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USE OF GTP TO DISTINGUISH CALCIUM TRANSPORTING ATPase ACTIVITY FROM OTHER CALCIUM DEPENDENT NUCLEOTIDE PHOSPHATASES IN HUMAN PLACENTAL BASAL PLASMA MEMBRANE

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SUMMARY: In many cells other than the erythrocyte, the relationship between ATP dependent calcium transport and calcium dependent ATP hydrolysis is complex. The characteristics of ATP hydrolysis often differ from those of calcium transport. Demonstration of a specific transport ATPase is complicated by heterogeneity and high background activity in the presence of magnesium. In basal plasma membrane of human placental syncytiotrophoblast, the addition of 5 mM GTP greatly reduces the background release of $^{32}P_i$ from 0.1 mM [γ,^{32}P]-ATP. The addition of GTP permits measurement of high affinity calcium dependent ATPase under conditions which support calcium uptake. GTP does not affect the velocity of calcium uptake, and in its presence the calcium and magnesium concentration dependence of calcium uptake and calcium dependent ATPase are similar.

The existence of a distinctive and essentially homogeneous high affinity calcium ATPase as the enzymatic basis of ATP dependent calcium transport was established in the erythrocyte plasma membrane and apparently confirmed from studies in muscle sarcolemma. This concept has been extended by implication to plasma membranes derived from a variety of cell types (1). Recent investigations, however, have demonstrated that in many cells the relationship between high affinity calcium ATPase activity and ATP dependent calcium transport is complex (1-5). Calcium uptake requires magnesium at a concentration close to that of ATP. Such a concentration markedly elevates total ATPase and renders calcium dependence difficult or impossible to demonstrate. Some investigators have dealt with these problems by measuring high affinity calcium dependent ATP hydrolysis in the absence of added magnesium using the chelator trans-cyclohexane-1,2-diamine-N,N,N',N'-tetraacetic acid to control magnesium. The interpretation of these experiments is highly questionable because this chelator is now known to have different affinities for calcium and magnesium than earlier thought (1).

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We have recently demonstrated a high affinity calcium transporter in the basal plasma membrane of human placenta with many of the established properties of plasma membrane calcium transport systems (6). Because of the problems described above, it has been impossible to measure a high affinity calcium dependent nucleotide hydrolytic activity under the same conditions which support calcium uptake. ATP dependent calcium uptake and high affinity calcium dependent nucleotide hydrolytic activity have recently been demonstrated in several tissues to differ in nucleotide specificity and sensitivity to inhibitors (3-5). Although these reports strongly suggest that high affinity calcium dependent ATP hydrolytic activity is heterogeneous, no means have been demonstrated for measuring the calcium transport component of ATP hydrolysis separately from other undefined nucleotide hydrolytic activity. In this report we describe the use of GTP as a competing nucleotide to inhibit non-specific ATP hydrolysis and permit the demonstration and measurement of a magnesium requiring calcium dependent ATPase with the properties expected of a calcium transporter.

METHODS

Materials: [γ,^{32}P]-ATP, $^{45}CaCl_2$, and Omnifluor were from Dupont, NEN (Boston, MA). ATP (Tris salt), GTP (Tris salt), ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), and other reagents were from Sigma (St. Louis, MO). Toluene, xylenes, and isobutanol were scintillation grade or better from Fisher (St. Louis, MO). Ethanol was from U.S. Industrial Chemicals Co. (Tuscola, IL).

Isolation of Membrane Vesicles: Basal plasma membrane was isolated from human placental trophoblast and its protein content measured as previously reported (6). Isolated basal membrane was resuspended in 250 mM sucrose, 50 mM Tris-HCl (pH 7.4 at 4 ^{o}C), quick frozen and stored at -70 ^{o}C. Vesicles could be stored for several months without loss of calcium uptake or ATP hydrolytic activity.

Calcium Uptake: The uptake of calcium was measured by a standard filtration assay (6). Sucrose was added to adjust the osmolarity to 340 mOsm. ATP dependent calcium uptake was calculated as the difference between uptake in the presence and absence of ATP.

ATP Hydrolysis: ATP hydrolysis was quantitated by the method of Seals et al. (7). The reaction mixture was similar to that for uptake. Calcium dependent ATP hydrolysis was calculated as the difference between ATP hydrolyzed in the presence and absence (EGTA alone) of calcium.

