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CHARACTERISTICS OF TRANSMURAL POTENTIAL CHANGES ASSOCIATED WITH THE PROTON–PEPTIDE CO-TRANSPORT IN TOAD SMALL INTESTINE

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SUMMARY

1. Ionic dependence and kinetic properties of the peptide-evoked potentials across everted toad intestine were investigated with eighteen dipeptides and four tripeptides. All peptides evoked saturable increases in the mucosal negativity regardless of the presence of Na^+.

2. The peptide-evoked potentials recorded in the absence of Na^+ were sensitive to external pH (pH_o); lowering pH_o from 7·4 to 6·5 and 5·5 caused stepwise increases in their amplitude.

3. Loading epithelial cells with 9-aminoacridine or acetate caused a significant increase or decrease in amplitude of the Gly-Gly-evoked potential, suggesting intracellular alkalinization or acidification also has a great influence on the peptide-evoked potential.

4. Kinetically, Na^+-independent peptide-evoked potentials conformed to simple Michaelis–Menten kinetics, and lowering pH_o caused a decrease of the half-saturation concentration (K_t) for Gly-Gly without changing the maximum potential difference increase. Similar affinity-type kinetic effect was also seen for Gly-Gly influx.

5. Simultaneous measurements of Gly-Gly-induced increase in short-circuit current and Gly-Gly influx revealed that the coupling ratio of H^+ and Gly-Gly flows was $1·78 \pm 0·12$, suggesting the stoichiometry of the H^+–peptide co-transport being $2:1$.

6. Kinetic analyses of the peptide-evoked potentials indicated that all glycyl-dipeptides tested (Gly-Gly, Gly-Pro, Gly-Sar, Gly-Leu, Gly-Phe) and other dipeptides (Ala-Ala, Ala-Phe, Phe-Ala) shared a common carrier. Gly-Gly-Gly and Ala-Ala-Ala were also found to share the same carrier, while Phe-Phe, Leu-Leu and Phe-Leu appeared to be transported by a different carrier.

7. K_t values for di- and tripeptides, which apparently shared a common carrier, fell in a narrow range (0·5–2·2 mM). There was no clear correlation between $1/K_t$ value and molecular weight.

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INTRODUCTION

Kohn, Smyth & Wright (1968) first demonstrated that, in isolated rat small intestine, addition of a dipeptide or tripeptide to the mucosal bathing solution caused an immediate and sustained increment of the transmural potential. They also showed that the increment of the transmural potential was saturable and conformed to Michaelis–Menten kinetics, like the sugar- and amino acid-evoked potentials. Regarding the sugar- and amino acid-induced changes in the transmural potential, many studies have established that the potential changes are closely related to the Na^+-coupled electrogenic transport of these substances across the luminal membranes (see Schultz, 1978; Hoshi & Himukai, 1982). However, the nature of the peptide-evoked potential has not been studied in detail until recently.

Since the discovery by Newey & Smyth (1959, 1960) of the carrier-mediated transport of intact dipeptides across the luminal membranes of intestinal cells, a number of studies have been carried out to characterize the transport system for peptides (see Matthews, 1975; Matthews & Payne, 1980). Although some of the early studies suggested its Na^+-dependence (Rubino, Field & Schwachman, 1971; Addison, Burston & Matthews, 1972; Addison, Burston, Matthews, Payne & Wilkinson, 1974), most of subsequent studies have more clearly shown that the transport of intact peptides across the luminal membrane is entirely independent of Na^+ (Cheeseman & Parsons, 1976; Himukai & Hoshi, 1980; Ganapathy, Mendicino & Leibach, 1981; Berteloot, Kahn & Ramaswamy, 1982; Himukai, Kameyama & Hoshi, 1983). Nevertheless, electrogenic nature of the peptide transport has repeatedly been demonstrated by some of the above investigators (Himukai *et al.* 1983) and others (Boyd & Ward, 1982).

Recently, the electrogenicity of intact peptide transport, thus far unexplained, has clearly been ascribed to its H^+-coupled co-transport by the studies in brush-border membrane vesicles from the intestine and the kidney (Ganapathy & Leibach, 1983; Ganapathy, Burckhardt & Leibach, 1984; Takuwa, Shimada, Matsumoto, Himukai & Hoshi, 1985*a*; Takuwa, Shimada, Matsumoto & Hoshi, 1985*b*). This led us to reinvestigation of fundamental properties of the peptide-induced potential changes across the intestinal wall.

The recording of the co-transport-related transmural potential changes is technically simple but quantitative analyses of the changes are quite useful for the studies of kinetic properties, specificity, distribution and comparative physiology of electrogenic co-transport systems. To establish the usefulness of recording of the peptide-evoked potential in studies of the peptide transport, it seemed of importance to study the basic properties of this particular evoked potential, e.g. ionic dependence, pH dependence and relation to membrane transport of peptides. Also, it seemed important to establish the recording conditions which could give pure information of the H^+-dependent transport of intact peptides, not interfered with by transport of amino acids liberated by the membrane digestion of peptides before transport. This is particularly desired in studies of the peptide transport since the electrical method can be used for many kinds of peptides of which radioactive compounds are not available. A part of the results of this study was presented elsewhere (Hoshi, 1986*a*).

<div align="center">METHODS</div>

Preparations and incubation media. The upper part of the small intestine of the toad (*Bufo vulgaris*) was used throughout the experiments of the present study. Our previous studies showed that this part of the toad intestine generated larger sugar- and amino acid-transport potentials than other parts (Hoshi & Komatsu, 1968) and had very high tolerance to Na^+-free conditions as revealed by long-lasting (over 5 h) and well-maintained ability to generate Na^+-independent H^+-dependent dipeptide-evoked potentials *in vitro* (Hoshi, 1968*a*). After anaesthetizing the animals by an intracisternal injection of 0·3 ml 25 % urethane solution, a short segment of the upper intestine, about 3 cm length from the entering portion of the hepatopancreatic duct, was removed. The isolated segment was rinsed with fresh Ringer solution and everted.

The everted intestine was tied over a multiple fenestrated area of a polyethylene tube of 5 mm in outer diameter and 10 cm in length. One end of the tube, near the fenestrated area was made blind. The everted intestine, thus supported by the tube, was immersed and pre-incubated in a Na^+-containing or a Na^+-free Ringer solution depending on the purpose of the subsequent experiments. The Na^+-containing solution had the following composition (in mM): Na_2SO_4, 50; K_2HPO_4, 1·24; KH_2PO_4, 0·42; $CaSO_4$, 1·5; $MgSO_4$, 1·0; mannitol, 102. The reason for the use of SO_4^{2-}-substituted solution was to reduce the medium conductivity, thereby, to increase the paracellular resistance of the tissue. This was useful for obtaining larger potential difference (p.d.) responses to added substrates (Hoshi, Suzuki, Kusachi & Igarashi, 1976). The Na^+-free Ringer solution used in the present study had the following composition (in mM): mannitol, 200; K_2HPO_4, 1·24; KH_2PO_4, 0·42; $CaSO_4$, 1·5; $MgSO_4$, 1·0. The pH of the solutions was adjusted to a desired value by adding small amounts of 0·2 M-tris(hydroxymethyl)aminomethane (Tris) or 0·2 M-2-(*N*-morpholino)ethanesulphonic acid solution (the final concentration of the buffer did not exceed 10 mM). During experiments, all solutions were bubbled with air.

