Evidence for Mg^{2+}-Dependent, $Na^+ + K^+$-Activated ATPase and Ca^{2+}-ATPase in the Human Term Placenta[1] (37265)

RICHARD K. MILLER[2] AND WILLIAM O. BERNDT
(Introduced by R. E. Gosselin)

Department of Pharmacology & Toxicology, Dartmouth Medical School, Hanover, New Hampshire 03755

Since the original observations of Skou (1), Mg^{2+}-dependent, Na^+ and K^+-activated ATPases[3] have been described for many different mammalian tissues, such as kidney (2) and heart (3), and for bacteria (4). Cerletti, Fronticelli and Zichella (5) have demonstrated ATPase activity and alkaline phosphatase activity in the human term placenta, but did not characterize the ATPase as the transport enzyme. Recently Shami and Radde (6, 7) have described a Ca^{2+}-ATPase in the guinea pig placenta; it was not clear from these reports whether or not this enzyme was the same as that described by Cerletti, Fronticelli and Zichella.

Indirect evidence for the presence of a transport ATPase was obtained in this laboratory when it was found that slices of human term placenta failed to accumulate α-amino-isobutyric acid in the absence of Na^+ or K^+, or in the presence of ouabain (8). It seemed likely that these effects might be related to a Mg^{2+}-dependent, $Na^+ + K^+$-activated ATPase as has been suggested for many transport systems (9). The present studies were undertaken to characterize the ATPase activity of human term placenta.

Materials and Methods. The placentas were obtained immediately upon delivery and placed in an iced chamber for transport to the laboratory. The preparation and purification of the ATPase were done using the method of Matsui and Schwartz (3) with minor modifications. The placental tissue used was at least 90% of fetal origin by histologic examination and was obtained as a cube by excluding the decidua basalis and chorionic plate. This block of tissue was further cubed and rinsed 3 times in 0.25 *M* sucrose and 1 m*M* EDTA–Tris at pH 7.4 to remove the blood before homogenization in a Waring blender. The procedures were performed at 0 to 4°. A centrifugation at 10,000*g* for 15 min produced a precipitate of crude tissue homogenate. The crude precipitate was resuspended in the sucrose solution containing 0.1% deoxycholate and a second centrifugation at 10,000*g* was performed for 15 min. The resulting supernatant was treated with deoxycholate (0.05%), and centrifuged for 15 min at 20,000*g*. The supernatant from this centrifugation was spun at 105,000*g* for 60 min, and the precipitate was incubated in a 2 *M* sodium iodide solution (pH 8.0), at 2° for 30 min. The sodium iodide-treated precipitate was centrifuged at 105,000*g* for 60 min. The supernatant was discarded and the sodium iodide-treated microsomes were washed in an EDTA solution (1 m*M*) and centrifuged 3 times at 105,000*g*. The final microsomal preparation was rapidly frozen using dry ice and acetone and was stored at −18°. The ATPase activity persisted for 3 wk or less; longer storage in the frozen state produced a reduction in enzyme activity.

The standard assay for ATPase involved the use of approximately 50 μg of protein

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[2] Present address: Stein Research Institute, Thomas Jefferson University Medical College, 920 Chancellor Street, Philadelphia, PA 19107.

[3] Abbreviations: ATP = adenosine triphosphate; ATPase = Adenosinetriphosphatase; ADP = adenosine diphosphate; GTP = quanosine triphosphate; ITP = inosine triphosphate; EDTA = ethylenediaminetetraacetic acid; and TCA = trichloroacetic acid.

(0.1 ml) in a total reaction volume of 1 ml. In addition the medium contained 5 m*M* $MgCl_2$, 10 m*M* Tris buffer at pH 7.4, 3 m*M* Tris–ATP, 100 m*M* NaCl and 15 m*M* KCl. The incubations were performed in duplicate at 37°. The protein was preincubated for 5 min, and the reaction was started with the addition of 0.1 ml of Tris–ATP or another suitable substrate. After 30 min, the reaction was terminated by the addition of 1 ml of ice-cold 10% TCA. The solution was centrifuged for 10 min after which a 1 ml aliquot was placed into 4 ml of 0.01 *N* sodium acetate.

The ATP utilization was measured as the release of inorganic phosphate as determined by the method of Lowry and Lopez (10). Protein concentration was measured by the Lowry *et al.* method (11). A Gilford microsample 300-N spectrometer was used to measure inorganic phosphate (675 n*M*) and protein (700 n*M*). Although the proteins were measured at a different wavelength than that reported originally (11), the procedure was verified by preparing appropriate standard curves. The data were presented as the mean ± the standard deviation, and the significance of the data was determined by the use of Student's *t* test.

