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NUCLEOTIDE-INDUCED ALTERATION OF RAT LIVER MICROSOME CALCIUM PUMP ACTIVITY

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Summary

Rat liver microsomes sequester calcium by an energy dependent process that may be a nonmuscle cell analog of the sarcoplasmic reticulum Ca^{2+} pump of skeletal muscle (Moore, L., Chen, T.S., Knapp, H.R., Jr. and Landon, E.J. (1975) J. Biol. Chem. 250, 4562—4568). Homogenization of rat liver in the presence of ATP (5 mM) results in a 2-fold increase of the specific activity of the microsome Ca^{2+} pump. The effect of ATP is concentration dependent and is detected at ATP levels as low as 0.1 mM. ATP will produce this effect if added before homogenization, after homogenization or after any of the centrifugation steps of microsome isolation. Homogenization of rat liver in the presence of ADP and AMP also increases specific activity of the microsome Ca^{2+} pump, but to a lesser extent than ATP. Other nucleoside triphosphates have been tested and are in general less effective than ATP in increasing microsome Ca^{2+} pump activity. The phosphate group of nucleotides appears to be important to this effect in that adenosine does not affect Ca^{2+} pump activity, while sodium pyrophosphate will increase pump activity but to a smaller extent than ATP. The presence of nucleotides or pyrophosphate during microsome isolation results in the release of a small amount of protein material from microsomes. These proteins can be detected in $105\,000 \times g$ supernatant by SDS-polyacrylamide gel electrophoresis. Three bands of molecular weight 46 000, 42 000 and 36 000 comprise the majority of protein material released from microsomes. The ability of nucleotides to release one of the these proteins, the 42 000 molecular weight band, from microsomes correlates with the ability of the nucleotide to increase microsome Ca^{2+} pump activity. Preliminary evidence indicates that the protein released from ATP-treated microsomes is able to sup-

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Abbreviations: ACTH, adrenocorticotropin; Pipes, 1,4-piperazinediethanesulfonic acid.

press the stimulated calcium uptake measured in ATP-treated microsomes. It is possible that this protein functions to regulate Ca^{2+} pump activity in the endoplasmic reticulum of liver.

Introduction

Because calcium is thought to play an important role in regulation of cellular function, the mechanisms by which nonmuscle cells control cytoplasmic Ca^{2+} levels has attracted increasing attention in the last few years. It is well known that mitochondria from a variety of tissues sequester calcium and it has been proposed that this organelle may modulate cytoplasmic Ca^{2+} levels [1]. Recent studies have shown that the microsome fraction isolated from a number of nonmuscle tissues can also sequester calcium and it has been suggested that this system may be a nonmuscle cell analog of the sarcoplasmic reticulum Ca^{2+} pump of skeletal muscle [2—9]. Although a number of nonmuscle cells and tissues have been found to have this activity relatively little is known about regulation of Ca^{2+} pump activity in nonmuscle cells. In liver, sex steroids [10] and peptide hormones [11,12] can alter pump activity. ACTH and nucleotides alter Ca^{2+} pump activity in adrenal microsomes [9]. In platelets, cAMP has been suggested to have a role in regulation of the Ca^{2+} pump activity [13]. In cultured fibroblasts cell density has a major effect on Ca^{2+} pump activity [8].

The present study demonstrates that treatment of liver microsomes with nucleotides increases Ca^{2+} pump activity of the microsome fraction and also releases protein material from microsomes. A correlation between the ability of nucleotides to release this protein material from microsomes and to increase microsome Ca^{2+} pump activity suggests that release of this protein material may be responsible for nucleotide-induced alteration of microsome Ca^{2+} pump activity. It is possible that these proteins interact with and thus regulate Ca^{2+} pump activity of liver microsomes.

Experimental Procedures

Subfractionation of liver homogenates. Microsomes were isolated from rat liver as previously described at 0—4°C [6]. Homogenates were prepared in 0.25 M sucrose or in 0.25 M sucrose containing a nucleoside, a nucleotide or sodium pyrophosphate. All nucleic acid derivatives were obtained from Sigma Chemical Co. (St. Louis, MO). In any experiment all homogenates were prepared from a single liver. Microsome pellets were resuspended in 0.25 M sucrose for protein and Ca^{2+} pump assays. In certain cases the final supernatant was retained for electrophoresis on polyacrylamide gels containing sodium lauryl sulfate.

In certain experiments the initial microsome pellet was washed by resuspension in 0.25 M sucrose and recentrifugation at 105 000 $\times g$ for 60 min. These washed pellets were resuspended in 0.25 M sucrose or 0.25 M sucrose containing 5 mM nucleotide, nucleoside or sodium pyrophosphate and once again centrifuged at 105 000 $\times g$ for 60 min. The final microsome pellets were resuspended in 0.25 M sucrose for the Ca^{2+} pump assay and the final supernatant prepared for SDS-polyacrylamide gel electrophoresis.

In certain studies the liver homogenate was subfractionated to a greater extent and all particulate fractions were examined for microsome-like (azide insensitive) Ca^{2+} pump activity. In these cases this centrifugation scheme was followed: 1500 × *g* for 10 min, followed by 4500 × *g* for 10 min, followed by 12 500 × *g* for 10 min, followed by 32 500 × *g* for 10 min and finally 105 000 × *g* for 60 min.

Calcium uptake assay. Ca^{2+} pump activity was measured as previously described [6] and the assay is described in the legend of Fig. 1.

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out in a slab gel system (Bio-Rad). Typically a 10 cm × 14 cm × 1.5 mm gel contained 12% acrylamide/0.32% bisacrylamide/0.25 M Tris/0.8 M glycine/0.1% SDS, pH 8.6. The gel was polymerized by addition of 0.06% *N,N,N',N'*-tetramethylethylenediamine and 0.1% ammonium persulfate. The running buffer consisted of 0.25 M Tris/0.8 M glycine (pH 8.6)/0.1% SDS. 30 μg protein was applied to each lane. The gels were run at 15 mA (constant current) for 0.5 h until the tracking dye had completely entered the gel. Current was then increased to 30 mA until the tracking dye had migrated off the gel. Electrophoresis was continued an additional 1.5 h. During electrophoresis cool water (14°C) was circulated through the system. The gels were fixed and stained in a 50% trichloroacetic acid solution containing 0.25% Coomassie blue R and destained in 7.5% acetic acid.

