Two Ca\(^{2+}\)-dependent ATPases in Rat Liver Plasma Membrane

THE PREVIOUSLY PURIFIED (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase IS NOT A Ca\(^{2+}\)-PUMP BUT AN ECTO-ATPase*

(Received for publication, March 21, 1988)

Sue-Hwa Lin‡
From the Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138

William E. Russell
From the Department of Pediatrics, Harvard Medical School, and Pediatric Endocrine-Metabolic Unit, Massachusetts General Hospital, Boston, Massachusetts 02114

We have shown that the rat liver plasma membrane has at least two (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPases. One of them has the properties of a plasma membrane Ca\(^{2+}\)-pump (Lin, S.-H. (1985) J. Biol. Chem. 260, 7850–7856); the other one, which we have purified (Lin, S.-H., and Fain, J. N. (1984) J. Biol. Chem. 259, 3016–3020) and characterized (Lin, S.-H. (1985) J. Biol. Chem. 260, 10976–10980) has no established function. In this study we present evidence that the purified (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase is a plasma membrane ecto-ATPase.

In hepatocytes in primary culture, we can detect Ca\(^{2+}\)-ATPase and Mg\(^{2+}\)-ATPase activities by addition of ATP to the intact cells. The external localization of the active site of the ATPase was confirmed by the observation that the Ca\(^{2+}\)-ATPase and Mg\(^{2+}\)-ATPase activities were the same for intact cells, saponin-treated cells, and cell homogenates. Less than 1% of total intracellular lactate dehydrogenase, a cytosolic enzyme, was released during a 30-min incubation of the hepatocytes with 2 mM ATP. This indicates that the hepatocytes maintained cytosolic membrane integrity during the 30-min incubation with ATP, and the Ca\(^{2+}\)-ATPase and Mg\(^{2+}\)-ATPase activity measured in the intact cell preparation was due to cell surface ATPase activity.

The possibility that the ecto-Ca\(^{2+}\)-ATPase and Mg\(^{2+}\)-ATPase may be the same protein as the previously purified (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase was tested by comparing the properties of the ecto-ATPase with those of (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase. Both the ecto-ATPase and the (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase have broad nucleotide-hydrolyzing activity, i.e. they both hydrolyze ATP, GTP, UTP, CTP, ADP, and GDP to a similar extent. The effect of Ca\(^{2+}\) and Mg\(^{2+}\) on the ecto-ATPase activity is not additive indicating that both Ca\(^{2+}\) and Mg\(^{2+}\)-ATPase activities are part of the same enzyme. The ecto-ATPase activity, like the (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase, is not sensitive to oligomycin, vanadate, N-ethylmaleimide and p-chloromercuribenzoate; and both the ecto-ATPase and purified (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase activities are insensitive to protease treatments. These properties indicate that the previously purified (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase is an ecto-ATPase and may function in regulating the effect of ATP and ADP on hepatocyte Ca\(^{2+}\) mobilization (Char- est, R., Blackmore, P. F., and Exton, J. H. (1985) J. Biol. Chem. 260, 15789–15794).

The cytosolic free calcium concentration of hepatocytes is in the range of 0.1 to 0.2 μM (Murphy et al., 1980). It is proposed that part of the Ca\(^{2+}\) gradient is maintained by a high affinity ATP-dependent Ca\(^{2+}\) transporter localized in the plasma membrane. The Ca\(^{2+}\) pumps of human erythrocyte membrane and rat heart sarcolemma have been characterized and purified (Niggli et al., 1979; Caroni and Carafoli, 1981). In those tissues the plasma membrane Ca\(^{2+}\) pumps, like the muscle sarcoplasmic reticulum Ca\(^{2+}\) pump, possess ATPase activity which can be activated by Ca\(^{2+}\) in the presence of Mg\(^{2+}\). However, no Ca\(^{2+}\)-stimulated ATPase activity could be found in rat liver plasma membrane under similar conditions. As a result, a high affinity Ca\(^{2+}\)-stimulated ATPase activity which was observed in the absence of exogenously added Mg\(^{2+}\) was thought to be the enzyme responsible for hepatocyte plasma membrane Ca\(^{2+}\) transport (Lotersztajn et al., 1981, 1984). We have purified the high affinity Ca\(^{2+}\)-stimulated ATPase (Lin and Fain, 1984). The properties of the high affinity (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase are different from those of the plasma membrane Ca\(^{2+}\) pump studied by reconstituting liver plasma membrane proteins into artificial liposomes (Lin, 1985a, 1985b). Further characterization of this ATPase demonstrated that this enzyme can be activated by either Ca\(^{2+}\) or Mg\(^{2+}\); and it was also shown that this enzyme has broad nucleotide specificity, and its activity is not inhibited by inhibitors of known ion transporters (Lin, 1985b).