Tris–ATP, ADP, GTP, ITP, and bovine serum albumin were purchased from the Sigma Chemical Co. Ouabain and sodium deoxycholate were obtained from Nutritional Biochemicals Corp., and the ethacrynic acid was a gift from Dr. John Baer of the Merck, Sharp and Dohme Research Laboratories.

Results. The data in Tables I and II indicate the presence of both a Mg^{2+}-dependent and Ca^{2+}-dependent ATPase. The Mg^{2+}-dependent enzyme demonstrated a decreased utilization of ATP when Na^+ or K^+ was removed from the bathing medium, although the extent of inhibition with Na^+ removal was less than with K^+ removal. The removal of Mg^{2+} reduced the ATPase activity to virtually zero. Ouabain (0.1 m*M*) reduced the $Na^+ + K^+$-ATPase activity by over 40%, while ethacrynic acid (5 m*M*) decreased it by over 75%.

In the presence of Ca^{2+} (Table II) at pH 7.4, the ATPase activity was decreased by 21% compared to that in the presence of

TABLE I. Mg^{2+} ATPase Activity in the Human Term Placenta.

	ATP hydrolysis[a] ($M \pm$ SD)	Change (%) from controls
Control	0.34 ± 0.14 (5)	—
No Na^+	0.29 ± 0.10 (5)	−14.7[b]
No K^+	0.23 ± 0.19 (5)	−32.4[b]
Ouabain (0.1 m*M*)	0.20 ± 0.12 (5)	−41.2[b]
Ethacrynic acid (5 m*M*)	0.08 ± 0.1 (3)	−76.5[b]
No Mg^{2+}	0.02 ± 0.03 (5)	−94.1[b]
Blank	0.015 ± 0.03 (5)	−95.6[b]

[a] Hydrolysis is expressed as micromoles of inorganic phosphate released per milligram protein per minute; conditions were 37°, pH 7.4, 5 m*M* $MgCl_2$, 3 m*M* Tris–ATP, 15 m*M* KCl, 100 NaCl, and 10 m*M* Tris unless otherwise stated. The blank was 1 ml of 10% TCA added during preincubation to inactivate the enzyme. The numbers in parentheses are the number of placentas used.

[b] Significantly different at $< .05$ level from Mg^{2+} control.

Mg^{2+}. Further, the Ca^{2+}-ATPase activity was insensitive to ouabain or the absence of Na^+or K^+. However, when both Na^+ and K^+ were deleted from the medium, there appeared to be a small but statistically significant increase over the Ca^{2+}-ATPase activity with the ions present. Ethacrynic acid was found to be as effective an inhibitor of this enzyme as of the Mg^{2+}-ATPase.

The enzymes can be further differentiated by their pH optima. The ouabain-sensitive, Mg^{2+}-dependent ATPase (*i.e.*, transport ATPase) had an optimal pH near 7.4–7.5, whereas the optima for the Mg^{2+}-ATPase and the Ca^{2+}-ATPase were above 8.0 (Fig. 1).

The ouabain-sensitive ATPase also had a substrate preference. ATP was hydrolyzed with greater facility than the other triphosphate nucleotides tested, with the order of preference being: ATP > GTP = ITP. Also ADP was hydrolyzed but to a considerably smaller degree than ATP (Table III). The ouabain-sensitive enzyme also showed an optimum substrate concentration (Fig. 2). In these experiments the enzyme activity

TABLE II. Ca^{2+} ATPase Activity in the Human Term Placenta.

	ATP hydrolysis[a] ($M \pm$ SD)		Change (%) from controls
Control (5 m*M* $MgCl_2$)	0.34 ± 0.14	(5)	—
Control (5 m*M* $CaCl_2$)	0.27 ± 0.075	(4)	−20.6[b]
No Na^+, K^+	0.30 ± 0.1	(3)	11.8[c]
No Na^+	0.27	(2)	—
No K^+	0.27	(2)	—
Ouabain (0.1 m*M*)	0.27 ± 0.09	(4)	—
Ethacrynic acid (5 m*M*)	0.07 ± 0.11	(4)	−74.1[c]
No Ca^{2+}	0.02 ± 0.04	(4)	−92.6[c]

[a] Hydrolysis is expressed as micromoles of inorganic phosphate released per milligram protein per minute; conditions were 37°, pH 7.4, 5 m*M* $CaCl_2$, 3 m*M* Tris–ATP, 15 m*M* KCl, 100 m*M* NaCl, and 10 m*M* Tris unless otherwise stated. The numbers in parentheses are the number of placentas used.