Recording of the transmural potential and short-circuit current. The techniques of recording the transmural potential difference were described in detail in a previous paper (Hoshi & Komatsu, 1968). Briefly, a pair of polyethylene bridges (2 mm o.d., filled with 2 % agar in 1 M-KCl) was used to lead out the p.d. across the intestinal wall and the p.d. was recorded on a high-sensitivity pen recorder (National VP-654B, Japan) via calomel half cells. Short-circuit current measurements were performed in the same way as described in a previous study (Hoshi *et al.* 1976). Briefly, the everted intestine was fixed over a polyethylene tube of which fenestrated area was replaced by four stainless-steel rods. The rods of 1–1·5 cm length were fixed 5 mm apart from each other. The reason for the use of such metal rods was to get a homogeneous potential field inside the everted preparation when external current was applied to nullify the transmural p.d. For passing the current, non-polarizable electrodes made of $Zn–ZnSO_4$ cells and polyethylene bridges filled with 2 % agar in 1 M-Tris-SO_4 were employed. The tip of one of the bridges was placed inside the supporting tube just above the portion covered with the intestine, while the tip of the p.d.-measuring bridge inside the tube was fixed around the centre of the everted intestine. The tip of the other bridge for passing current was placed at a remote portion in the external solution, while that of the external p.d.-measuring bridge was placed in the vicinity of the mucosal surface just outside the tip of the internal p.d.-recording one. Fluid resistance between the p.d.-measuring electrodes was measured in the absence of the tissue before or after the measurements with the tissue. The resistance was taken into consideration when short-circuit current was determined.

Influx measurements. [1-^{14}C]Glycylglycine was used for the measurements of glycylglycine (Gly-Gly) influxes. The extracellular fluid volume of the tissue was measured with D-(1-^{3}H(N))-mannitol, and Gly-Gly uptake was corrected for this. The radioactive Gly-Gly was obtained from Amersham International (custom-made); [^{3}H]mannitol was from New England Nuclear. In the experiment in which kinetic effect of external pH (pH$_o$) was examined, strips of the upper intestine were incubated with radioactive and non-radioactive Gly-Gly and radioactive mannitol without any fixing device. In this series of experiments, at least four preparations were needed in a single experiment, but, because of relatively marked axial (longitudinal) difference in transport activity, four segments obtained from a single animal could not be used. Therefore, the upper intestine was divided longitudinally into four strips and these were used for four different test concentrations of Gly-Gly. In the case of simultaneous measurements of short-circuit current increase and Gly-Gly influx, we used the rod-supported fenestrated tube for the fixation of the preparation as described above. In this case, the preparation was immersed in 20 ml Na^+-free Ringer solution and both

radioactive and non-radioactive Gly-Gly were added to the mucosal solution. The concentrations of [^{14}C]Gly-Gly and [^{3}H]mannitol were 0·2 and 2 μCi/ml, respectively.

Our previous studies showed that Gly-Gly uptake by guinea-pig intestinal tissue linearly increased with time up to 2 min after the start of uptake at 37 °C (Himukai & Hoshi, 1980). In the present study, all experiments were performed at 25 ± 1 °C. Therefore, 2 min uptake was measured for obtaining influx values. At the end of the incubation for flux measurements, the preparations were picked up from the incubation medium, rinsed with a cold Ringer solution for about 10 s, blotted on filter paper, and weighed on a torsion balance. Thereafter, the peptide and [^{3}H]mannitol
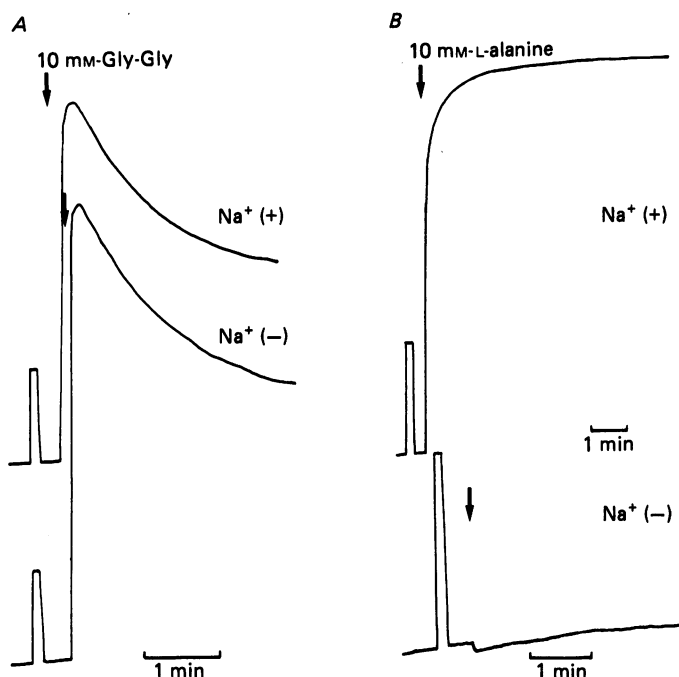

Fig. 1. Typical examples of the Gly-Gly and L-alanine-evoked changes in the transmural potential in the presence and absence of Na$^+$ in the bathing medium. *A*, the patterns of the Gly-Gly (10 mM)-evoked potentials in the presence of 100 mM-Na$^+$ (upper trace) and in the absence of Na$^+$ (lower trace) in the medium. *B*, L-alanine (10 mM)-induced p.d. changes in the presence of 100 mM-Na$^+$ (upper trace), and the absence of response in the Na$^+$-free medium (lower trace). Square waves just before the p.d. responses indicate 5 mV calibration.

were extracted in 1 ml 0·1 M-HNO$_3$ solution overnight. The extraction fluids were counted in a liquid scintillation counter (Aloka LSC-601, Tokyo).

Chemicals. All chemicals used were of reagent grade. Dipeptides, including glycyl-, leucyl-, alanyl- and phenylalanyl-dipeptides, were obtained from Sigma. Also, tripeptides (triglycine and trialanine) were purchased from Sigma. Some other dipeptides, such as glutamylglutamate and carnosine were obtained from Nakarai (Tokyo). All peptides examined in the present study are naturally occurring ones and comprised of L-type amino acids. 9-Aminoacridine was also supplied from Sigma.

