Vasopressin-, Angiotensin II-, and \( \alpha_1 \)-Adrenergic-induced Inhibition of Ca\(^{2+} \) Transport by Rat Liver Plasma Membrane Vesicles*

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A rapid method for isolating highly purified rat liver plasma membrane vesicles using isotonic medium and Percoll self-forming gradient centrifugation is described. The vesicles were characterized by enzyme markers and electron microscopy. The method also yielded a fraction rich in nuclei. The vesicles transported Ca\(^{2+} \) in an ATP-dependent manner and this was enhanced by oxalate. The \( V_{\text{max}} \) for Ca\(^{2+} \) uptake was 0.65 ± 0.08 nmol/mg·min, which was approximately 18-fold higher than for other liver plasma membrane preparations, and the \( K_m \) for Ca\(^{2+} \) was 5.2 ± 0.4 nm.

Calcium uptake was inhibited by 40–50% in vesicles isolated from rat livers perfused for 3 min with \( 10^{-7} \) M vasopressin. The half-maximally effective concentration of vasopressin was \( 5 \times 10^{-10} \) M which correlates with that for raising cytosolic Ca\(^{2+} \) and phosphorylase a. Inhibition was not significant in vesicles from livers perfused with vasopressin for only 1 min, indicating that inhibition of the Ca\(^{2+} \) pump may not be involved in the rise in cytosolic Ca\(^{2+} \) observed at 1–2 s with this hormone. Epinephrine (\( 10^{-8} \) M) and angiotensin II (\( 10^{-7} \) M) inhibited Ca\(^{2+} \) uptake by 31 ± 10 and 26 ± 5%, respectively, at 3 min. Glucagon (\( 10^{-7} \) M) had no effect.

It is proposed that the inhibitory action of the Ca\(^{2+} \)-dependent hormones on the plasma membrane Ca\(^{2+} \) pump plays an important role in the actions of these hormones by prolonging the elevation in cytosolic Ca\(^{2+} \).

The mobilization of intracellular Ca\(^{2+} \) (mainly from mitochondria) by \( \alpha_1 \)-adrenergic agonists, vasopressin and angiotensin II, in rat liver hepatocytes is a well established phenomenon (see Refs. 1 and 2). A large fraction of the Ca\(^{2+} \) mobilized is extruded from the cell presumably by a plasma membrane (Ca\(^{2+}\)-Mg\(^{2+} \))-ATPase, accounting for the efflux of Ca\(^{2+} \) observed in isolated hepatocytes and perfused liver (3–6). The postulated elevation in cytosolic Ca\(^{2+} \) concentration ([Ca\(^{2+} \)]) has been confirmed utilizing hepatocytes loaded with the Ca\(^{2+} \) indicator Quin-2 (7). However, since the intracellular Ca\(^{2+} \) stores are limited, other changes must occur to prolong the actions of the Ca\(^{2+} \)-dependent hormones. Consequently, it has been postulated that either the plasma membrane (Ca\(^{2+}\)-Mg\(^{2+} \))-ATPase is inhibited and/or the influx of extracellular Ca\(^{2+} \) is stimulated (1). Indeed, \( \alpha_1 \)-adrenergic stimulation has been shown to stimulate the influx of Ca\(^{2+} \) from the external medium (8–10).

In this communication, we describe a rapid method to prepare vesicles from rat liver plasma membranes utilizing Percoll gradient centrifugation. These vesicles are able to transport Ca\(^{2+} \) in an energy-dependent manner. When they are isolated from rat livers perfused with Ca\(^{2+} \)-dependent hormones, the Ca\(^{2+} \) transport activity is inhibited up to 50%. It is postulated that this effect ensures that the Ca\(^{2+} \) mobilizing hormones maintain an elevated [Ca\(^{2+} \)], and hence produce prolonged physiological responses.

**EXPERIMENTAL PROCEDURES**

**Plasma Membrane Isolation**—Male 200–250-g body weight, Sprague-Dawley rats (Harlan Industries, Indianapolis, IN) fed ad libitum on Purina Lab Chow were killed by cervical dislocation and their livers rapidly removed and placed in ice-cold medium containing 250 mM sucrose, 5 mM HEPES-KOH, and 1 mM EGTA, pH 7.4. They were minced with scissors and homogenized by 10 passes with a loose-fitting Dounce homogenizer (Wheaton) followed by 3 passes with a tight-fitting homogenizer, then diluted to give a 6% (w/v) homogenate. The homogenate was then centrifuged at 1,464 × g for 10 min, and the resulting pellet was resuspended in the isolation medium and diluted to give a 6% (w/v) suspension. A volume (10.4 ml) of this was mixed with 1.4 ml of Percoll (Pharmacia) in 16-ml Corex tubes and centrifuged at 34,540 × g for 30 min. Two distinct layers close to the top of the tube were revealed. These were harvested and washed in 5 volumes of 250 mM sucrose, 50 mM Tris-Cl, pH 8.0, and the resulting pellets were resuspended in the same medium. The protein content of the membranes was measured according to Lowry et al. (11).