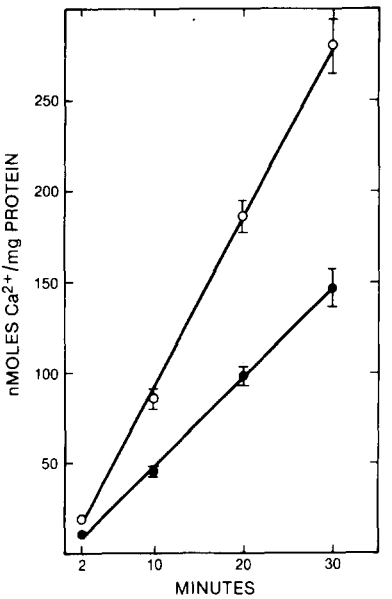
In some cases a stacking/resolving SDS-polyacrylamide gel electrophoresis system was used. In this case the 7.5 cm × 14 cm × 1.5 mm resolving gel (lower gel) contained 10% acrylamide/0.25% bisacrylamide/0.375 M Tris-HCl (pH 8.9)/0.1% SDS/2 mM EDTA. The 1.5 cm × 10 cm × 1.5 mm stacking gel (upper gel) contained 3% acrylamide/0.08% bisacrylamide/50 mM Tris-phosphate (pH 6.7/0.1% SDS/2 mM EDTA. Both gels were polymerized as described above. The running buffer was the same as described above. 8—12 μg protein were applied to the lane containing material released by ATP and a current of 15 mA was applied until the tracking dye entered the resolving gel. All other lanes received an equal volume of material. Current was increased to 30 mA electrophoresis continued until the tracking dye had just migrated to the end of the gel. These gels were fixed, stained and destained as described above.

The following proteins were used as molecular weight standards to determine the apparent subunit molecular weight of the bands on SDS-polyacrylamide gel electrophoresis: β-galactosidase (130 000), phosphorylase *a* (93 500), albumin (68 000), γ-globulin heavy chain (50 000), ovalbumin (43 000) and γ-globulin light chain (23 500).

Results

Effect of ATP in homogenization media on microsome Ca^{2+} pump activity

When ATP (5 mM) was added to 0.25 M sucrose used to homogenize rat liver, Ca^{2+} pump specific activity was increased in the isolated microsome fraction (Fig. 1). At all time points examined, specific activity of the microsome Ca^{2+} pump was increased approx. 2-fold. Under the assay conditions employed, the increase of Ca^{2+} pump activity was stable throughout the assay period. The effect of homogenization in the presence of ATP was not mimicked by homo-



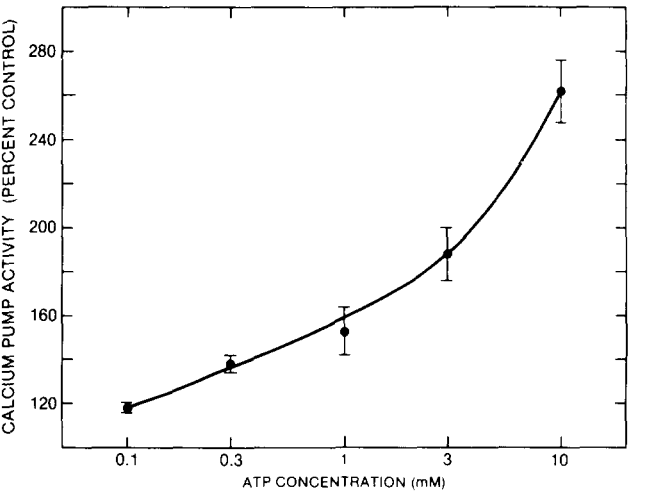


Fig. 1. Effect of homogenization in sucrose-ATP on liver microsome calcium uptake. Liver was homogenized in 0.25 M sucrose or 0.25 M sucrose with 5 mM ATP as described in Experimental Procedures. Both microsome pellets were resuspended in 0.25 M sucrose and calcium uptake activity of the microsomes was determined in a medium containing 100 mM KCl, 30 mM imidazole-histidine buffer pH 6.8, 5 mM $MgCl_2$, 5 mM ATP (pH adjusted to 6.8 with imidazole), 5 mM ammonium oxalate, 5 mM sodium azide, 20 μM $CaCl_2$ (0.1 μCi/ml $^{45}Ca^{2+}$) and 20—50 μg/ml microsome protein at 37° C. At timed intervals 0.5 ml samples were removed, filtered through 0.45 μm nitro-cellulose filters and ^{45}calcium trapped in microsomes retained on the filter determined by liquid scintillation spectrophotometry. The data represent the mean ± S.E. of the determination in five separate experiments. Sucrose microsomes (●——●), Sucrose + ATP microsomes (○——○).

Fig. 2. Effect of ATP concentration in homogenization medium on calcium uptake by liver microsomes. Liver was homogenized in 0.25 M sucrose or 0.25 M sucrose containing the concentration of ATP indicated in the figure. Microsomes were isolated, resuspended in 0.25 M sucrose and Ca^{2+} pump activity determined as described in Experimental Procedures. Data are expressed as percent of control activity (microsomes isolated from 0.25 M sucrose) ±S.E. and represent four separate experiments.

genization in the presence of KCl (0.15 and 0.6 M) or in the presence of another chelator (EDTA 5 mM).

This ATP-induced alteration of Ca^{2+} pump activity was dependent upon the concentration of ATP added to the homogenization medium (Fig. 2). Ca^{2+} pump activity was enhanced at ATP concentrations as low as 0.1 mM.

