REVISTA ESPAÑOLA DE FISIOLOGÍA, 48 (3), 171-176, 1992

$HgCl_2$ Inhibition of Na-Independent L-Proline Transport in Chicken Proximal Cecum

C. Amat, A. Puchal, J. M. Planas and M. Moretó*

Grup d'Absorció Intestinal
Dep. Ciències Fisiològiques Humanes i de la Nutrició, Facultat de Farmàcia
Universitat de Barcelona
08028-Barcelona (Spain)

(Received on February 2, 1992)

C. AMAT, A. PUCHAL, J. M. PLANAS and M. MORETÓ. *$HgCl_2$ Inhibition of Na-Independent L-Proline Transport in Chicken Proximal Cecum.* Rev. esp. Fisiol., 48 (3), 171-176, 1992.

The proximal cecum of 5- and 13-wk-old chickens takes up L-proline (Pro) by two saturable pathways that differ in their requirement of Na^+. The kinetic properties of Pro influx in cecal segments incubated in Na^+-free conditions, have been studied and the effect of $HgCl_2$ on the uptake process has been tested. Experiments were carried out using an *in vitro* everted-sleeve method. Kinetic parameters were estimated by nonlinear regression analysis. One min Pro fluxes in tissues incubated in presence of $HgCl_2$ fit a straight line, indicating that a Na-independent saturable component was inhibited. Estimated Kd* values are the same in 5- and 13-wk-old chickens, 0.023 ± 0.001 and 0.027 ± 0.001 $\mu l \cdot mg^{-1} \cdot min^{-1}$, respectively. $HgCl_2$-sensitive fluxes fit a Michaelis hyperbola, with similar Km* values, 4.85 ± 1.86 (5-wk) and 9.47 ± 3.0 (13-wk) mmol/l. However, Vmax* in 5-wk chickens (0.662 ± 0.053 $nmol \cdot mg^{-1} \cdot min^{-1}$) is higher than in 13-wk birds (0.420 ± 0.039), in accordance with previous results. The present data give further support to the existence of a Na-independent L-proline carrier in the chicken proximal cecum which is inhibitable by $HgCl_2$.

Key words: L-Proline Transport, Chicken proximal cecum, Hg.

Amino acids are transported across the intestinal epithelium via multiple transport pathways (16). Amino acid transport in birds has been reviewed by LERNER (7) who showed that, in the small intestine of the domestic fowl, amino acids can be absorbed by means of Na-dependent and Na-independent systems.

The proximal cecum of the chicken can accumulate amino acids such as valine, phenylanaline, leucine, and proline (11). At high lumenal concentrations, the ab-

* To whom all correspondence should be addressed: Laboratori de Fisiologia, Facultat de Farmàcia, Av. Joan XXIII s/n. 08028 - Barcelona (Spain).

sorption of L-proline (Pro) in the cecum is 23 % by a Na-dependent mediated mechanism, and the remaining 77 % by a Na-independent process, which was believed to be diffusional (13). MORETÓ *et al.* (10), in a study of cecal Pro absorption during development suggested, however, a Na-independent carrier-mediated system for Pro from the 4th week after the hatch. This conclusion was deduced indirectly, splitting up the Na-independent influx rates into diffusive and saturable components by kinetic analysis.

In the present paper we have studied whether the saturable component of the Na-independent Pro influx in the chicken proximal cecum can also be demonstrated by the chemical inactivation of carrier proteins. The agent chosen was $HgCl_2$ because it is an effective inhibitor of carrier-mediated sugar and amino acid uptake in mammalian, as well as in avian species (1, 9, 14, 15, 17). Our results give additional support to the view that the chicken cecum has a PRO carrier that does not require sodium.

Materials and Methods

Animals. — Male white Leghorn chickens 4- to 7-wk-old (5- wk-old group) and 12- to 15-wk-old (13-wk-old group) were used. Birds had free access to water and a commercial diet (Gordina, Gallina Blanca Purina, Madrid, Spain).

Uptake measurements and kinetic constants. — The experiments were carried out on the proximal cecal region, as defined in a previous paper (3). One cm long segments were everted and fixed to metal rods of the appropriate diameter, and incubated as described by KARASOV and DIAMOND (4). Tissues were first preincubated for 5 min in a nutrient- and Na^+-free Ringer solution with or without 3 mmol/L $HgCl_2$. To measure rates of L-proline (Pro) uptake, sleeves were incubated for 1 min in a Na^+-free Ringer to which Pro was added at concentrations ranging from 0.1 to 50 mmol/L. Trace amounts of U- ^{14}C-labeled Pro were added to quantify the nutrient associated with the tissue. D-[1-^3H]mannitol was used to correct Pro uptake for adherent fluid. During both preincubation and incubation the medium was strongly stirred with a magnetic bar (1,200 rpm) to minimize unstirred layer effects.