<div align="center">RESULTS</div>

Na$^+$-independent, pH-dependent nature of the dipeptide-evoked potentials

In the present study, more than twenty naturally occurring peptides were examined. All the dipeptides and tripeptides tested, including glycyl-dipeptides and other groups of di- and tripeptides, evoked essentially the same type of transmural potential changes regardless of the presence of Na$^+$, when added to the mucosal

bathing solution. Some aspects of the evoked potentials were similar to those of the D-glucose- and L-alanine-evoked potentials which were recorded in the presence of Na^+. For example the peptides caused an immediate increase in the negativity of the mucosal side with respect to the serosal one and the amplitude of the evoked potential was saturable when examined by stepwise increases in peptide con-
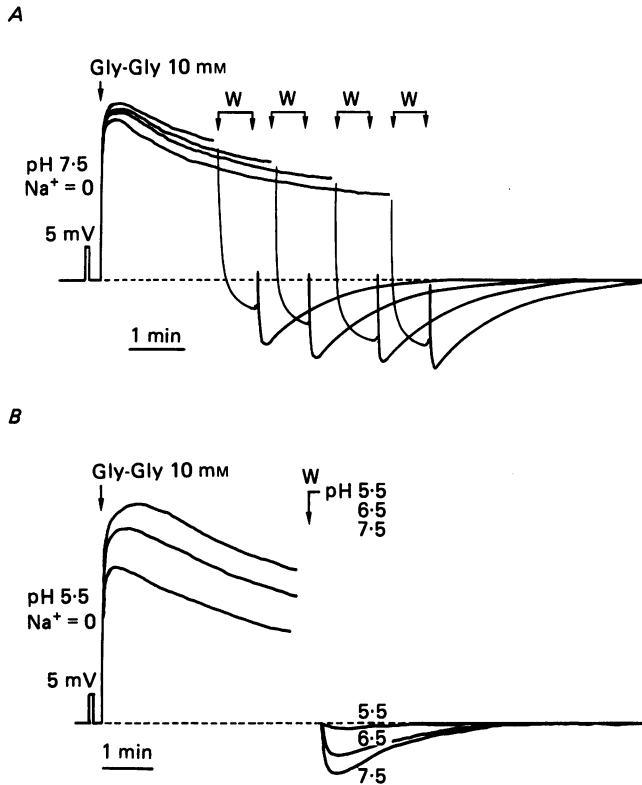

Fig. 2. Undershooting p.d. responses to washing-out of added Gly-Gly. *A*, responses at different time points after generation of the Gly-Gly-evoked potential in the absence of Na^+. The added Gly-Gly was washed out with fresh Ringer solution having the same composition as that of the incubation medium. Wash-out (W) was repeated twice in each experiment. Tracings of four successive experiments made in a single preparation are superimposed. *B*, effect of pH of wash-out solution on the undershooting response. Wash-out was made twice as shown in *A* and only the responses to the second wash-out are presented. The stabilized p.d. levels were different when the pH of the wash-out solution was different from that of the incubation medium. For the sake of easier comparison, the stabilized levels were shifted to the same level. Tracings of three successive experiments made in a single preparation (different from that in *A*) were superimposed. Since this series of experiments was time consuming, time-dependent decrease in amplitude of the Gly-Gly-evoked potential was relatively large.

centration. However, some striking differences were seen as compared to the sugar- and amino acid-evoked potentials as described below.

One of the marked differences was that the increased potential declined relatively rapidly when the concentration of the added peptide was relatively high, e.g. above several millimolar. The sugar- and amino acid-evoked potentials recorded in the presence of Na^+ usually form a plateau after reaching a peak even when a

concentration of saturation level is examined. The rapidity and the degree of the decline after reaching a peak varied from tissue to tissue, but in most cases, the augmented p.d. declined to about 60–70 % of the peak amplitude within 5 min when tested at 10 mM (saturation level) of peptide concentration (Fig. 1 *A*).

The most striking difference, as compared to the amino acid-evoked changes of potential, was the complete independence of Na^+. Namely, a p.d. change of entirely
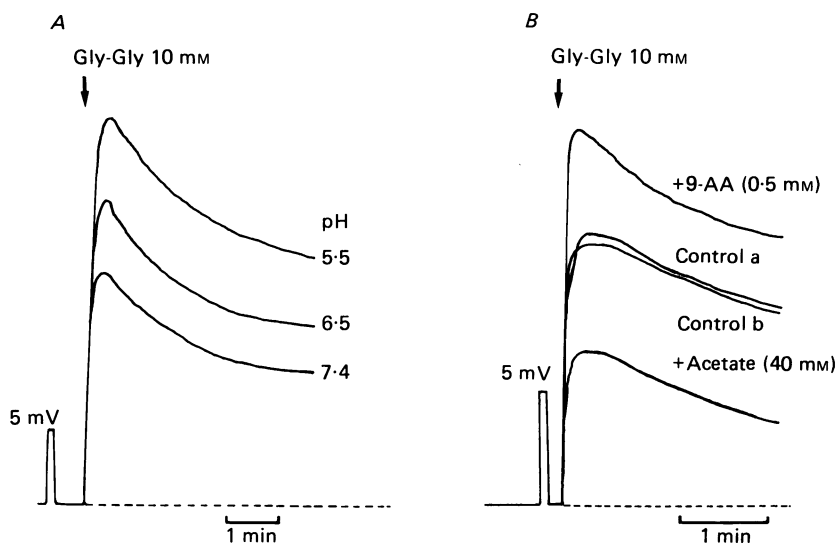

Fig. 3. Effects of external medium pH and modulation of intracellular pH on the Gly-Gly (10 mM)-evoked potential in the absence of Na^+. *A*, effect of external pH. Three tracings recorded in three successive experiments at different pH from a single preparation were superimposed. *B*, effect of modulation of intracellular pH. Two sets of tracings were superimposed. One is for the experiment with 9-aminoacridine (9-AA). After recording control response (control a), the preparation was incubated with 9-AA for 5 min, thereafter the response to Gly-Gly was recorded. The other is of acetate effect; control recording (control b) was made in the presence of 40 mM-NaCl, thereafter the preparation was incubated with 40 mM-sodium acetate, instead of NaCl, and the response was recorded.

the same time course and of a rather increased amplitude was invariably recorded after changing the medium from the Na^+-containing to the Na^+-free solution (Fig. 1 *A*). Moreover, quite reproducible evoked potentials were recorded repeatedly for many hours in the Na^+-free solution, although slight time-dependent decreases in amplitude were noted when repeated observations were made for many hours. In most preparations, about 30 % decrease occurred during 3 h period of observation. In some experiments, the preparations were pre-incubated in the Na^+-free solution for many hours in order to assure the complete absence of Na^+. After such a prolonged incubation in the Na^+-free medium, the peptides induced large p.d. changes quite reproducibly. In contrast, the amino acid-evoked potential rapidly became smaller and smaller after immersion in a Na^+-free solution, and after 1–2 h incubation in the Na^+-free solution, no p.d. change was recorded as far as tested with L-alanine, L-leucine and L-proline (Fig. 1 *B*). D-Glucose-evoked potential also rapidly decreased in its amplitude after switching to the Na^+-free solution, but a small change remained

even after a prolonged incubation and the evoked p.d. change exhibited dependence on external pH (see Discussion). The Na^+-independent nature of the peptide-induced p.d. change indicates that the electrogenic mechanism does not require Na^+.

