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Rapid Report

H^+/glycyl-glycine cotransport in eel intestinal brush-border membrane vesicles: studies with the pH-sensitive dye Acridine orange

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Monitoring the fluorescence quenching of the pH-sensitive dye Acridine orange, proton accumulation in the presence of an inside-negative transmembrane potential was measured in eel (*Anguilla anguilla*) intestinal brush-border membrane vesicles. It was demonstrated that the proton accumulation was specifically increased by the presence of the dipeptide glycyl-glycine in the extravesicular space, showing saturation kinetics at increasing dipeptide concentrations and was specifically inhibited by diethylpyrocarbonate. Data reported suggest the presence of an electrical-potential-dependent H^+/glycyl-glycine cotransport system in the eel intestinal brush-border membrane vesicles.

Although it has been reported that the transport of di- and tripeptides through the luminal membrane of the intestinal absorbing cells strongly depended on the presence of a transmembrane pH-gradient ($[H^+]_{out} > [H^+]_{in}$) [1–3], until now no direct evidence has been produced indicating that proton ions were translocated in association with the carrier-mediated movement of the dipeptides. In the present investigation, the use of the fluorescent dye Acridine orange, permitted the demonstration of proton uptake in eel intestinal BBMV, specifically induced by the dipeptide glycyl-glycine.

Brush-border membrane vesicles (BBMV) were prepared from the intestine of yellow eels, by the Mg^{2+}-precipitation technique, as described elsewhere [4]. Protein concentration was measured with the Bio-Rad kit (using lyophilized bovine plasma gamma globulins as a standard) and adjusted to the final concentration of 12.5 mg/ml for each experiment.

Acridine orange fluorescence was measured with a Perkin-Elmer LS-5 spectrofluorometer, equipped with an electronic stirring system and a thermostatized (25°C) cuvette holder. Fluorescence signals were continuously recorded with a Hitachi Perkin-Elmer 561 recorder. The excitation and emission wavelengths were 498 and 530 nm, respectively and both slits were set to 5 nm. In a glass cuvette, 10 μl of a 0.6 mM Acridine orange solution (in water), 10 μl of a 1 mM valinomycin solution (in ethanol) and 1960 μl of a cuvette buffer were placed, giving the final composition indicated in the legends of the figures. The fluorescence value was set to 90 arbitrary fluorescence units. Then, 20 μl of vesicle suspension were added to the cuvette to start the experiment. With this experimental set-up, any movement of proton ions inside the vesicular space (acidification) gave rise to an intravesicular accumulation of the protonated form of Acridine orange molecules and could be followed as a quenching in the fluorescence signal [5].

For the inhibition experiments with diethylpyrocarbonate (DEP), 200 μl of brush-border membrane vesicles suspension (protein concentration 25 mg/ml), preloaded in 100 mM mannitol, 100 mM KCl, 2 mM Hepes (adjusted to pH 7.4 with Tris) (buffer A), were incubated with 1 mM DEP (from a 100 mM stock solution in ethanol), 280 mM mannitol, 20 mM potassium phosphate (pH 6.4) (buffer B) at 20°C for 1 h. As a control, the same amount of brush-border membrane vesicles were incubated in buffer B without DEP. The final ethanol concentration in the incubation media was 1%. After incubation, the vesicle suspensions were diluted 35-fold with ice-cold buffer A and centrifuged at 50000 × *g* for 30 min. The washing procedure was

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminoethane; DEP, diethylpyrocarbonate; BBMV, brush-border membrane vesicles.

repeated twice. After resuspension in buffer A (final concentration 12.5 mg/ml), the fluorescence quenching of Acridine orange was measured in DEP-treated and untreated (i.e., incubated with buffer B without DEP as a control) brush-border membrane vesicles.

In every experiment, intravesicular and extravesicular buffers had the same ionic strength, pH, anion concentration and osmolarity. Data points are the mean results of at least triplicate determinations. Standard errors are shown whenever they exceeded the size of the symbols.

All chemicals were of reagent grade and were purchased from Merck (Darmstadt, Germany); valinomycin and diethylpyrocarbonate were obtained from Sigma (St. Louis, MO, USA).

