDER MEMBRANES

DIFFERENTIAL ENZYMATIC MODIFICATION OF HIGH-AFFINITY RECEPTORS AND UNSPECIFIC BINDING SITES

HARTMUT GLOSSMANN and DAVID M. NEVILLE, JR

Laboratory of Neurochemistry, Section on Biophysical Chemistry, National Institute of Mental Health, Bethesda, Md. 20014 (U.S.A.)

(Received July 2nd, 1973)

SUMMARY

High-affinity phlorizin receptors in isolated kidney brush border membranes are destroyed by the proteolytic enzymes trypsin and papain. The digested membranes show increased unspecific phlorizin binding. It is proposed, that both enzymes expose a deeper, more hydrophobic layer in the brush border membrane to explain the latter finding.

INTRODUCTION

Isolated kidney brush membranes (rat) contain high-affinity receptors for the potent sugar transport inhibitor phlorizin[1–5].

A strictly sodium-dependent receptor ($K_1 = 5\,\mu M^{-1}$, $N_1 = 40$–50 pmoles/mg protein) in the brush border membrane has affinity only for D-glucose > D-galactose > 2-deoxy-D-glucose while a second receptor ($K_2 \approx 0.15\,\mu M^{-1}$, $N_2 \approx 60$ pmoles/mg protein), shows sensitivity to D-mannose and several other sugars[4]. The high affinity of phlorizin for the glucose carrier *in vivo* ($5\,\mu M^{-1}$)[6] and the perfect agreement of binding-inhibition and transport-inhibition data obtained with phlorizin analogues *in vitro* and *in vivo* by Diedrich and coworkers[5,6] strongly suggest that phlorizin receptors may represent the glucose carrier. We have recently shown that trypsin treatment of the brush border membrane led to a loss of phlorizin binding when tested at low concentrations of tracer phlorizin[4]. We will report here that treatment with proteolytic enzymes reduces the number of high-affinity sites in digested membranes but increases the phlorizin binding for those sites which do not bind monosaccharides.

MATERIALS AND METHODS

The preparation of brush border membrane, enzyme determination and the phlorizin binding assay using millipore filtration have been described in detail

Abbreviation: TPCK, [L-(1-tosylamido-2-phenyl)ethylchloromethyl ketone]-trypsin.

elsewhere[4,7–9]. In this study binding of phlorizin was also measured with an ultracentrifugation technique as follows.

Brush border membranes were incubated for 5 min at 22 °C with tracer phlorizin in a final volume of 0.2 ml. Membrane particles were sedimented by centrifugation ($50000 \times g$ for 30 min at 22 °C). The supernatants were discarded and the upper surface of the membrane pellet was rinsed by quick addition and removal of 250 μl ice-cold 10% (w/v) sucrose in assay buffer (20 mM Tris–HCl, 140 mM NaCl, 5 mM disodium ethylenediaminetetraacetate, pH 7.4).

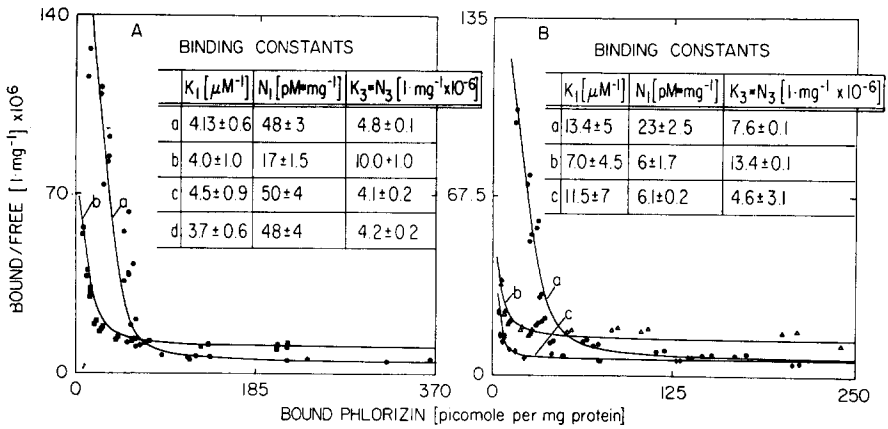
[^{3}H]Phlorizin (5 Ci/mmole, 1 pmole = 11100 dpm) was from New England Nuclear, TPCK [L-(1-tosylamido-2-phenyl)ethylchloromethyl ketone]-trypsin, papain (15.7 units/mg), carboxypeptidase A and B (treated with DFP) were from Worthington, pancreatic trypsin inhibitor and neuraminidase were from Sigma. Binding constants were calculated with a computer program[4]. In the results reported here, binding curves were fitted assuming one set of high affinity sites (K_1, N_1) which show competition with D-glucose and one set of sites (K_3, N_3) which are unsaturated over the range of free phlorizin tested and show no affinity for monosaccharides. We will refer to these as unspecific sites.