Determination of Free Calcium and Magnesium: The concentration of calcium chloride and magnesium chloride stock solutions was determined by atomic absorption spectroscopy. Estimates of free calcium and magnesium were obtained as previously described (6) using published affinity constants (8) for the significant complexes present. The concentration of

the stock solution of EGTA and its affinity for calcium was verified as previously described (6) using a calcium selective electrode (Orion Research, Cambridge, MA). Magnesium added at concentrations approximating the free magnesium in the incubation media (up to 0.3 mM) had only slight interference in the measurement of free calcium in the submicromolar range. Free calcium concentrations measured in incubation media correlated well with those predicted by calculation.

RESULTS

ATP Hydrolysis of Placental Basal Membrane and Effect of Magnesium and GTP: In the isolated basal plasma membrane of human placental syncytiotrophoblast under conditions which support calcium transport activity (magnesium concentrations close to those of ATP, free calcium ~1 μM), background ATP hydrolysis was very high and there was no measurable significant calcium dependent activity (Table 1). Calcium dependent ATP hydrolysis could however, be measured in the absence of added magnesium.

We have observed, in keeping with reports in the literature (3-5), that in addition to differing in their response to magnesium, calcium transport and calcium dependent ATP hydrolysis differ in nucleotide specificity. GTP as well as ATP are substrates for calcium dependent hydrolysis whereas transport is specific for ATP (Kelley and Smith, in preparation). In the absence of magnesium, the addition of GTP caused a very substantial decrease in calcium stimulation of ATP hydrolysis (Table 1). In the presence of magnesium, GTP decreased the background

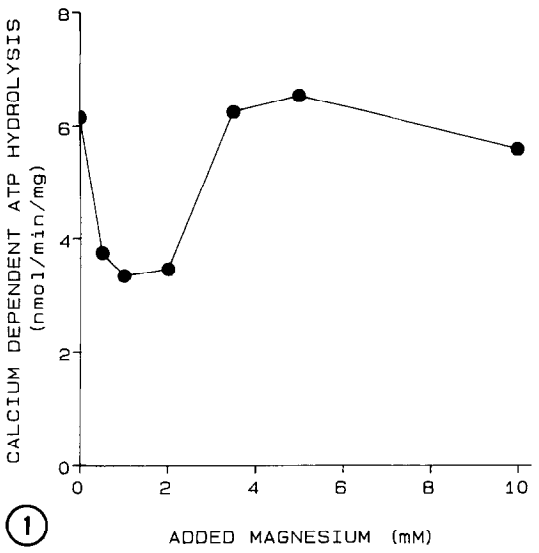
Table 1. ATP Hydrolysis of Placental Basal Plasma Membrane and Effects of Magnesium and GTP[a]

GTP (mM)	$MgCl_2$ (mM)	ATP Hydrolysis $+Ca^{+2}$	ATP Hydrolysis $-Ca^{+2}$	Ca^{+2} Dependent
0	0	39.6±13.7[b]	17.5±6.8	22.1±0.2
0	0.3[c]	113.5±24.5	111.7±26.1	1.8±1.7
5	0	6.1±2.2	0.3±0.2	5.8±2.0
5	3.5[c]	25.6±1.6	23.1±1.3	2.5±0.6

[a]Data is given in specific activity (nmol/min/mg protein) and standard deviation of results from 3 placentas. Data from each placenta is the mean of duplicate determinations. The calcium dependent activity was determined by individual subtraction of $-Ca^{+2}$ from $+Ca^{+2}$ values for each placenta.

[b]Total calcium was 200 μM and EGTA was 200 μM giving 1 μM free calcium.

[c]Magnesium concentrations were selected that maximized calcium uptake and gave similar free magnesium concentrations.



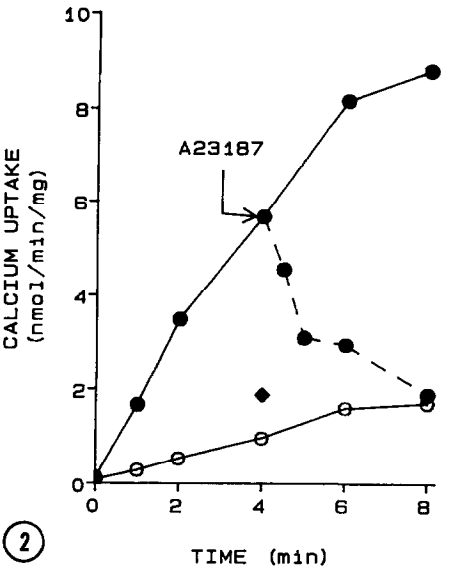


Figure 1. Effect of added magnesium on calcium dependent ATP hydrolysis: Calcium dependent hydrolysis (4 min) of 0.10 mM ATP was measured in the presence of 5 mM GTP, 0.20 mM EGTA, 0 and 0.20 mM $CaCl_2$ (0 and 0.5-3 µM free calcium). Calcium dependent activity is initially lowered by the addition of magnesium. At concentrations above 1 mM, magnesium stimulates calcium dependent activity reaching a maximum at concentrations near the total nucleotide concentration.