Since the high affinity (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase is not the liver plasma membrane Ca\(^{2+}\) pump, its physiological function remains unknown. Recently, Charest et al. (1985) reported that stimulation of isolated hepatocytes with ATP or ADP induced a rapid but transient increase of free cytosolic Ca\(^{2+}\) concentration indicating the existence of P2-purinergic receptor(s) in the hepatocyte plasma membranes. Further study also showed that the transient response was probably due to rapid hydrolysis of the ATP by a plasma membrane ecto-ATPase. As part of the effort to study the function of the (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase, we tested the possibility that this enzyme is a plasma membrane ecto-enzyme with its nucleotide hydrolyzing site facing the outside of the cell. In this communication, we present evidence that Ca\(^{2+}\)-ATPase and Mg\(^{2+}\)-ATPase activity can be detected by addition of ATP to the outside of intact hepatocytes. The properties of this enzyme indicate that it is the same enzyme that we previously purified (Lin and Fain, 1984).

* This work was supported by Grant HL08893 from the National Institutes of Health (to G. G.) and by a Medical Research Career Starter grant from the Human Growth Foundation (to W. E. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Medical Foundation (Boston, MA) Research Fellow.

12253

This is an Open Access article under the CC BY license.
Expt.-Ca**+-Mg**+-nucleotidase of Hepatocytes

1984) and characterized (Lin, 1985b). Our finding that the (Ca**+-Mg**+-)ATPase hydrolyzes extracellular ATP and ADP suggests that it may play a role in terminating the effect of ATP and ADP on hepatocyte Ca**+-mobilization.

**EXPERIMENTAL PROCEDURES**

**Materials—**ATP (disodium salt), AMP-PNP, 1, EGTA, ADP, AMP, GTP, GDP, ouabain, alginolycine, and papain were obtained from Sigma. Trypsin and chymotrypsin were from Cooper Biomedical.

**Assay of ATPase Activities in Suspensions and Homogenates of Hepatocytes—**Hepatocytes were isolated from Sprague-Dawley rats (Charles River, CD strain) by collagenase digestion, as described by Seglen (1976). At the beginning of each experiment, the hepatocytes were washed twice with buffer A which contained 120 mM NaCl, 5 mM KCl, 20 mM Heps-Tris (pH 7.4), and 2 mM EGTA. The reactions were started by addition of ATP to a final concentration of 2 mM. The reactions were stopped by addition of 0.5 M perchloric acid; the inorganic phosphate released was determined as described above. One ml of each reaction mixture was taken for inorganic phosphate determination at 0 min was used for background subtraction. The total inorganic phosphate released in up to 10 min is the "measured inorganic phosphate" (Mm) adjusted for a volume factor (1.6/0.2) plus the amount withdrawn for the previous measurement, i.e. Mm. By the same token, Mm and Mm should be added back to the measurement at 15 min after the adjustment was made for the volume factor. The ATPase activities of homogenates were measured on 0.2 ml aliquots of the hepatocyte homogenate withdrawn at different periods of time. Only a volume factor of 10 is required for the ATPase activity calculation in these cases.

**Affinities for Ca**+- and Mg**+-**—The affinity for Ca**+ of the ecto-Ca**+-ATPase activity was determined by incubating the hepatocytes in primary culture with buffer A containing different amounts of Ca**+. The reactions were started by addition of ATP to a concentration of 2 mM. The free Ca**+ concentrations were calculated with the association constant of KCa**+ = 4.28 × 10^-6 M^-1. The amount of Mg**+ contaminating the assay mixtures was 1 μM as determined by atomic absorption. In the presence of 2 mM ATP, this amount of Mg**+ gives absorption less than 0.02 μM free Mg**+.

The affinity for Mg**+ of the ecto-Mg**+-ATPase activity was determined by incubating the hepatocytes in primary culture with buffer A containing different amounts of Mg**+. The reactions were started by addition of ATP to a concentration of 2 mM. The free Mg**+ concentrations were calculated with the association constant of KMg**+ = 3.8 × 10^-6 M^-1.