The effect of ATP did not depend upon the presence of the nucleotide during homogenization (Table I). In these experiments ATP (5 mM) was added to the homogenization medium before homogenization, or to the homogenate, or to the supernatant after the 1500 × g, 10 min centrifugation or 12 500 × g, 20 min centrifugation. Resuspension of liver microsomes in 0.25 M sucrose containing 5 mM ATP and isolation of a 105 000 × g, 60 min pellet also resulted in higher Ca^{2+} pump activity (Table I).

The effect of ATP could be due to an alteration of the affinity of the pump system for one of the substrates, i.e., calcium or Mg-ATP. Examination of the kinetics of the liver microsome Ca^{2+} pump as previously described [6] indi-

TABLE I

THE EFFECT OF ADDITION OF ATP AT VARIOUS POINTS DURING ISOLATION OF LIVER MICROSOMES ON MICROSOME Ca^{2+} PUMP ACTIVITY

Liver was homogenized in 0.25 M sucrose and microsomes isolated as described in Experimental Procedures. ATP was added to liver material at various points during microsome isolation or microsome pellets were resuspended in 0.25 M sucrose or 0.25 sucrose with 5 mM ATP. All microsome pellets were resuspended in 0.25 M sucrose and Ca^{2+} pump activity determined as described in Experimental Procedures. The results are expressed as percent of control (sucrose) microsomes ± S.E. and represent four independent experiments.

	ATP added				
	Before homogenization	After homogenization	After 1500 × *g* pellet	After 12 500 × *g* pellet	After 105 000 × *g* pellet
Ca^{2+} pump specific activity (percent control)	202 ± 7.6	191 ± 10	216 ± 18	216 ± 11	191 ± 26

cated that homogenization in sucrose-ATP resulted in a change of 2—3-fold in maximal velocity of pumping but no significant change of apparent affinity of the pump system for either calcium or Mg-ATP (Table II).

Distribution of Ca^{2+} pump activity after homogenization in medium containing ATP

In one series of experiments the liver homogenate was subfractionated into five membrane fractions by differential centrifugation (Table III). Homogenization of liver in an ATP-containing medium resulted in a greater percentage of protein (about 8%) in the low-speed pellet. In all subsequent pellets, except the 12 500 × *g*, 10 min pellet, protein recovery was slightly decreased. All five pellets were examined for microsome-like (azide insensitive) Ca^{2+} pump activity. Sodium azide has been shown to inhibit mitochondrial calcium uptake and at the concentration employed azide has been shown to produce virtually com-

TABLE II

THE EFFECT OF HOMOGENIZATION IN SUCROSE-ATP ON KINETICS OF LIVER MICROSOME Ca^{2+} PUMP

Liver was homogenized in 0.25 M sucrose or 0.25 M sucrose containing 5 mM ATP and microsomes isolated as described in Experimental Procedures. Kinetic parameters for MgATP were calculated after determination of Ca^{2+} pump activity over the range 0.625—10 mM MgATP. Kinetic parameters for calcium were calculated after determination of Ca^{2+} pump activity over the range of 0.2—20 μM Ca^{2+}, as previously described [6]. The parameters for MgATP were determined in five independent pairs of preparations. The parameters for calcium were determined in another set of five independent pairs of preparations. The values reported are the mean ± S.E.

Homogenization condition	Mg ATP		Calcium	
	K_m (mM)	*V* (nmol Ca/mg per min)	K_m (μM)	*V* (nmol Ca/mg per min)
Sucrose	1.0 ± 0.2	5.3 ± 0.5	2.7 ± 0.3	8.3 ± 1.6
Sucrose-ATP	1.3 ± 0.2	14 ± 1	2.4 ± 0.5	18 ± 4

TABLE III

THE EFFECT OF HOMOGENIZATION IN SUCROSE-ATP ON LIVER HOMOGENATE FRACTIONATION AND Ca^{2+} PUMP ACTIVITY

Liver was homogenized in 0.25 M sucrose or 0.25 M sucrose containing 5 mM ATP and fractionated as described in Experimental Procedures. All particulate fractions were resuspended in 0.25 M sucrose and Ca^{2+} pump activity determined as described in Experimental Procedures. Pump activity was determined in the presence of sodium azide to inhibit mitochondrial calcium uptake [6,8]. The data represent four independent experiments and are reported as the mean ± S.E.

Homogenization condition	Liver subcellular fractions				
	1500 × *g* pellet	4500 × *g* pellet	12 500 × *g* pellet	32 500 × *g* pellet	105 000 × *g* pellet
	Protein distribution (percent in fraction)				
Sucrose	40 ± 4	15 ± 3	9 ± 1	11 ± 1	25 ± 2
Sucrose-ATP	48 ± 2	14 ± 1	10 ± 1	10 ± 0.5	20 ± 1
	Ca^{2+} pump activity distribution (percent in fraction)				
Sucrose	9 ± 2	2 ± 0.5	4 ± 1	32 ± 6	53 ± 7
Sucrose-ATP	11 ± 3	3 ± 0.5	9 ± 1	27 ± 7	50 ± 8
	Ca^{2+} pump specific activity (percent control)				
Sucrose-ATP	209 ± 22	256 ± 58	431 ± 94	142 ± 15	181 ± 14

plete inhibition of ATP-dependent mitochondrial calcium uptake [6,8]. 75—85% of the microsome-like (azide insensitive) Ca^{2+} pump activity was isolated in the least rapidly sedimenting fractions, i.e., in membrane fractions that were sedimented with forces greater than 12 500 × *g*. The distribution of pump activity was not altered by homogenization in the presence of ATP. In all fractions collected specific activity of the microsome-like Ca^{2+} pump was higher in material isolated from livers homogenized with 5 mM ATP added to the homogenization medium (Table III). When total pump activity in all five membrane fractions was considered, microsome-like pump activity recovered in the experiments described in Table III totaled 10 171 ± 818 nmol Ca^{2+}/30 min per g liver if homogenized in 0.25 M sucrose and 17 295 ± 1233 nmol Ca^{2+}/30 min per g liver if homogenized in 0.25 M sucrose with 5 mM ATP. Thus, homogenization in a medium containing 5 mM ATP resulted in microsome-like pump activity of 172 ± 13% of control. This represents both an increase of specific activity of the Ca^{2+} pump activity recovered in each fraction and an increase of total pump activity recovered from a gram of liver.