It has previously been demonstrated in eel renal BBMV that, in the presence of a transmembrane electrical potential, the fluorescence quenching of Acridine orange can be used to monitor the diffusional proton movement into the vesicular space [6]. Data reported in Fig. 1A show that a trans-membrane electrical potential across eel intestinal BBMV could induce not only a proton movement due to simple diffusion, but also the activation of an additional proton uptake dependent on the presence of glycyl-glycine in the extravesicular medium. In Fig. 1A, it can be observed that under short-circuited membrane potential condition, obtained by using equal K^+ concentrations in the intra- and extravesicular media ($[K^+]_{in} = [K^+]_{out} = 100$ mM) and valinomycin, the addition of 20 μl of vesicle suspension (250 μg protein) into the cuvette, did not produce transient fluorescence quenching (trace a). Under the same experimental conditions, no significant change in the fluorescence quenching was observed when 40 mM glycyl-glycine substituted the same amount of mannitol in the extravesicular medium (trace b). On the other hand, when an inside-negative electrical potential, created by an asymmetrical distribution of K^+ ions in the intra- and extravesicular media ($[K^+]_{in} = 100$ mM, $[K^+]_{out} = 1$ mM) and valinomycin, was imposed across the vesicles membranes, a transient fluorescence quenching was observed (trace c), indicating intravesicular acidification. Under the experimental conditions used in Fig. 1A, the fluorescence quenching shown in trace c could be easily explained by proton diffusion inside the vesicular space, driven by the electrical gradient present across the brush-border membrane vesicles. Interestingly, in the presence of an electrical potential difference across the membrane vesicles, when the final extravesicular medium contained 40 mM of glycyl-glycine, the resulting fluorescence quenching was significantly ($P < 0.01$) higher than that obtained in the absence of the dipeptide (trace d). The increase of the fluorescence quenching seemed be specifically related to the presence of the dipeptide in the extravesicular medium, since no effect on base-line (i.e., on trace c) was observed when glycyl-glycine was substituted by the same amount (40 mM final concentration) of glycine, L-proline or D-glucose (trace e). In every experimental condition shown in Fig. 1A, the injection of 20 μl of 3 M KCl dissipated the electrical membrane potential across the vesicle membranes and