**Assay of Lactate Dehydrogenase Activity—**The lactate dehydrogenase activity was assayed by addition of 100 μl of cell medium or homogenate to 2 ml of solution containing 6.6 mM NADH, 0.5 mM Tris-HCl, pH 7.4. One ml of the mixture was pipetted into each cuvette containing 33 μl of distilled water or 30 μl of 30 mM sodium pyruvate. The difference in absorbance at 340 nm was recorded continuously in a Kontron Uvikon 810 spectrophotometer. One unit represents the oxidation of 1 μmol of NADH/min at 25°C.

**RESULTS**

**Ca**+-ATPase and Mg**+-ATPase Activities of Intact and Disrupted Hepatocytes—In the initial experiments, hepatocytes freshly prepared by liver perfusion and collagenase digestion were used. Although Ca**+-stimulated and Mg**+-stimulated ATPase activities could be detected by incubating intact hepatocytes with ATP, the lactate dehydrogenase activity of the cell supernatant was about 50% of that of the homogenate, indicating that freshly isolated hepatocytes were very leacy. In order to unambiguously demonstrate the side-ness of this plasma membrane enzyme, a hepatocyte preparation with minimum membrane leakage was required. Therefore, hepatocytes in primary culture were used in later experiments. Hepatocytes in primary culture were prepared by collagenase perfusion of rat livers. The method employed yielded a cell suspension containing greater than 95% hepatocytes, which were then plated on collagen-coated dishes. Under this condition, viable hepatocytes attach to the dish and the majority of damaged cells are washed off the dish at the beginning of the experiments.

In Fig. IA shows the time course of Ca**+-stimulated and Mg**+-stimulated ATPase activities of hepatocytes in primary culture. These activities were detected in the presence of 1.6μM free Ca**+ or 25 μM free Mg**+, respectively. Disruption of the hepatocytes by scraping the cells off the collagen-coated dishes followed by sonication for 1 min did not increase either the Ca**+-ATPase or Mg**+-ATPase activities (Fig. IB). At the end of a 30 min incubation, aliquots of cell medium from the intact cells, and the disrupted cell preparations were taken for determination of lactate dehydrogenase activity. The lactate dehydrogenase activity of the cell medium from the intact cells was 14% of that of the disrupted cells (0.27 vs 1.92

1 The abbreviations used are: AMP-PNP, 5'-adenosylpyrophosphate; ATP, adenosine triphosphate; Ca**+, calcium ion; Heps, 4-(2-hydroxyethyl)-1-piperazinethesulonic acid; SDS, sodium dodecyl sulfate.
ATPase activities of hepatocytes in primary culture and of disrupted hepatocytes. ATPase activities are the same for both intact and sonically disrupted hepatocytes. The ATPase activities were calculated as described under "Experimental Procedures." The amount of inorganic phosphate determined. The ATPase activities were calculated as described under "Experimental Procedures." B, hepatocytes in primary culture were removed from dishes and homogenized as described under "Experimental Procedures." Buffers A and B, in the intact cell. The hydrolysis of AMP-PNP in the presence of Ca2+ may be due to the presence of 5'-nucleotidase, which is known to be a hepatocyte ecto-enzyme, in the intact cell. The hydrolysis of AMP-PNP in the presence of Ca2+ may be due to the sequential hydrolysis of AMP-PNP by the Ca2+-dependent adenosine triphosphate pyrophosphohydrolase activity (Floidgaard and Torp-Pedersen, 1978) and the 5'-nucleotidase.

Ecto-(Ca2+-Mg2+)-nucleotidase of Hepatocytes

**Fig. 1.** Time course of Ca2+-stimulated and Mg2+-stimulated ATPase activities of hepatocytes in primary culture and of disrupted hepatocytes. A, the hepatocytes in primary culture were incubated in the presence of 120 mM NaCl, 5 mM KCl, 20 mM Hepes/Tris (pH 7.4), 2 mM EGTA without added Ca2+ or Mg2+ (O), or with 5 mM Ca2+ ( ), or with 1 mM Mg2+ ( ), or with 2 mM Ca2+ plus 1 mM Mg2+ (A), respectively. The reactions were started by the addition of ATP to a final concentration of 2 mM. After incubating the cells with ATP at room temperature for different periods of time as indicated, aliquots of cell medium were withdrawn and the amount of inorganic phosphate determined. The ATPase activities were calculated as described under "Experimental Procedures." B, hepatocytes in primary culture were removed from dishes and homogenized as described under "Experimental Procedures." Buffers A and B, in the intact cell. The hydrolysis of AMP-PNP in the presence of Ca2+ may be due to the presence of 5'-nucleotidase, which is known to be a hepatocyte ecto-enzyme, in the intact cell. The hydrolysis of AMP-PNP in the presence of Ca2+ may be due to the sequential hydrolysis of AMP-PNP by the Ca2+-dependent adenosine triphosphate pyrophosphohydrolase activity (Floidgaard and Torp-Pedersen, 1978) and the 5'-nucleotidase.

