$(Ca^{2+} + Mg^{2+})$-ATPase AND CALMODULIN ACTIVITY OF RAT RED BLOOD CELLS: LACK OF DEPENDENCE ON VITAMIN D*

Bernard P. HALLORAN, H. F. DeLUCA and Frank F. VINCENZI*

Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, WI 53706 and **Department of Pharmacology, University of Washington, Seattle, WA 98105, USA*

Received 10 March 1980

1. Introduction

Calmodulin is the name recently coined for an ubiquitous Ca^{2+}-binding protein. CaM is a heat-stable, 18 000 mol. wt, acidic protein containing 4 Ca^{2+} binding sites/molecule [1,2]. CaM has been implicated in a number of cellular processes including Ca^{2+}-dependent activation of phosphodiesterase [3], adenylate cyclase [4], and $(Ca^{2+} + Mg^{2+})$-ATPase [5]. It has also been implicated as a modulator of active calcium transport across the plasma membrane [6].

The absorption of calcium in the intestine is another process that may involve the transport of calcium across the plasma and other membranes and has been shown to be mediated by vitamin D [7,8]. Vitamin D stimulates not only active transport in the intestine, but also the activity of alkaline phosphatase and $(Ca^{2+} + Mg^{2+})$-ATPase [9,10]. In addition, vitamin D increases the concentration of an acidic, heat-stable protein (CaBP) having 4 high affinity calcium binding sites/molecule and localized in the intestine [11,12].

The similarities in the physico–chemical and perhaps the immunological [13] properties of CaM and CaBP, and their possible involvement in the regulation of calcium transport led to the question as to whether these proteins have interchangeable functions. Unlike CaM, CaBP failed to stimulate red blood cell plasma membrane $(Ca^{2+} + Mg^{2+})$-ATPase activity (F.F.V., unpublished). Another question, is whether or not the activity of RBC plasma membrane $(Ca^{2+} + Mg^{2+})$-ATPase and/or the activity of CaM is dependent on vitamin D. Results presented here show no dependence of these parameters on the vitamin D status of rats.

Abbreviations: CaM, calmodulin; CaBP, calcium-binding protein; RBC, red blood cell; 25-OH-D_3, 25-hydroxyvitamin D_3, 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; SDS, sodium dodecyl sulfate; P_i, inorganic phosphate

2. Materials and methods

Female weanling rats from Holtzman (Madison, WI) were maintained on a vitamin D-deficient diet containing 0.44% calcium and 0.3% phosphorus [14] for $\geqslant 4$ months before experimentation. Control animals received the same diet supplemented with 25 IU vitamin D/day.

Blood was collected from the abdominal aorta from several animals, pooled, and the plasma analyzed for calcium, 25-hydroxyvitamin D_3 (25-OH-D_3) and 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) [15–17]. Blood samples were counted on an electronic particle counter (Coulter, model Z_{BI}). As noted below, ATPase activities of red cell membranes and the CaM activity of red cell hemolysates were determined in duplicate. Two separate experiments were conducted in this manner and the results were the same in each case. The results from one such experiment are reported. Human RBC membranes were prepared from blood freshly drawn or stored on ice up to 2 days. The resultant membranes were tested in parallel with the membranes from rat RBCs. Red blood cells were washed 3 times and the buffy coat was removed by aspiration. Isolated RBC membranes were prepared essentially as in [18].

ATPase activities were determined at 37°C as in [18] with minor modifications. Mg^{2+} activity was defined as the hydrolysis of ATP (above blanks) in the presence of 0.1 mM EGTA. $(Ca^{2+} + Mg^{2+})$-ATPase

was defined as the extra hydrolysis caused by the addition of 0.2 mM $CaCl_2$. Basal (Ca^{2+} + Mg^{2+})-ATPase was that measured in the absence of added CaM or hemolysate. Activated (Ca^{2+} + Mg^{2+})-ATPase was that measured in the presence of added CaM or hemolysate. The reaction was stopped after 60 or 90 min by addition of 1 ml 10% SDS . P_i released from ATP was determined by an automated colorimetric assay (F&S) [19]. When CaM activity of hemolysates was tested, the hemolysates was tested, the hemolysates were added to the (Ca^{2+} + Mg^{2+})-ATPase assay system at 10–200 μl (per 2 ml assay). 'Crossover' experiments were performed in which the hemolysate from vitamin D-deficient or vitamin D-replete rats, respectively, was added to membranes of normal human RBCs, and membranes of RBCs from vitamin D-replete and vitamin D-deficient rats. Purified CaM was prepared from beef RBCs by a method adapted from that in [20,21].