Rat liver plasma membranes were also prepared by the method of Song et al. (12) for comparison purposes.

**Rat Liver Perfusion**—Livers of rats were perfused as described previously with bicarbonate buffer containing 2.4 mM Ca\(^{2+} \) (13). Livers were initially perfused with hormone-free medium for 6 min. The median lobe was then tied off and rapidly removed for preparation of "time zero" plasma membranes. Perfusion of the remaining liver lobes was continued with hormone or saline being continuously infused into the portal cannula line. At designated times, the left and right lobes were ligated and rapidly removed for preparation of plasma membranes.

**Enzyme Assays**—5′-Nucleotidase, glucose-6-phosphatase and cytochrome c oxidase were measured as described previously (14). (Ca\(^{2+}\)-Mg\(^{2+} \))-ATPase was measured under conditions described by Loten et al. (15) except that phosphate release was determined as in Ref. 14.

**Measurement of Ca\(^{2+} \) Transport**—Ca\(^{2+} \) uptake by liver plasma membrane vesicles was assayed essentially as described by Chan and Junger (16), unless stated otherwise, in 500 μl of medium containing 180 mM sucrose, 50 mM Tris-Cl, pH 8.0, 0.2 mM EGTA, 18 μCi/ml of \(^{45}\)Ca\(^{2+} \) (0.18 mM total, 7.5 nM free), 10 mM MgCl\(_2\), 20 mM sodium azide, 1 μM ruthenium red, 10 mM Tris-ATP, and 4 mM Tris oxalate. The free concentration of Ca\(^{2+} \) was varied in some experiments and was calculated using the COMICS program (17). Association constants

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2. The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N',N' '-tetraacetic acid; TRIS, tris(hydroxymethyl)-aminomethane.
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Stents were taken from Sillen and Martell (18). The reaction was started by adding 100 \(\mu\)g of plasma membrane protein and the mixture was incubated at 37 °C in a shaking water bath. At appropriate times, 450-\(\mu\)l samples were removed and filtered under vacuum on 0.45-\(\mu\)m pore Millipore filters (type HA) that had been soaked in 0.25 M KCl for 1 h. The filters were washed three times with 5 ml of 250 mM sucrose and 40 mM NaCl. The membrane vesicles trapped on the filter were dried and counted in 10 ml of ACS aqueous counting scintillant (Amersham).

RESULTS

Characteristics of Plasma Membrane Vesicles Prepared by Percoll Gradient Centrifugation—Preliminary differential centrifugation of rat liver homogenates showed that the bulk of the (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase and 5'-nucleotidase activities sedimented at low speed (1,464 \(\times g\) for 10 min) while most of the glucose-6-phosphatase and cytochrome c oxidase sedimentsed at higher g forces (data not shown). Therefore, the low speed pellet was resuspended and added to Percoll (11.9% final concentration) and centrifuged at 34,540 \(g\) for 30 min to prepare plasma membranes. Two distinct layers appeared close to the top of the tube while all other material remained on the bottom. The two top layers were analyzed for enzyme markers and by electron microscopy. The very top layer was identified as rich in nuclei on the basis of electron microscope analysis. This fraction was low in 5'-nucleotidase, glucose-6-phosphatase, (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase, and cytochrome c oxidase (Table I). The second layer showed a large enrichment in two plasma membrane markers, namely 5'-nucleotidase and (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase. These were enriched 24- and 11-fold, respectively, over the total homogenate (Table I). Contamination of the fraction by microsomes and mitochondria was minimal since glucose-6-phosphate and cytochrome c oxidase showed 40 and 60% decreases in specific activity over the homogenate. For comparison, data from plasma membranes prepared by the Song et al. (12) procedure are shown in Table I. It is evident that the membranes prepared using Percoll are more enriched in 5'-nucleotidase and (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase. Electron microscopy revealed the presence of vesicles of different sizes in the Percoll-derived membranes and the virtual absence of mitochondria (data not shown). In general, these membranes had more vesicles and were less contaminated with other organelles than were Song et al. (12) membranes.