**Effect of Proteases on Ecto-ATPase and Purified ATPase Activities**—Since the ATP-hydrolyzing site of the ecto-ATPase is extracellular, it was interesting to see whether proteolysis would destroy the ATPase activity from the outside of the cells. As shown in Table II, the ecto-ATPase activity of hepatocytes in primary culture is not sensitive to

units/10^6 cells). Addition of saponin (40 μg/ml) to the intact cell preparation after a 30-min incubation with ATP caused release of all the lactate dehydrogenase activity into the cell medium (2.03 units/10^6 cells). These results indicate that the hepatocytes in primary culture were still intact after 30 min of incubation with 2 mM ATP, and the Ca2+-ATPase and Mg2+-ATPase activities measured in the whole cell preparation were due to cell surface ATPase activities.

In some experiments, the Mg2+-ATPase activity in the disrupted cell preparations was much higher than that of intact cells (about 4-fold higher), although the Ca2+-ATPase activity was the same for both intact and disrupted cell preparations (data not shown). The higher Mg2+-ATPase activity in the disrupted cell preparations probably is mitochondrial ATPase which became accessible after disrupting the cells by freeze-thaw and sonication. This view is supported by the observation that when oligomycin (50 μg/ml) was included in the assay media, the Mg2+-ATPase activities of both intact cells and disrupted cells became the same (data not shown). These results also indicate that the ecto-Mg2+-ATPase activity is different from mitochondrial ATPase which is oligomycin-sensitive.

One might suppose that the Mg2+- and Ca2+-ATPase activities observed with intact cells might derive from a small population of dead cells with permeable plasma membrane. In this case, the observation that Ca2+-ATPase and Mg2+-ATPase activities are the same for both intact and sonically disrupted cells could be inconclusive if sonication were to generate membrane vesicles with the same orientation as in intact cells and give no further exposure of the inner surface of the plasma membrane to the substrate, ATP. To test this hypothesis, hepatocytes in primary culture were also disrupted by addition of 40 μg/ml saponin, which is known to permeabilize hepatocyte plasma membranes without disrupting mitochondria and endoplasmic reticulum (Joseph et al., 1984).

As shown in Fig. 2, the Ca2+-ATPase and Mg2+-ATPase activities in intact, saponin-treated, and homogenized cells are about the same. The lactate dehydrogenase activities at the end of a 60-min incubation were 0.10, 1.49, and 0.80 units/10^6 cells for intact, saponin-treated, and homogenized cells, respectively. This indicates that, under the conditions used for ATPase assays, no further Ca2+-ATPase and Mg2+-ATPase can be detected by permeabilizing the cell.

**Nucleotide Specificity of Ecto-ATPase Activities**—In order to test the possibility that the ecto-Ca2+-ATPase and Mg2+-ATPase may be the same protein we previously purified (Lin and Fain, 1984) and characterized (Lin, 1985b), several properties of the previously characterized high affinity (Ca2+- Mg2+)-nucleotidase were determined with the enzyme in intact hepatocytes. The nucleotide specificity of the ecto-ATPase activities is shown in Table I. Both Ca2+- and Mg2+-stimulated activities have broad substrate specificities. The relative nucleotide-hydrolyzing rates are about the same as that of the purified (Ca2+-Mg2+)-ATPase (Lin, 1985b) except with AMP and AMP-PNP. The hydrolysis of AMP may be due to the presence of 5'-nucleotidase, which is known to be a hepatocyte ecto-enzyme, in the intact cell. The hydrolysis of AMP-PNP in the presence of Ca2+ may be due to the sequential hydrolysis of AMP-PNP by the Ca2+-dependent adenosine triphosphate pyrophosphohydrolase activity (Floidgaard and Torp-Pedersen, 1978) and the 5'-nucleotidase.

**Effect of Proteases on Ecto-ATPase and Purified ATPase Activities**—Since the ATP-hydrolyzing site of the ecto-ATPase is extracellular, it was interesting to see whether proteolysis would destroy the ATPase activity from the outside of the cells. As shown in Table II, the ecto-ATPase activity of hepatocytes in primary culture is not sensitive to...
p-Nitrophenyl phosphate (p-NPP) was used as substrate, and the accumulation of p-NPP in the supernatant was measured. The results represent the average of duplicate determinations.

