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Citric acid as an adjuvant for transepithelial transport

M.J. Cho, J.F. Scieszka and P.S. Burton

Drug Delivery Systems Research, The Upjohn Company, Kalamazoo, MI 49001 (U.S.A.)

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Citric acid has been reported to promote the GI absorption of heparin (Sue et al., 1976) and the vaginal absorption of a luteinizing hormone-releasing hormone analog (Okada et al., 1982). Recently a synthetic peptidyl renin inhibitor was reported to be orally active when coadministered with citric acid (Pals et al., 1986). This putative adjuvant effect of citric acid for transepithelial transport has become a popular subject, at least in Japan, in the patent literature (Jap. Patent). In this communication, we would like to demonstrate that the observations cited above are consistent with a hypothesis that citrate opens up tight junctions of an epithelium thereby enhancing paracellular shunt pathway of a solute transport. We further speculate that citrate serves as a Ca^{2+} chelator.

Requirement of Ca^{2+} in maintaining the normal "gate" and "fence" functions of epithelial tight junctions has been well documented (Gumbiner et al., 1987). In addition, an experimental model has been available for some time for modulating the tight junctions developed in a confluent epithelial cell monolayer. In this model, any change in the paracellular shunt is monitored by the electrical resistance across the cell monolayer (Martinez-Palomo et al., 1980). Essentially an identical system was adopted in the present study. A detailed description can be found elsewhere (Cho et al., 1989). Briefly, the so-called highly resistant strain I of Madin Darby canine kidney (MDCK) epithelial cells were grown on a 4.7-cm^2 polycarbonate membrane with 3-μm pores (Transwell from Costar) to confluence in a modified Eagles' MEM under 5% CO_2 atmosphere at 37 °C. Transepithelial electrical resistance (TEER) was then monitored with various treatments of the cell monolayer.

Experiments similar to the one shown in Fig. 1 were repeated numerous times. A detailed explanation of Fig. 1 should, therefore, serve as a general description of the experimental procedure. Once the MDCK cells reach confluence, usually four or five days after seeding at 7.5×10^4 cells/cm^2, the nutrient-rich medium was replaced with Hanks' balanced salt solution (HBSS) at time point A. After 60 min incubation, at B, TEER was determined again, and HBSS was replaced with various test media both in the apical and basal sides of the MDCK cell monolayer. At time point C, the cell layer was placed back in the modified Eagles' MEM and incubated further overnight. The study shown in Fig. 1 employed a total of 22 cell monolayer preparations. The absolute TEER was 397 ± 87 (SD) $\Omega \cdot cm^2$ at time point B. Each test employed 3 monolayers, except for HBSS

Correspondence: M.J. Cho, The Upjohn Co. (7271-209-6), Kalamazoo, Michigan 49001, U.S.A.

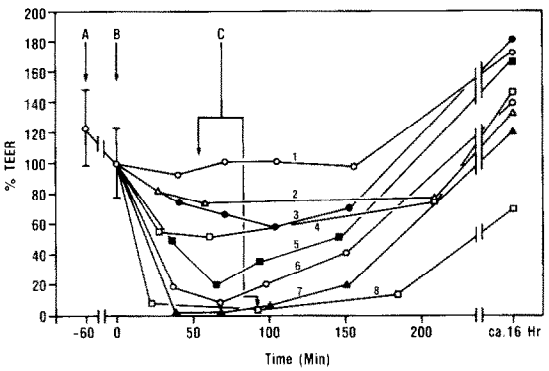

Fig. 1. Transepithelial electrical resistance (TEER) across the MDCK cell monolayers incubated in various test media. All values were normalized against those at $t = 0$, or point B. At A, the modified Eagles' MEM was replaced with Hanks' balanced salt solution (HBSS), which was in turn replaced at B with: (1) HBSS; (2) 1.0 mM succinate; (3) Ca^{2+}/Mg^{2+}-free HBSS (CMF); (4) 1.0 mM salicylate; (5) 1.0 mM EGTA; (6) 10 mM citrate; (7) 1.0 mM citrate; and (8) 0.1 mM citrate, all in CMF. At C, these test media were replaced with the modified Eagles' MEM. Incubation and TEER measurement were carried out at 37 °C and room temperature, respectively.

(No. 1 on Fig. 1) and 0.1 mM citrate (No. 8). Here only two monolayers were used for each test solution. Average TEER was normalized against the value at $t = 0$, or time point B. Test solutions were all prepared in Ca^{2+}/Mg^{2+}-free HBSS (CMF), except for the case of HBSS. In CMF, Ca^{2+} concentration was found to be $\leq 5 \times 10^{-6}$M, approximately the lowest detection limit for a typical Ca^{2+} electrode (Orion, Model 93–20). The final tonicity and pH were approximately 290 mOsm/kg and 7.4, respectively.

The decrease in TEER associated with the initial 60-min incubation in HBSS is attributed to a sudden change in medium composition. Note that the cells had been grown in a nutrient-rich medium for 4 or 5 days up to point A. As shown by the samples which were continuously incubated in HBSS (No. 1 on Fig. 1), TEER was very much stabilized after point B. When the cells were exposed to CMF (No. 3), TEER decreased steadily. Presence of 1.0 mM succinic acid (No. 2) changes neither the rate nor the extent of the TEER decrease, serving as a negative control. Succinic acid at pH 7.4 is almost exclusively dianionic, while citric acid would be triply charged. Both are members of Krebs' TCA cycle. Although it is a dicarboxylic acid, succinic acid is not expected to form a stable metal chelate due to favorable staggered conformation which would keep both carboxyl functions in a *trans* position. Similarly, 1.0 mM salicylate (No. 4 in Fig. 1), a putative adjuvant for transepithelial transport (Nishihata et al., 1983; Kajii et al., 1986), produced only a small decrease in TEER.

In contrast to these samples, citrate and a typical divalent metal chelator EGTA caused a TEER decrease by more than 80% within an hour (Nos. 5–8). As in all other cases studied, the effect of both citrate and EGTA on TEER was reversible in that the TEER values were fully recovered after overnight incubation in the modified Eagles' MEM. It was noticed, however, that the recovery rate was critically dependent on the duration in which the cells were exposed to a given test medium, confirming the literature finding (Martinez-Palomo et al., 1980). Effect of citrate was indistinguishable for a concentration range from 0.1 to 10 mM. Also citrate was as effective as EGTA, although the latter is much superior to citrate in terms of chelate formation with Ca^{2+} (Bell, 1977; Perrin, 1977; Gabriel et al., 1983). If the mechanism by which citrate induces TEER decrease is via ability to chelate Ca^{2+}, then these observations imply that a finite Ca^{2+} concentration is required in the extracellular medium for maintaining the junctional integrity and that even as low as 0.1 mM citrate could abolish the required level of Ca^{2+}. Such a requirement was reported in the case of *Necturus* gallbladder (Palant et al., 1983).

In accordance with the observed decrease in TEER, our preliminary data on the transport of a few selected fluid-phase markers indicated a significant increase in permeability of the cell monolayer in the presence of citrate. For example, as much at 15% of the total [^{14}C]-sucrose added to the apical side of an MDCK cell layer was found within 60 min in the basal side in the presence of 1.0 mM citrate. Under the same conditions, the cells exposed to HBSS, 1.0 mM EGTA, and CMF allowed approximately 1.4%, 6.0% and 9.3% of the total sucrose to pass through, respectively. However, these data should be interpreted with extreme caution, for CMF itself affects the junc-

tional integrity, to result in highly permeable cell monolayers as shown in this study and by others (Palant et al., 1983). This experimental difficulty in studying effects of potential adjuvants on the transport process per se is being critically assessed at the present time.

In conclusion, the results from TEER measurements suggest that citrate may serve as a Ca^{2+}-chelator thereby opening tight junctions in an epithelium. As such, the present study supports the mechanism proposed for the adjuvant effects observed with Ca^{2+}-chelating enamine derivatives in the rectal absorption of β-lactam antibiotics (Murakami et al., 1982).

References

Sue, T.K., Jaques, L.B. and Yuen, E., Effects of acidity, cations and alcoholic fractionation on absorption of heparin from gastrointestinal tract. *Can. J. Physiol. Pharmacol.*, 54 (1976) 613–617.

Okada, H., Yamazaki, I., Ogawa, Y., Hirai, S., Yashiki, T., and Mima, H., Vaginal absorption of a potent lutenizing hormone-releasing hormone analog (leuprolide) in rats. I. Absorption by various routes and absorption enhancement. *J. Pharm. Sci.*, 71 (1982) 1367–1371.

Pals, D.T., Thaisrivongs, S., Lawson, J.A., Kati, W.M., Turner, S.R., DeGraaf, G.L., Harris, D.W., and Johnson, G.A., Evaluation of an orally-active inhibitor of renin. *Hypertension*, 8 (1986) 1105–1112.

Japanese Patents 60023326, 56138110, and 55055120, abstracted through Derwent No. 85–066395/11, 90298D/49, and 68395C/39, respectively.

Gumbiner, B., Structure, biochemistry, and assembly of epithelial tight junctions. *Am. J. Physiol.*, 253 (1987) C749–C758.

Martinez-Palomo, A., Meza, I., Beaty, G., and Cereijido, M., Experimental modulation of occluding junctions in a cultured transporting epithelium. *J. Cell Biol.*, 87 (1980) 736–745.

Cho, M.J., Thompson, D.P., Cramer, C.T., Vidmar, T.J. and Scieszka, J.F., MDCK epithelial cell monolayer as a model cellular transport barrier. *Pharm. Res.*, 6 (1989) 71–77.

Bell, C.F., *Principles and Applications of Metal Chelation*, Clarendon, Oxford, 1977, p. 133.

Perrin, D.D., *Stability Constants of Metal-Ion Complexes, Part B*, Pergamon, Oxford, 1977, p. 374 and p. 1006.

Gabriel, J.L., Aogaichi, T., Dearolf, C.R. and Plaut, W.E., Apparent stability constants of magnesium and calcium complexes of tricarboxylates. *Anal. Lett.*, 16 (1983) 113–127.

Palant, C.E., Duffey, M.E., Mookerjee, B.K., Ho, S., and Bentzel, C.J., Ca^{2+} regulation of tight-junction permeability and structure in *Necturus* gallbladder. *Am. J. Physiol.*, 245 (1983) C203–C212.

Nishihata, T., Rytting, H., Kamada, A., Higuchi, T., Routh, M. and Caldwell, L., Enhancement of rectal absorption of insulin using salicylates in dogs. *J. Pharm. Pharmacol.*, 35 (1983) 148–151.

Kajii, H., Horie, T., Hayashi, M., and Awazu, S., Effects of salicylic acid on the permeability of the plasma membrane of the small intestine of the rat: a fluorescence spectroscopic approach to elucidate the mechanism of promoted drug absorption. *J. Pharm. Sci.*, 75 (1986) 475–478.

Murakami, T., Yata, N., Tamauchi, H., and Kamada, A., Studies of absorption promotors for rectal preparations. II. A possible mechanism of promoting efficacy of enamine derivatives in rectal absorption. *Chem. Pharm. Bull.*, 30 (1982) 659–665.