Effect of related compounds on Ca^{2+} pump activity

Other nucleotides in the adenine series had a smaller effect on liver microsome Ca^{2+} pump activity as shown in Table IV. All compounds were tested at a concentration of 5 mM. Although inclusion of adenosine in the homogenization medium was without effect on Ca^{2+} pump activity, all three adenine nucleotides tested (ATP, ADP and AMP) resulted in Ca^{2+} pump activities higher than control. The effect of ADP and AMP was less than that produced by ATP.

TABLE IV

EFFECT OF HOMOGENIZATION IN A MEDIUM CONTAINING ADENINE DERIVATIVES OR SODIUM PYROPHOSPHATE ON LIVER MICROSOME CALCIUM UPTAKE ACTIVITY

Liver was homogenized in 0.25 M sucrose or 0.25 M sucrose containing the indicated compound at 5 mM. Microsomes were isolated and Ca^{2+} pump activity was determined as described in Experimental Procedures. Data are expressed as percent of control activity (microsomes isolated from 0.25 M sucrose) ± S.E. and represent six separate experiments.

	Test compound (5 mM)				
	ATP	ADP	AMP	Ade	PP_i
Ca^{2+} pump activity (percent control)	219 ± 24	167 ± 14	136 ± 4	102 ± 6	159 ± 8

Inclusion of sodium pyrophosphate (5 mM) in the homogenization medium also resulted in Ca^{2+} pump activity significantly higher than control (Table IV), suggesting the importance of the phosphate group. Other nucleoside triphosphates were tested at 5 mM in the homogenization medium and the results are reported in Table V. Only GTP and UTP produced calcium uptake activities significantly greater than control, demonstrating that the effect was not limited to adenine nucleotides and also that it was not strictly limited to purine nucleotides.

Electrophoresis of subcellular fractions isolated from homogenates containing sucrose or sucrose and nucleotides

Both liver microsomes and final supernatants have been examined by SDS-polyacrylamide gel electrophoresis to determine if removal of protein material from microsomes by nucleotides correlated with the ability of the nucleotides to alter Ca^{2+} pump activity. Preliminary experiments with microsome fraction suggested that at least three bands observed on SDS-polyacrylamide gel electrophoresis were either absent or somewhat diminished in microsomes prepared from a homogenate containing ATP. This can be seen by comparing the microsome protein bands indicated by the three arrows in Fig. 3 (compare lanes 1 and 3, from the left). Microsome material isolated from sucrose homogenized liver had two distinct bands in the molecular weight range of 46 000 (upper arrow, lane 3, Fig. 3). The lower of these two bands was found to be absent in

TABLE V

EFFECT OF HOMOGENIZATION IN A MEDIUM CONTAINING VARIOUS NUCLEOTIDE TRIPHOSPHATES ON LIVER MICROSOME CALCIUM UPTAKE ACTIVITY

Liver was homogenized in 0.25 M sucrose or 0.25 M sucrose containing the indicated indicated nucleotide triphosphate at a concentration of 5 mM. Microsomes were isolated and Ca^{2+} pump activity was determined as described in Experimental Procedures. Data are expressed as percent of control activity (microsomes isolated from 0.25 M sucrose). The mean ± S.E. of five independent experiments are reported.

	Nucleotide triphosphate (5 mM)				
	ATP	GTP	UTP	ITP	CTP
Ca^{2+} pump activity (percent control)	184 ± 5	147 ± 12	123 ± 9	118 ± 10	104 ± 12

the microsome fraction isolated from a homogenate prepared with added 5 mM ATP (lane 1, Fig. 3). Another band, molecular weight about 42 000, was diminished in microsomes prepared from the homogenate containing ATP (middle arrow). Finally, a third band (molecular weight about 36 000, lower arrow) was also diminished in the microsome fraction isolated from the ATP-containing homogenate.