No significant amount of Ca2+-ATPase activity was detected in the supernatant fraction (data not shown), suggesting that the ecto-ATPase enzyme was still membrane-bound after the protease treatment.

Effect of Inhibitors on Ecto-ATPase Activity—One of the distinct properties of the previously purified (Ca2+-Mg2+)-ATPase was that its Ca2+-stimulated ATPase activity was not sensitive to several known ATPase inhibitors (Lin, 1985b). The ecto-ATPase activity, like the previously purified (Ca2+-Mg2+)-ATPase, was not inhibited by 0.5 mM vanadate, 5 mM N-ethylmaleimide, 1 mM ouabain, and 50 μM 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (not shown).

Effect of Ca2+ and Mg2+ on Ecto-ATPase Activity—The high affinity (Ca2+-Mg2+)-ATPase of rat liver plasma membrane can be activated by either Ca2+ or Mg2+ (Lin, 1985b). Addition of both Ca2+ and Mg2+ to the ATPase assay medium gave the same ATPase activity as Mg2+ alone in both whole cell and disrupted cell preparations (Fig. 1, A and B). The nonadditive effect of Ca2+ and Mg2+ indicates that both activities are from the same enzyme.

As shown in Fig. 3A, in the absence of Mg2+ (the free Mg2+ concentration was less than 0.02 μM) the ecto-ATPase activity of intact hepatocytes is stimulated by Ca2+ in a concentration-dependent fashion. The Ca2+ concentration dependence curve is best fitted by one component which has the affinity for Ca2+ of 5.2 μM. In the absence of added Ca2+, the ecto-ATPase activity was not changed upon examination by SDS-polyacrylamide gel electrophoresis (data not shown).

Further, after trypsin treatment of the purified enzyme, its size was not changed upon examination by SDS-polyacrylamide gel electrophoresis (data not shown).

### TABLE I

Substrate specificity of ecto-Ca2+-ATPase and Mg2+-ATPase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Ca2+-ATPase</th>
<th>Mg2+-ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ADP</td>
<td>31.7</td>
<td>40.6</td>
</tr>
<tr>
<td>AMP</td>
<td>26.8</td>
<td>13.2</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>20.1</td>
<td>2.7</td>
</tr>
<tr>
<td>GTP</td>
<td>93.2</td>
<td>120.9</td>
</tr>
<tr>
<td>GDP</td>
<td>39.3</td>
<td>40.5</td>
</tr>
<tr>
<td>UTP</td>
<td>76.1</td>
<td>157.7</td>
</tr>
<tr>
<td>CTP</td>
<td>78.3</td>
<td>120.2</td>
</tr>
<tr>
<td>p-Nitrophenol phosphate</td>
<td>6.3</td>
<td>2.4</td>
</tr>
</tbody>
</table>

### TABLE II

Effect of protease treatments on hepatocyte ecto-Ca2+-ATPase activity and on purified (Ca2+-Mg2+)-ATPase activity

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Ca2+-ATPase</th>
<th>Mg2+-ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ADP</td>
<td>31.7</td>
<td>40.6</td>
</tr>
<tr>
<td>AMP</td>
<td>26.8</td>
<td>13.2</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>20.1</td>
<td>2.7</td>
</tr>
<tr>
<td>GTP</td>
<td>93.2</td>
<td>120.9</td>
</tr>
<tr>
<td>GDP</td>
<td>39.3</td>
<td>40.5</td>
</tr>
<tr>
<td>UTP</td>
<td>76.1</td>
<td>157.7</td>
</tr>
<tr>
<td>CTP</td>
<td>78.3</td>
<td>120.2</td>
</tr>
<tr>
<td>p-Nitrophenol phosphate</td>
<td>6.3</td>
<td>2.4</td>
</tr>
</tbody>
</table>

trypsin, chymotrypsin, or papain treatment. Although treatment of the hepatocytes in primary culture with proteases causes dissociation of the cells from the collagen-coated culture dishes, less than 25% of the ecto-Ca2+-ATPase activity was lost with 50 μg/ml trypsin, chymotrypsin, or papain for 90 min at room temperature. When the purified high affinity (Ca2+-Mg2+)-ATPase was treated with proteases under the same conditions, the purified Ca2+-ATPase activity was similarly insensitive to protease treatment (Table II). Furthermore, after trypsin treatment of the purified enzyme, its size was not changed upon examination by SDS-polyacrylamide gel electrophoresis (data not shown).