RESULTS

When kidney brush border membranes were treated with trypsin (ratio 1:10–1:20; mg trypsin/mg brush border protein) for 15–25 min at 37 °C, 10–20% of the total brush border protein was released and could not be sedimented by centrifugation at $50000 \times g$ (30 min) or $120000 \times g$ (60 min).

The trypsin-digested membranes, collected by centrifugation ($50000 \times g$, 30 min) demonstrated peculiar binding properties for phlorizin when compared with untreated or "control" membranes (membranes incubated in buffer or incubated with trypsin in the presence of trypsin inhibitor). At low initial concentrations of phlorizin a decrease of bound phlorizin was observed. At high initial concentrations of phlorizin a two-fold increase in bound phlorizin occurred, while only small changes at intermediate concentrations were found. A comparison of binding data in a Scatchard plot (Fig. 1a) suggested that trypsin had two actions. It affected high-affinity phlorizin receptors and changed unspecific phlorizin binding of the digested membrane. An analysis of binding data with computer fitting confirmed, that trypsin treatment markedly decreases the number of high-affinity phlorizin receptors, while increasing unspecific phlorizin binding (characterized by the term $K_3 \times N_3$) two-fold. The effects of trypsin were strictly dependent on its proteolytic activity; trypsin inactivated by diisopropylphosphofluoridate was completely ineffective. Since disc electrophoresis[10] revealed, that most of the proteins released by trypsin treatment coelectrophoresed with the main glycopeptides of the kidney brush border membrane[11] we treated the brush border membranes with neuraminidase. However, neuraminidase treatment did not alter the binding curve (Fig. 1a). Hence, if phlorizin receptors are glycoproteins accessible to neuraminidase, terminal sialic acid does not seem to be important in phlorizin binding.

The proteolytic enzyme, papain, had similar actions on the brush border membrane as trypsin (Fig. 1b), but is more potent than the latter, compared on a weight to weight basis (Tables I and II).



	K_1 [μM^{-1}]	N_1 [pM·mg^{-1}]	$K_3 \cdot N_3$ [l·mg^{-1} x10^{-6}]
a	4.13±0.6	48±3	4.8±0.1
b	4.0±1.0	17±1.5	10.0±1.0
c	4.5±0.9	50±4	4.1±0.2
d	3.7±0.6	48±4	4.2±0.2

	K_1 [μM^{-1}]	N_1 [pM·mg^{-1}]	$K_3 \cdot N_3$ [l·mg^{-1} x10^{-6}]
a	13.4±5	23±2.5	7.6±0.1
b	7.0±4.5	6±1.7	13.4±0.1
c	11.5±7	6.1±0.2	4.6±3.1

Fig. 1A. Effect of trypsin treatment on phlorizin binding of the brush border membrane (Millipore technique, 37 °C). Brush border membranes were digested at 37° C for 15 min with trypsin (1 mg/20 mg brush border protein) followed by trypsin inhibitor; and neuraminidase (1 mg/10 mg brush border protein). Digested membranes were diluted tenfold with ice-cold assay buffer, centrifuged at 50000 $\times g$ for 30 min at 2 °C and the pellets after resuspension in assay buffer were used for the binding tests. Only the results for the trypsin treated membranes (b) and control membranes (membranes incubated in buffer) (a) are shown in the graph. Membranes incubated with trypsin + trypsin inhibitor (c) or neuraminidase (d) yielded binding data which were not significantly different from control membranes. B. Comparison of the effects of papain and *N*-ethylmaleimide treatment on phlorizin receptors (Millipore technique, 37 °C). Brush border membranes were treated with *N*-ethylmaleimide and papain as indicated in Table IIC. a, membranes incubated in buffer; b, membranes treated with papain; c, membranes treated with *N*-ethylmaleimide. Each point in A and B is a mean value of a duplicate determination. For reasons of space the right part of the graph in A has been cut off at 370 pmoles bound per mg of protein and in B at 250 pmoles per mg of protein though extending to 600 or 800 pmoles per mg of protein.