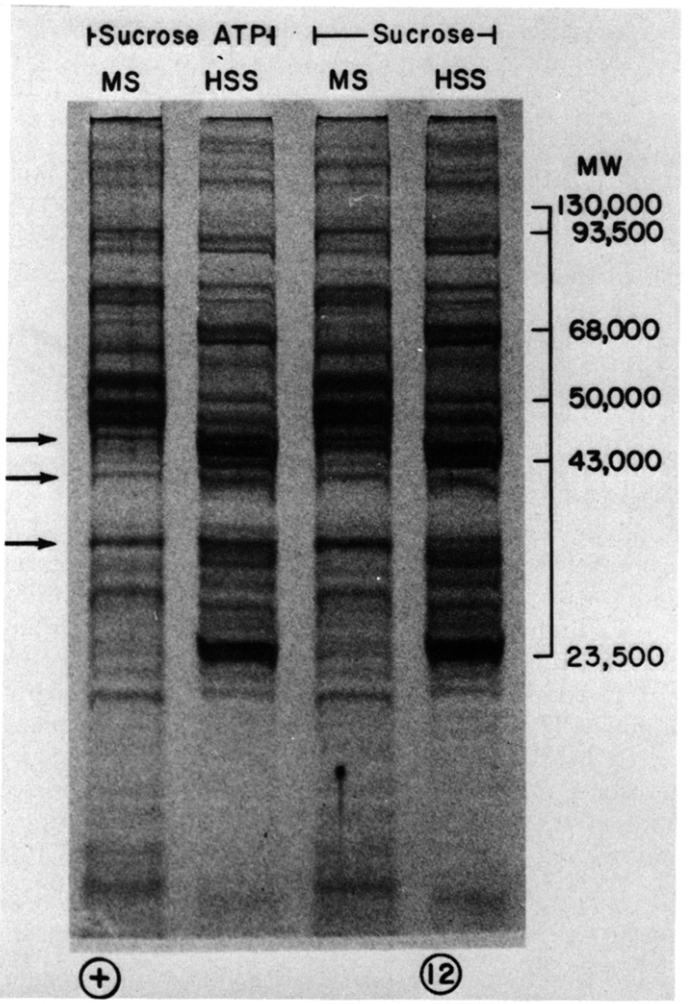


Fig. 3. Effect of homogenization of liver in sucrose-ATP on electrophoretic pattern of microsomes and high speed supernatant (cytosol). Microsomes and cytosol were isolated from liver homogenized on 0.25 M sucrose or 0.25 M sucrose containing 5 mM ATP as described in Experimental Procedures. 30 μg protein were applied to each lane of a polyacrylamide gel prepared from 12% acrylamide containing 0.1% SDS. Electrophoresis was conducted as described in Experimental Procedures. β-Galactosidase (130 000), phosphorylase *a* (93 500), albumin (68 000), γ-globulin heavy chain (50 000), ovalbumin (43 000) and γ-globulin light chain (23 500) were applied as molecular weight markers. Migration positions of these marker proteins are indicated on the figure. Microsomes (MS) and cytosol (HSS) isolated from liver homogenized in sucrose-ATP were applied to the two left lanes. Microsomes and cytosol isolated from liver homogenized in sucrose were applied to the two right lanes. From top to bottom the arrows indicate bands with apparent molecular weights of 46 000, 42 000 and 36 000, respectively.

These differences were more apparent when samples of final supernatants were electrophoresed as shown in lanes 2 and 4, Fig. 3. It would appear that proteins removed from microsomes prepared from an ATP-containing homogenate are found in the corresponding final supernatant (cytosol).

Experiments with washed microsomes confirmed that these protein bands did in fact originate from microsome material. Microsomes were isolated in the normal manner from liver homogenized in 0.25 M sucrose. These microsomes were washed by resuspension in 0.25 M sucrose and centrifugation at 105 000 × *g* for 60 min. The washed microsome pellets were resuspended in 0.25 M sucrose or a sucrose solution containing adenine nucleotide, adenosine or sodium pyrophosphate at 5 mM. After centrifugation at 105 000 × *g* for 60 min, the final supernatants were collected and prepared for SDS-polyacrylamide gel electrophoresis. Equal volumes of each final supernatant (wash) were applied to the stacking/separating gel system described in Experimental Procedures. Because equal volumes of the final supernatants were applied to each lane, the intensity of the bands seen on SDS-polyacrylamide gel electrophoresis should be proportional to the amount of protein released by the nucleotide, nucleoside or inorganic pyrophosphate. The volume of sample applied to the gel was chosen so that 8 μg protein from the ATP-containing final supernatant was applied to the SDS-polyacrylamide gel electrophoresis system. As shown in Fig. 4 approx. 20 bands were observed when this material was electrophoresed and stained. Bands corresponding to the three bands observed in SDS-polyacrylamide gel electrophoresis of cytosol were readily detected. As with cytosol, these bands were most prominent in preparations from the ATP-, ADP- and sodium pyrophosphate-treated material. Less prominent bands were observed with AMP-derived material. Protein was virtually absent from the final supernatants (washes) obtained from washed microsomes resuspended in 0.25 M sucrose or sucrose with adenosine.

Because only very small amounts of protein appeared in the final supernatants, from washed microsomes resuspended in 0.25 M sucrose with or without adenosine, the washed microsomes were not contaminated with substantial amounts of cytosol proteins that could be released by resuspension. In those lanes with final supernatant material from nucleotide-treated, washed microsomes the amount of Coomassie blue stained material on the gel increased in the order AMP < ADP < ATP. Determination of total protein in these final supernatants showed that AMP released approx. 4 μg protein/mg microsome protein, ADP released 13 μg protein/mg microsome protein and ATP released 22 μg protein/mg microsome protein. The quantity released by the nucleotide roughly correlated with the increase of Ca^{2+} pump activity produced by the nucleotide (compare with Table IV).

Final supernatants obtained from washed microsomes treated with other nucleoside triphosphates have also been subjected to SDS-polyacrylamide gel electrophoresis. The results are shown in Fig. 5. In general CTP, GTP, ITP and UTP released the same protein bands as did ATP. However, the intensity of protein bands on SDS-polyacrylamide gel electrophoresis varied depending upon the nucleoside triphosphate used to treat washed microsomes. As an example all nucleotides released the 46 000 molecular weight band; but CTP, a nucleoside triphosphate which was unable to increase Ca^{2+} transport activity (Table V),