It is possible that the nucleotide-hydrolyzing site of the ecto-ATPase might be released from the plasma membrane by the proteases. To test this hypothesis, purified liver plasma membranes were treated with 50 μg/ml trypsin, chymotrypsin, or papain, respectively, at 37°C for 1 h. The membranes were then spun down with an Airfuge for 5 min, and the Ca2+-ATPase activities were measured in both supernatant and pellet fractions. No significant amount of Ca2+-ATPase activity was detected in the supernatant fraction (data not shown), suggesting that the ecto-ATPase enzyme was still membrane-bound after the protease treatment.

**FIG. 3. Effect of Ca2+ and Mg2+ concentrations on ATPase activity. A, effect of Ca2+. The hepatocytes in primary culture were incubated with different concentrations of Ca2+ at room temperature for 60 min. The free Ca2+ concentrations were calculated by using the association constant of K_{CaATP} = 4.28 × 10^7 M^-1 and K_{CaMgATP} = 7.39 × 10^8 M^-1. The curve is the best fit of the function V_0(Ca)/[K + (Ca)]. A K of 5.2 μM with a correlation coefficient (r^2) of 0.996 was obtained. B, effect of Mg2+. The free Mg2+ concentrations were calculated by using the association constant of K_{MgATP} = 3.8 × 10^10 M^-1. The hepatocytes in primary culture were incubated with different concentrations of Mg2+ at room temperature. Aliquots of cell medium were withdrawn after 15- and 30-min incubations with ATP. Error bars indicate ranges of determinations. The curve is the best fit of the function V_0(Mg)/[K + (Mg)]. A K of 5.0 μM with a correlation coefficient (r^2) of 0.986 was obtained.**
activity was stimulated by Mg\(^2+\), with \(K_a\) for Mg\(^2+\) of around 5 \(\mu\)M free Mg\(^2+\) (Fig. 3B).

**DISCUSSION**

This paper reports the presence of an ecto-Ca\(^2+\)- and Mg\(^2+\)-stimulated ATPase in hepatocytes in primary culture. The external localization of the nucleotide-hydrolyzing site is supported by the observation that the Ca\(^2+\)-ATPase and Mg\(^2+\)-ATPase activities are the same for both intact cells, saponin-treated cells, and homogenized cells. The properties of this ecto-ATPase suggest that the previously purified high affinity (Ca\(^2+\)-Mg\(^2+\))-ATPase of rat liver plasma membrane is the ecto-ATPase. First, the nucleotide specificity of the ecto-ATPase is the same as that of the purified enzyme; both of them are able to hydrolyze ATP, GTP, UTP, CTP, ADP, and GDP to a similar extent. Second, the ecto-ATPase activity can be activated by either Ca\(^2+\) or Mg\(^2+\), and the effects of Ca\(^2+\) and Mg\(^2+\) on this ecto-ATPase activity are not additive, indicating that both Ca\(^2+\)- and Mg\(^2+\)-ATPase activities reside on the same enzyme. This is consistent with the property of the purified enzyme in which a nonadditive effect of Ca\(^2+\) and Mg\(^2+\) on the enzymatic activity was observed. Third, the ecto-ATPase, like the purified enzyme, is not affected by oligomycin, vanadate, N-ethylmaleimide, and \(\beta\)-chloromercuribenzoate. Furthermore, the activities of both the ecto-ATPase and purified ATPase are quite insensitive to protease treatments. Consistent with this conclusion is the recent finding by molecular cloning and sequencing of the gene for the (Ca\(^2+\)-Mg\(^2+\))-ATPase that in this protein there is one hydrophobic segment which is localized near the C-terminal end of the protein and that there are many putative N-glycosylation sites in the rest of the protein. The molecular arrangement of this protein is similar to that of other membrane ecto-enzymes which have been studied, i.e. alkaline phosphatase (Millan, 1986; Kam et al., 1985; Hentzorn et al., 1986; Övitt et al., 1986; Berger et al., 1987) and \(\gamma\)-glutamyltransferrase (Laperche et al., 1986).