[b] Significantly different at $<$ .05 level from Mg^{2+} control. Only the control data obtained in the presence of $CaCl_2$ were compared to the $MgCl_2$ data. The data from all other experimental manipulations were compared to the $CaCl_2$ control.

[c] Significantly different at $<$ .05 level from Ca^{2+} control.

was measured under optimal conditions and the degree of activity at each substrate concentration was determined by inhibition with ouabain (0.1 m*M*). The maximum inhibition by ouabain was noted in the ATP concentration range of 3 to 5 m*M*, whereas a higher concentration actually decreased the ouabain sensitivity of the preparation.

Discussion. This study presents direct evidence for a Mg^{2+}-dependent, Na^+ + K^+-activated, ouabain-sensitive ATPase in the human term placenta as well as evidence for a Ca^{2+}-ATPase. These data corroborate the amino acid accumulation studies using this tissue (8) thus maintaining the concept of an association of the Mg^{2+}-dependent, Na^+ + K^+-activated, ouabain-sensitive ATPase with transport processes. The characteristics of this ouabain-sensitive enzyme are similar to those noted for other preparations, such as those from brain (12), intestine (13), and kidney (17). For example, the pH optimum is similar to values obtained with other ATPase preparations (14).

The substrate preference demonstrated here also has been noted for other ATPase enzymes. Schoner, Beusch and Kramer (15) demonstrated that an ox brain microsomal preparation utilized ATP preferentially. ADP is hydrolyzed by the human placental enzyme preparation, but to a lesser extent than is ATP. Can the ADPase-like activity account for the inorganic phosphate levels reached in the ATPase activity measurements due to the further hydrolysis of ATP to ADP to AMP? As shown in Table III the ADPase-like activity could account for only a small part of the breakdown of ATP. Of the ouabain-sensitive ATP hydrolysis only 23% could be accounted for by an ADPase that is similarly inhibited. Whether or not ADP inhibits ATP hydrolysis by the human placental enzymes was not studied, but on the basis of the ox brain experiments (15) this result might be anticipated.

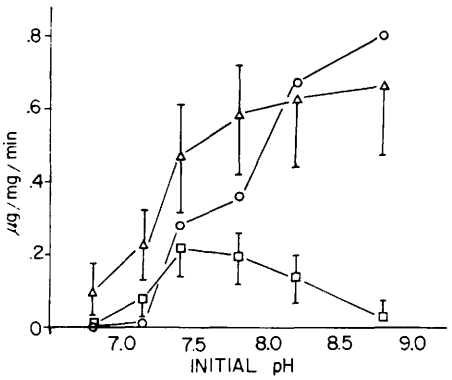

FIG. 1. The effect of pH on ATPase activity of human term placenta. Each point is the mean of 3 to 4 experiments and the bars are the standard deviations, except for the Ca^{2+}-dependent ATPase where the points are the means of two experiments. The pH values are presented as those at the beginning of the incubation. Conditions were 37°, 3 m*M* Tris–ATP, 15 m*M* KCl, 100 m*M* NaCl, 10 m*M* Tris and either 5 m*M* $MgCl_2$ or $CaCl_2$; the ouabain concentration was 0.1 m*M*. (○) Ca^{2+} ATPase; (□) Mg^{2+} ouabain-sensitive ATPase; (△) Mg^{2+} ouabain-insensitive ATPase.

TABLE III. The Effect of Different Substrates on Human Term Placental Mg^{2+}-Dependent, Ouabain-Sensitive ATPase Activity.

Substrate (3 m*M*)		Substrate hydrolysis[a]			Ouabain-sensitive activity (%)
		Control	Ouabain (0.1 m*M*)		
			Insensitive	Sensitive	
ATP	A	0.35	0.19	0.16	46
	B	0.25	0.11	0.14	56
GTP	A	0.24	0.21	0.03	12
	B	0.19	0.17	0.02	11
ITP	A	0.22	0.20	0.02	9
	B	0.14	0.12	0.02	14
ADP	A	0.18	0.14	0.04	22
	B	0.12	0.09	0.03	25

[a] Hydrolysis is expressed at micromoles of inorganic phosphate released per milligram protein per minute; conditions were 37°, pH 7.4, 5 m*M* $MgCl_2$, 3 m*M* substrate, 15 m*M* KCl, 100 m*M* NaCl and 10 m*M* Tris. The data are from two experiments, A and B, each run in duplicate.