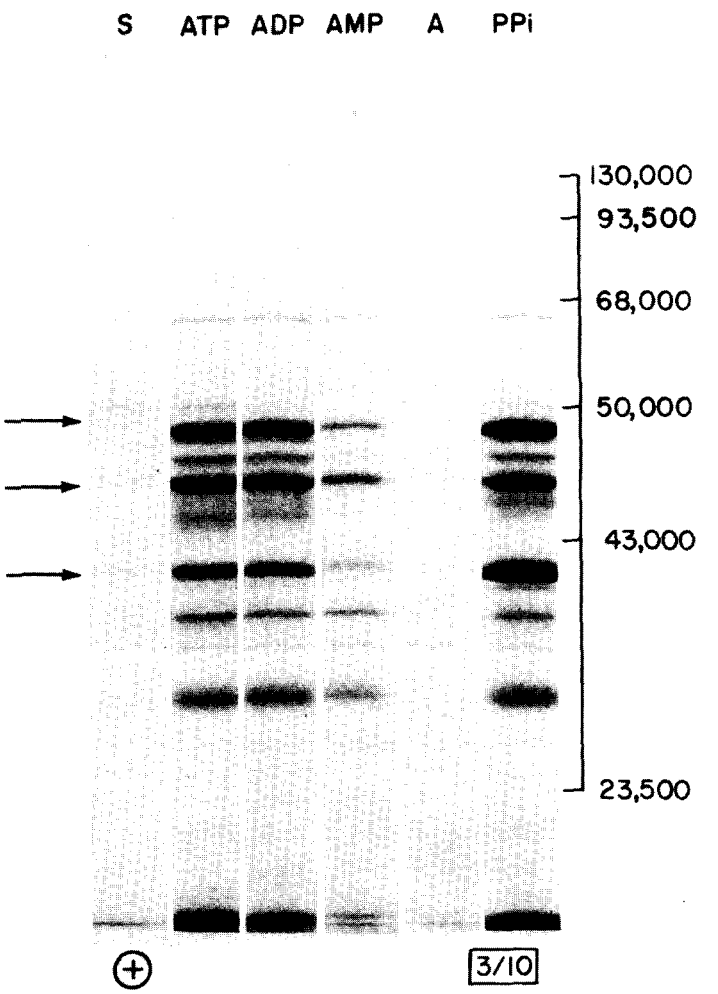


Fig. 4. Proteins released from liver microsomes resuspended in adenine derivatives or sodium pyrophosphate. Liver microsomes were isolated and washed in 0.25 M sucrose as described in Experimental Procedures. The washed microsomes pellets were resuspended in 0.25 M sucrose containing the indicated adenine derivative or sodium pyrophosphate (PP_i) at a concentration of 5 mM. After centrifugation at 105 000 × *g* for 60 min the supernatants were prepared for SDS-polyacrylamide gel electrophoresis in the stacking/separating gel system described in Experimental Procedures. 8 μg protein from the sucrose-ATP supernatant were applied to the gel. An equal volume of each of the other final supernatants was applied to the appropriate lane. The migration position of the molecular weight marker proteins (Fig. 5) is indicated by the scale to the right of the gel. The arrows indicate protein bands described in Fig. 3.

released a relatively large amount of the 36 000 molecular weight band but a relatively small amount of the 42 000 molecular weight band. The appearance of the 42 000 molecular weight band in the supernatant from washed microsomes treated with nucleotides, or with sodium pyrophosphate, appeared to correlate with increased activity of the Ca^{2+} pump. This is documented in Table VI with data obtained by scanning these gels.

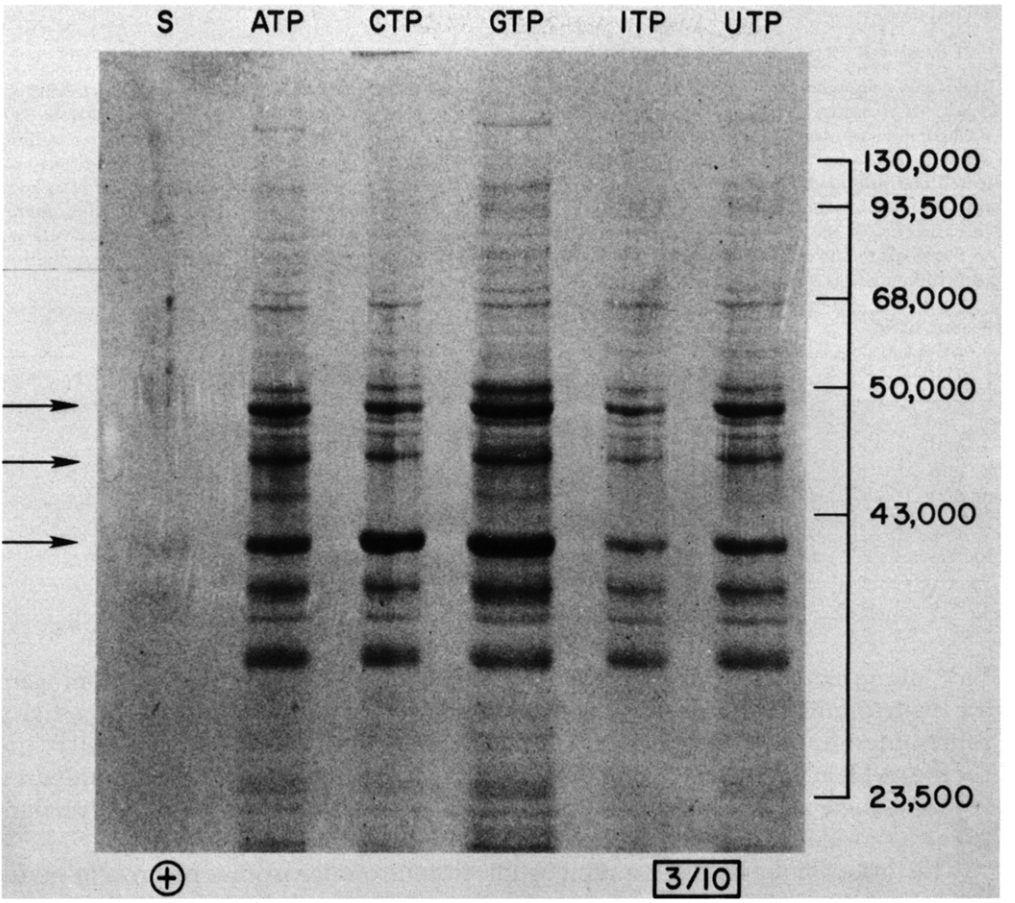


Fig. 5. Protein released from liver microsomes resuspended in various nucleoside triphosphates. Liver microsomes were isolated and washed in 0.25 M sucrose as described in Experimental Procedures. The washed microsome pellets were resuspended in 0.25 M sucrose or in 0.25 M sucrose containing the indicated nucleoside triphosphate at a concentration of 5 mM. After centrifugation at 105 000 × *g* for 60 min the supernatants were prepared for SDS-polyacrylamide gel electrophoresis in the stacking/separating gel system described in Experimental Procedures. 8 μg protein were applied to the sucrose-ATP lane. An equal volume of the other final supernatants was applied to the appropriate lane. Migration positions of the molecular weight marker proteins (see legend for Fig. 3) are indicated by the scale to the right of the gel. The arrows indicate protein bands described in Fig. 3.