TABLE I

DESTRUCTION OF D-GLUCOSE SENSITIVE PHLORIZIN BINDING SITES BY PROTEOLYTIC ENZYMES IN THE BRUSH BORDER MEMBRANE

Kidney brush border membranes were digested with trypsin (1 mg per 10 mg brush border protein), papain (1 mg per 25 mg brush border protein), carboxypeptidase A (1 mg per 25 mg brush border protein) and carboxypeptidase B (1 mg per 25 mg brush border protein) for 15 min at 37 °C. Digests and control samples incubated in buffer without enzymes were diluted ten-fold with ice-cold asssay buffer and centrifuged at 50000 $\times g$ for 30 min at 2°C . Resuspended pellets were adjusted to the same protein concentration and binding tests were performed in the absence or presence of 18 mM D-glucose using the millipore filtration technique at 37 °C. 18 pmoles/mg protein phlorizin was bound to control membranes in the absence of D-glucose and 9 pmoles per 1 mg protein were bound in the presence of D-glucose. [^{3}H]Phlorizin concentration was 0.13 μM. Values are expressed as percent of D-glucose inhibitable binding seen in controls.

Pretreatment conditions at 37 °C	*% of D-glucose inhibited phlorizin binding*
Buffer (control)	100
Trypsin	43
Papain	16
Carboxypeptidase A	86
Carboxypeptidase B	94
Carboxypeptidase A + carboxypeptidase B	61

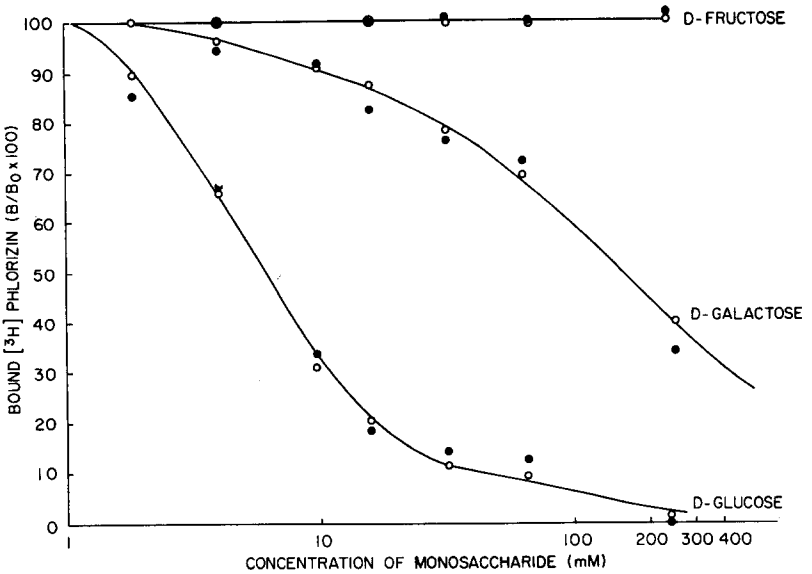


Fig. 2. Kidney brush border membranes were incubated with trypsin (1 mg per 10 mg brush border protein) for 15 min at 37 °C followed by pancreatic trypsin inhibitor (1 mg per mg trypsin) or with trypsin + trypsin inhibitor present. Digests were diluted ten-fold with ice-cold assay buffer, centrifuged at 50000 × *g* for 30 min at 2 °C and after removal of the supernatant pellets were adjusted to the same protein concentration (1 mg/ml) and binding tests performed (Millipore technique, 37 °C). Trypsin had removed 16% of the total brush border protein. [^{3}H]Phlorizin was 0.041 μM. The graph shows the inhibition of phlorizin binding by D-glucose and D-galactose for membranes digested with trypsin (●—●) and control membranes (exposed to trypsin + trypsin inhibitor) (○—○). B, [^{3}H]phlorizin bound in the presence of monosaccharide; B_0, [^{3}H]phlorizin bound in the absence of monosaccharide. B_0 was 14% of the total tracer initially present for control membranes and 4.5% for trypsin treated membranes. Binding data in the presence of D-fructose, a monosaccharide which does not affect phlorizin binding at these low phlorizin concentrations[4] are also included. Each point is a mean value of a duplicate determination.