When the added peptide was washed out from the mucosal solution, the transmural potential returned to the original level after showing an undershoot. The amplitude of the undershoot was larger when the time of wash-out was later so that a greater decline had already occurred (Fig. 2*A*). Also, the undershoot was found to be dependent on the pH of the wash-out solution. When the pH of the wash-out
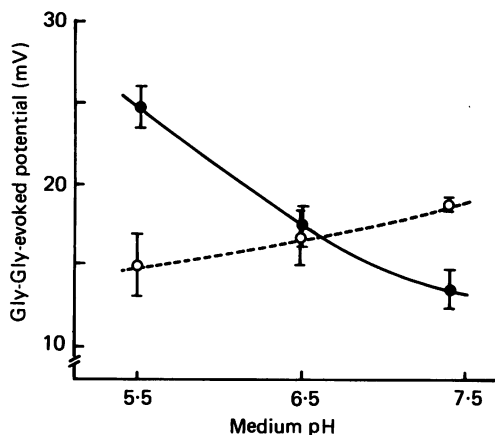

Fig. 4. Summarized data on the effects of medium pH on the peak amplitude of the Gly-Gly-evoked potential in the absence (heavy line) and presence (dashed line) of Na^+ in the medium. The relative magnitudes of the p.d. responses in the presence and absence of Na^+ do not directly indicate the relative magnitudes of the peptide-induced apical membrane current since the evoked potential is dependent on the transmural resistance which is greatly influenced by the medium conductivity.

solution was lowered, the amplitude of the undershoot became smaller and, usually, was virtually abolished when a solution of pH 5·5 was used (Fig. 2*B*). This seems to indicate that a H^+ concentration gradient across the luminal membrane is one of the important factors determining the amplitude of the undershoot. This further implies that the spontaneous decline of the potential after reaching a peak is related, at least in part, to accumulation of H^+ within the cells during the transport of dipeptides. Another possibility is that the dipeptides fail to leave the epithelial cells across the basolateral membrane since peptide transporters appear to be absent at this face of the epithelium. The build up of intracellular peptide concentration might then result in a transient response.

As the Na^+–H^+ antiport system is known to exist in the brush-border membranes of mammalian intestine (Murer, Hopfer & Kinne, 1976; Knickelbein, Aronson, Atherton & Dobbins, 1983), addition of Na^+ to the Na^+-free mucosal solution during the course of decline of the potential was expected to retard the decline or restore the p.d. toward the peak level. However, addition of NaCl to a final concentration of 10, 20 and 30 mM was found to have no effect on the time course (data not shown).

The Na^+-independent dipeptide-evoked potential was found to be quite sensitive to both external and intracellular pH (pH_o and pH_i). When pH_o was lowered from

7·4 to 6·5 and 5·5, the amplitude of the evoked potential was significantly increased in a stepwise fashion. Figures 3*A* and 4 show the effect of pH_o on the Gly-Gly-evoked potential in the absence of Na^+. Such an effect of pH_o was not clearly seen in the presence of Na^+ (Fig. 4). The reason for this will be discussed later.

Addition of a weak base, 9-aminoacridine, or a weak acid, acetate, to the external solution was used in order to modify intracellular pH. These substances are known
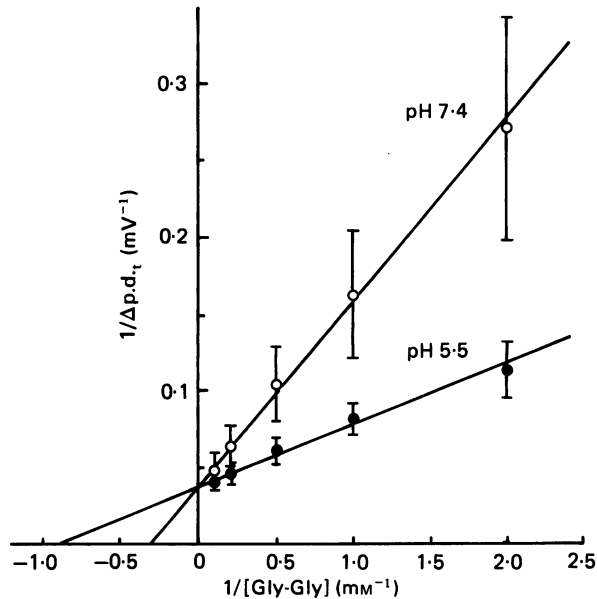

Fig. 5. Lineweaver–Burk plot of the relationship between the amplitude of the Gly-Gly-evoked potential (Δp.d.$_t$) and the final medium concentration of added Gly-Gly at two different pH values; 7·4 and 5·5. For each preparation fixed over a fenestrated tube, two different pH conditions were tested and the average values from five different preparations were presented with S.E.M.

to cross the cell membrane by non-ionic diffusion and act within the cells as proton acceptors or proton donors, respectively (Bramhall, 1986; Jentch, Janicke, Sorgenfrei, Keller & Wiederholt, 1986). Accordingly, the presence of 9-aminoacridine in the bathing solution is expected to cause alkalinization of the intracellular fluid, whereas acetate may cause intracellular acidification. Figure 3*B* shows a typical experiment with these substances. Aminoacridine caused a significant increase in amplitude of the Gly-Gly-evoked potential, whereas 40 mM-acetate depressed the amplitude significantly. The effects of these substances were dose dependent; 0·5 mM-9-aminoacridine and 40 mM-acetate caused almost the maximum effects. When either 9-aminoacridine or acetate was removed, the response to Gly-Gly recovered completely within 10–15 min.

Kinetic effects of external pH

The experimental data described above clearly show the pH dependence of the peptide-evoked potentials in the absence of Na^+. To study kinetic effects of pH_o, the concentration dependence of the Gly-Gly-evoked potential was compared at different

pH values, 7·4 and 5·5, under Na$^+$-free conditions. Similarly the kinetics of Gly-Gly influx was compared at different values of pH$_0$ in separate experiments using the different preparations, i.e. longitudinal strips of the upper intestine described in the Methods section. The Gly-Gly-evoked potentials conformed to Michaelis–Menten kinetics, so that the double-reciprocal (Lineweaver–Burk) plot of the data gave a straight line. Two lines for respective pH values intercepted the ordinate at a
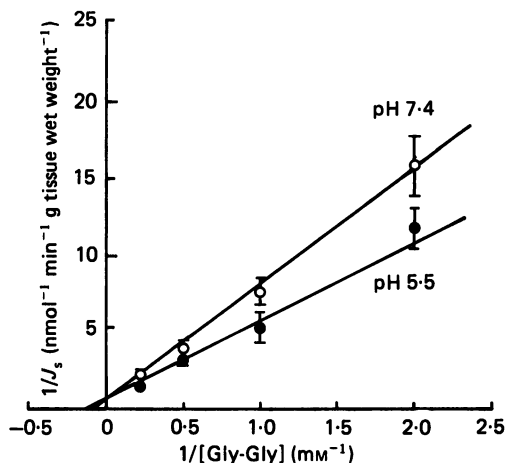