Kinetic analysis was performed with Pro fluxes obtained in Na^+-free conditions without (Na-independent uptake) and with $HgCl_2$ ($HgCl_2$-insensitive uptake). The difference between the two fluxes (the Na-independent $HgCl_2$-sensitive Pro uptake) was also evaluated. Kinetic parameters, apparent Km, Vm and Kd (expressed here as Km*, Vm* and Kd*, respectively), were estimated by nonlinear regression analysis from plots generated by the Enzfitter statistical package (Biosoft, Cambridge, UK). Whether the Na-independent fluxes better fitted a straight line or an equation comprising the sum of saturable and diffusional components was tested. The decision as to the best fit was taken from the value of the average deviation (AD) of the curve from experimental points ($AD = [\Sigma E(x-\bar{x})^2/df]^{1/2}$), as recommended by MOTULSKY and RANSNAS (12).

Composition of the solutions. — The composition of the Ringer solution was (in mmol/L): 93.4 choline chloride, 6.2 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 7 H_2O, 24.9 $KHCO_3$ and 50 D-mannitol. Osmolarity was routinely checked and maintained by equimolar substitution of mannitol when necessary.

Statistical analyses. — Results are given as means ± SE. Fluxes shown were from «n» different chickens, and the standard error accounts for interanimal variability. Kinetic constants were estimated from pooled flux values, and the standard error

therefore reflects adequacy of fitting. The Student *t* test was applied when required and the differences were considered significant at $P < 0.05$.

Results

The Na-independent Pro fluxes in the 5 wk-old and the 13 wk-old chickens are shown in figures 1 and 2. These values were subjected to a similar analysis to the one applied before (10), and the results again show that in both groups the curves obtained in Na^+-free conditions fit a Michaelis hyperbola plus a straight line better than a simple diffusion equation (AD = 0.283 and 0.626, respectively, in 5-wk-old chickens; AD = 0.275 and 0.410, respectively, in 13-wk-old chickens). The kinetic analysis of the saturable and non saturable components yields kinetic constants that do not differ from those previously reported and are shown in table I.

Fluxes of Pro in tissues incubated in Na-free conditions and in the presence of

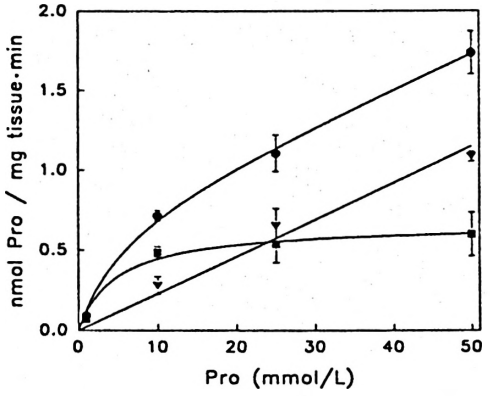

Fig. 1. *Na-independent L-Proline (Pro) uptake by proximal cecum of 5 wk-old chickens.* Total influx of Pro as a function of Pro concentration (●), n = 5-8. Na-independent $HgCl_2$-insensitive uptake (▼), n = 5-8. Na-independent $HgCl_2$-sensitive uptake calculated by subtracting fluxes in the presence of $HgCl_2$ from total uptake in Na^+-free conditions (■), n = 5-8. Error bars smaller than size of symbols are omitted.

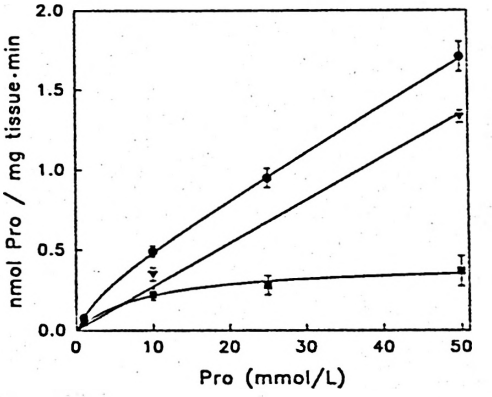

Fig. 2. *Na-independent L-Proline (Pro) uptake by proximal cecum of 13 wk-old chickens.* Total influx of Pro as a function of Pro concentration (●), n = 5-18. Na-independent $HgCl_2$-insensitive uptake (▼), n = 5-6. Na-independent $HgCl_2$-sensitive uptake calculated by subtracting fluxes in the presence of $HgCl_2$ from total uptake in Na^+-free conditions (■), n = 5-18. Error bars smaller than size of symbols are omitted.

Table I. *Kinetic constants for the Na-independent uptake of L-Proline (Pro) by chicken proximal cecum*
Values are means ± S.E. Kinetic constants were estimated by nonlinear regression analysis.

Constant	This study		Moretó et al. (10)	
	5 wk	13 wk	5 wk	13 wk
Km*, mmol/L	4.85 ± 1.86	9.47 ± 3.0	6.22 ± 2.26	4.02 ± 1.86
Vmax*, nmol · mg^{-1} · min^{-1}	0.662 ± 0.053	0.420 ± 0.039**	0.763 ± 0.10	0.266 ± 0.049**
Kd*, µL · mg^{-1} · min^{-1}	0.023 ± 0.001	0.027 ± 0.001	0.021 ± 0.002	0.029 ± 0.009

** $p < 0.05$

$HgCl_2$ fit a straight line with regression coefficients of 0.994 (fig. 1) and 0.998 (fig. 2) for 5 and 13 wk-old chickens, respectively. The slopes of these lines yield values of 0.023 µl/mg · min in 5 wk-old chickens and 0.027 µl/mg · min in 13 wk-old birds.

The flux components obtained when subtracting the $HgCl_2$-insensitive fluxes from the Na-independent uptake of Pro were fitted to a Michaelis hyperbola. The resulting kinetic constants are in table I.

Discussion

In a previous study (10) a Na-independent carrier system for Pro was proposed, on the basis of kinetic analysis, in the proximal cecum of 5 and 13 wk-old birds. In 1-3 day-old birds, fluxes obtained in the absence of Na were fitted to a Michaelis hyperbola plus a diffusional component, yielding a Km* of 2,400 mmol/L (unpublished results). This value has no physiological meaning considering that amino acid concentration in the cecum is low (13). Since experimental points showed a good fit to a straight line it was concluded that 1-3 day-old chickens lack a Pro Na-independent saturable carrier.

During the period comprised between 1-3 day and 5 wk after the hatch, cecal enterocytes express a Na-independent carrier that is also present in 13 wk-old chickens. The time when transporters are first expressed varies with species and with trasporter which can be attributed to genetic or dietary signals, this allowing functional adaptations to nutritional demands (2). The present study has tested, in 5 and 13 wk-old chickens, whether the saturable component of the Na-independent cecal Pro influx can be demonstrated by the inactivation of carrier proteins with $HgCl_2$.

Mercurials are widely used to inhibit transport systems because they have strong avidity for -SH groups. BIHLER and CYBULSKY (1) demonstrated in the mouse intestine that a short (2 min) treatment with 3 mmol/L $HgCl_2$ abolished sugar transport at the apical side of intestinal cells. MILLER (8) observed that 1 mmol/L $HgCl_2$ increases Km for glucose in brush border vesicles of fish intestine. There is also evidence indicating that $HgCl_2$ can inhibit the phloridzin-sensitive glucose uptake in the chicken intestine (9) and the facilitated diffusion sugar transport (5). Amino acid transport systems have also been shown to be sensitive to Hg^{++} inhibition in the rabbit ileum (17).

Since the inhibitory effects of Hg^{++} are exerted rapidly (9, 14), preincubation of the tissues in 3 mmol/l of $HgCl_2$ for 5 min in a well stirred medium was considered to be sufficient to abolish Pro transport in the chicken intestine. The possible effects of Hg^{++} on the Na-gradient across the membrane (1, 6, 8) are not relevant because the process under study is independent of the Na^+ gradient.

The slope of the lines plotting the Na-independent $HgCl_2$-insensitive Pro influx *vs* concentration are 0.023 ± 0.001 in 5 wk-old chickens and 0.027 ± 0.021 in 13-wk-old birds. Neither slope differs from the Kd* previously calculated by kinetic means (10), indicating that in both cases the fluxes measured correspond to the passive movement of the substrate. Since Hg^{++} does not change the passive permeability of organic substrates (6, 8, 15) the $HgCl_2$-insensitive Pro influx may be assumed to be equivalent to the passive flux.

The kinetic analysis of the Na-independent $HgCl_2$-sensitive Pro fluxes demonstrated that they fit a Michaelis hyperbola in both age groups, indicating that Hg^{++} inhibits a saturable component of Na-independent Pro absorption. In addition, the kinetic constants obtained by this method do not differ from those obtained in the previous study, and they maintain the same statistical differences between age groups. Thus, Vmax* is lower in 13-

wk-old than in 5-wk-old chickens ($p < 0.05$) without differences in the apparent affinity constant ($p > 0.1$).

From the values of total Pro absorption at 50 mmol/L obtained before (10) and from the fluxes obtained in the absence of Na^+ (present study), the contribution of the Na-dependent pathway can be estimated to be 45-50 % of total uptake, while OBST and DIAMOND (13) reported a 23 % contribution in similar experimental conditions. Albeit there may be quantitative differences in the estimation of the relative contribution of either mechanism, both studies agree that the absorption of Pro in the absence of sodium makes a significant contribution to total Pro absorption. The question arising is whether the Na-independent Pro absorption is entirely due to a pure simple diffusion process or, rather, to the presence of a saturable component. The evidence obtained by kinetic analysis and from Hg^{++} sensitivity supports the view that about 10-20 % of Pro absorption takes place through a saturable process.