3. Results

Plasma samples taken from the vitamin D-deficient group of rats had 5.6–5.8 mg calcium/100 ml, while those taken from the control group of animals had 10.5–10.8 mg calcium/100 ml. No 25-OH-D_3 (<2 ng/ml) or 1,25-$(OH)_2D_3$ (<5 pg/ml) was found in any of the rats from the vitamin D-deficient group, verifying that the animals were, in fact, vitamin D deficient.

It was observed that rat RBCs were somewhat more resistant to hemolysis than fresh human RBCs. The fraction of RBCs which did not hemolyze was discarded. This fraction was estimated to be 5% of the cells in the case of rat and ⩽1% for human cells. In an electronic particle counter, rat cells had a smaller apparent volume than human RBCs. The apparent volumes of RBCs of the 2 groups of rat cells (D-deplete vs D-replete) were indistinguishable, although vitamin D-deficient rat blood showed a modest anemia.

Membranes of rat RBCs exhibit (Mg^{2+})-ATPase, (Na^+ + K^+ + Mg^{2+})-ATPase, and (Ca^{2+} + Mg^{2+})-ATPase activities. The magnitudes of the (Mg^{2+})-ATPase activity were somewhat higher and the magnitudes of (Ca^{2+} + Mg^{2+})-ATPase were lower than the corresponding activities in human RBC membranes (table 1). The sensitivity to and extent of activation by CaM was similar in membranes from both groups of rats. Hemolysates of rat RBCs contained CaM-like activity. When added to membranes isolated from human RBCs, rat hemolysates caused concentration-dependent activity of (Ca^{2+} + Mg^{2+})-ATPase. However, neither the extent of activation, nor the apparent CaM activity of the hemolysates was influenced by the vitamin D status of the rats from which the hemolysates were derived (fig.1).

When hemolysates from vitamin D-deplete rat RBCs were added to membranes isolated from vitamin D-replete rat RBCs and vice versa, concentration-dependent activation of the (Ca^{2+} + Mg^{2+})-ATPase was observed. No differences were observed in these crossover experiments (data not shown). Thus, it appears that neither the (Ca^{2+} + Mg^{2+})-ATPase, nor the CaM activity of RBCs is influenced by the vitamin D status of rats. Furthermore, the interaction between CaM and the (Ca^{2+} + Mg^{2+})-ATPase does not appear to be influenced by vitamin D.

Table 1
RBC membrane ATPase activities

ATPase activity[a]	Normal human	Rat	
		Vitamin D-deplete	Vitamin D-replete
Mg^{2+}-ATPase	3.8[b]	26.0	25.7
(Na^+ + K^+ + Mg^{2+})-ATPase	12.4	12.9	14.4
Basal (Ca^{2+} + Mg^{2+})-ATPase[c]	12.6	5.5	6.1
Maximal activated (Ca^{2+} + Mg^{2+})-ATPase[d]	41.8	21.8	27.1

[a] See methods for operational definitions of various ATPase activities
[b] Entries are expressed in nmol P_i released · mg membrane protein^{-1} . min^{-1} and represent the average of duplicate determinations
[c] No added CaM
[d] CaM added at 1 μg/ml

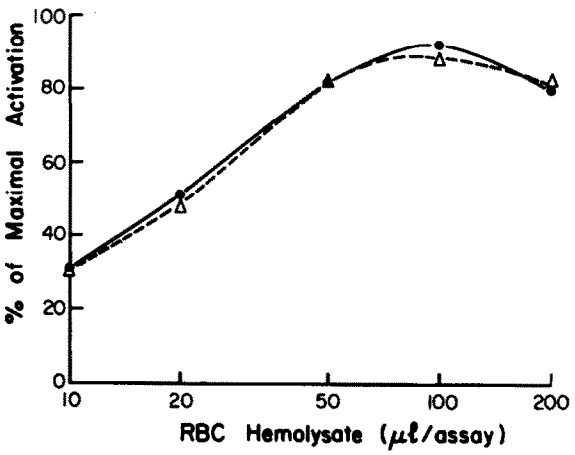

Fig.1. Activation by rat RBC hemolysates of human RBC membrane (Ca^{2+} + Mg^{2+})-ATPase. Activity of these membranes increased from 12.5–58.8 nmol P_i . mg protein^{-1} . min^{-1} upon addition of 1.0 μg beef CaM/ml. Rat RBC hemolysates also produced activations and data are plotted as % of the maximal activation. Hemolysates of both vitamin D-deplete (•——•) and vitamin D-replete (△——△) rats gave comparable activation. All values are the result of duplicate determinations.

4. Discussion

Ca^{2+}-binding proteins are now recognized as important intracellular factors in biological regulation. CaM has been implicated in a wide variety of cellular functions, including plasma membrane Ca^{2+} transport [6]. The precise role of CaM in the regulation of plasma membrane Ca^{2+} transport, however, remains to be determined. Likewise, the role of CaBP in the intestinal transport of Ca^{2+} is not clear.