Fig. 6. The effect of medium pH on kinetics of Gly-Gly uptake rate. Lineweaver–Burk plot. Four longitudinal strips were obtained from a single upper intestine and each was incubated at a different Gly-Gly concentration. J_s denotes the influx of Gly-Gly as determined from linear uptake of the dipeptide during initial 2 min. The pH values examined are given in the Figure. Each figure is the mean ± S.E.M. from six different animals.

common point. It is seen that lowering pH$_0$ causes a decrease of value of the half-saturation concentration (K_t) for the dipeptide without changing the maximum p.d. change. The K_t value decreased from 3·3 mM at pH 7·4 to 1·1 mM at pH 5·5, thus the kinetic effect is of the 'affinity-type' (Fig. 5). A similar affinity-type effect was seen for Gly-Gly influx when measured at pH 7·4 and 5·5. As we used strips in the latter series of experiments, the values obtained for K_t were significantly higher (12·8 mM at pH 7·4 and 8·2 mM at pH 5·5) than in the preparations fixed over supporting tubes. The higher values of K_t in such unfixed strips are probably due to the greater thickness of unstirred layers. Nevertheless, similar affinity-type kinetics were seen when pH$_0$ was lowered (Fig. 6). Affinity-type kinetic effects are seen for other cation-coupled co-transport systems when the cation concentration is changed (Heinz, 1978).

Relationship between short-circuit current increase and Gly-Gly influx

The data described above support the concept that dipeptides are co-transported with H$^+$ across the luminal membrane of the intestinal and renal epithelium (see Ganapathy & Leibach, 1985; Hoshi, 1985). Overshooting uptake of dipeptides in the presence of a sufficiently large H$^+$-gradient has been demonstrated in membrane vesicle experiments (Takuwa *et al.* 1985*a*; Miyamoto, Ganapathy & Leibach, 1985).

Such H^+ gradient-driven uphill transport of dipeptides is strong evidence for H^+-coupled co-transport. The coupling ratio of H^+–dipeptide co-transport, however, has not yet been determined.

In order to study the stoichiometry of the co-transport, Gly-Gly-induced short-circuit current increase and Gly-Gly influx were measured simultaneously using the same tissue mounted on a specially designed polyethylene tube as described in

TABLE 1. Coupling ratio of Gly-Gly-induced cation flow and Gly-Gly influx

Experiment	Uptake medium conditions		Mean ΔI_{sc}* (μA g tissue wet weight^{-1})	(a) Net cation movement† (nmol min^{-1} g tissue weight^{-1})	(b) Gly-Gly influx (nmol min^{-1} g tissue weight^{-1})	Ratio of a to b
	pH	[Gly-Gly] (mM)				
1	5·5	1	43·7	27·2	11·9	2·29
2	5·5	2	139·1	86·5	48·5	1·78
3	7·4	1	74·4	46·3	33·2	1·39
4	7·4	1	117·3	72·9	38·9	1·87
5	5·5	1	156·6	97·4	50·0	1·95
6	5·5	2	182·4	113·4	71·8	1·58
7	5·5	1	164·6	102·3	42·7	2·40
8	7·4	1	70·6	43·9	31·8	1·38
9	7·4	1	75·2	46·8	33·5	1·40
Mean ± S.E.M.						1·78±0·12

* Short-circuit current increase (ΔI_{sc}) is the mean value of 2 min measurement period.

† As all measurements were performed under Na^+-free conditions, net cation movement can be assigned to H^+ flow. Net cation movement was calculated by multiplying mean ΔI_{sc} by a factor of 60, then dividing by the Faraday constant.

Methods. The measurements were performed at different concentrations of Gly-Gly and at different pH. The increase in short-circuit current caused by Gly-Gly was converted to the amount of monovalent cation transferred per unit time per unit tissue wet weight by dividing the increased current by the Faraday constant, and thus the ratio of the cation flow to Gly-Gly influx was obtained. The data are summarized in Table 1. The average ratio was $1·78±0·12$ ($n = 9$), suggesting that two H^+ are co-transported with one dipeptide. When plotted on a linear scale the regression line could be expressed as $Y = 0·48 X + 6·54$, where Y is the Gly-Gly influx and X is the amount of cation transferred simultaneously, as reported by one of the authors in a preliminary communication (Hoshi, 1986a). Since the incubation medium contained no Na^+ and only small amounts of K^+, Ca^{2+} and Mg^{2+}, which were proven to have no effect on the evoked potential (data not shown), the monovalent cation transferred can be assigned to H^+. Our previous determination of the coupling ratio of D-galactose and Na^+ which was carried out by essentially the same method gave the value of $1:1$ (Hoshi *et al.* 1976). Therefore, it is certain that about twice the number of cations are co-transported per mole of substrate in the case of the H^+–peptide co-transport as compared with the Na^+–sugar co-transport.

Specificity pattern of the electrogenic peptide carrier system(s)

In this series of experiments, an attempt has been made to see whether other dipeptides and tripeptides share a common electrogenic transport mechanism with Gly-Gly. For this purpose, kinetic properties of the evoked potentials induced by
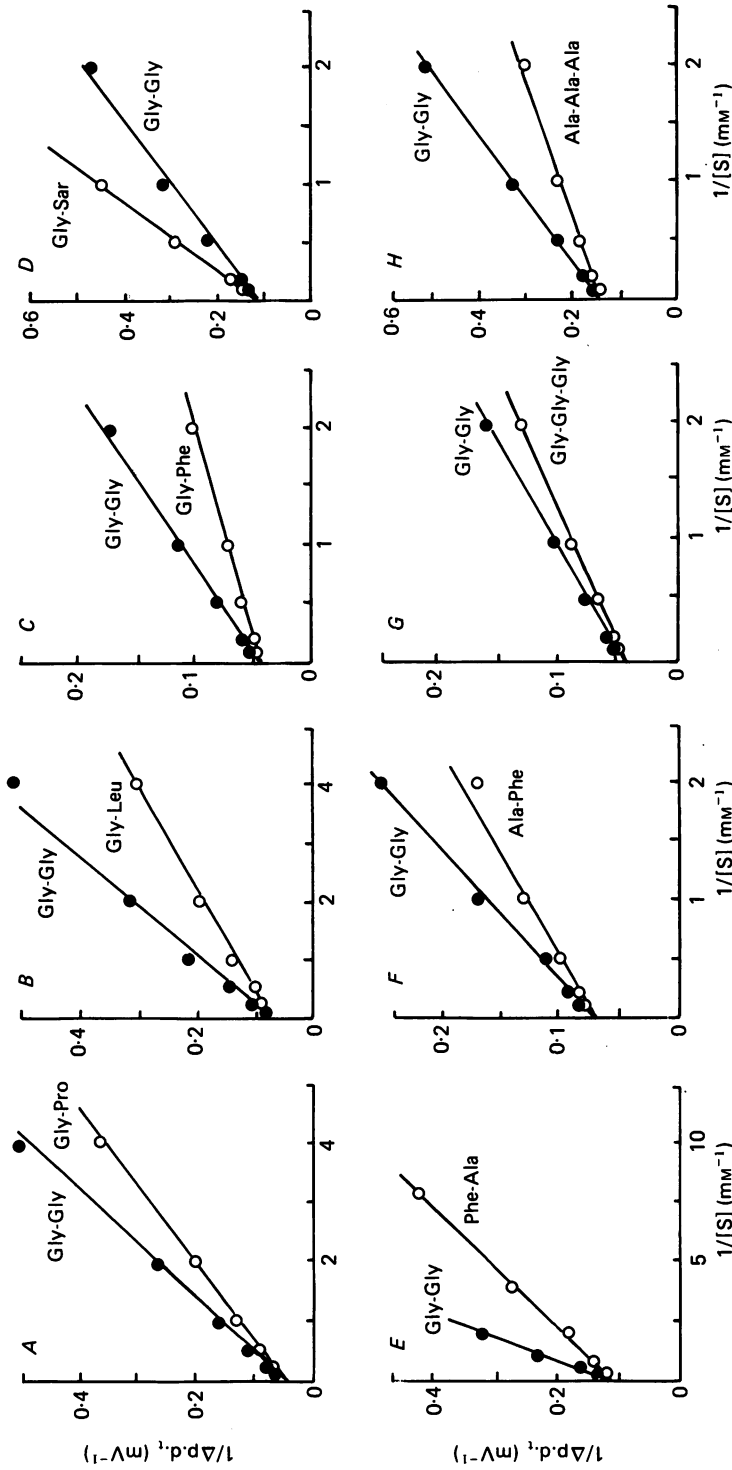
Fig. 7. Comparison of the Lineweaver–Burk plot of the Gly-Gly-evoked potential and those of the evoked potentials (Δp.d.$_t$) induced by eight different peptides. The p.d. measurements were performed in the Na$^+$-free medium of pH 5·5. All the peptides shown in this Figure gave the same maximum p.d. change (Δp.d.$_{t,\,max}$) as that for Gly-Gly. [S]: peptide concentration.