Again, as was the case with trypsin, unspecific binding for phlorizin in the digested membrane is increased. The residual high-affinity receptors present in the digested membrane show the same sensitivity to D-glucose and D-galactose as untreated membranes (Fig. 2). Besides papain and trypsin, a combination of carboxypeptidase A and B was capable of destroying D-glucose-sensitive phlorizin binding in the brush border membrane (Table I). Since both enzymes are diisopropylphosphofluoridate treated, this action cannot be attributed to trypsin or chymotrypsin contamination. The millipore filtration technique has disadvantages. It could be argued, that proteolytic digestion of the brush border membrane leads to smaller particles which sediment after centrifugation (50000 × *g*, 30 min), but are not retained on the millipore filters. This was tested by comparing phlorizin binding of untreated and digested membranes with the millipore and ultracentrifugation technique.

The results with both methods were very similar (Table II), assuring us that we are not measuring artifacts observable only with millipore filtration.

One could suspect that destruction or removal of high-affinity phlorizin binding sites in the brush border membrane is always correlated with a change in unspecific

phlorizin binding. This is clearly not the case as exemplified by the action of *N*-ethylmaleimide: Treatment with *N*-ethylmaleimide reduces the number of high-affinity brush border membranes without changing unspecific phlorizin binding significantly (Fig. 1B, Table IIC).

TABLE II

EFFECT OF PAPAIN AND TRYPSIN ON PHLORIZIN BINDING OF THE KIDNEY BRUSH BORDER MEMBRANE

A. Brush border membranes were digested with trypsin (1 mg per 10 mg brush border protein) for 15 min at 37 °C. Pancreatic trypsin inhibitor was added (1 mg per mg trypsin) and it was proceeded as described in Table I. The protein concentration in the resuspended pellets was adjusted to the same value (1 mg/ml) and phlorizin binding measured with a millipore technique at 37 °C and 22 °C or with ultracentrifugation at 22 °C as described under Methods. [^{3}H]Phlorizin was 0.74 μM. Note that phlorizin binding at 22 °C is only 40–50% of the value at 37 °C which is due to the lower association constant of the high-affinity receptor at 22 °C[5]. B. Brush border membranes were digested with papain (1 mg per 60 mg brush border protein) for 10 min at 37 °C. This was followed by a 10-fold dilution of the digest with ice-cold assay buffer and centrifugation as described above. C. Brush border membranes were digested with papain (1 mg per 40 mg brush border protein) or reacted with *N*-ethylmaleimide (1 mM) for 10 min at 37 °C. Centrifugation and binding tests were performed as described above. Values are mean values ±1 S.D. Number of experiments are in parenthesis.

	Pretreatment conditions (at 37 °C)	*[^{3}H]Phlorizin bound (pmoles/mg protein)*		
		Millipore filtration		*Ultracentrifugation (22 °C)**
A	Buffer, 15 min	6.4±0.2 (4)	at 22 °C	
	Buffer, 15 min	16.5±0.5 (4)	37 °C	6.8±0.4 (4)
	Trypsin, 15 min	7.3±0.4 (4)	37 °C	3.4±0.1 (4)
	Trypsin + trypsin inhibitor, 15 min	17.0±0.4 (4)	37 °C	6.9±0.3 (4)
B	Buffer, 10 min	16.9±1.1 (4)	37 °C	8.9±0.5 (3)
	Papain, 10 min	9.8±0.6 (4)	37 °C	4.7±0.3 (4)
C	Buffer, 10 min	15.6±1.2 (3)	37 °C	6.4±0.1 (3)
	Papain, 10 min	5.9±0.7 (3)	37 °C	3.1±0.2 (3)
	N-Ethylmaleimide, 10 min	4.2±0.2 (3)	37 °C	3.0±0.2 (3)

* Expressed as the difference between [^{3}H]phlorizin bound in the absence or presence of 1.5 mM unlabeled phlorizin.