Figure 2. Time course of calcium uptake: ATP dependence and A23187 release: Calcium uptake was measured in the presence of 5 mM GTP, 3.5 mM $MgCl_2$, 0.20 mM EGTA, 0.20 mM $CaCl_2$ (0.89 µM free calcium) both in the presence (filled circles) and absence (open circles) of 0.10 mM ATP. A23187 (10 µM) was added at 0 (diamond) and 4 minutes (filled circles, dashed line). Uptake was stopped by filtration at various times. Calcium is taken up in a time dependent manner both in the presence and absence of ATP. Ionophore release shows that the ATP dependent component of uptake is into an internal vesicular space.

activity and allowed the measurement of calcium stimulatable ATP hydrolysis. When the magnesium concentration was varied over a wide range in the presence of GTP, calcium stimulated activity first decreased and then increased reaching a maximum at magnesium concentrations approaching total nucleotide concentration (figure 1).

Effect of GTP on Calcium Uptake: The time course of calcium uptake measured in the presence of GTP in placental basal membrane vesicles was similar to that seen in the absence of GTP in results published from this laboratory (6) (figure 2). ATP independent uptake was a minor fraction of total uptake and the addition of the ionophore A23187 caused ATP stimulated uptake to decrease to the level achieved in the absence of ATP. Magnesium stimulated calcium dependent uptake with a plateau in the millimolar range of total magnesium which was approximately equal to the concentration of total nucleotide (figure 3). This stimulation of uptake occured in the

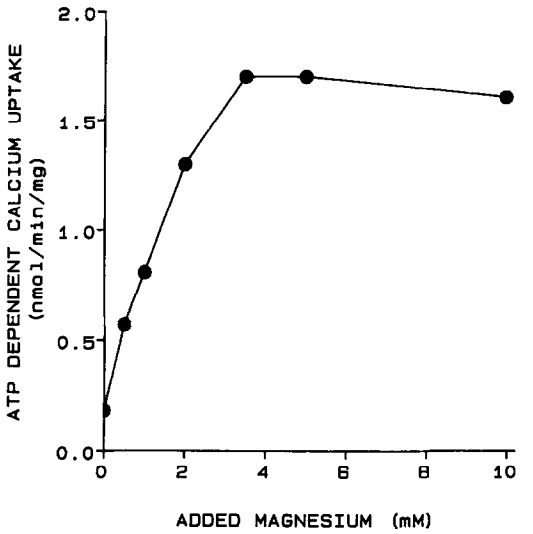


Figure 3. Magnesium requirement of ATP dependent calcium uptake: Calcium uptake (2 min) was measured in the presence of 5 mM GTP, 0.20 mM EGTA, 0.20 mM $CaCl_2$ (0.5-3 μM free calcium) in the presence and absence of 0.10 mM ATP. ATP dependent calcium uptake requires the addition of magnesium to concentrations approximating total nucleotide concentration.

same range as the magnesium stimulation of calcium dependent ATPase measured under the same conditions (compare with figure 1).

Calcium Concentration Dependence of Calcium Uptake and ATPase Activity: In placental basal membrane, calcium uptake was dependent on concentration with high affinity saturation in the nanomolar range both in the presence and absence of GTP (figure 4a). In the presence of GTP, measured calcium dependent ATPase activity demonstrated the same high affinity calcium stimulation as did uptake (figure 4b).