many different kinds of peptides were investigated under Na^+-free conditions and compared with those of Gly-Gly. It is known that di- and tripeptides are partly hydrolysed by brush-border membrane oligopeptidases before transport. However, as the amino acid-evoked potentials are absolutely dependent on the presence of Na^+, use of Na^+-free conditions eliminates involvement of Na^+–amino acid co-
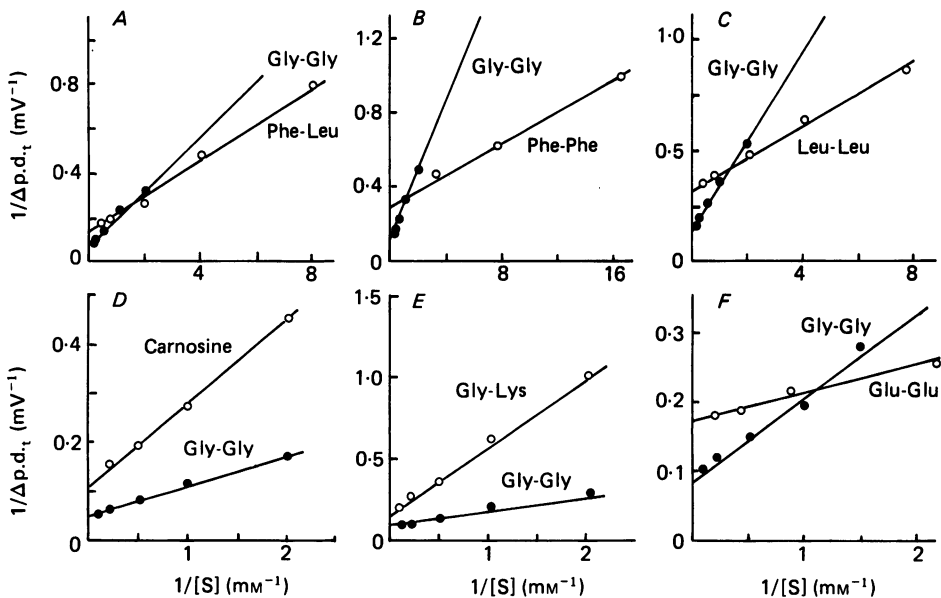

Fig. 8. Comparison of the Lineweaver–Burk plot of the Gly-Gly-evoked potential and those of the evoked potentials (Δp.d.$_t$) induced by another six peptides including charged peptides. The p.d. measurements were performed in the Na^+-free solution of pH 5·5. All peptides shown in this Figure gave different values of Δp.d.$_{t,max}$ from that for Gly-Gly. [S]: peptide concentration.

transport (Himukai & Hoshi, 1980). Accordingly, the present method gave information concerning the H^+-coupled transport of intact peptides across the luminal membranes.

As the preparations exhibited a slight time-dependent decrease in the amplitude of the evoked potential, Gly-Gly-evoked potentials caused by stepwise increases in Gly-Gly concentration were recorded just before and after the recording of the responses to another test peptide in the same preparation. The average values of the Gly-Gly-evoked potentials for each concentration were plotted double reciprocally and the line obtained was compared to that for another peptide. By doing this, both time-dependent and tissue-specific differences in kinetics could be eliminated, and the precise coincidence of the intercepts on the ordinate of the double-reciprocal plot could therefore be evaluated.

The results are summarized in Figs 7 and 8. The lines for all glycyl-dipeptides tested, such as Gly-Pro, Gly-Leu, Gly-Phe, Gly-Sar and some other dipeptides, such as Phe-Ala, Ala-Phe and Ala-Ala, were found to intercept the ordinate at the same point as that for the Gly-Gly-evoked potential. Tripeptides, such as Gly-Gly-Gly and Ala-Ala-Ala, had exactly the same intercept as that for Gly-Gly. These data indicate

that a wide variety of dipeptides and also tripeptides share a common electrogenic (H$^+$-coupled) transport mechanism. From early studies of mutual transport inhibition (Das & Radhakrishnan, 1975), the intestinal brush-border membrane has been considered to possess a common peptide carrier for a wide variety of di- and tripeptides (Matthews & Payne, 1980). The results of the present study seem to give

TABLE 2. Values of K_t (mM) for peptides and amino acids determined by the electrical measurements*

Peptides exhibited the same Δp.d.$_{t,max}$ as that for Gly-Gly		Peptides exhibited Δp.d.$_{t,max}$ different from that for Gly-Gly		Amino acids‖	
Gly-Gly	1·21±0·10†	Phe-Phe	2·20 (1·39)	Pro	17·4±2·3
Gly-Sar	2·15 (1·21)‡	Phe-Leu	0·48 (1·39)	Gly	10·9±2·2
Gly-Pro	1·50 (2·10)	Leu-Leu	0·23 (1·38)	Ser	1·7±0·1
Gly-Leu	0·77 (1·67)	Carnosine	1·59 (1·38)	Ala	1·4±0·1
Gly-Phe	0·65 (1·50)	Gly-Lys	2·77 (1·01)	Thr	1·3±0·3
Ala-Pro	1·25 (0·58)	Glu-Glu	0·26 (0·99)	Gln	0·9±0·1
Ala-Phe	1·25 (0·83)	----------------§		Asn	0·9±0·1
Phe-Ala	0·65 (1·33)	Leu-Ala	0·37 (1·74)	Val	0·5±0·04
Phe-Gly	0·51 (0·92)	Leu-Gly	1·20 (1·59)	Phe	0·5±0·03
Ala-Ala	0·58 (1·43)	Ala-Gly-Gly	1·10 (0·75)	Try	0·4±0·06
Gly-Gly-Gly	1·25 (0·58)	Phe-Gly-Gly	2·00 (0·45)	Leu	0·3±0·06
Ala-Ala-Ala	0·59 (1·34)			Met	0·3±0·06
				Ile	0·2±0·01

* All peptide-evoked potentials were recorded in the Na$^+$-free solution of pH 5·5, whereas amino acid-evoked potentials were recorded in the Na$^+$-containing (Na$^+$ 100 mM) solution of pH 7·4.