One approach to determine the sidedness of a membrane protein is to use specific antibodies. If the enzymatic reaction can be inhibited in the intact cell by an antibody specific for the enzyme, then one can conclude that the enzyme has its active site located on the outside of the cell. In order to use such an approach, an antibody against purified (Ca\(^2+\)-Mg\(^2+\))-ATPase was prepared by inoculating purified protein into the popliteal lymph nodes of a rabbit (Sigel et al., 1983). The antisera reacts with the purified (Ca\(^2+\)-Mg\(^2+\))-ATPase by immunoblotting (Towbin et al., 1979) and immunoprecipitation. By indirect immunofluorescence, the antibodies recognize a protein localized in the bile canalicular domain of hepatocytes. The antisera binds to the detergent (polyoxyethylene 9-lauryl ether)-solubilized, enzymatically active form of the (Ca\(^2+\)-Mg\(^2+\))-ATPase and depletes the (Ca\(^2+\)-Mg\(^2+\))-ATPase activity from solution when the antibody-enzyme complex was precipitated by protein A-Sepharose. The (Ca\(^2+\)-Mg\(^2+\))-ATPase activity was found to be associated with the precipitates. However, the antisera inhibits the (Ca\(^2+\)-Mg\(^2+\))-ATPase activity weakly, indicating that the antibodies may not recognize the nucleotide-hydrolyzing site of the enzyme. A series of monoclonal antibodies against the purified ecto-ATPase was also prepared, and none of the monoclonal antibodies inhibited the ATPase activity.

The insensitivity of the ecto-ATPase activity to protease treatments is quite surprising. Besides trypsin, chymotrypsin, and papain, other proteases (subtilisin, elastase, *Staphylococcus aureus* V8 protease, thermolysin, submaxillary protease, pronase, bromelin, and *Streptomyces griseus* protease) were also tested for their effect on the Ca\(^2+\)-ATPase and Mg\(^2+\)-ATPase activity. In each case, there was no significant loss (less than 20%) of Ca\(^2+\)-ATPase or Mg\(^2+\)-ATPase activity. The high resistance of the ecto-ATPase activity to proteolysis may be a protective mechanism of the enzyme against extracellular proteases. Since the ecto-ATPase is a glycoprotein (Lin and Pain, 1984), it is possible that the insensitivity of the active site to proteolysis is due to the presence of carbohydrate on the protein. The nucleotide sequence of the cDNA for the gene of the ecto-ATPase shows that there are more than 15 potential asparagine glycosylation sites in the ecto-ATPase protein.

The ecto-ATPase of liver plasma membranes was first found to have a high affinity (Ca\(^2+\)-Mg\(^2+\))-ATPase with a dissociation constant, \(K_a\) for Ca\(^2+\) in the range of 0.01 to 0.2 \(\mu\)M (Lotersztajn et al., 1981; Iwasa et al., 1982). The values of \(K_a\) for the detergent-solubilized and purified enzyme are 0.09 and 0.16 \(\mu\)M for Ca\(^2+\) and Mg\(^2+\), respectively (Lin, 1985b). In hepatocytes in primary culture, however, the \(K_a\) values for Ca\(^2+\) and Mg\(^2+\) are 5.2 and 5.0 \(\mu\)M, respectively, which are higher than those of the purified enzyme. The factor(s) which gives such a difference between the purified enzyme and the enzyme in intact cells is not clear. It has been reported that there is an inhibitor (Lotersztajn and Pecker, 1982; Lotersztajn et al., 1985) and an activator (Lotersztajn et al., 1981) for the high affinity (Ca\(^2+\)-Mg\(^2+\))-ATPase of rat liver plasma membranes. Whether these two putative regulators are involved in the change of affinity for Ca\(^2+\) is unknown. Also it is possible that a GTP-binding protein may be involved in such a phenomenon. Consistent with this hypothesis is the recent finding by Lotersztajn et al. (1987) that the effect of glucagon on the (Ca\(^2+\)-Mg\(^2+\))-ATPase activity is mediated by a GTP-binding protein as revealed by the sensitivity of the effect to cholera toxin. Furthermore, the change of affinity for Ca\(^2+\) may be due to the difference between the oxidation-reduction status of the intracellular medium and the preparation medium. Finally, the local Ca\(^2+\) and Mg\(^2+\) concentrations for the enzyme may be quite different between the intact cells and the solubilized enzyme. Results from molecular cloning and sequencing of the gene for this ecto-ATPase showed that the C terminus of the postulated sequence for the ecto-ATPase contains a unique cAMP-dependent serine phosphorylation consensus sequence (Lys-Arg-X-X-Ser) (Kreb's and Beavo, 1979). Whether phosphorylation or dephosphorylation of the ecto-ATPase is involved in changing the affinity of this enzyme for Ca\(^2+\) and Mg\(^2+\) is presently under investigation. As a result of the unexpected lower affinity for Ca\(^2+\) in intact cells, the concentration of Ca\(^2+\) used in the measurement of Ca\(^2+\)-ATPase activity, i.e. 1.5 \(\mu\)M free [Ca\(^2+\)], is lower than the \(K_a\) for the enzyme for Ca\(^2+\) in intact cells, while the concentration of Mg\(^2+\) used, i.e. 25 \(\mu\)M free [Mg\(^2+\)], is close to the concentration for maximal activation of Mg\(^2+\)-ATPase activity. This accounts for the observation that the Mg\(^2+\)-ATPase activity is higher than the Ca\(^2+\)-ATPase activity in several studies. In fact, in the presence of saturating amounts of Ca\(^2+\) (i.e. 100 \(\mu\)M free Ca\(^2+\)) or Mg\(^2+\) (i.e. 200 \(\mu\)M free Mg\(^2+\)), the Ca\(^2+\)-ATPase and Mg\(^2+\)-ATPase activities are the same (data not shown).