Our results strengthen the existence of a saturable Na-independent Pro transport in the proximal cecum of the chicken; furthermore, they confirm that this system has lower capacity (Vmax*) than the Na-dependent system, and that it decreases with age. Finally, the present study indicates that Hg^{++} can be used as a tool for the study of the Na-independent Pro transport.

Acknowledgements

This study was supported by grants PB87-0140 and PB88-0219 from Dirección General de Investigación Científica y Técnica, Ministerio de Educación y Ciencia, Spain.

Resumen

El ciego proximal de pollos de 5 y 13 semanas de edad es capaz de transportar L-prolina (Pro) mediante dos sistemas de transporte que difieren en sus requerimientos de Na^+. Hemos estudiado las propiedades cinéticas del transporte de Pro a través de la membrana apical en segmentos cecales incubados en ausencia de Na^+, y hemos probado el efecto del $HgCl_2$ en este proceso. Los experimentos fueron realizados mediante la técnica *in vitro* de los segmentos evertidos. Los parámetros cinéticos fueron estimados por regresión no lineal. Los flujos de Pro en tejidos incubados durante un minuto en presencia de $HgCl_2$ se ajustan a una línea recta, indicando la inhibición de un componente saturable independiente de Na^+. Los valores estimados de Kd* son similares en pollos de 5 y de 13 semanas, 0,023 ± 0,001 y 0,027 ± 0,001 $\mu l \cdot mg^{-1} \cdot min^{-1}$, respectivamente. Los flujos sensibles al $HgCl_2$ se ajustan a una hipérbola de Michaelis, con valores similares de Km*, 4,85 ± 1,86 (5 semanas) y 9,47 ± 3,0 (13 semanas) mmol/l. No obstante, la Vmax* en pollos de 5 semanas (0,662 ± 0,053 $mmol \cdot mg^{-1} \cdot min^{-1}$) es mayor que en los de 13 semanas (0,420 ± 0,039), de acuerdo con estudios previos. Los resultados obtenidos confirman la existencia de un sistema de transporte para la L-prolina independiente de Na^+ en el ciego proximal, inhibible por $HgCl_2$.

Palabras clave: Transporte de L-prolina, Ciego proximal de pollo, Mercurio.

References

1. Bihler, I. and Cybulsky, R.: *Biochim. Biophys. Acta*, 298, 429-437, 1973.
2. Buddington, R. K. and Diamond, J. M.: *Ann. Rev. Physiol.*, 51, 601-619, 1989.
3. Ferrer, R., Planas, J. M. and Moretó, M.: *Brit. Poultry Sci.*, 32, 679-691, 1991.
4. Karasov, W. H. and Diamond, J. M.: *J. Comp. Physiol.*, 152, 105-116, 1983.
5. Kinter, W. B. and Pritchard, J. B.: In «Handbook of Physiology». American Physiological Society, Bethesda, 1977, pp. 563-576.
6. Klip, A., Grinstein, S. and Semenza, G.: *Biochim. Biophys. Acta*, 558, 233-245, 1979.
7. Lerner, J.: *Comp. Biochem. Physiol.*, 78A, 205-215, 1984.

8. Miller, D. S.: *J. Pharmacol. Exp. Ther.*, 216, 70-76, 1981.
9. Miller, D. S., Shehata, A. T. and Lerner, J.: *J. Pharmacol. Exp. Ther.*, 214, 101-105, 1980.
10. Moretó, M., Amat, C., Puchal, A., Buddington, R. K. and Planas, J. M.: *Am. J. Physiol.*, 260, G457-G463, 1991.
11. Moretó, M. and Planas, J. M.: *J. Exp. Zool.*, Suppl. 3, 111-116, 1989.
12. Motulsky, H. J. and Ransnas, L. A.: *FASEB J.*, 1, 365-374, 1987.
13. Obst, B. S. and Diamond, J. M.: *J. Exp. Zool.*, Suppl. 3, 117-126, 1989.
14. Rodríguez-Yoldi, M. J., Lluch, M. and Ponz, F.: *Rev. esp. Fisiol.*, 43, 239-244, 1987.
15. Rodríguez-Yoldi, M. J., Lugea, A., Barber, A., Lluch, M. and Ponz, F.: *Rev. esp. Fisiol.*, 45, 207-214, 1989.
16. Stevens, B. R., Kaunitz, J. D. and Wright, E. M.: *Ann. Rev. Physiol.*, 46, 417-433, 1984.
17. Stirling, C. E.: *J. Membr. Biol.*, 23, 33-56, 1975.