Inhibition of microsome Ca^{2+} pump by ATP-released proteins

An initial attempt has been made to demonstrate that the proteins removed from microsomes by resuspension in an ATP-containing medium could inhibit microsome Ca^{2+} pump activity. Washed microsomes were treated with 5 mM ATP in 0.25 M sucrose and the supernatant recovered after centrifugation at 105 000 × *g* for 60 min. The supernatant was dialyzed against two changes of buffered sucrose overnight. The dialyzed final supernatant was added to the calcium uptake assay before addition of fresh microsome material isolated from an ATP-containing homogenate. As shown in Table VII partial inhibition of Ca^{2+} pump activity was produced by addition of the dialyzed supernatant. Inhibition was dependent upon the amount of final supernatant added. Because

TABLE VI

RELEASE OF PROTEINS FROM MICROSOMES BY NUCLEOTIDES AND PYROPHOSPHATE

Liver was homogenized in 0.25 M sucrose and microsomes isolated as described in Experimental Procedures. Microsomes were washed by resuspension in 0.25 M sucrose and reisolated by centrifugation. The washed microsomes were resuspended in 0.25 M sucrose containing the indicated nucleotide or sodium pyrophosphate at 5 mM. After centrifugation at 105 000 × *g* for 60 min the final supernatant was collected and prepared for SDS-polyacrylamide gel electrophoresis. Electrophoresis was on the discontinuous system described in Experimental Procedures. Stained gels were scanned on an Ortec 4310 densitometer and areas under the curve for the three primary protein bands determined. The areas are presented relative to the peak in the supernatant prepared with ATP. The results represent the mean of the determination with three or four preparations.

Protein band (apparent molecular weight)	Test compound							
	ATP	ADP	AMP	CTP	GTP	ITP	UTP	PP_i
	Relative peak area							
46 000	1	0.8	0.3	0.8	0.8	0.4	0.6	1.5
42 000	1	0.7	0.2	0.3	0.6	0.3	0.4	0.5
36 000	1	0.7	0.3	2.7	1.4	0.7	1.0	2.7
	Relative increase							
Increase of Ca^{2+} pump activity	1	0.7	0.4	0	0.5	0.1	0.2	0.6

Ca^{2+} pump activity was approximately doubled by treatment of the homogenate with 5 mM ATP, the observed 25% inhibition of total Ca^{2+} pump activity represented approx. 50% inhibition of the increase of Ca^{2+} pump activity due to the ATP effect. No inhibition was seen if a dialyzed supernatant prepared from washed microsomes resuspended in a sucrose solution without a nucleotide was added to the calcium uptake assay.

This effect may be due to reincorporation into microsomes of protein material released by ATP. When microsomes isolated from an ATP-containing homogenate were incubated with the dialyzed supernatant described above, rebinding of the released protein occurred. This is demonstrated in Fig. 6. Apparently

TABLE VII

INHIBITION OF MICROSOME Ca^{2+} PUMP BY MATERIAL RELEASED BY TREATMENT OF WASHED MICROSOMES WITH ATP

Microsomes were isolated and washed as described in Experimental Procedures. The washed microsomes were resuspended in 0.25 M sucrose or in 0.25 M sucrose containing 5 mM ATP. After centrifugation at 105 000 × *g* for 60 min the final supernatant (HSW) was collected. Both final supernatants were dialyzed against two changes of 0.25 M sucrose 10 mM Pipes, pH 6.8, (100 vols. each charge) overnight. Protein concentration of the sucrose-ATP HSW averaged 0.79 ± 0.03 mg/ml after dialysis. 50 or 100 µl of each HSW were added to the calcium uptake assay described in Experimental Procedures before initiation of the assay with microsome protein. The results are expressed as the mean ± S.E. for the determination with five microsome and five HSW preparations.

	Control	Sucrose-ATP HSW		Sucrose HSW 100 µl
		50 µl	100 µl	
Ca^{2+} pump specific activity (nmol Ca^{2+}/mg protein per 30 min)	391 ± 27	330 ± 21	298 ± 28	373 ± 28