Ca\(^{2+}\) Transport by Plasma Membrane Vesicles—The time course of Ca\(^{2+}\) uptake by a preparation of Percoll-derived liver plasma membrane vesicles is shown in Fig. 1 (left). There was little uptake in the absence of ATP, whereas the ATP-dependent calcium uptake proceeded linearly for about 10-15 min. The ATP-dependent Ca\(^{2+}\) uptake at the end of 30 min for 3 separate preparations was 7.0 ± 1.3 nmol/mg of protein in the presence of 4 mM oxalate and 4.5 ± 0.7 nmol/mg of protein in its absence. The ATP-independent Ca\(^{2+}\) uptake or binding accounted for approximately 7% of the total Ca\(^{2+}\) accumulated at 30 min and oxalate had negligible effect on the basal activity.

For comparison, Ca\(^{2+}\) uptake by Song et al. (12) membranes (Fig. 1, right) was measured under identical conditions to those in Fig. 1, left. It is very evident that these membranes showed much lower ATP-dependent Ca\(^{2+}\) transport, with rates similar to those reported by Chan and Junger (16).

Calcium uptake by Percoll-derived membrane vesicles was not altered by ruthenium red or NaN\(_{3}\), known inhibitors of mitochondrial Ca\(^{2+}\) transport. However, it was abolished by the ionophore A23187, indicating uptake into membrane bound vesicles (data not shown).

The ATP-dependent Ca\(^{2+}\) uptake of Percoll-derived vesicles was measured as a function of Mg\(^{2+}\) concentration at a constant ATP concentration of 10 mM and free Ca\(^{2+}\) concentration of 7.5 nM. No calcium uptake occurred when Mg\(^{2+}\) was omitted. At 5 mM Mg\(^{2+}\), calcium uptake proceeded half-maximally, and it reached a maximum at 10-15 mM Mg\(^{2+}\). Ca\(^{2+}\) uptake was also studied as a function of ATP concentration. In these studies, approximately 20% of the initial ATP was hydrolyzed in 5 min as measured by [(\(\gamma\)-\(\beta\))-\(^{32}\)P]ATP hydrolysis. The ATP concentrations for half-maximal and maximal activity were approximately 2 and 10-15 mM, respectively. The ATP-dependent calcium uptake by the vesicles was also measured as a function of free Ca\(^{2+}\) concentration (Fig. 2). The \(K_a\) and \(V_{max}\) values derived from 4 separate experiments using Eadie-Hofstee plots were 5.2 ± 0.4 nM and 0.65 ± 0.08 nmol/mg of protein, respectively.

Effect of Vasopressin on Ca\(^{2+}\) Transport by Plasma Membrane Vesicles—Direct addition of vasopressin (10\(^{-8}\)-10\(^{-7}\) M) to plasma membrane vesicles 10 min before or simultaneously with ATP did not alter Ca\(^{2+}\) transport (data not shown). Therefore vesicles were prepared from the lobes of rat livers that had been perfused with 10\(^{-7}\) M vasopressin. As shown in Fig. 3 (left), little difference was observed in the Ca\(^{2+}\) transport activity of lobes perfused with saline (control). However, the infusion of 10\(^{-7}\) M vasopressin markedly inhibited Ca\(^{2+}\) uptake by the vesicles as shown in Fig. 3 (left). At 2, 3, and 6 min, there was a 32, 39, and 50% inhibition, respectively, of Ca\(^{2+}\) uptake compared with control at each time. The effect of vasopressin at 1 min was not statistically significant.

Rat liver lobes were also perfused for 3 min with concentrations of vasopressin ranging from 10\(^{-10}\) to 10\(^{-7}\) M (Fig. 3, right). Maximal effects were seen at 10\(^{-8}\) M vasopressin and the concentration for half-maximal effects was 5 × 10\(^{-10}\) M (Fig. 3, right). These concentrations are very similar to those for vasopressin effects on [Ca\(^{2+}\)], Ca\(^{2+}\) efflux, and phospholipase in hepatocytes (e.g. see Ref. 3).

### Table I

| Fraction       | 5'-Nucleotidase | Glucose-6-phosphatase | (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase | Cytochrome c oxidase |
|----------------|-----------------|-----------------------|-------------------------------|---------------------|
|                | \(\text{pmol/mg protein - 30 min}\) | \(\text{pmol/mg protein - 20 min}\) | \(\text{pmol/mg protein - 10 min}\) | \(\text{pmol/mg protein - min}\) |
| Total homogenate | 1.02 ± 0.15     | 1.50 ± 0.23           | 0.95 ± 0.14                   | 46.9 ± 2.0          |
| Low speed pellet | 1.53 ± 0.05     | 2.15 ± 0.21           | 2.35 ± 0.55                   | 57.7 ± 7.1          |
| Plasma membrane | 24.21 ± 2.00     | 3.90 ± 0.13           | 10.50 ± 0.93                  | 18.9 ± 3.6          |
| Nuclei          | 3.36 ± 0.44     | 0.91 ± 0.11           | 0.98 ± 0.14                   | 20.9 ± 2.6          |
| Plasma membrane* | 11.41 ± 1.18     | 0.72 ± 0.13           | 4.70 ± 0.50                   | ND                  |

*Prepared by the method of Song et al. (12).
thus indicating that the epinephrine effect was mediated by produce a slight vasopressin, a representative experiments. Calcium uptake represents the difference in Ca\textsuperscript{2+} uptake in vesicles. Other hormones were tested to see if they also caused inhibition at 3 min. Epinephrine (10\textsuperscript{-5} M) produced a 31 ± 10% (n = 4, p < 0.01) inhibition of Ca\textsuperscript{2+} uptake, which was reduced by the \( \alpha_1 \)-adrenergic antagonist prazosin (10\textsuperscript{-6} M) to produce a slight 2 ± 12% (n = 3) increase in Ca\textsuperscript{2+} uptake, thus indicating that the epinephrine effect was mediated by \( \alpha_1 \)-adrenergic receptors. Angiotensin II (10\textsuperscript{-7} M) produced a 26 ± 5% inhibition (n = 3, p < 0.01), whereas glucagon (10\textsuperscript{-7} M) was ineffective with 8 ± 7% inhibition (n = 4, not significant). When glucagon 10\textsuperscript{-7} M was added together with 10\textsuperscript{-7} M vasopressin, a 45 ± 7% (n = 4) inhibition was obtained.

**DISCUSSION**

This report describes a method for isolating rat liver plasma membrane vesicles capable of high ATP-dependent Ca\textsuperscript{2+} transport activity. The entire procedure can be performed within 1 h and involves centrifugation in a Percoll self-forming gradient of the low speed pellet obtained by homogenization of liver in isotonic medium. Plasma membranes prepared in this way are purer with respect to enzyme markers and electron microscopic appearance compared with the other well characterized liver plasma membrane preparations (Table I and Ref. 12). Another interesting feature of the method is that another fraction enriched in nuclei can be separated at the same time.

The plasma membrane vesicles prepared using Percoll transport approximately 5-fold more Ca\textsuperscript{2+} than other membrane preparations (Fig. 1 and Ref. 16). One explanation may be that the Percoll-derived membranes have higher proportion of inside-out polarity. Another reason may be that the method involves isotonic medium and requires a relatively short time, whereas other methods use hypotonic media and/or take longer (4–16 h). Under the latter conditions, some peripheral proteins may be lost from the membranes and there may be less formation of well sealed vesicles.

The characterization of Ca\textsuperscript{2+} transport in hepatic membranes with a \( K_v \) of 14–17 nM for Ca\textsuperscript{2+} has been reported previously (16, 19). Our measurements on the \( K_v \) for Ca\textsuperscript{2+} uptake, namely 5.2 nM (Fig. 2), agree with these studies. However, all of these \( K_v \) values are below the concentration of cytosolic Ca\textsuperscript{2+} in unstimulated hepatocytes, which is approximately 200 nM (7). This suggests that factors in the intact cell may decrease the affinity of the Ca\textsuperscript{2+} pump.

In this work, we report for the first time the marked effects of vasopressin, angiotensin II, and epinephrine on liver Ca\textsuperscript{2+} transport activity in subsequently isolated plasma membrane vesicles. Although the dose response for vasopressin inhibition of Ca\textsuperscript{2+} uptake by plasma membrane vesicles correlates well with that for the other effects of vasopressin in liver, it is not likely that this inhibition is involved in the primary signal(ing) by which Ca\textsuperscript{2+}-mobilizing hormones elevate cytosolic Ca\textsuperscript{2+}. This elevation takes place 1–2 h after hormone addition to the liver (5, 7), whereas the inhibition of the plasma membrane Ca\textsuperscript{2+} pump is not significant at 1 min. On the other hand, this inhibition may be related to the maintenance of elevated cytosolic Ca\textsuperscript{2+}. This is possible because during hormone stimulation cytosolic Ca\textsuperscript{2+} remains elevated for some time before it returns to its resting level i.e. well beyond the time at which Ca\textsuperscript{2+} efflux from the liver cell ceases (3, 7). It is interesting to note that the time frame of vasopressin inhibition of Ca\textsuperscript{2+} uptake by plasma membrane vesicles correlates with that for phosphatidylinositol hydrolysis.
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in liver (20). Phosphoinositides are known to influence Ca\textsuperscript{2+}-ATPase activity in some tissues (21–23). The nature of the alterations induced in the liver (Ca\textsuperscript{2+}-Mg\textsuperscript{2+})-ATPase pump by the Ca\textsuperscript{2+}-dependent hormones is under further study.

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