Calcium and Adenosine Triphosphate Binding to Renal Membranes

ROGER F. PALMER and VIRGINIA A. POSEY

From the Department of Pharmacology and Therapeutics, University of Florida College of Medicine, Gainesville 32601. Dr. Palmer is a Markle Scholar in Academic Medicine. His present address is Department of Medicine, University of Miami School of Medicine, Miami, Florida 33152.

ABSTRACT Calcium binds to membranous structures isolated from rabbit kidney cortex homogenates. The binding is enhanced by ATP and Mg$^{++}$ in combination. Other nucleotides, ITP and GTP, do not have this property. In contrast to similar preparations of nerve and muscle, the binding is not augmented by oxalate (3–100 mM). Also, binding of calcium cannot be correlated with ATP hydrolysis. p-Chloromercuribenzoate and the mercurial diuretic agent mercaptomerin inhibit the binding of calcium. This system can be distinguished from the binding of calcium by mitochondria by lack of azide inhibition and by failure of ADP-succinate to substitute for ATP. $^{14}$C- and $\gamma^{32}$P-labeled ATP bind to the renal membranes in the absence of calcium, but only the $^{32}$P binding increases when calcium is added. The ratio of $^{32}$P bound to $^{45}$Ca bound is 2:1. The above data are consistent with a hypothesis that calcium is metabolically bound to renal membranes and that this binding is associated with membrane phosphorylation. Such a formulation may have pertinence to the conformational state of renal membranes and subsequent permeability characteristics. It also allows for the concept that membrane stability requires metabolic participation, as well as calcium ions.

INTRODUCTION

The interaction of calcium with membranes is of extreme biological importance. Calcium binds to natural and artificial membranes, altering permeability characteristics and electrical properties (1). With whole membranes or membrane fragments of muscle (2) or nerve (3), a portion of the binding is active; that is, an energy source, usually ATP, is required and the uptake proceeds against electrochemical gradients. In muscle, fragments of membranes of the sarcoplasmic reticulum bind calcium passively and, in addition, accumulate it actively, utilizing ATP as the energy source. A role in muscle relaxation has been ascribed to this latter property (4). Membrane fragments from peripheral nerve (3) and brain (5, 6) have similar characteristics. However, the physiological role is obscure.
It is the purpose of this report to describe calcium binding of a somewhat
different sort to membrane fragments from kidney, a noncontractile and
(as far as is known) nonexcitable tissue. Binding occurs in the absence of
ATP, but proceeds to a greater extent in its presence. The ATP-dependent
binding, in contrast to nerve or muscle, is not enhanced by oxalate, nor does
it appear to be associated with ATP hydrolysis. The uptake is associated with
binding of $^{32}$P from labeled ATP-$\gamma$-$^{32}$P, but not $^{14}$C from $^{14}$C-ATP. These re-
results are discussed in relation to a calcium-dependent phosphorylated com-
plex that may be related to the renal membrane conformational state.

METHODS

Grana Preparation  Rabbits were killed by a blow on the head. The kidneys
were quickly removed, and the cortex was dissected and minced with scissors, using
cold (4°C) 0.25 M sucrose as the homogenizing medium. The mince was homogenized
in 10:1 (w/v) homogenizing medium with all-glass TenBroeck homogenizers and
centrifuged at 1000 g for 20 min in a Lourdes model AA-C centrifuge (Lourdes In-
strument Corp., Brooklyn, N. Y.). The pellet was washed with 2.5:1 (w/v) 0.25 M
sucrose at 1000 g for 20 min and discarded. The supernatant, with washings added,
was centrifuged at 10,000 g for 20 min in a Spinco model L ultracentrifuge at 2°C.
This pellet was used for mitochondrial assays. The remaining supernatant was cen-
trifuged at 78,000 g for 45 min. The resulting pellet was rinsed with 5 ml of homogeniz-
ing medium and then resuspended in 10:1(w/v) 0.25 M sucrose. This suspension
contained 0.8–1.3 mg protein/ml as determined by the method of Lowry et al. (7),
and was used for grana$^1$ assays. All preparations were used within $\frac{1}{2}$ hr after final
resuspension.

$^{45}$Ca Uptake  Grana suspensions (1.2 ml) were added to 10.8 ml of an incuba-
tion mixture containing reagents as reported in the tables and figures. All final
mixtures contained 3 mM histidine, pH 6.8. These mixtures, now containing 0.08–
0.13 mg protein/ml, were incubated at 37°C for 10 min in a water bath, except where
noted. Then 5 ml aliquots were pipetted onto a Millipore suction apparatus with 25
mm diameter, 0.45 μ pore size, type HA Millipore filters. These filters had previously
been rinsed with 250 mM KCl and washed with 10 ml glass-distilled water. It was
found that batches of filters received from the manufacturer bound calcium to the
filters nonspecifically to varying degrees. With the KCl treatment of the filters, much
smaller and more uniform amounts of calcium were bound to the filter papers in the
absence of microsomes. The mixture was completely filtered within 5 sec by vacuum.

The sediment on each filter was washed with 10 ml of cold (4°C) 0.25 M sucrose.
The filters were removed, dried under heat lamps, and placed in glass scintillation
vials containing 15 ml fluid containing 70% toluene, 30% methyl alcohol, 0.1 g/liter
POPOP [1,4-bis[2-(5-phenyloxazolyl)]benzene, scintillation grade, Packard In-
strument Co., Chicago, Ill.] and 4.0 g/liter PPO [2,5-diphenyloxazole, scintillation
grade, Packard].

$^1$ Grana, microsomes, and 78,000 g pellet are used interchangeably, and all refer to the ultracen-
trifugal fraction that sediments at 78,000 g.
Standards of $^{45}$Ca were prepared by absorbing 50 μl of the radioactive solution on a Millipore filter, drying, and placing it in the scintillation vial, as above. The samples were counted in a Beckman model LS-200 counter (Fullerton, Calif.). Controls containing all reagents except grana were run simultaneously in each experiment, being treated in the same manner as those with grana, and were used in correcting for the amount of $^{45}$Ca bound nonspecifically to the filter papers. The amount bound to the filter papers in the absence of grana was always less than 5 % of the total calcium taken up.

Adenosine triphosphatase activity was measured using the incubated mixture. 1 ml of incubated mixture was added at 10 min to 0.1 ml 50 % trichloroacetic acid Inorganic phosphorus (Pi) was then determined by the method of Fiske and Subbarow (8).

Rate of Calcium Uptake Mixtures were prepared as above and warmed to 37°C in a water bath. Grana were then added at time 0. At timed intervals, 2.5 ml aliquots were taken, filtered, washed with 5 ml of cold (4°C) 0.25 M sucrose, and counted in the usual manner.

$^{32}$P and $^{14}$C Uptake Labeled ATP, either as $^{14}$C or as $^{32}$P, was checked for purity (see below). Approximately 1 μc/ml was incubated and sampled in the same manner as the procedure for $^{45}$Ca, using the appropriate controls.

A potassium phosphate buffer, pH 6.8, containing tracer amounts of Na$_2$H$_2$PO$_4$, was used in measuring phosphate uptake. The radioactivity of this solution was adjusted to approximately 1000 cpm/mumole. Sampling was done according to the method described for $^{45}$Ca, using the appropriate controls.

Washing Procedures We had previously determined that when ions are present in the experimental tube in excess of the isotope, washing with sucrose alone is as effective in removing nonspecific counts as washing with “cold” solutions identical with the experimental solution (9). This does not mean that there is not a slow exchange of $^{45}$Ca for bound $^{45}$Ca or ATP for bound ATP-$^3$P, but the washing procedure is so short that insufficient time elapses for significant exchange to occur (<30 sec). Consequently, sucrose washes, which were as effective as any other in removing nonspecific counts, were used throughout.

Chemicals All solutions were prepared with glass-distilled water. L-Histidine HCl (Nutritional Biochemicals Corp., Cleveland, Ohio) was neutralized to pH 6.8 with imidazole (Matheson, Coleman, and Bell, Cincinnati, Ohio). All salts were analytical grade chlorides (Mallinckrodt Chemical Works, St. Louis, Mo.). $^{45}$Ca (Abbott Laboratories, North Chicago, Ill.) was used with cold CaCl$_2$ to give final counts between 5000 and 100,000 cpm/filter. Disodium ATP (Sigma Chemical Co., St. Louis, Mo.) was rendered free of sodium and calcium by passage through Dowex 50W-X8 in the H$^+$ form, and it was then neutralized with solid Tris (Trizma, Sigma Chemical Co.) to pH 7.0. Thin layer chromatography, according to the method presented by Randerath (10) with silica gel and solution 2, was used to check the purity of the ATP, ATP-$^3$P, and $^{14}$C-ATP (Nuclear-Chicago Corp., Des Plaines, Ill.). Disodium phosphate-$^{32}$P (New England Nuclear Corp., Boston, Mass.) was used
with KHPO₄ buffer. ITP, GTP, and ADP (Sigma Chemical Co.) were the trisodium, sodium, and sodium salts, respectively.

**Electron Microscopy**  Electron micrographs of the 78,000 g pellet were made according to a slightly modified method of Luft (11), using 600 Å sections and a Porter-Blum MT-2 ultramicrotome.

**RESULTS**

**Electron Microscopy of 78,000 g Pellet**

Fig. 1 illustrates the typical appearance of a section of the pellet in question. There are many vesicular structures, and frequent longitudinal masses resembling brush borders or microvilli. No mitochondria or mitochondrial fragments can be specifically identified. However, damaged fragments may be present.

**Characteristics of Centrifugal Fractions**

The absence of functioning mitochondria is confirmed by biochemical differences between the two fractions presented in Table I. Under the conditions employed, the mitochondrial fraction exhibits marked azide sensitivity to calcium uptake, whereas with the membrane fraction 5 mM azide has no effect. Succinate plus ADP restores calcium uptake by the mitochondrial fraction in the absence of exogenously added ATP. Yet this combination has no effect on the lighter precipitate. These facts support the view that mitochondrial fragment contamination is not an important feature contributing to the results obtained.

There is a small but insignificant amount of ouabain-sensitive Na⁺ + K⁺ ATPase in the microsomal and mitochondrial fraction under the conditions of isolation employed (Table I). The activity of this system in the lighter precipitate is much less than previously reported (12), most likely because of the absence of deoxycholate and EDTA from the homogenizing medium. Potassium oxalate, whether added to the incubation tube or included in the homogenizing medium, does not enhance calcium uptake in the lighter fraction. This observation represents a marked difference from similar systems in muscle (13) or nerve (3) fragments. The explanation offered for the enhancement of calcium binding by oxalate involves the accumulation of free calcium inside the vesicle to a concentration greater than the outside concentration. The free calcium concentration reached inside is sufficient to precipitate calcium as the insoluble oxalate salt. The data presented here have not demonstrated a “pump” similar to the muscle sarcoplasmic reticulum pump in this fraction. An alternative explanation would be that oxalate does not permeate the vesicle. The latter prospect seems unlikely, in view of the fact
Figure 1. Electron micrograph of an osmium tetraoxide-fixed pellet. *V* = vesicle; *M* = microvillus or brush border. × 35,000.
that the same negative result is seen even when oxalate (3–100 mM) is present
during homogenization of the tissue (data not shown).

TABLE I
UPTAKE OF CALCIUM AND ATPase ACTIVITY IN MITOCHONDRIAL
AND MICROSOMAL FRACTION OF RABBIT KIDNEY CORTEX
Results are means ± standard errors of three experiments.

| Conditions | 3 mM ATP, 20 µM 45CaCl2, 3 mM MgCl2, plus | 78,000 g ppt | 10,000 g ppt |
|------------|------------------------------------------|---------------|---------------|
|            | µmoles/g protein | µmoles/hr/mg protein | µmoles/g protein | µmoles/hr/mg protein |
| No addition | 5.47 ± 0.71 | 15.25 ± 1.56 | 69.88 ± 8.50 | 18.38 ± 0.56 |
| 3 mM K oxalate | 5.81 ± 0.18 | 16.75 ± 1.58 | 40.04 ± 2.41 | 20.91 ± 0.79 |
| 5 mM Na azide | 4.82 ± 0.30 | 12.50 ± 0.34 | 2.76 ± 0.31 | 10.09 ± 0.29 |
| 100 mM NaCl | 5.66 ± 0.49 | 16.69 ± 0.89 | 52.32 ± 7.69 | 20.00 ± 0.71 |
| 100 mM KCl | 6.67 ± 0.51 | 16.81 ± 1.11 | 78.16 ± 16.99 | 20.53 ± 0.81 |
| No ATP | 1.37 ± 0.12 | 0 | 1.07 ± 0.11 | 0 |
| 100 mM NaCl, 20 mM KCl | 5.85 ± 0.54 | 19.06 ± 0.44 | 61.90 ± 8.96 | 24.25 ± 0.56 |
| 100 mM NaCl, 20 mM KCl, 10^{-4} M ouabain | 5.19 ± 0.43 | 17.41 ± 0.34 | 62.20 ± 10.69 | 22.41 ± 0.69 |
| No ATP; 3 mM Na succinate, 3 mM ADP | 1.68 ± 0.14 | 55.71 ± 7.2 |

TABLE II
EFFECT OF VARIOUS NUCLEOTIDES ON CALCIUM
UPTAKE IN THE MICROSOMAL FRACTION
Results are the means ± standard errors of three experiments.

| Conditions | 20 µM 45CaCl2, 3 mM MgCl2, plus | 78,000 g ppt | 10,000 g ppt |
|------------|---------------------------------|---------------|---------------|
|            | µmoles/g protein | µmoles/hr/mg protein |
| No addition | 1.27 ± 0.05 | 0 |
| 3 mM GTP | 0.77 ± 0.11 | 10.66 ± 0.67 |
| 3 mM ITP | 1.08 ± 0.14 | 15.75 ± 1.09 |
| 3 mM ATP | 4.27 ± 0.23 | 15.00 ± 0.75 |

Nucleotide Specificity
As shown in Table II, GTP, ITP, and ATP were hydrolyzed, but only ATP was capable of increasing calcium uptake above control levels. Inosine triphosphate was hydrolyzed to the same extent as ATP, yet was incapable of increasing calcium uptake.

Table III shows a requirement for Mg ++ as a cofactor for the phenomenon observed, as in other ATP-dependent reactions (14).
TABLE III

Mg++ AND ATP REQUIREMENT FOR CALCIUM BINDING OF THE MICROSOMAL FRACTION OF KIDNEY CORTEX

Results are the means ± standard errors of 12 separate preparations unless otherwise noted.

| Conditions: 20 μM CaCl₂ plus | Ca²⁺ uptake μmoles/g protein |
|-------------------------------|-----------------------------|
| 3 mM ATP                      | 0.79 ± 0.23*                |
| 3 mM MgCl₂                    | 1.40 ± 0.17                 |
| 3 mM ATP, 3 mM MgCl₂          | 4.82 ± 0.63                 |
| 3 mM ATP, 3 mM MgCl₂, 3 mM K oxalate | 5.47 ± 0.71               |

* Mean of three experiments in three preparations.

Rate of Calcium Uptake and Calcium Concentration Dependence of the Reaction

In Fig. 2 the initial rate of accumulation, as well as the amount of calcium taken up after 45 min, is a function of the initial Ca²⁺ concentration of the medium. The plot also depicts the time course of binding in the absence of ATP. The binding prior to 30 sec is the same whether ATP is present or not.

![Time course of calcium uptake](image-url)
Further uptake of calcium does not occur unless ATP is present. Binding to the membrane fraction is virtually complete within the first sampling period (30 sec) in the absence of ATP. Further uptake in the presence of ATP (Fig. 2) suggests a time-dependent chemical reaction, and the shape of the curves suggest saturation kinetics; i.e. as the calcium in the medium is increased, the initial rate increases toward a maximum value.

**ATP Hydrolysis**

In other calcium-accumulating systems, the uptake is proportional to the ATP hydrolyzed (13). In Table IV, increasing quantities of calcium taken up are not associated with enhanced ATP hydrolysis.

**TABLE IV**

FORMATION OF INORGANIC PHOSPHORUS FROM ATP UNDER VARYING INITIAL CALCIUM CONCENTRATIONS

Results are the means ± standard errors of three experiments.

| Conditions: 3 mM MgCl₂, 3 mM ATP, plus Ca²⁺ uptake Pi released | μmoles/g protein | μmoles/hr/mg protein |
|-------------------------------------------------------------|-------------------|----------------------|
| 20 μM Ca⁺⁺                                                  | 5.32 ± 0.96       | 17.13 ± 0.33         |
| 50 μM Ca⁺⁺                                                  | 6.77 ± 1.30       | 16.69 ± 0.68         |
| 100 μM Ca⁺⁺                                                 | 9.27 ± 2.05       | 16.25 ± 0.50         |
| No Ca⁺⁺                                                     | 0                 | 16.75 ± 0.25         |

**Effect of Monovalent Cations**

In other systems, monovalent cations depress or stimulate calcium uptake. Sodium, in particular, has been observed to compete with calcium for certain sites in brain, heart, and peripheral nerve (see ref. 15 for discussion). It was of interest, therefore, to examine effects of these cations by including them in the incubation medium (Table V). There may be some stimulation by K⁺ (p ≤ 0.05); however, there is no inhibition by Na⁺, which represents a difference between this preparation and heart microsomes, where calcium uptake is depressed by Na⁺ (9).

**Inhibitors**

Sulfhydryl-binding agents depress calcium uptake, as does the mercurial diuretic mercaptomerin (Table VI). Ouabain at 10⁻⁴ M has no effect (Table I).

**Effect of Temperature**

In Fig. 3, rate experiments as described in Methods are presented at the three temperatures noted. Uptake rates after 1 min are profoundly influenced by
Calcium and ATP Binding

When \(^{14}\)C-labeled ATP is incubated with renal membranes without Ca\(^{++}\), approximately 7 \(\mu\)moles are taken up in 10 min (Table VII). The same amount of labeled ATP-\(^{32}\)P is bound. However, when 20 \(\mu\)M CaCl\(_2\) is added to the preparation, there is no further uptake of \(^{14}\)C-ATP, but \(^{32}\)P labeling doubles (Table VII). Neither Na\(^+\), K\(^+\), nor Mg\(^{++}\) has this property (Table VIII), as it seems specific for calcium. This and other features distinguish

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**TABLE V**

EFFECT OF MONOVALENT CATIONS

Results are the means \(\pm\) standard errors of 12 experiments.

| Conditions: 3 mM ATP, 20 \(\mu\)M CaCl\(_2\), 3 mM MgCl\(_2\), plus | Ca\(^{++}\) uptake \(\mu\)moles/g protein |
| --- | --- |
| No addition | 4.82 \(\pm\) 0.63 |
| 100 mM NaCl | 4.96 \(\pm\) 0.53 |
| 100 mM KCl | 6.12 \(\pm\) 0.72 |
| 100 mM choline Cl | 4.78 \(\pm\) 0.92* |

*Mean of three experiments.

**TABLE VI**

EFFECT OF VARIOUS INHIBITORS ON INORGANIC PHOSPHORUS FORMATION AND CALCIUM UPTAKE IN MITOCHONDRIAL AND MICROSONAL FRACTIONS

Results are the means \(\pm\) standard errors of three experiments.

| Conditions: 3 mM ATP, 20 \(\mu\)M CaCl\(_2\), 3 mM MgCl\(_2\), plus | 76,000 g ppt | 10,000 g ppt | 76,000 g ppt | 10,000 g ppt |
| --- | --- | --- | --- | --- |
| | \(\mu\)moles/g protein | \(\mu\)moles/hr/mg protein | \(\mu\)moles/g protein | \(\mu\)moles/hr/mg protein |
| No addition | 5.90 \(\pm\) 0.60 | 16.00 \(\pm\) 1.46 | 68.94 \(\pm\) 7.98 | 15.56 \(\pm\) 0.51 |
| 10\(^{-5}\) M PCMB* | 1.41 \(\pm\) 0.12 | 13.69 \(\pm\) 1.12 | 2.40 \(\pm\) 0.28 | 14.25 \(\pm\) 0.97 |
| 20 mM KCl, 10\(^{-5}\) M PCMB, 100 mM NaCl | 1.09 \(\pm\) 0.10 | 15.72 \(\pm\) 1.20 | 0.46 \(\pm\) 0.45 | 14.83 \(\pm\) 0.73 |
| 10\(^{-2}\) M Na mercapto-mercapto-merin | 1.85 \(\pm\) 0.12 | 14.72 \(\pm\) 1.15 | 3.70 \(\pm\) 0.50 | 13.31 \(\pm\) 0.89 |
| 20 mM KCl, 10\(^{-2}\) M Na mercapto-mercapto-merin, 100 mM NaCl | 1.05 \(\pm\) 0.09 | 16.72 \(\pm\) 1.30 | 1.56 \(\pm\) 0.10 | 15.60 \(\pm\) 1.08 |
| 100 mM NaCl, 20 mM KCl | 6.65 \(\pm\) 0.51 | 19.47 \(\pm\) 1.35 | 66.96 \(\pm\) 5.46 | 21.19 \(\pm\) 1.23 |
| No ATP | 1.63 \(\pm\) 0.11 | 0 | 1.02 \(\pm\) 0.09 | 0 |

*PCMB = \(\beta\)-chloromercuribenzoate.

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**ATP Binding and \(^{32}\)P Incorporation**

When \(^{14}\)C-labeled ATP is incubated with renal membranes without Ca\(^{++}\), approximately 7 \(\mu\)moles are taken up in 10 min (Table VII). The same amount of labeled ATP-\(^{32}\)P is bound. However, when 20 \(\mu\)M CaCl\(_2\) is added to the preparation, there is no further uptake of \(^{14}\)C-ATP, but \(^{32}\)P labeling doubles (Table VII). Neither Na\(^+\), K\(^+\), nor Mg\(^{++}\) has this property (Table VIII), as it seems specific for calcium. This and other features distinguish
this incorporation from that described by Post, Sen, and Rosenthal (16).

There is a significant depression of \(^{32}\)P binding by 100 mM Na\(^+\) (Table VIII), but no effect on calcium uptake (Table I). There would appear,

![Graph](image)

**Figure 3.** Time course of calcium uptake in the 78,000 g fraction from kidney cortex at three different temperatures. Initial CaCl\(_2\) concentration was 20 \(\mu\)M; ATP and MgCl\(_2\) concentrations were 3 mM.

**Table VII**

| Conditions | \(\text{\(^{32}\)P uptake}\) (\(\mu\)moles/g protein) | \(\text{\(^{4}\)C uptake}\) (\(\mu\)moles/g protein) |
|------------|--------------------------------------------------|----------------------------------|
| No addition | 7.07 ± 1.52                                       | 7.34 ± 0.65                      |
| 20 \(\mu\)M CaCl\(_2\) | 16.96 ± 1.06                                      | 8.01 ± 1.03                      |

then, to be a discrepancy between \(^{32}\)P uptake and calcium binding. This apparent discrepancy can be explained by the effect of sodium on ATP-\(^{32}\)P binding in the absence of calcium [(7.07 ± 1.52) - (3.47 ± 1.45), Table VIII], accounting for the difference.
TABLE VIII
INCORPORATION OF $^{32}$P FROM $\gamma$-Labeled ATP$^{32}$P INTO
MICROSOMAL MEMBRANES UNDER VARIOUS IONIC INFLUENCES

Results are the means ± standard errors of three determinations on three separate preparations.

| Conditions: 3 mM ATP-$\gamma$-$^{32}$P, 3 mM MgCl$_2$, plus | $^{32}$P incorporated | μmol/g protein |
|-----------------------------------------------------------|------------------------|---------------|
| No addition                                               | 7.07 ± 1.52            |               |
| 20 μM CaCl$_2$                                            | 16.96 ± 1.06           |               |
| 20 μM CaCl$_2$, 100 mM KCl                               | 12.83 ± 0.83           |               |
| 20 μM CaCl$_2$, 100 mM NaCl                              | 10.61 ± 0.44           |               |
| 100 mM KCl                                                | 6.02 ± 1.21            |               |
| 100 mM NaCl                                               | 3.47 ± 1.45            |               |
| 20 μM NaCl                                                | 4.26 ± 0.72            |               |
| 100 mM KCl, 100 mM NaCl                                  | 3.23 ± 0.38            |               |

Rate of ATP-$\gamma$-$^{32}$P Binding

The time course of $^{32}$P incorporation into grana from ATP-$\gamma$-$^{32}$P in the presence and absence of calcium is presented in Fig. 4. The rate of $^{32}$P incorporation parallels the rate of $^{45}$Ca uptake. No further binding of $^{32}$P occurs beyond the 2 min value when calcium is absent.

![Figure 4](chart.png)

**Figure 4.** Time course of $^{32}$P uptake from 3 mM ATP-$\gamma$-$^{32}$P and 3 mM MgCl$_2$ in the presence (○—○) and absence (×—×) of 20 μM CaCl$_2$ (left ordinate). $^{45}$Ca uptake (20 μM CaCl$_2$), measured in the same preparations (▲—▲), is depicted on right ordinate. Each point is the mean of three experiments. Other conditions as in Methods.
An explanation which might be offered for the data presented is that, as ATP is hydrolyzed, it releases \(^{32}\)P, which precipitates when calcium is present and is then trapped on the filter. It would seem that this possibility is unlikely at such low concentrations of Ca\(^{++}\) and PO\(_4\)\(^{-}\); moreover, ITP does not enhance calcium uptake although it is hydrolyzed to the same extent as ATP (Table II). Nevertheless, the possibility was further studied as follows. \(^{32}\)P\(_i\) at various concentrations was incubated with 20 \(\mu\)M calcium (Table IX).

**TABLE IX**

INCUBATION OF \(^{32}\)P\(_i\) AT VARIOUS CONCENTRATIONS OF THE SAME SPECIFIC ACTIVITY (1000 CPM/\(\mu\)MOLE)

Radioactivity was assayed as described in the text, at 37°C. The buffer was at pH 6.8. Results are the means \(\pm\) standard errors of four experiments.

| Concentration         | \(^{32}\)P incorporated (\(\mu\)moles/g protein) |
|-----------------------|-----------------------------------------------|
| 3 mM KH\(_2\)\(^{32}\)PO\(_4\)\(^{-}\) | 43 \(\pm\) 3.05                               |
| 3 mM KH\(_2\)\(^{32}\)PO\(_4\)\(^{-}\), 20 \(\mu\)M CaCl\(_2\) | 36 \(\pm\) 4.12                               |
| 300 \(\mu\)M KH\(_2\)\(^{32}\)PO\(_4\)\(^{-}\) | 0.8 \(\pm\) 0.15                               |
| 300 \(\mu\)M KH\(_2\)\(^{32}\)PO\(_4\)\(^{-}\), 20 \(\mu\)M CaCl\(_2\) | 0.65 \(\pm\) 0.10                               |

![Figure 5](image-url)

**Figure 5.** Phospholipase C, 50 \(\mu\)g/ml, and membranes, 0.4 mg protein/ml, were incubated for the time periods shown on the abscissa at 37°C. Then the solution was centrifuged and washed free of phospholipase C. The treated, resuspended membranes were assayed for calcium binding with 20 \(\mu\)M calcium at 37°C, in the presence of 3 mM ATP and Mg\(^{++}\). Membranes treated identically with 0.25 M sucrose served as controls.
After 10 min of incubation the concentration of $\text{PO}_4^{3-}$ hydrolyzed from 3 mM ATP is approximately 10% of the ATP initially present, or 300 $\mu$M (Table I). When this concentration of $^{32}\text{P}_i$ is incubated with 20 $\mu$M CaCl$_2$ and grana, only 0.65 $\mu$ mole of $^{32}$P is bound per gram of protein, whereas 17 $\mu$ moles would be expected if ATP-$\gamma$-$^{32}$P were present (Table VII). When the concentration of $^{32}$P$_i$ was increased to 3 mM, no excess uptake of $^{32}$P occurred when calcium was present, although total binding increased (Table IX). Thus the thesis that calcium precipitates with phosphate hydrolyzed from ATP is not supported.

In 10 min approximately 5 $\mu$ moles of $^{45}$Ca are bound (Table I), and in that same period approximately 10 $\mu$ moles of $^{32}$P are incorporated above the amount of $^{14}$C labeling (Table VII). Approximately 2 moles of phosphate are bound from the terminal phosphate of ATP for each mole of calcium taken up.

**Incubation of Renal Membranes with Phospholipase C**

Fig. 5 depicts results of preincubation of membranes in phospholipase C (Sigma Chemical Co., St. Louis, Mo.) on calcium binding at various intervals. Phospholipase C reduces calcium uptake when membranes are thus pretreated. The addition of lysolecithin in varying concentrations did not restore calcium-binding ability (data not shown).

**DISCUSSION**

The literature embodies suggestions that calcium is necessary for the functional stability of membranes. Most of the evidence for the interaction of calcium and membranes comes from work on nerve or muscle. The work presented here deals with this problem for the first time in a noncontractile, nonexcitable tissue. Calcium does bind to these membrane fragments, and MgATP is required for maximum binding.

Much of the pertinence of these observations to the discussion below hinges on the purity of the preparation. Binding of calcium to mitochondrial fragments seems unlikely, owing to lack of inhibition by azide. However, calcium and $^{32}$P could bind to ribonucleoprotein or other contaminants. Phospholipase C inhibits calcium binding, suggesting that membrane structures are involved (Fig. 5), although binding could not be restored by the addition of lysolecithin.

Unquestionably, calcium in other systems binds to the anionic groups of phospholipids without the participation of ATP. Rojas and Tobias (17) have presented compelling evidence that the binding alters permeability of artificial membranes. Van Breemen has further extended these observations, and has shown that calcium decreases cation permselectivity, suggesting binding to anionic groups of phospholipids (18). Whether a similar process
occurs in natural membranes is speculative, although Martonosi has recently shown that phospholipase C will inhibit membrane function in skeletal muscle sarcoplasmic reticulum as measured by active calcium uptake, and the addition of phospholipids restores calcium pumping (19). He concluded that hydrolysis of phospholipids prevents initial calcium binding, requisite for subsequent uptake.

Kleinzeller et al. (20) have amply demonstrated that calcium lack enhances ion and water “leakage” in kidney slices. They concluded that a calcium-dependent mechanism (contractile or elastic?) contributes to the regulation of cell volume. The observations presented here, in their similarity to muscle systems, are perhaps pertinent to their conclusions.

Our data are consistent with the following scheme (RM = renal membrane).

\[
2 \text{ATP} + \text{RM} \rightleftharpoons \text{RM} \hspace{0.5cm} \begin{array}{c} P^- \\ P^- \end{array} \rightleftharpoons 2 \text{ADP} \tag{1}
\]

then

\[
\begin{array}{c} P^- \\ P^- \end{array} \rightleftharpoons \text{RM} + \text{Ca}^{++} \rightleftharpoons \text{RM} \hspace{0.5cm} \begin{array}{c} P^- \\ P^- \end{array} \text{Ca} \tag{2}
\]

The stoichiometry is presented in Results. From the scheme, we would not expect to find enhanced inorganic phosphate formation from ATP when calcium is bound (Table IV). The phosphorylated membrane complex would have a high affinity for calcium, as proposed (13, 19), and shift reaction 2 to the right.

The final product would be responsible for conformational stability, and actually a conformational change might be induced by reaction 1 or 2; this is pertinent, perhaps, to the proposal of Kleinzeller et al. (20).

The attractiveness of this hypothesis resides in its ability to link observations that implicate calcium, as well as metabolism, as factors in determining membrane stability.

The fact that calcium uptake is depressed by mercurial diuretics suggests a potential site for the action of these drugs.

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REFERENCES

1. Manery, J. F. 1966. Effects of Ca ions on membranes. *Fed. Proc.* 25:1804.
2. Carvalho, A. P., and B. Leo. 1967. Effects of ATP on the interaction of Ca++, Mg++,
and K+ with fragmented sarcoplasmic reticulum isolated from rabbit skeletal muscle.
J. Gen. Physiol. 50:1327.

3. LEIBERMAN, E. M., R. F. PALMER, and G. H. COLLINS. 1967. Calcium ion uptake by crustacean peripheral nerve subcellular particles. Exp. Cell Res. 46:412.

4. SANDOW, A. 1965. Excitation-contraction coupling in skeletal muscle. Pharmacol. Rev. 17: 265.

5. ROBINSON, J. D., and W. D. LUST. 1968. Adenosine triphosphate-dependent calcium accumulation by brain microsomes. Arch. Biochem. Biophys. 125:286.

6. OTSUKA, M., I. OHYUKI, and S. EBRASHI. 1965. ATP-dependent Ca binding of brain microsomes. J. Biochem. (Tokyo). 58:188.

7. LOWRY, O. H., N. J. ROSSBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265.

8. FEKE, C. F., and Y. SUBBAROW. 1925. The colorimetric determination of phosphorus. J. Biol. Chem. 66:375.

9. PALMER, R. F., and V. A. POSEY. 1967. Ion effects on calcium accumulation by cardiac sarcoplasmic reticulum. J. Gen. Physiol. 50:2085.

10. RANDORF, K. 1966. Thin-Layer Chromatography. Academic Press, New York. 223.

11. LUFT, J. H. 1961. Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9:409.

12. NECHAY, B. R., R. F. PALMER, D. A. CHINOY, and V. A. POSEY. 1967. Problem of Na+ + K+ adenosine triphosphatase as the receptor for diuretic action of mercurials and ethacrynic acid. J. Pharmacol. Exp. Ther. 157:599.

13. HASELBACH, W. 1964. Relieving factor and the relaxation of muscle. Progr. Biophys. Mol. Biol. 14:167.

14. SKOU, J. C. 1965. Enzymatic basis for transport of Na+ and K+ across cell membrane. Physiol. Rev. 45:596.

15. ALONSO, G., and M. WALSER. 1968. ATP splitting and calcium binding by brain microsomes measured with a rapid perfusion method. J. Gen. Physiol. 52:111.

16. POST, R. L., A. K. SEN, and A. S. ROSENTHAL. 1965. A phosphorylated intermediate in adenosine triphosphate-dependent sodium and potassium transport across kidney membranes. J. Biol. Chem. 240:1437.

17. ROJAS, E., and J. M. TOBIAS. 1965. Membrane model: association of inorganic cations with phospholipid monolayers. Biochim. Biophys. Acta. 94: 394.

18. VAN BREEKEN, C. 1968. Permeability of a porous phospholipid cholesterol artificial membrane. Calcium and lanthanum effects. Biochem. Biophys. Res. Commun. 32:977.

19. MARTONOSI, A. 1967. Role of phospholipids in the ATP-ase activity of skeletal muscle microsomes. Biochem. Biophys. Res. Commun. 29:753.

20. KLEINZELLER, A., A. KNOTKOVA, and J. NEDVIDEKOVA. 1968. The effect of calcium ions on the steady-state ionic distribution in kidney cortex cells. J. Gen. Physiol. 51:326s.