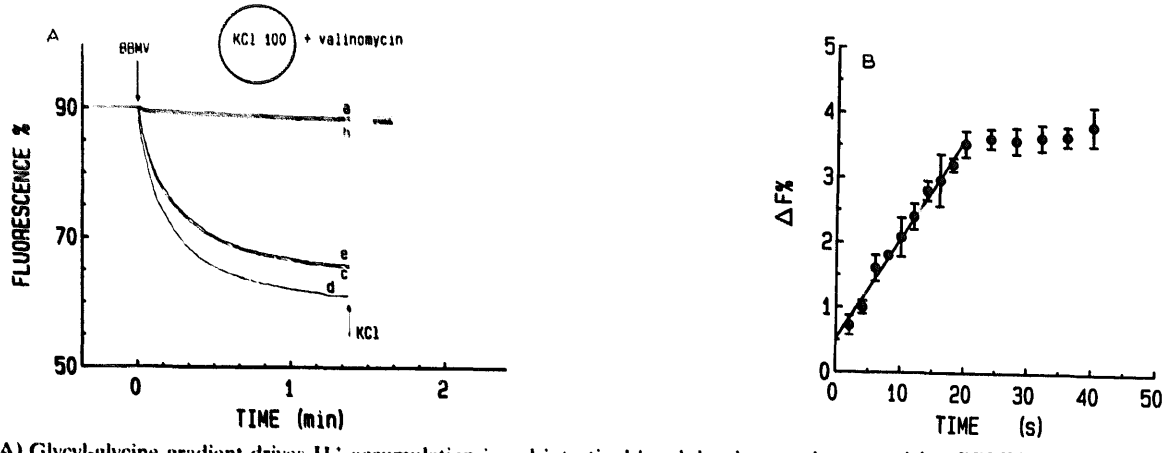

Fig. 1. (A) Glycyl-glycine gradient drives H^+-accumulation in eel intestinal brush-border membrane vesicles. BBMV were prepared in a buffer containing mannitol 100, KCl 100, Hepes 2 (adjusted to pH 7.4 with Tris). Unless otherwise indicated, concentrations are in mM. To start the experiment 20 μl of BBMV (250 μg protein) were injected into 1980 μl of a cuvette buffer containing 3 μM Acridine orange, 5 μM valinomycin, 0.5% ethanol, mannitol 100, Hepes 2 (adjusted to pH 7.4 with Tris) and: (1), KCl 100 (trace a); (2), KCl 100, glycyl-glycine 40 (trace b); (3), choline chloride 100 (trace c); (4) choline chloride 100, glycyl-glycine 40 (trace d); (5), choline chloride 100, glycine or L-proline or D-glucose 40 mM (trace e). Where substrates were present, they iso-osmotically replaced mannitol. To obtain faster re-equilibration of transmembrane pH gradient, 20 μl of a 3 M KCl solution were added into the cuvette at the time indicated. (B) Time-course of glycyl-glycine-dependent H^+ accumulation in BBMV from the eel intestine. The initial rate of fluorescence quenching (i.e., of proton accumulation) was followed for 40 s from the beginning of the reaction; data points were obtained by subtracting second by second the fluorescence quenching observed in the absence of glycyl-glycine (trace c of (A)) from the fluorescence quenching observed in the presence of 40 mM glycyl-glycine (trace d of (A)). $\Delta F\%$ values reported in the figure are the mean results of three determinations.

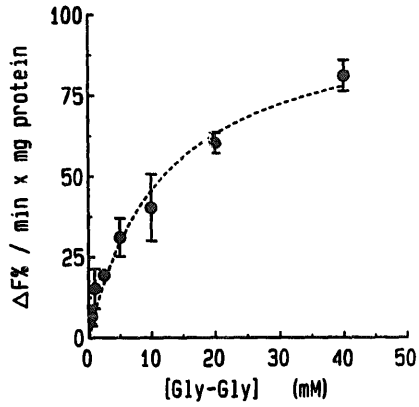

Fig. 2. Dependence of the initial rate of proton accumulation (measured at 15 s after the start of the reaction) on increasing extravesicular glycyl-glycine concentrations. Vesicles were prepared in a medium containing (in mM) mannitol 100, KCl 100, Hepes 2 (adjusted to pH 7.4 with Tris). 20 μl of a vesicle suspension were injected into a cuvette buffer containing 3 μM Acridine orange, 5 μM valinomycin, 0.5% ethanol, 100 mM mannitol, 100 mM choline chloride, 2 mM Hepes (adjusted to pH 7.4 with Tris) and increasing glycyl-glycine concentrations from 0.25 to 40 mM osmotically compensated by decreasing mannitol concentrations. The dashed line represents the best curve fitting the data and was obtained with an iterative nonlinear regression program. Kinetic parameters were expressed as K_{app} (in mM) and ΔF_{max} (in $\Delta F\%$/min per mg protein) and were 12.36 ± 3.13 mM and 102.27 ± 10.66 $\Delta F\%$/min per mg protein in this experiment, respectively.

rapidly reversed the fluorescence quenching signals to similar, equilibrium values, thus indicating the close relationship between the membrane electrical potential and the H^+ movement through the BBMV.

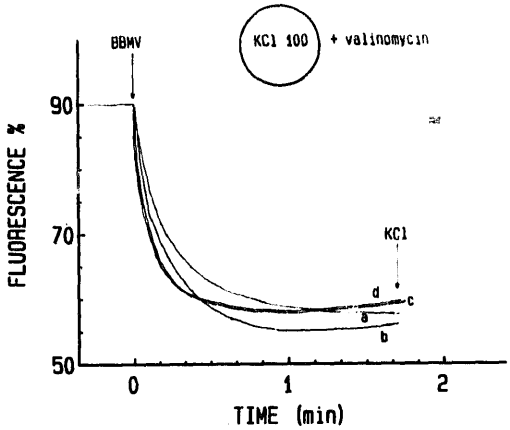

Fig. 3. DEP inhibition of proton accumulation in BBMV from the eel intestine. BBMV were incubated with (trace c and trace d) or without (trace a and trace b) 1 mM DEP as described in the paper. Experimental conditions were the same as described in Fig. 1A. BBMV, preloaded in (in mM) mannitol 100, KCl 100, Hepes 2 (adjusted to pH 7.4 with Tris), were injected into a cuvette buffer containing: (1), mannitol 100, choline chloride 100, Hepes 2 (adjusted to pH 7.4 with Tris) (trace a and trace c) or (2), mannitol 60, choline chloride 100, glycyl-glycine 40, Hepes 2 (adjusted to pH 7.4 with Tris) (trace b and trace d).

In Fig. 1B, the time-course of glycyl-glycine-dependent fluorescence changes is reported, estimated by subtracting the fluorescence quenching values in the absence of glycyl-glycine (trace c of Fig. 1A) from those in the presence of glycyl-glycine 40 mM (trace d of Fig. 1A). The dipeptide-dependent fluorescence-quenching values linearly increased with time for 20 s after BBMV injection, so that the fluorescence changes at 15 s could be reasonably considered as an estimate of the initial rate of glycyl-glycine-dependent intravesicular proton-accumulation.

When the initial rates of the dipeptide-dependent fluorescence quenching were plotted vs. increasing concentrations of glycyl-glycine (0.25–40 mM), they showed a saturation behaviour (Fig. 2). Furthermore, when data reported in Fig. 2 were fitted to a Michaelis-Menten-type equation and the kinetic parameters were estimated by a non-linear regression program (Statgraphics, STSC, Rockville, MD), the kinetic parameters reported in the legend of Fig. 2 were obtained.

Data reported in Fig. 1A and Fig. 2 strongly suggest that a carrier-mediated H^+/glycyl-glycine cotransport system is present on eel intestinal BBMV. To further support this statement, BBMV were incubated with diethylpyrocarbonate (DEP) which has been reported to inhibit the dipeptide transporter in rabbit intestine [7,8]. Results reported in Fig. 3 show that, in the presence of an inside-negative membrane potential, glycyl-glycine-dependent proton accumulation observed in non-DEP-treated BBMV (trace b) was significantly inhibited in DEP-treated BBMV (trace d). This effect seems to be specific for this dipeptide transporter, since in the same membrane-vesicle preparation D-glucose, glycine and L-proline Na^+-dependent transports were not inhibited by DEP (data not shown). All these observations clearly indicate a direct involvement of the histidine residues of the glycyl-glycine transporter in the translocation of H^+ ions.

In conclusion, results shown in the present report strongly suggest that at the luminal side of eel enterocytes, proton ions are cotransported with the dipeptide glycyl-glycine by a carrier-mediated transport, energized by the transmembrane electrical potential.

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References

1 Ganapathy, V., Burckhardt, G. and Leibach, F.H. (1984) J. Biol. Chem. 259, 8954–8959.
2 Ganapathy, V., Burckhardt, G. and Leibach, F.H. (1985) Biochim. Biophys. Acta 816, 234–240.
3 Ganapathy, V. and Leibach, F.H. (1985) Am. J. Physiol. 249, G153–G160.

4 Storelli, C., Vilella, S. and Cassano, G. (1986) Am. J. Physiol. 251, R463–R469.
5 Warnock, D.G., Reenstra, W.W. and Yee, V.J. (1982) Am. J. Physiol. 242, F733–F739.
6 Vilella, S., Zonno, V., Cassano, G., Maffia, M. and Storelli, C. (1991) Comp. Biochem. Physiol. 100A, 455–460.
7 Miyamoto, Y., Ganapathy, V. and Leibach, F.H. (1986) J. Biol. Chem. 261, 16133–16140.
8 Kramer, W., Durckheimer, W., Girbig, F., Gutjahr, U., Leipe, I. and Oekonomopulos, R. (1990) Biochim. Biophys. Acta 1028, 174–182.