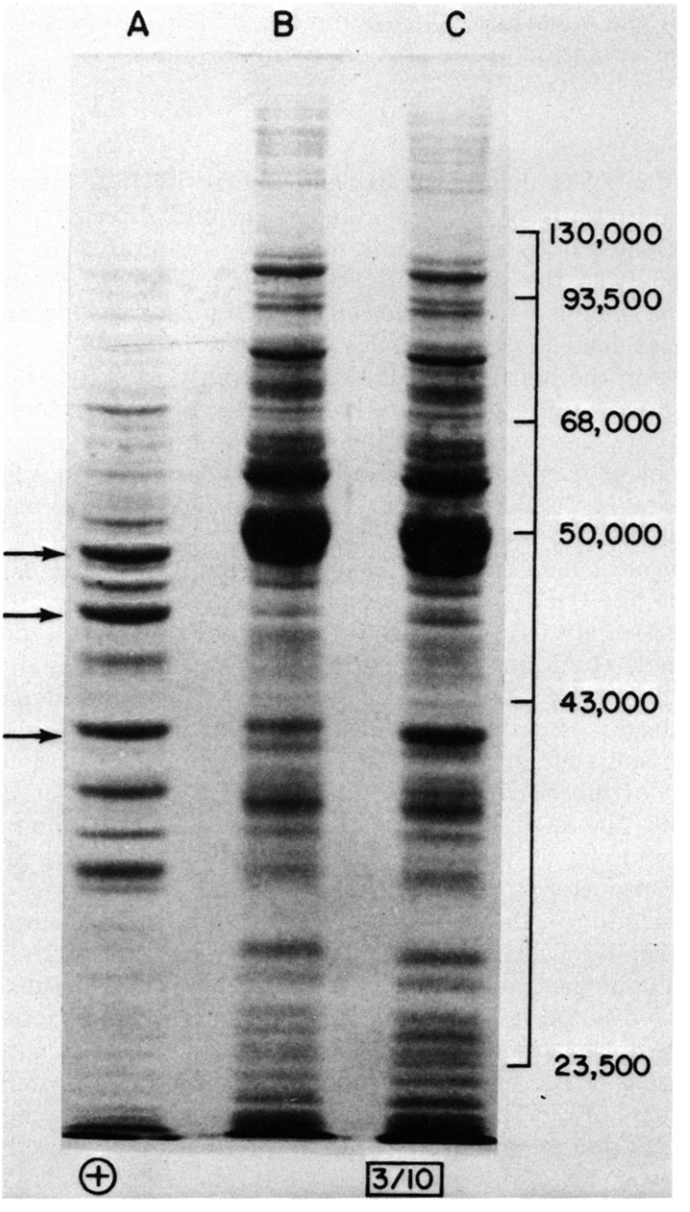


Fig. 6. Rebinding of proteins released from microsomes by treatment with ATP. Washed microsomes prepared in 0.25 M sucrose were resuspended with 5 mM ATP as described in Experimental Procedures. After centrifugation at 105 000 × *g* for 60 min the final supernatant was collected and dialyzed overnight against two changes of 0.25 M sucrose, 10 mM Pipes, pH 6.8, (100 vols. each change). Microsomes were then prepared from a homogenate containing 0.25 M sucrose with 5 mM ATP. These sucrose-ATP microsomes were resuspended in either 0.25 M sucrose or in the dialyzed sucrose-ATP final supernatant described above. After centrifugation at 105 000 × *g* for 60 min the two microsomal pellets were rinsed and prepared for SDS-polyacrylamide gel electrophoresis. Samples were electrophoresed in the stacking/separating gel system described in Experimental Procedures. 30 μg of each microsome preparation were applied to the gel. Lane A contains the dialyzed sucrose-ATP final supernatant. Lane B contains microsome material resuspended in and reisolated from 0.25 M sucrose, while Lane C contains microsome material resuspended in and reisolated from the dialyzed sucrose-ATP final supernatant. Arrows indicate protein bands described in Fig. 3.

the basis for inhibition of the microsome Ca^{2+} pump by the released proteins involved rebinding or reincorporation of the proteins by microsomes.

Discussion

It has been suggested that ATP-dependent calcium uptake by liver microsomes represents Ca^{2+} pump activity in liver endoplasmic reticulum [6,12]. Ca^{2+} pump activity is associated both with smooth membranes and rough endoplasmic reticulum isolated from the microsome fraction [6]. The Ca^{2+} pump activity in liver microsomes may be a nonmuscle cell analog of the sarcoplasmic reticulum Ca^{2+} pump of skeletal muscle and may participate in regulation of cytoplasmic calcium levels in the hepatocyte. If this Ca^{2+} transport system has a role in regulation of calcium in the cytoplasm it is of interest to understand how the hepatocyte regulates the transport system. The studies reported here demonstrate that treatment of liver microsomes or liver homogenate with ATP increases calcium uptake or Ca^{2+} pump activity of isolated liver microsomes. Although homogenization in the presence of ATP results in a small difference in protein distribution in particulate fractions, there is no significant change in distribution of microsome-like Ca^{2+} pump activity in particulate fractions isolated from liver. In all particulate fractions specific activity of the microsome-like Ca^{2+} pump is increased. Total pump activity isolated from 1 g liver is also increased by homogenization in the presence of ATP. When considered together these findings suggest that the effect of homogenization in the presence of ATP results from some effect on the Ca^{2+} transport process and does not result from alteration of the distribution of pump activity during isolation of the microsome fraction. The effect of ATP results from an increase of maximal velocity of calcium pumping by microsomes, but there is no effect on the apparent affinity of the transport system for calcium or MgATP.

The effect of homogenization in the presence of nucleotides on Ca^{2+} pump activity was first described by Laychock et al. [9], in adrenal microsomes. These workers found that homogenization of the adrenal cortex in the presence of a variety of nucleotides resulted in a 4—5-fold increase of Ca^{2+} pump activity. The present study demonstrates that a similar phenomenon occurs with liver and suggests that some effect of the nucleotides on microsomal protein may account for the increase of Ca^{2+} pump activity. When isolated microsomes are treated with nucleotides a small amount of protein is released into the subsequent supernatant. Approx. 2% of microsome protein is released when 5 mM ATP is used. While all nucleotides tested release some protein from microsomes, the nucleoside adenosine is virtually without effect. Three protein bands of apparent molecular weights 46 000, 42 000 and 36 000 comprise the majority of the protein released by nucleotides. These three proteins appear to originate from microsomes in that they are released from washed microsomes by treatment with a nucleotide. Different nucleotides vary in their ability to release these three protein bands. The ability of the nucleotides to release one of these bands, the band with an apparent molecular weight of 42 000, approximately correlates with the ability of the nucleotide to increase Ca^{2+} pump activity (Table VI). At least part of the protein released by treatment of microsomes with a nucleotide appears to be an inhibitor of microsome Ca^{2+} pump

activity. When this protein material is added back to sucrose-ATP microsomes it partially inhibits Ca^{2+} pump activity, possibly by rebinding to microsomes.

The data presented show that these proteins can be released from microsomes by treatment with ATP in vitro. The data do not show that ATP acts in vivo to release these proteins and to alter microsome Ca^{2+} pump activity. The physiological importance of this phenomenon is unknown. However, the data presented suggest that the protein with an apparent molecular weight of 42 000 can inhibit microsome Ca^{2+} pump activity when the protein is associated with microsomes. It is possible that this protein serves as a physiological regulator of the Ca^{2+} pump activity found in the microsome fraction. One can speculate, that this protein could be important in the response of the hepatocyte to hormones that have been shown to alter Ca^{2+} pump activity [10—12]. It is possible that these hormones regulate pump activity in the liver by modulating interaction of the 42 000 molecular weight protein with the Ca^{2+} transport system. Proof of this speculation will not be possible until the protein is isolated and identified.

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