† K_t value for Gly-Gly is given as mean±s.e.m. of thirty-two different preparations.

‡ Figures in parentheses are the K_t values for Gly-Gly determined in the respective preparations in which the listed dipeptides or tripeptides were examined for kinetic properties.

§ Four peptides listed below a dashed line gave a slightly different Δp.d.$_{t,max}$ value from that for Gly-Gly, but further experiments seem necessary until definitive conclusion is made in regard to coincidence of Δp.d.$_{t,max}$.

‖ K_t values for amino acids are means±s.e.m. of five different preparations.

strong support to this concept. However, there were some dipeptides which had a different intercept from that for Gly-Gly. These include Phe-Leu, Phe-Phe, Leu-Leu, carnosine, Gly-Lys and Glu-Glu (Fig. 8).

The K_t values for dipeptides and tripeptides determined by the evoked potential measurements fell in a relatively narrow range as compared with those for neutral amino acids determined by similar electrical measurements but in the presence of Na$^+$ (Table 2). The K_t values for hydrophilic amino acids, such as L-proline (Pro) and glycine (Gly), are very high (10–20 mM), whereas hydrophobic amino acids such as L-isoleucine (Ile), L-leucine (Leu), L-methionine (Met) had a relatively low value of K_t (0·2–0·3 mM), thus the K_t values distribute over two or three orders of magnitude. In contrast, many dipeptides, including glycyl-dipeptides comprised of a hydrophobic amino acid (e.g. Phe and Leu) had very similar K_t values, ranging from 0·65 to 2·15 mM. The relationship between molecular weight and K_t value for peptides was markedly different from that for neutral amino acids (Fig. 9). In the case of amino acids, a steep linear relationship is seen between the values of $1/K_t$ and molecular weight as Hajjar & Curran (1970) pointed out previously. In contrast, such a

correlation is far less marked in the case of peptides, and almost no tendency for an increase in $1/K_t$ value with increase in molecular weight was seen. This indicates that the hydrophobic interactions which are prominent in amino acid–carrier interaction (Preston, Schaeffer & Curran, 1974) are unimportant in the case of peptide transport.
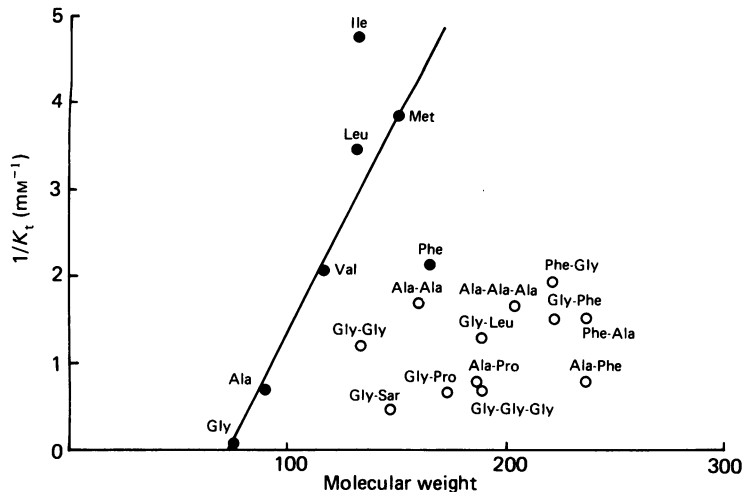

Fig. 9. The relationship between electrically measured K_t values and molecular weight for amino acids (filled circles) and peptides (open circles). The K_t values for amino acids were determined from concentration dependence of the evoked potentials in the presence of Na^+ (pH 7·4), and those for peptides were measured from the evoked potentials recorded in the absence of Na^+ and at pH 5·5.

DISCUSSION

The results of the present study clearly indicate that the transmural p.d. changes induced by dipeptides and tripeptides are independent of Na^+ and related to the H^+-coupled transport of the peptides across the luminal membrane. This is in accord with the results of recent studies on dipeptide transport across the brush-border membrane vesicles (Ganapathy, Mendicino, Pashley & Leibach, 1980; Ganapathy et al. 1984; Takuwa et al. 1985 a, b; Miyamoto et al. 1985). In such vesicles, dipeptide transport has been shown to be entirely independent of Na^+, but to be pH gradient-dependent and electrogenic. In these vesicles, glycyl-dipeptides, such as Gly-Pro, Gly-Sar and Gly-Gly, have been shown to cause depolarization of the membrane potential which can be visualized by a change in fluorescence of a membrane potential-sensitive dye, such as diS-C_3-(5), added to the suspension medium (Ganapathy et al. 1984; Takuwa et al. 1985 a, b). Also, Boyd & Ward (1982) and Shimada & Hoshi (1986) demonstrated that the transmembrane potential across the luminal membrane of intact amphibian intestinal epithelia was depolarized when dipeptides, such as Gly-Leu, Gly-Gly and carnosine, were added to Na^+-free mucosal bathing solution, and that the depolarization was accompanied by a reduction of the input resistance. The pH$_o$ dependence of the amplitude of the depolarization was demonstrated by the latter authors (Shimada & Hoshi, 1986). The changes in the

transmural potential observed in the present study are all closely related to such H^+-dependent transport of peptides across the luminal membrane.