DISCUSSION

Our results suggest that high-affinity phlorizin receptors in kidney brush border membranes (rat) are easily attacked by the proteolytic enzymes trypsin and papain. Surprisingly, both enzymes had an additional, complicating effect on the phlorizin binding properties of the digested membrane. They increased unspecific binding for phlorizin. We[4], following others[12] have defined "unspecific" sites as those which appear unsaturable under assay conditions (for instance, the range of "free" ligand which was used) and then the term $K_3 \times N_3$ is similar to a distribution coefficient. This definition, however, does not exclude that these sites can be modified or altered

in their binding properties. Phlorizin is strongly retarded on Sephadex gels, and forms complexes with detergents (unpublished results). These properties are most likely due to the aromatic hydrophobic phloretin part of the molecule and any changes in the adsorption properties of the brush border membrane, which favor these interactions, would tend to increase unspecific binding. It seems, therefore, reasonable to propose, that both proteolytic enzymes after removal of attackable proteins and highly charged glycopeptides expose a deeper, more hydrophobic layer in the brush border membrane. This might express itself as an increase in unspecific phlorizin binding.

We have, in addition, demonstrated that high-affinity receptors can be blocked, *e.g.* with *N*-ethylmaleimide, without producing changes in unspecific phlorizin binding of the brush border membrane.

Thus, the adsorption characteristics of the brush border membrane for phlorizin offer interesting insight in "unspecific" binding, a term which is widely used but poorly understood.

The more general conclusion from this work is that in order to assess the perturbation of membrane receptors a wide range of free ligand concentrations should be used. Drastic changes in the binding properties of the perturbed receptors may be overlooked or even misinterpreted, if incomplete or incorrect analysis of the binding data is performed*. For instance, had we tested the phlorizin binding of digested brush borders only at 0.05 or 2 or 10 μM phlorizin, a "loss", no change or "enhancement" of binding would be found.

In contrast to the findings here, which were obtained with brush borders isolated in hypotonic media, it was recently reported that papain treatment did not affect the affinity of phlorizin nor the number of high-affinity sites in digested brush border membranes isolated under isotonic conditions[13,14]. It is possible that these differences are due to the different isolation technique used although the physico-chemical characteristics of the phlorizin receptors in both membrane preparations are very similar (compare refs 1, 2, 5 with 3, 4).

ACKNOWLEDGEMENTS

One of us (H.G.) was supported by a grant from Deutsche Forschungsgemeinschaft. We want to thank Dr D. Diedrich and M. Silverman for helpful discussions, and Mr J. Boone for excellent help in preparing brush border membranes.

REFERENCES

1 Bode, F., Baumann, K., Frasch, N. and Kinne, R. (1970) *Pfluegers Arch. Ges. Physiol.* 315, 53–65
2 Frasch, N., Frohnert, P. P., Bode, F., Baumann, K. and Kinne, R. (1970) *Pfluegers Arch. Ges. Physiol.* 320, 265–284
3 Glossmann, H. and Neville, Jr, D. M. (1972) *Z. Physiol. Chem.* 353, 708–709
4 Glossmann, H. and Neville, Jr, D. M. (1972) *J. Biol. Chem.* 247, 7779–7789

* We have deliberately neglected the presence of another brush border phlorizin receptor ($K_2 \approx 0.15\ \mu M^{-1}$, $N_2 \approx 60$ pmoles/mg protein) in our calculations for reasons of simplicity. Its inclusion for the curve fitting process did not change the basic results reported here, namely that papain and trypsin destroy high-affinity, D-glucose-sensitive phlorizin receptors and increase unspecific phlorizin binding of the digested brush border membrane.

5 Bode, F., Baumann, K. and Diedrich, D. F. (1972), *Biochim. Biophys. Acta* 290, 134–149
6 Vick, A., Diedrich, D. F. and Baumann, K. (1973) *Am. J. Physiol.* 224, 552–557
7 Wilfong, R. F. and Neville, Jr, D. M. (1970) *J. Biol. Chem.* 245, 6106–6112
8 Neville, Jr, D. M. (in the press) in *Methods in Enzymology*, (Fleischer, S., Packer, L. and Estabrook, R. W., eds)
9 Glossmann, H. and Neville, Jr, D. M. (1972) *FEBS Lett.* 19, 340–344
10 Neville, Jr, D. M. and Glossmann, H. (1971) *J. Biol. Chem.* 246, 6335–6338
11 Glossmann, H. and Neville, Jr, D. M. (1971) *J. Biol. Chem.* 246, 6339–6346
12 Baulieu, E. E. and Raynaud, J. P. (1970) *Eur. J. Biochem.* 13, 293–304
13 Thomas, L. and Kinne, R. (1972) *FEBS Lett.* 25, 242–244
14 Thomas, L. (1972) *FEBS Lett.* 25, 245–248