If the ATP concentration is raised above its optimum range of 3 to 5 m*M* then a reduction of ouabain inhibition is observed. This is taken to mean that there is a decrease in the available transport ATPase activity. This reduction may occur because of a decrease in free Mg^{2+} levels due to complexing with ATP, or to competition between ATP and Mg^{2+}-ATP, or both (16).

In addition, evidence also is presented for a Ca^{2+}-ATPase in the human term placenta. This preparation appears to be similar to the Ca^{2+}-ATPase in guinea pig placenta (6, 7). The pH optima for both (*i.e.*, human and guinea pig) Ca^{2+}-ATPases are above 8.0; they are insensitive to a high concentration of ouabain, but are sensitive to the diuretic, ethacrynic acid, as are the Mg^{2+}-ATPases. The present experiments were not performed at the pH optimum for Ca^{2+}-ATPase, yet the results are like those for the guinea pig placental enzyme run at higher pH values (6). These data indicate that the enzymes for human and guinea pig placentas are qualitatively similar.

The inhibition of ATP hydrolysis by ethacrynic acid was demonstrated by Duggan and Noll (17) in the renal cortex and has been confirmed by other workers (18, 19). It was also shown (17) that nonspecific Mg^{2+}-ATPases were also inhibited. As Shami and Radde (6, 7) and this study demonstrate, the Ca^{2+}-ATPases also are inhibited by ethacrynic acid. This action may reflect a nonspecific effect of ethacrynic acid, perhaps related to the metabolic effects of this compound noted in other tissues (20–23). Also sulfhydryl group inhibitors, *e.g.*, *N*-ethylmaleimide, did not block the binding or up-

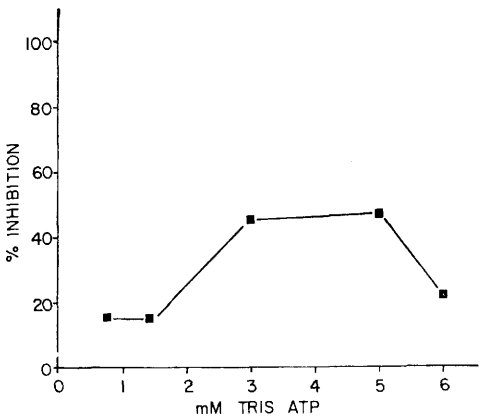

FIG. 2. The effect of Tris-ATP concentration on Mg^{2+}-dependent, Na^+ + K^+-activated ATPase activity. The degree of enzyme activity was determined by producing inhibition with 0.1 m*M* ouabain and this percentage inhibition is present on the abscissa. Each point is the mean of two experiments. Conditions were 37°, pH 7.4, 5 m*M* $MgCl_2$, Tris–ATP, 15 m*M* KCl, 100 m*M* NaCl, and 10 m*M* Tris.

take of ethacrynic acid in the rabbit renal cortex (24). These inhibitors also failed to reduce or prevent the gross membrane alterations in the red blood cells caused by ethacrynic acid. These membrane effects were not observed in red blood cells treated with ouabain (25). Thus with all these other effects reported, it might be concluded that ethacrynic acid is not specific for transport ATPases, although the diuretic will inhibit the activity of these enzymes.

Since there is alkaline phosphatase present in placental tissue (26), how much ATPase activity could this enzyme have in the present preparation? The alkaline phosphatase activity was not measured in this study, but from other studies (26, 27) it can be concluded that the activity is only minor at pH 7.4; the pH optimum for this enzyme is 9.5 to 10.5.

In conclusion, there appear to be at least two ATPases in the human term placenta, which may be involved with transport processes: (a) Ca^{2+}-ATPase which may be associated with Ca^{2+} transport from mother to fetus (6), and (b) a Mg^{2+}-dependent, $Na^+ + K^+$-activated, ouabain-sensitive ATPase which may be associated with the transport of organic compounds (8). This latter enzyme is presumed to be the so-called transport ATPase found in many tissues.

Summary. Mg^{2+}-dependent and Ca^{2+}-ATPases were isolated from human term placentas. The Mg^{2+}-dependent ATPase activity was partially inhibited by the absence of Na^+ or K^+ from the incubation medium or the presence of ouabain (0.1 m*M*) or ethacrynic acid (5 m*M*). This Mg^{2+}-dependent, ouabain-sensitive enzyme has a pH optimum near 7.4–7.5, and a substrate specificity for ATP > ITP = GTP. The Mg^{2+}-dependent ATPases were compared with a Ca^{2+}-ATPase, which has a pH optimum above 8.0 and was not inhibited by the absence of Na^+ or K^+ or the presence of ouabain, but was inhibited by ethacrynic acid.

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