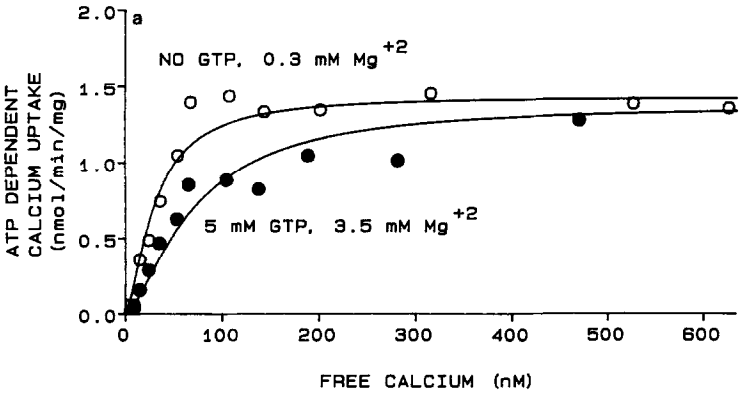


Figure 4a. Concentration dependence of ATP dependent calcium uptake in the presence or absence of GTP: Calcium uptake (4 min) was measured in the presence of 0.20 mM EGTA, varying concentrations (0-0.20 mM) of $CaCl_2$ in the presence and absence of 0.10 mM ATP. In both the presence and absence of GTP, calcium is taken up with a maximum velocity of 1.5 nmol/min/mg. The apparent $K_{0.5}$ for calcium in the absence of GTP was 30 nM and in the presence of 5 mM GTP it was 70 nM.

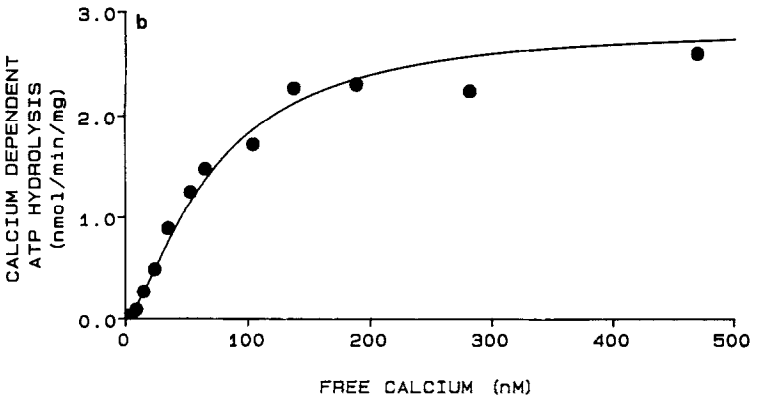


Figure 4b. Concentration dependence of calcium dependent ATP hydrolysis in the presence of GTP: Calcium dependent 0.10 mM ATP hydrolysis (4 min) was measured in the presence of 5 mM GTP, 3.5 mM $MgCl_2$, 0.20 mM EGTA and varying calcium concentrations (0-0.20 mM). Under conditions which support calcium uptake, ATP is hydrolyzed with a $K_{0.5}$ calcium of 70 nM and V_{max} of 3.2 nmol/min/mg. In preparations from different placentas, the maximum activity attained ranged from 3 to 6 nmol/min/mg.

DISCUSSION

The addition of GTP makes possible the demonstration and measurement of the component of ATP hydrolysis associated with the calcium transport ATPase. The high affinity activity so measured possesses properties corresponding to those of ATP dependent calcium transport. ATP hydrolysis occurs in the same range of free calcium as ATP dependent calcium transport; its maximum velocity is similar, and its magnesium dependence corresponds to that of transport. In the absence of GTP, calcium dependent ATP hydrolytic activity is several fold higher and is inhibited by added magnesium. While complex mechanisms may be present, the activity measured in the absence of GTP is almost certainly heterogeneous. GTP may act at more than one site to decrease heterogeneity. One action is apparently to inhibit a magnesium independent or magnesium inhabitable calcium dependent activity. This activity probably results from a non-specific divalent cation activated nucleotidase whose activation with either calcium or magnesium is diminished by the addition of the other competing metal.

Heterogeneity is apparent in membranes of other cells and has given rise to substantial controversy concerning the role of calcium activated ATPases in calcium transport (1-5,9). In the kidney, it has been difficult to distinguish between magnesium dependent and magnesium independent transporting and non-transporting ATPases (2); in the liver, there is disagreement as to whether previously reported calcium activated ATPases play any role in calcium transport (4,5,9). The use of an inhibitor such as GTP to inhibit non-transporting ATPases may be helpful in resolving such

controversies and identifying the particular ATPase which mediates calcium transport. It may also be helpful in identifying transporting ATPases under conditions where the measurement of transport is difficult or impossible, as with right side out or unsealed membranes or with soluble ATPases during purification.

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