It is known on the one hand that intestinal Ca^{2+} transport is regulated by vitamin D and a close relationship has been shown to exist between vitamin D and CaBP. On the other hand, these results show that neither plasma membrane (Ca^{2+} + Mg^{2+})-ATPase, nor the CaM activity of RBC hemolysates are influenced by the vitamin D status of experimental rats. Furthermore, the activation of (Ca^{2+} + Mg^{2+})-ATPase by CaM, which is most likely based on direct interaction between CaM and (Ca^{2+} + Mg^{2+})-ATPase [22], is not affected by vitamin D.

Based on these results, it is concluded that neither the plasma membrane Ca^{2+} pump nor the concentration of CaM in the red blood cell (and presumably in other cells) is dependent on the vitamin D status of the animal. Since the plasma membrane Ca^{2+} pump is regulated by internal Ca^{2+} [23], it is also likely that the Ca^{2+} content of most cells is little affected by vitamin D.

The ubiquitous nature and highly conserved sequence of CaM [1,24,25] suggests that CaM represents a very primitive calcium-binding protein. On the other hand, CaBP and its dependence on vitamin D may have evolved later. Thus, CaM and CaBP may be related structurally, and may each have regulatory roles in different Ca^{2+}-transport processes. However, the former does not appear to be sensitive to vitamin D.

Acknowledgements

This research was supported by USPHS grants AM-16436 to F.F.V., AM-14881 to H.F.D. and postdoctoral fellowship AM-05714 to B.P.H.; Department of Energy contract EY-76-S-02-1668, and the Harry Steenbock Research Fund of the Wisconsin Alumni Research Foundation. Dr Robert Wasserman kindly provided a sample of chick CaBP.

References

[1] Waterson, D. M., Garrelson, W. S., Keller, P. M., Sharief, F. and Vanaman, T. C. (1976) J. Biol. Chem. 251, 4501–4513.
[2] Dedman, J. R., Potter, J. D., Jackson, R. L., Johnson, J. D. and Means, A. R. (1977) J. Biol. Chem. 252, 8415–8422.
[3] Cheung, W. Y. (1970) Biochem. Biophys. Res. Commun. 38, 533–538.
[4] Brostrom, M. A., Huang, Y., Breckenridge, B. M. and Wolff, D. J. (1975) Proc. Natl. Acad. Sci. USA 72, 64–68.
[5] Gopinath, R. M. and Vincenzi, F. F. (1977) Biochem. Biophys. Res. Commun. 77, 1203–1209.
[6] Larsen, F. L. and Vincenzi, F. F. (1979) Science 204, 306–309.
[7] DeLuca, H. F. (1977) Adv. Clin. Chem. 19, 125–174.
[8] Kodicek, E. (1974) Lancet i, 325–329.
[9] Morrissey, R. L., Zolock, D. T., Bikle, D. D., Empson, R. N. and Bucci, T. J. (1978) Biochim. Biophys. Acta 538, 23–33.
[10] Melancon, M. J. and DeLuca, H. F. (1970) Biochemistry 9, 1658–1664.
[11] Wasserman, R. H. and Corradino, R. A. (1973) Vitamins and Hormones 31, 43–103.
[12] Bredderman, P. J. and Wasserman, R. H. (1974) Biochemistry 13, 1687–1694.

[13] Taylor, A. N. (1974) Arch. Biochem. Biophys. 161 100–108.
[14] Suda, T., DeLuca, H. F. and Tanaka, Y. (1970) J. Nutr. 100, 1049–1054.
[15] Halloran, B. P. and DeLuca, H. F. (1979) Science 204, 73–74.
[16] Eisman, J. A., Shepard, R. M. and DeLuca, H. F. (1977) Anal. Biochem. 80, 298–305.
[17] Eisman, J. A., Hamstra, A. J., Kream, B. E. and DeLuca, H. F. (1976) Arch. Biochem. Biophys. 176, 235–243.
[18] Farrance, M. L. and Vincenzi, F. F. (1977) Biochim. Biophys. Acta 471, 49–58.
[19] Raess, B. U. and Vincenzi, F. F. (1980) submitted.
[20] Jung, N. S. G. T. (1978) Master's Thesis, University of Washington.
[21] Jarrett, K. W. and Penniston, J. T. (1978) J. Biol. Chem. 253, 4676–4682.
[22] Lynch, T. J. and Cheung, W. Y. (1979) Arch. Biochem. Biophys. 194, 165–170.
[23] Schatzmann, H. J. (1973) J. Physiol. (London) 235, 551–569.
[24] Anderson, J. M. and Cormier, M. J. (1978) Biochem. Biophys. Res. Commun. 84, 595–602.
[25] Waisman, D., Stevens, F. C. and Wang, J. H. (1975) Biochem. Biophys. Res. Commun. 65, 975–982.