Most tissues contain ecto-ATPase activity which can be detected as a Ca\(^2+\)-stimulated ATPase activity. This ecto-ATPase activity on the plasma membrane is higher than Ca\(^2+\)-pump ATPase activity. In rat liver plasma membrane, the ATP-hydrolyzing activity of the ecto-ATPase is about 10 times that of the Ca\(^2+\)-pump ATPase (Lin, 1985a; Lin and...
Ecto-(Ca\(^{2+}\)-Mg\(^{2+}\))-nucleotidase of Hepatocytes

Acknowledgment—We thank Professor Guido Guidotti, in whose laboratory this work was done, for his support and advice during the course of this work.

REFERENCES


Fain, 1984). And in rat corpus luteum, a 1000-fold difference between the transport rate and the rate of the Ca\(^{2+}\)-ATPase was observed (Minami and Penniston, 1987). As a result, there is great confusion in deducing the significance of plasma membrane Ca\(^{2+}\)-stimulated ATPase activity. In the plasma membrane of several tissues, i.e. rat liver (Lin, 1958b), rat corpus luteum (Minami and Penniston, 1987), rat kidney (Ghijsen et al., 1984) and intestinal basolateral membrane (Moy et al., 1986), and neutrophil plasma membranes (Ochs and Reed, 1984), two different Ca\(^{2+}\)-ATPase activities have been reported. In a recent report by Pavoine et al. (1987), it was claimed that the high affinity (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase of liver plasma membrane is a Ca\(^{2+}\) pump by reconstituting partially purified (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase into artificial liposomes and demonstrating a small amount of Ca\(^{2+}\) transport activity in such a preparation. The (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase preparation in that study, however, was not highly purified, since the specific activity of the enzyme was only 20-fold greater than that of plasma membrane (Lotersztajn et al., 1986). In our previous study, a 300-fold purification was obtained (Lin and Fain, 1984). As a result, it is possible that the Ca\(^{2+}\)-pumping activity observed in the partially purified preparation may be due to contamination of the preparation with the Ca\(^{2+}\)-pump protein. Furthermore, the nucleotide specificity and vanadate sensitivity of the transport system, two critical criteria which distinguish the Ca\(^{2+}\) pump from the (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase, were not reported in that study. Therefore, the claim that the (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase is a Ca\(^{2+}\) pump is dubious. The function of the (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase which is not a calcium pump is not known.

Similar ecto-(Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase activities have been found in several other tissues (for references see Lin, 1985b). Several observations (Charest et al., 1985; Dubrov and De Young, 1985) suggest that possible roles for the ecto-ATPase may be to terminate the effect of ATP on the cells or to participate in the ATP effect via its phosphatase activity. It was also interesting to find that the P\(_2\)-purinergic effect has broad nucleotide specificity (Dubrov and De Young, 1985; Buxton et al., 1986; Okajima et al., 1987) as does the nucleotide-hydrolyzing activity of the ecto-ATPase. These correlations between the properties of the P\(_2\)-purinergic effect and the ecto-ATPase activity raise the possibility that the ecto-ATPase protein may be the P\(_2\)-purinergic receptor. In the J774 mouse macrophage cell line, Steinberg and Silverstein (1987) showed that ecto-ATPase does not mediate the effects of ATP on these cells. In hepatocytes, this possibility is presently under investigation. In this report, we show that the major (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase in the plasma membrane is a membrane ecto-ATPase and suggest that its function may be in the regulation of P\(_2\)-purinergic receptor function. This result should be helpful in clarifying the confusion about the plasma membrane (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPases.