Biochimica et Biophysica Acta, 1068 (1991) 1–8

ADONIS 000527369100321M

BBAMEM 75334

Identification of calmodulin-sensitive Ca^{2+}-transporting ATPase in the plasma membrane of bovine corneal epithelial cell

Peter S. Reinach [1,*], Nels Holmberg [1] and Raúl Chiesa [2]

[1] *Department of Ophthalmology, Washington University School of Medicine, St. Louis, MO (U.S.A.)* and [2] *Department of Ophthalmology, Columbia University, Physicians and Surgeons, New York, NY (U.S.A.)*

(Received 8 April 1991)

Key words: Cornea; Calmodulin; Epithelium; ATPase; Plasma membrane; Calcium ion transport

ATP-dependent Ca^{2+} uptake was characterized in a plasma membrane enriched fraction obtained from the bovine corneal epithelium. This uptake essentially represented intravesicular accumulation because 72% of the Ca^{2+} content was releasable following exposure to 10^{-6} M A23187. The substrate and Ca^{2+} requirements for maximal transport activity were similar to those described in the red blood cell because: (1) exogenous calmodulin (3 μM) significantly decreased the apparent K_m for Ca^{2+} to 0.31 μM and increased the rate of Ca^{2+} uptake; (2) a hydroxylamine labile Ca^{2+}-dependent phosphoenzyme intermediate was identified with an apparent molecular size of 140 kDa; (3) Ca^{2+}-dependent binding of ^{125}I-labelled calmodulin to this protein was demonstrated which could be antagonized with a calmodulin antagonist, trifluoperazine. These results show that the plasma membrane contains an ATP-dependent Ca^{2+} transporter. However, its relationship to a previously described high affinity form of Ca^{2+}-stimulated Mg^{2+}-dependent ATPase is not apparent because their $[Mg^{2+}]$ requirements to elicit maximal activity differed by two orders of magnitude.

Introduction

In the red blood cell, the Ca^{2+} transporter is a high affinity form of a Ca^{2+}-stimulated Mg^{2+}-dependent ATPase because the results of reconstitution studies showed that maximal enzymatic and transport activities could be elicited under the same assay condition [1,2]. Maximal activities were observed with physiological levels of $[Ca^{2+}]$ and millimolar levels of Mg^{2+} and ATP. This transporter has an apparent molecular size of 140 kDa and it requires Ca^{2+} either for the formation of an alkali-labile phosphorylated intermediate or binding of calmodulin [3–6]. Calmodulin selectively stimulates this transport activity through a decrease in the apparent K_m for Ca^{2+} and an increase in the V_{max} [7,8]. These properties of the red blood cell Ca^{2+} transporter are used as criteria to identify plasma membrane derived Ca^{2+} transport by other tissues.

In a wide variety of eukaryotes, there is plasma membrane derived Ca^{2+} transport activity [9]. The transporter has roles in the maintenance of Ca^{2+} homeostasis and receptor-effector coupling which are dependent on pump interaction with intracellular mediators such as calmodulin. An interaction with calmodulin can occur as a result of an increase in cytosolic $[Ca^{2+}]$ following activation of a Ca^{2+} mobilizing pathway. Stimulation of pump activity serves to restore the $[Ca^{2+}]$ to its control level and thereby terminate a response [10]. Therefore, a characterization of the properties of Ca^{2+} pump activity and the parameters which affect it are pertinent for a better understanding of both Ca^{2+} homeostasis and receptor-effector coupling.

In a plasma membrane enriched fraction of bovine corneal epithelium, high affinity Ca^{2+}-stimulated Mg^{2+}-dependent ATPase activity was identified provided there was no exogenous Mg^{2+}. Its activity could be further stimulated by calmodulin at physiological levels of $[Ca^{2+}]$ [11]. This enzymatic activity is not necessarily reflective of Ca^{2+} transport because in adipocytes a plasma membrane Ca^{2+} pump requires millimolar levels of Mg^{2+} for maximal activity whereas the identification of a high affinity form of Ca^{2+}-

* Current address: Medical College of Georgia, Physiology and Endocrinology, Augusta, GA 30912-3000, U.S.A.

Correspondence: P.S. Reinach, Medical College of Georgia, Physiology and Endocrinology, Augusta, GA 30912-3000, U.S.A.

stimulated Mg^{2+}-dependent ATPase was only possible without any exogenous (i.e., about 10 μM) Mg^{2+} [12,13]. It is not known if this difference in Mg^{2+} requirements reflects a type of plasma membrane Ca^{2+} transporter different than that in the red blood cell because there are no reports on the molecular size of this transporter in the adipocyte [9]. If the adipocyte and the red blood cell transporter are the same protein, this difference could mean that the adipocyte has more than one type of a high affinity form of Ca^{2+}-stimulated Mg^{2+}-dependent ATPase and only some of them are coupled to active Ca^{2+} transport.

In isolated bovine corneal epithelial cells, it was not possible to identify a role for Ca^{2+} as a second messenger even though there are different types of other evidence which are supportive of such a function for Ca^{2+} [14]. This evidence includes: (1) in the rabbit corneal epithelium and bovine corneal epithelial cells, an increase in phosphoinositide turnover was demonstrated following activation of phospholipase C by either norepinephrine or sodium fluoride [15,16]; (2) in a microsomal fraction from the bovine corneal epithelium, the identification of IP_3 binding sites which may be coupled to Ca^{2+} release channels [17]. Our uncertainties regarding any relationship between a high affinity form of Ca^{2+}-stimulated Mg^{2+}-dependent ATPase and active Ca^{2+} transport as well as the role of Ca^{2+} as a second messenger prompted us to study Ca^{2+} transport.

We demonstrate here and characterize ATP-dependent Ca^{2+} uptake in a plasma membrane enriched fraction of bovine corneal epithelium. As in the red blood cell, this transporter has a molecular size of 140 kDa and is stimulated by calmodulin. Therefore, the transporters appear to be identical even though maximal transport activity required about an 100-fold higher $[Mg^{2+}]$ than that needed to activate a previously described high affinity form of Ca^{2+}-stimulated Mg^{2+}-dependent ATPase. In the corneal epithelium, such a difference suggests that some of the forms of this enzymatic activity are not coupled to Ca^{2+} transport.

Materials and Methods

All reagents were analytical grade and were purchased from the following sources: New England Nuclear, [γ-^{32}P] ATP and $^{45}CaCl_2$; Amersham, ^{125}I-labelled calmodulin; Calbiochem, calmodulin; Bethesda Research Laboratories, ^{14}C-labelled protein molecular weight standards. All other chemicals were purchased from Sigma Chemical Company.

Bovine eyes were enucleated at a local abattoir within 1 h after death and transported to the laboratory in an ice cold NaCl Ringer's solution (pH 7.4) for immediate processing. The whole globe was rinsed with saline and the corneal epithelial cells were obtained by scraping away the surface layer of the cornea with a scalpel blade.

The epithelial cells collected from 70–90 eyes were placed in a solution containing 250 mM sucrose, 10 mM Hepes (4-(2-hydroxyethyl)-1-piperazinesulphonic acid), 30 μM PMSF (phenylmethylsulphonyl fluoride), 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM *N*-ethylmaleimide, 1.1 IU/ml aprotinin, and 1 mM EGTA (ethylene glycol bis (β-aminoethyl ether) tetraacetic acid), following adjustment of the pH to 7.5 with KOH. The corneal epithelial cells were initially homogenized at 1000 rpm with a motor driven Teflon pestle and a 10 ml homogenizer (Wheaton). After 5 strokes, this step was repeated with aliquots of the homogenate in a 5 ml homogenizer. The resulting homogenate was centrifuged for 10 min at $900 \times g$. This step was repeated following resuspension of the pellet. The supernatants (S-1) were pooled and then centrifuged at $20000 \times g$ for 30 min. The resulting supernatant (S-2) was then centrifuged either at $150000 \times g$ for 90 min to obtain an endoplasmic reticulum enriched pellet or at $56000 \times g$ for 60 min to obtain a different pellet (P-3) which was further processed with sucrose density gradient centrifugation. To obtain a plasma membrane enriched fraction, P-3 was resuspended with a 5 ml homogenizer in 0.5 ml of a solution containing 50 mM Hepes-KOH (pH 7.5), 250 mM sucrose, 1 mM DTT (dithiothreitol) (pH 7.5), and applied to the top of a discontinuous sucrose gradient in a 5 ml tube. The volume of each of the four layers was 1 ml and their specific gravities were 1.14, 1.16, 1.18 and 1.20. These solutions also contained 10 mM Hepes and 1 mM EGTA (pH 7.5). Samples of the resuspended P-3 pellet were centrifuged for 90 min at $150000 \times g$ in a SW 50.1 rotor. Each of the four fractions (B-1 to B-4) were collected by aspirating them from beneath the individual layers with a syringe connected to a bent needle. The four fractions were then individually pooled and diluted with a sucrose-free solution of the same composition (1:4 v/v). These fractions were centrifuged at $150000 \times g$ for 90 min and then resuspended in 1 ml of 50 mM Hepes and 250 mM sucrose (pH 7.5) and stored at −70 °C.

The plasma membrane, endoplasmic reticulum and mitochondrial marker assays used were 5'-nucleotidase, glucose-6-phosphatase and succinate–cytochrome-*c* reductase, respectively [18–20]. The ATP-dependent Ca^{2+} uptake assay was essentially the same as previously described [21]. The standard assay medium contained 50 mM Pipes-Tris buffer (at 37 °C), 80 mM K_2SO_4, 200 mM sucrose, 4 mM Tris oxalate, 1 mM $MgCl_2$, 0.1 mM EGTA, and 1.75 nCi $^{45}CaCl_2$. Its pH was adjusted to 7.5 with Tris base. The ratio of $[Ca^{2+}]$/[EGTA] was varied as previously described to obtain a desired free Ca^{2+} concentration [11,12]. Membrane protein was assayed as previously described us-

ing bovine serum albumin as a standard [22]. The medium had a volume of 350 μl which contained between 5 and 10 μg protein. Ca^{2+} uptake was initiated upon the addition of ATP from a stock solution and terminated by adding 50 μl of the assay medium to 2.8 ml of 1 mM $LaCl_3$ in 40 mM Pipes (pH 7.5), as it filtered through a 0.22 μm type GS Millipore filter, which was then rinsed twice with 3.0 ml of the same solution. Radioactivity contained on the filter was measured with a scintillation counter following solubilization of the filter in Filtron-X (National Diagnostics, Summerville, NJ). Active Ca^{2+} uptake was calculated from the difference in nmoles of Ca^{2+} accumulated per mg of protein in the presence and absence of ATP.

The methods for membrane phosphorylation, hydroxylamine reaction, SDS-PAGE and radioautography were essentially the same as those previously described [23]. The detection of calmodulin binding proteins, which were separated by SDS-PAGE according to the method of Laemmli [24], was carried out using ^{125}I-calmodulin and a gel overlay technique [25]. Membrane preparations of both corneal epithelium and red blood cells were fractionated with SDS-PAGE in four identical gels. One pair of gels was incubated in a buffer containing 2 μCi/ml ^{125}I-calmodulin and 2.5 mM trifluoperazine, one gel in the presence of 1 mM $CaCl_2$ and the other gel instead in the presence of 1 mM EGTA. As a control, a second pair of gels was incubated in a buffer containing only 2 μCi/ml ^{125}I-calmodulin, one gel in the presence of 1 mM $CaCl_2$ and the other gel instead in the presence of 1 mM EGTA. The binding of ^{125}I-calmodulin to the protein components in the gels was detected by autoradiography.

All values are shown as means $\pm$ S.E. Statistical significance was determined using a paired Student's *t*-test.

Results

To identify an appropriate fraction for a study on plasma membrane net Ca^{2+} uptake, we compared in four membrane fractions (B-1-4), the enrichments of 5'-nucleotidase activity, glucose-6-phosphatase and succinate–cytochrome-*c* reductase with respect to the initial homogenate. In Table I, the specific activities of 5'-nucleotidase and succinate-cytochrome-*c* reductase in B-1 are compared to those in S-1, S-2 and P-3 because B-1 was the most enriched fraction (i.e., 6.5-fold) in plasma membrane activity. Consistent with this result, B-1 was the only fraction that was slightly deenriched in succinate-cytochrome-*c* reductase. It was the least enriched with glucose-6-phosphatase and its specific activity was 8.3 $\pm$ 1.1 mmol P_i/min per mg protein which was 1.2-fold greater than in the homogenate but was only 35 $\pm$ 5% of the value in a microsomal enriched fraction (see Methods). Therefore, the B-1 fraction was used to study plasma membrane associated ATP-dependent Ca^{2+} uptake.

TABLE I

Marker activities in plasma membrane containing fractions

Data are given as the mean values with standard error of the mean for six preparations.

Fraction	5'-Nucleotidase (nmol/mg protein per h)	Succinate–cytochrome-c reductase (nmol/mg protein per min)
Homogenate	0.46 $\pm$ 0.14	0.78 $\pm$ 0.34
S1	0.44 $\pm$ 0.11	0.87 $\pm$ 0.19
S2	0.40 $\pm$ 0.11	0.58 $\pm$ 0.16
P3	2.12 $\pm$ 1.66	2.05 $\pm$ 0.99
B1	2.97 $\pm$ 0.93	0.70 $\pm$ 0.55

The time course for ATP-dependent Ca^{2+} uptake, in the presence of 5 mM ATP and 1.2 μM Ca^{2+}, is shown in Fig. 1. Under these conditions, there was maximal stimulation of the high affinity form of Ca^{2+}-stimulated Mg^{2+}-dependent ATPase [11]. Ca^{2+} uptake increased nearly linearly during the first 5 min followed by a slower pseudosaturating phase. Neither of these uptakes were ATP-limited because, with 1.25 instead of 5 mM ATP, the time courses were identical to one

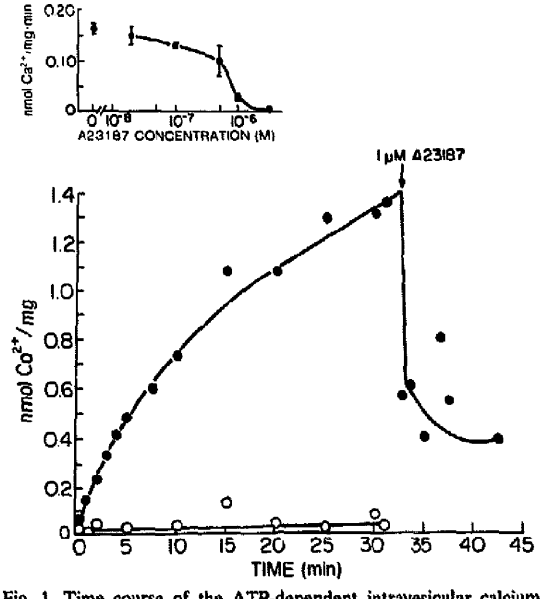

Fig. 1. Time course of the ATP-dependent intravesicular calcium accumulation and its release by the Ca^{2+} ionophore, A23187. Calcium uptake was measured at various times in the presence (closed symbols) or absence (open symbols) of 5 mM ATP. At 32.5 min, 1 μM A23187 was added. Inset: concentration-dependent effects of A23187 on the ATP-dependent intravesicular accumulation of calcium. The points are the means $\pm$ S.E. of measurements in three experiments each performed in triplicate.

another (data not shown). Ca^{2+} uptake was ATP dependent because in its absence the uptake was much smaller and invariant with time. On the average, in this and seven other experiments, 5 mM ATP significantly stimulated Ca^{2+} uptake by 12.24 ± 1.12-fold at 5 min. If either 5 mM UTP, TTP or CTP was instead substituted for ATP, the Ca^{2+} uptake in three different membrane preparations (each assay performed in duplicate) never exceeded 15% of the value measured with ATP.

To determine if Ca^{2+} uptake represented intravesicular accumulation, we measured the effects of preincubation with the Ca^{2+} ionophore, A23187, on Ca^{2+} uptake (c.f. Fig. 1). Thirty two and one half minutes after the initiation of Ca^{2+} uptake, 1 μM A23187 was added and Ca^{2+} uptake continued for another 10 min. Upon addition of this ionophore, the content of intravesicular calcium decreased to 28% of the value measured in other vesicles whose Ca^{2+} uptake was instead terminated at 30 min. This decrement indicates that most of the calcium accumulation occurred into an intravesicular compartment whereas some remained bound to the membranes and filters after washings. In other membrane preparations, the same protocol was followed as with 10^{-6} M A23187, except that the concentration of A23187 was varied between 5 nM and 5 μM. The results, shown in the inset to Fig. 1, indicated a concentration dependent decrease in calcium content which substantiates that intravesicular accumulation resulted from ATP-dependent Ca^{2+} uptake.

The dependence of changes in Ca^{2+} concentration on calcium uptake was studied to determine whether or not the ionic requirements for stimulation of intravesicular Ca^{2+} uptake are comparable to those of a high affinity Ca^{2+}-stimulated Mg^{2+}-dependent ATPase described in other tissues as well as the corneal epithelium [8,11]. In Fig. 2A, is shown the relationship between the concentration of Ca^{2+} and ATP-dependent Ca^{2+} accumulation at 5 min. Uptake increased almost linearly by about 125% between 0.15 and 0.5 μM Ca^{2+} whereas at higher concentrations it appeared to approach saturation. This Ca^{2+}-dependent increase in Ca^{2+} uptake occurred over the same concentration range known to stimulate the high affinity form of Ca^{2+}-stimulated Mg^{2+}-dependent ATPase [11].

The effects of exogenous $MgCl_2$ concentration on Ca^{2+} uptake are shown in Fig. 2B. Ca^{2+} uptake increased by 10-fold following an elevation in $MgCl_2$ concentration from 10 μM to 1 mM. At 1 mM $MgCl_2$, Ca^{2+} uptake was maximal because there was no further elevation in uptake following another 2-fold increase in $MgCl_2$ concentration. This increase in Ca^{2+} uptake was at variance with the Mg^{2+} requirement for maximal activation of a previously described Ca^{2+}-stimulated Mg^{2+}-dependent ATPase activity which was

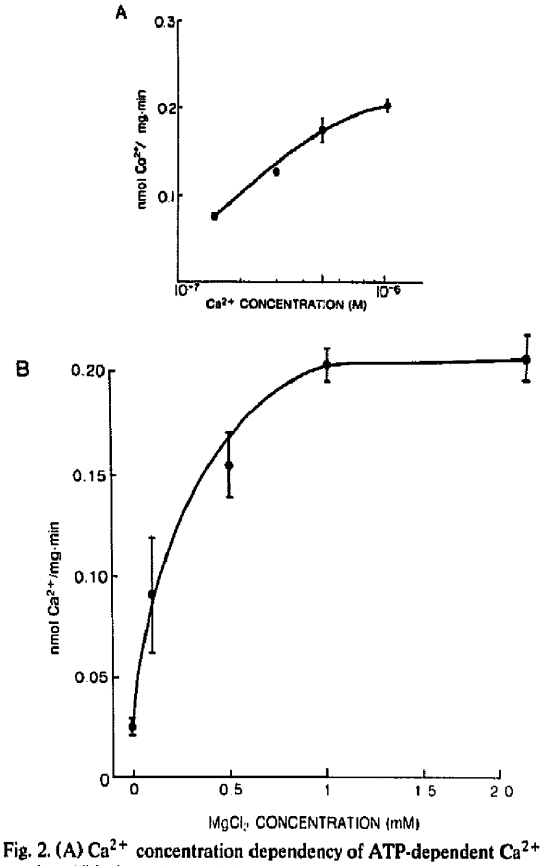

Fig. 2. (A) Ca^{2+} concentration dependency of ATP-dependent Ca^{2+} uptake. ATP-dependent calcium uptake was measured after 5 min incubation of membranes. Values are the means ± S.E. of four different membrane preparations each assayed in duplicate. (B) $MgCl_2$ concentration dependency of ATP-dependent Ca^{2+} uptake. Uptake was measured after 5 min following incubation in the assay medium with 5 mM ATP, 1.04 μM Ca^{2+} and as described in Methods. Values are the means ± S.E. of four different membrane preparations each assayed in duplicate.

instead maximally activated with 1 μM Ca^{2+} and endogenous (i.e., about 10 μM) Mg^{2+} [11].

We previously showed that 6 μM calmodulin maximally stimulated by about 200% ATP hydrolysis by a high affinity form of Ca^{2+}-stimulated Mg^{2+}-dependent ATPase. This was a selective effect because a preincubation with 300 μM trifluoperazine, a calmodulin antagonist, effectively suppressed calmodulin stimulation [11]. These effects suggested a plasma membrane origin for this activity because the only pump protein with which calmodulin is known to interact is a plasma membrane ATPase [7].

To substantiate that this ATPase activity is plasma membrane derived and mediates Ca^{2+} uptake, we measured the effects of exogenous calmodulin on Ca^{2+} uptake. With 0.3 μM Ca^{2+} (i.e., a submaximal concentration), the results shown in Fig. 3 indicate that be-

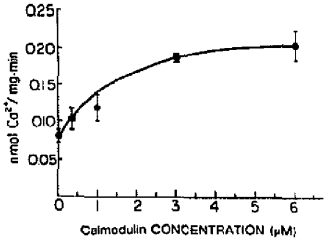

Fig. 3. ATP-dependent Ca^{2+} uptake after 5 min of incubation as a function of calmodulin concentration in the presence of 0.3 μM Ca^{2+}. Values are the means ± S.E. of four different membrane preparations each performed in triplicate.

tween 0.4 and 3 μM calmodulin, ATP-dependent Ca^{2+} uptake increased by 115%. No additional significant stimulation was measured with 6 μM calmodulin. These corresponding effects of calmodulin substantiate the notion that the plasma membrane preparation contains an ATPase which mediates Ca^{2+} uptake.

The results shown in Table II indicate that 3 μM calmodulin decreased the apparent K_m for Ca^{2+} of the ATP-dependent Ca^{2+} uptake without significantly changing the V_{max}. This decrease in the apparent K_m is consistent with the mechanism of stimulation of Ca^{2+} uptake by calmodulin in red blood cell plasma membranes [26–29]. In other studies employing red blood cells, however, calmodulin was reported to affect both of these kinetic parameters [9].

Characteristic of ion transporting ATPases is the formation of hydroxylamine labile acyl phosphate intermediates [30,31]. The phosphorylated intermediate of the Ca-transporting ATPase in the red blood cell membrane requires Ca^{2+} for its formation and has an apparent molecular size of 138 kDa [32]. Similarly the phosphorylated intermediate of the Na/K-ATPase (the α-subunit) requires Na^+ for its formation, is quickly hydrolyzed in the presence of K^+ and it has an apparent molecular size of 93 kDa [33]. These phosphorylated intermediates can be detected in membrane

TABLE 2

Effect of 3 μM calmodulin on kinetic parameters for ATP-dependent Ca^{2+} uptake

Values are means ± S.E. of three different membrane preparations; each of which were assayed in triplicate. ATP-dependent Ca^{2+} uptake was assayed in standard assay medium described in Methods except that the vehicle either contained calcium alone or in addition 3 μg calmodulin.

	K_m (Ca^{2+}) (μM)	V_{max} (nmol/min per mg)
Control	0.51 ± 0.13	0.31 ± 0.11
Calmodulin	0.31 ± 0.11 [a]	0.34 ± 0.07 [b]

[a] $P < 0.05$.
[b] *N.S.*

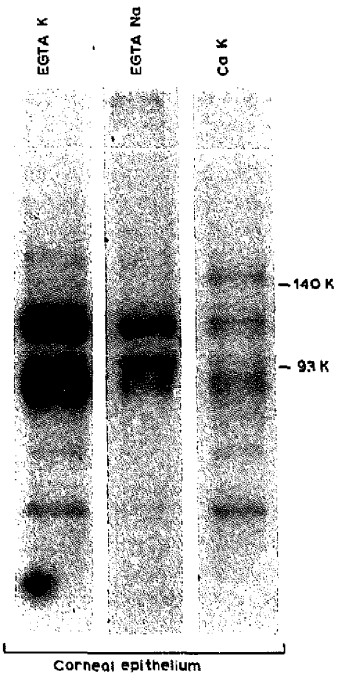

Fig. 4. SDS-PAGE analysis of the corneal epithelium B-1 membrane fraction after phosphorylation with [γ-^{32}P]ATP. The autoradiograph of a 6% gel is shown. A Ca-dependent phosphoprotein with molecular mass 140 kDa (3rd lane), and a Na-stimulated/K-inhibited phosphoprotein with molecular mass 93 kDa (2nd lane) are apparent. The broad and diffuse band with a molecular mass lower than 93 kDa (1st lane) was consistently observed. This component unrelated to the phosphorylated intermediates is probably a polypeptide phosphorylated at Ser, Thr or Tyr residues by a protein kinase because it is not hydroxylamine sensitive.

preparations incubated with [γ-^{32}P]ATP in the presence or absence of the appropriate ion, with SDS-PAGE analysis of the reaction mixture followed by autoradiography [23]. Accordingly, phosphorylation experiments were carried out to investigate the formation of a Ca^{2+}-dependent phosphorylated intermediate in the plasma membrane enriched fraction and determine its apparent molecular size with SDS-PAGE. Since the plasma membranes of all eukaryotes contain Na/K-ATPase, it was also of interest to investigate the presence of a Na^+-dependent phosphorylated intermediate, which would validate the plasma membrane origin of the preparation.

The results of the phosphorylation experiments are shown in Fig. 4. Unique Ca^+ and Na^+-dependent bands were detected in the corneal epithelial membrane fraction. A 140 kDa band was observed in the presence of Ca^{2+} but it was absent in the presence of EGTA (i.e., Ca^{2+}-free). A 93 kDa band was detected in the presence of Na^+ but it was absent when NaCl

was replaced with KCl. The Ca^{2+} dependency and the apparent molecular size of the 140 kDa band are suggestive of the phosphorylated intermediate of a red blood cell-like Ca^{2+}-stimulated Mg^{2+}-dependent ATPase. As expected, a Na^+-dependent phosphoprotein with a M_r of 93 kDa was also detected, corresponding to the alpha subunit of the Na/K-ATPase, validating the plasma membrane origin of the preparation. To verify the acyl phosphate character, of the Ca^{2+}-dependent phosphorylated intermediate, a preparation was phosphorylated in the presence of Ca^{2+} and then exposed to hydroxylamine.

The ^{32}P-phosphorylated products were analyzed with SDS-PAGE. The autoradiograms are shown in Fig. 5 and indicate that one protein ($M_r = 140$ kDa) contained an acylphosphate bond. Its hydroxylamine lability and apparent molecular size suggest that this phosphoprotein is the phosphorylated intermediate of a plasma membrane Ca^{2+}-dependent ATPase.

Direct assessment of a calmodulin interaction with a plasma membrane ATPase pump protein was performed. The proteins were separated with SDS-PAGE and by an overlay technique they were then incubated with ^{125}I-calmodulin either in the presence of Ca^{2+} or EGTA. The proteins which bound ^{125}I-calmodulin in a Ca^{2+}-dependent manner were identified by autoradiography. The lane of the autoradiogram shown in Fig. 6, which is labeled CE, indicates that several components in the B-1 fraction bound calmodulin in a Ca^{2+}-dependent manner. This effect was the basis for comparing calmodulin binding with that of a pure red blood cell plasma membrane preparation. In the adjacent lane, is shown the pattern of calmodulin binding of the red blood cell plasma membrane preparation. This preparation contained two prominent bands both of which bound calmodulin provided Ca^{2+} was present. Note, however, that the only band common to both preparations, which bound calmodulin, was the 140 kDa band (i.e., Ca^{2+} transporting ATPase). The selectivity of these interactions with calmodulin was validated through a determination of the effect on calmodulin binding of preincubation of the membranes with a calmodulin antagonist (2.5 mM trifluoperazine). In both the plasma membrane of the corneal epithelium and the red blood cell, trifluoperazine preincubation completely prevented calmodulin binding to the 140 kDa band (Fig. 6). These results show that the 140 kDa protein in the corneal epithelium, whose binding to calmodulin was Ca^{2+}-dependent, is homologous to the Ca^{2+} transporting ATPase previously characterized in plasma membrane preparations of red blood cells [3].

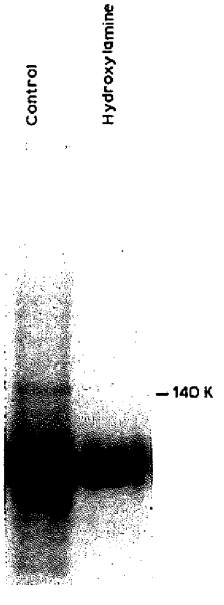

Fig. 5. SDS-PAGE analysis of a ^{32}P-labelled corneal epithelium B-1 membrane preparation after hydroxylamine reaction at pH 5.3. The autoradiograph of a 6% gel is shown. The Ca-dependent phosphoprotein with molecular mass 140 kDa is hydroxylamine sensitive and corresponds therefore to an acyl-phosphate. The intense broad band with lower molecular mass was consistently resistant to hydroxylamine. Therefore, it is unrelated to any of the labile phosphorylated intermediates.

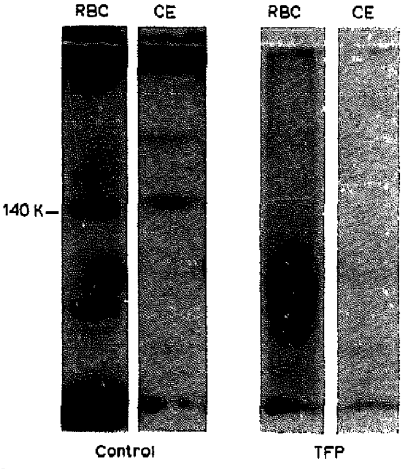

Fig. 6. ^{125}I-calmodulin binding to corneal epithelium (CE) and red blood cell (RBC) membrane preparations fractionated by SDS-PAGE in 6% gels. The autoradiographs of the gels after binding in the presence of 1 mM $CaCl_2$ are shown. In the presence of 1 mM EGTA, instead of $CaCl_2$, no ^{125}I-calmodulin binding was detected (not shown).

Discussion

A plasma membrane Ca^{2+} transporter was identified in the bovine corneal epithelium because its requirements for maximal activation are comparable to those in the red blood cell, namely: (1) strict require-

ment for ATP and millimolar levels of Mg^{2+}; (2) physiological levels of $[Ca^{2+}]$; (3) stimulation by exogenous calmodulin which was associated with a decrease in the apparent K_m for Ca^{2+}. Note that neither the magnitude of the decrease in the K_m nor the small and insignificant increase in V_{max} are in close agreement with the effects observed in reconstitution studies employing $(Ca^{2+}+Mg^{2+})$-ATPase purified from erythrocyte membrane and heart sarcolemma [7,35]. However, the apparent K_m values for Ca^{2+} (i.e., 0.4 μM) in the presence of calmodulin in these systems is in excellent agreement with our value of 0.3 μM. Therefore, our observed smaller changes in values of the kinetic parameters may simply mean that the membranes still contain effectors of ATPase activity which have an activating effect. Such an effector could include endogenous calmodulin which is tightly bound despite our efforts to completely strip it away. More direct evidence for the cellular origin of this activity stems from a characterization of some of the molecular properties of the protein. As in the red blood cell, both its binding to calmodulin and formation of a phosphorylated intermediate were Ca^{2+}-dependent [9]. These considerations along with the close correspondence to the molecular size of the red blood cell Ca^{2+} transporter (i.e., 140 kDa) make it apparent that the plasma membrane of the corneal epithelium contains a Ca^{2+} transporter [7]. Additional support for the plasma membrane origin of the transporter is that the B-1 fraction was coenriched in Na/K-ATPase activity (data not shown) and contained the alpha subunit of Na/K-ATPase. Consistent with the very low level of contamination of the B-1 fraction with the ER marker, glucose-6-phosphatase, an endoplasmic reticulum Ca^{2+} pump could not be identified. Were this pump present following SDS-PAGE, a Ca^{2+}-dependent and hydroxylamine-sensitive phosphorylated intermediate would have appeared in the region of 100 kDa [34].

In a number of tissues, in which plasma membrane active Ca^{2+} transport was characterized, the conditions needed for maximal activation of active Ca^{2+} transport and $(Ca^{2+}+Mg^{2+})$-ATPase were identical to one another [9]. However, in liver and neutrophils even though there is plasma membrane Ca^{2+} transport it is mediated by what appears to be a different protein than in erythrocyte membranes and heart sarcolemma [7,9,35]. In the liver, the labile phosphorylated intermediate of the transporter has a molecular mass of 70–105 kDa instead of about 140 kDa. This difference is reflected in its lack of binding to calmodulin and different substrate and ionic requirements for maximal activity. However, in the plasma membranes of the rat myometrium and the human placenta, even though there is a plasma membrane Ca^{2+} transporter nearly identical to that of the red blood cell and heart sarcolemma, the identity of the $(Ca^{2+}+Mg^{2+})$-ATPase coupled to active Ca^{2+} transport was not apparent. This masking effect suggests the presence of other $(Ca^{2+}+Mg^{2+})$ ATPases of unknown function [36,37]. Similarly, in the corneal epithelium the properties of the plasma membrane $(Ca^{2+}+Mg^{2+})$-ATPase coupled to active Ca^{2+} transport could not be resolved because of the 100-fold difference between the Mg^{2+} concentrations that are required to elicit their maximal activation.

The identification of plasma membrane Ca^{2+} transport in the corneal epithelium is relevant to elucidating receptor-effector coupling. Some physiological responses may be linked to the activation of receptors which are documented, in other tissues, to elicit the mobilization of Ca^{2+} from intracellular stores [10]. However, the evidence in the corneal epithelium that Ca^{2+} serves as a second messenger is somewhat contradictory. Inasmuch as, there is convincing evidence for α_1-adrenergic mediated pathways that are linked to endoplasmic reticulum Ca^{2+} release channels, it was unexpected that no increases in intracellular Ca^{2+} concentration could be elicited from an exposure to a Ca^{2+}-mobilizing adrenergic or cholinergic agonist [14,15–17]. One possible explanation for this negative result, based on the current study, is that any elicited increase in Ca^{2+} was very short lived due to rapid activation of a plasma membrane Ca^{2+} pump which served to quickly restore Ca^{2+} to its control level.

Acknowledgements

This research was supported by a NIH grant, EY04795 (P.R.). The helpful comments of Dr. Madeleine Kirchberger on a preliminary version of this manuscript are deeply appreciated.

References

1 Niggli, V., Penniston, J.T. and Carafoli, E. (1979) J. Biol. Chem. 254, 9955–9958.
2 Carafoli, E., Zurini, M., Niggli, V. and Krebs, J. (1982) Ann. NY Acad. Sci. 402, 304–328.
3 Graf, E., Verma, A.K., Gorski, J.P., Lapaschuk, G., Niggli, V., Zurinc, M., Carafoli, E. and Penniston, J.T. (1982) Biochem. 21, 4511–4516.
4 Ghijsen, W.E.J.M, DeLung, M.D. and Van Os, C.H. (1982) Biochim. Biophys. Acta 689, 327–336.
5 Bygrave, F.L. (1978) Biochem. J. 170, 87–91.
6 Selinger, Z., Naim, E. and Lasser, M. (1970) Biochim. Biophys Acta 203, 326–334.
7 Niggli, V., Adunyah, E.S., Penniston, J.T. and Carafoli, E. (1981) J. Biol. Chem. 256, 395–401.
8 Carafoli, E. (1987) Annu. Rev. Biochem. 56, 395–433.
9 Carafoli, E. (1991) Physiol. Rev. 71, 129–153.
10 Abdel Latif, A.A. (1986) Pharm. Rev. 38, 228–257.
11 Reinach, P. and Holmberg, N. (1987) Curr. Eye Res. 6, 399–405.
12 Pershadsingh, H. and McDonald, J.M. (1980) J. Biol. Chem. 255, 4087–4093.
13 Pershadsingh, H.A. and McDonald, J.M. (1979) Nature 288, 492–495.

14 Cork, R.J., Reinach, P., Moses, J. and Robinson, K.R. (1987) Curr. Eye Res. 6, 1309–1317.
15 Akhtar, R.A. (1987) Exp. Eye Res. 44, 849–862.
16 Akhtar, R.A. (1988) Curr. Eye Res. 7, 487–495.
17 Akhtar, R.A. and Abdel Latif, A.A. (1990) Curr. Eye Res. 9, 387–392.
18 Avruch, J. and Wallach, D.F.H. (1971) Biochim. Biophys. Acta 233, 334–337.
19 Aronson, N.N., Jr. and Touster, O. (1974) Methods Enzymol. 31, 90–102.
20 Linhardt, K. and Walter, K. (1965) Phosphatases, in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.), pp. 779–785, Academic Press, New York, NY.
21 Colca, J.R., Kotagal, N., Lacey,, P.E., McDaniel, M.L. (1983) Biochim. Biophys. Acta 729, 176–184.
22 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1981) J. Biol. Chem. 193, 265–275.
23 Chiesa, R., Sredy, J. and Spector, A. (1985) Curr. Eye Res. 4, 897–903.
24 Laemmli, U.K. (1970) Nature 227, 680–685.
25 Carlin, R.K., Grab, D.J. and Sickevitz, P. (1981) J. Cell Biol. 89, 449–455.
26 Klinger, R., Wetzker, R., Fleisher, I. and Frunder, H. (1980) Cell Calcium 1, 229–240.
27 Gopinath, R.M. and Vincenzi, F.F. (1977) Biochem. Biophys. Res. Commun. 77, 1203–1209.
28 Roufogalis, B. (1979) Can. J. Physiol. Pharm. 57, 1331–1349.
29 Scharff, O. and Foder, R. (1978) Biochim. Biophys. Acta 509, 67–77.
30 Pedersen, P.L. and Carafoli, E. (1987) Trends Biochem. Sci. 12, 146–150.
31 Pederson, P.L. and Carafoli, E. (1987) Trends Biochem. Sci. 12, 186–189.
32 Penniston, J.T. (1982) Ann. NY Acad. Sci. 402, 296–302.
33 Skou, J.C. (1975) Q. Rev. Biophys. 7, 401–434.
34 MacLennan, D.H., Brandl, C.J., Korczak, B. and Green, N.M. (1985) Nature 316, 696–700.
35 Caroni, P., Zurini, M., Clark, A. and Carafoli, E. (1983) J. Biol. Chem. 258, 7305–7310.
36 Enyedi, A., Minami, J., Caride, A. and Penniston, J.T. (1988) Biochem. J. 252, 215–220.
37 Kelley, L., Borke, J.L., Verma, A.K., Kumar, R., Penniston, J.T. and Smith, C.H. (1990) J. Biol. Chem. 265, 5453–5459.