In the present study, the coupling ratio of H^+ current to dipeptide flux was determined to be approximately 2 ($1\cdot78\pm0\cdot12$). A linear relationship between H^+ and Gly-Gly influxes supports the idea that Gly-Gly transport is mediated by an H^+-coupled co-transport, similar in mechanism of energy coupling to the Na^+-coupled transport of D-glucose and amino acids. As to the true stoichiometry of the H^+–peptide co-transport, however, a problem remains to be solved before any final conclusion is reached. With regard to the relationship between luminal membrane ionic current and short-circuit current, Ginzburg & Hogg (1967) previously stressed that active transcellular transport of Na^+ coincided exactly with the short-circuit current across a transporting epithelium only when the mucosal entry and basolateral exit of Na^+ reached a steady state. In the case of the H^+-coupled transport, H^+ current flows only across the luminal membrane, and a steady transepithelial flow of H^+ is not attained. Therefore special analyses of the relationship between luminal membrane co-transport current (i_m) and the increase in transepithelial short-circuit current increase (ΔI_{sc}) under transient conditions are needed. Our analyses (Hoshi, 1986b) revealed that $\Delta I_{sc}/i_m$ ratio is a function of the resistance components of epithelial cells (the luminal and basolateral membrane resistances, R_a and R_b, and the paracellular resistance, R_1). Theoretically, $\Delta I_{sc}/i_m$ ratio is unity only when $R_a/(R_a+R_b)$ and $R_1/(R_a+R_b)$ is sufficiently high, otherwise the ratio is less than unity. For example, when $R_a/(R_a+R_b)$ is 0·8 and $R_1/(R_a+R_b)$ is about 1, the ratio is nearly unity. The value of R_1 was $1210\pm100\ \Omega\ \mathrm{cm}^2$ in our experiments. Although the value of $R_a/(R_a+R_b)$ was determined to be 0·63 in *Necturus* small intestine in the presence of Na^+ (Gunter-Smith, Grasset & Schultz, 1982), exact values of the resistance components of the present preparation under Na^+-free conditions have not yet been obtained. However, these data and the results of our analyses suggest that the true flow coupling ratio may be slightly higher than 1·78, suggesting the H^+–peptide stoichiometry being 2:1. Similar measurements using the electrical and tracer flux techniques for the Na^+–sugar co-transport in guinea-pig intestine gave the value of coupling ratio of approximately 1:1 (Hoshi *et al.* 1976). Thus, in the case of H^+-coupled peptide transport, twice as many cations are transported per mole of substrate when compared with Na^+-coupled sugar transport.

Although the results of the present study showed that the peptide-induced transmural potential changes were independent of Na^+ and dependent on H^+, the pH_o effects were clearly seen only under Na^+-free conditions. A similar masking effect of Na^+ on pH_o effect was also seen in studies on the uptake of dipeptide by intact epithelial sheets of guinea-pig intestine (Himukai *et al.* 1983), but not in isolated membrane vesicles. A possible reason for this is the presence of a 'microclimate' pH layer in the intact intestinal epithelium (Lucas, Schneider, Haberich & Blair, 1975) which is known to be significantly influenced by the presence or absence of Na^+ from the bathing medium (Lucas, 1984). Therefore, it seems of particular importance to eliminate Na^+ from the medium if observations of H^+- (or pH-) dependence of transport of peptide-evoked potentials are intended. Furthermore, the use of a Na^+-free medium is very important to eliminate the involvement of transport of amino acids liberated by membrane digestion. By doing this, we can observe by the

p.d. recording method the properties of the H^+–peptide co-transport uncontaminated by other electrogenic transporters in this membrane.

Under Na^+-free conditions, it was possible to compare the kinetics of the evoked potentials induced by many different peptides without the complication of electrogenic amino acid co-transport. Our results indicate that many glycyl- and alanyl-dipeptides share a common electrogenic (H^+-coupled) co-transport system. Also tripeptides such as Gly-Gly-Gly and Ala-Ala-Ala were shown to share the same carrier. This supports the idea that the intestinal peptide carrier has an extremely broad specificity. This concept, however, was derived from transport inhibition experiments (Das & Radhakrishnan, 1975) and, in such experiments in the presence of Na^+, it was difficult to determine whether the inhibition was competitive, non-competitive or heterologous due to competition for the driving force (H^+ gradient). Our data indicate more clearly that a variety of dipeptides and tripeptides share a common carrier. This broad specificity of the carrier seems to be an important characteristic of the peptide transport system of the brush-border membrane.

Some other peptides, including carnosine, Leu-Leu, Phe-Leu and Phe-Phe appeared to be transported by a separate carrier(s). Among these peptides, however, carnosine seems to be of particular interest since kinetic analysis of influx measurements in guinea-pig intestine clearly shows that the mutual inhibition between carnosine and Gly-Gly is purely competitive (Himukai, 1985). However, his and our p.d. measurements revealed that the maximum p.d. change induced by carnosine was always about half that for Gly-Gly. This seems to indicate that the H^+–carnosine stoichiometry is rather exceptional and probably 1:1. As for the dipeptides comprised only of hydrophobic amino acids, e.g. Phe-Leu, Leu-Leu and Phe-Phe, the present results suggest the presence of a separate specific carrier for these. Matthews & Burston (1983) showed that Leu-Leu uptake by hamster small intestine was not inhibited by Gly-Sar, supporting this possibility.

Glu-Glu and Gly-Lys, which carry a net charge at normal pH, also exhibited quite different kinetics from that of Gly-Gly. It was rather surprising that an anionic dipeptide, such as Glu-Glu, generated an evoked potential of the same polarity as that seen with neutral dipeptides. This indicates that excess H^+ is co-transported with the anionic dipeptide so that a net cation current is generated. Whether these charged dipeptides are transported by the same carrier as that for neutral dipeptides is still in dispute. Although an earlier study by Addison, Matthews & Burston (1974) concerning the mutual inhibition among carnosine, Lys-Lys and Glu-Glu suggested the presence of separate carriers for charged and non-charged peptides, a later study by Taylor, Burston & Matthews (1980) showed that uptake of Gly-Sar was inhibitable by Glu-Glu and Lys-Lys. Further studies seem to be needed for solving this point.

It seems of interest to point out that the K_t values for those peptides which are transferred by a common carrier fall in a relatively narrow range as compared to those of their constituent amino acids. The relationship between the molecular weight of these peptides and their affinity for the carrier ($1/K_t$) indicates that hydrophobic interactions are not so important as for amino acids. Both the broad specificity and the narrow affinity range exhibited by the peptide transport system are unique properties. These characteristics may have special physiological

significance since non-preferential absorption of amino acids must be of nutritional importance.

Finally, a comment should be made about the effect of Na^+-free conditions on the D-glucose-evoked potential. In contrast to the amino acid-evoked potential, the glucose-evoked potential was not completely suppressed by the Na^+-free conditions as described in Results. Parallel experiments disclosed that the glucose-evoked potential exhibited features partly similar to those of the peptide-induced potential under the Na^+-free conditions. Namely, the amplitude of the glucose-evoked potential was markedly increased by lowering pH_o, and the pH-dependent glucose potential was completely inhibited by a low concentration of phlorizin (Hoshi, Takuwa, Abe & Tajima, 1986). Such properties of the glucose-evoked potential together with voltage-stimulated uptake of D-glucose by brush-border membrane vesicles in the absence of Na^+ (Hilden & Sacktor, 1982) may be explained in terms of H^+-coupled co-transport of D-glucose by phlorizin-sensitive D_1-glucose carrier (so-called Na^+–D-glucose co-transporter). In other words, when Na^+ is absent, its role appears to be filled by H^+. This finding taken together with the other results presented in this report indicates that there are three different types of cation-coupled co-transport systems in the intestinal brush-border membrane in regard to the cation-specificity; one is the absolutely Na^+-dependent neutral amino acid transport system, secondly the absolutely H^+-dependent peptide transport system, and thirdly the Na^+-dependent but partly H^+-substitutable hexose transport system.

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