Inhibition of Na\(^+\)K\(^+\)ATPase Activity in Membranes of Sindbis Virus-Infected Chick Cells

INTRODUCTION

Sindbis virus is a small, enveloped RNA virus that replicates in and kills vertebrate cells grown in culture. Cell killing induced by Sindbis virus and other members of the alphavirus group is characterized by the rapid inhibition of host-directed macromolecular synthesis, alterations in the intracellular concentrations of metabolites and ions, and structural and enzymatic changes in the cell membrane (reviewed in Kääriäinen and Ranki, 1984; Ulug et al., 1987). Ultimately, cells productively infected with alphaviruses enter programmed cell death or apoptosis (Levine et al., 1993; Ubol et al., 1994). Recent studies using alphavirus replicon systems have revealed that although expression of early, nonstructural viral proteins is sufficient to account for inhibition of host protein synthesis, this event per se cannot account for the rapid development of cytopathic changes in cells infected by alphaviruses (Liljestro¨m and Garoff, 1991; Frolov and Schlesinger, 1994). Instead, the rapid induction of cytopathic lesions in cells infected by alphaviruses may result from damage to the cell membrane. Rapid development of cytopathic changes in Sindbis virus-infected cultures is prevented by treatment with tunicamycin (Ulug and Bose, 1985), a glycosylation inhibitor that blocks maturation of the viral envelope proteins (Leavitt et al., 1977).

Consistent with these findings, it has been suggested that appearance of the two viral glycoproteins at the surface of Sindbis virus-infected cells temporally correlates with rapid development of cytopathic lesions (Frolov and Schlesinger, 1994).

An alteration in monovalent cation transport is among the earliest cytopathic responses that are detected in Sindbis virus-infected cells (Ulug et al., 1984), and ensuing changes in the intracellular concentrations of Na\(^+\) and K\(^+\) may contribute to the rapid killing of virus-infected cells (Garry et al., 1979; Ulug and Bose, 1985; Garry, 1994). Alterations in intracellular monovalent cation levels in Sindbis virus-infected chick cells result at least in part, from a reduction in the activity of the ouabain-sensitive Na\(^+\)K\(^+\)ATPase, or Na\(^+\) pump (Ulug et al., 1984). Inhibition of Na\(^+\) pump activity in cells infected with Sindbis virus was defined by a reduction in the V\(_{\text{max}}\) for ouabain-sensitive K\(^+\) influx. The number of ouabain binding sites present on the cell surface was unaltered as a consequence of Sindbis virus infection, suggesting that inhibition of Na\(^+\) pump activity results from a reduction in the specific activity of the enzyme.

The Na\(^+\)K\(^+\)ATPase catalyzes active transport of Na\(^+\) out of cells and K\(^+\) into cells with an apparent stoichiometry of 3Na\(^+\):2K\(^+\):1ATP (reviewed in Kyte, 1981; Jorgensen, 1982; Cantley et al., 1983; Farley et al., 1994). In addition to maintaining a high intracellular concentration of K\(^+\) and a low intracellular concentration of Na\(^+\), the Na\(^+\)K\(^+\)ATPase is electrogenic, contributing...
to the formation of an ionic potential across the cell membrane. Purified preparations of the Na⁺K⁺-ATPase consist of at least two polypeptide species, an alpha subunit of 94–106 kDa and a beta subunit of 41–52 kDa, although a third or gamma subunit may also be present (Mercer et al., 1993). The alpha subunit is the catalytic component of the Na⁺K⁺-ATPase and contains the ATP binding site (Castro and Fairley, 1979; Smith et al., 1980; Ponzié et al., 1983; Shull et al., 1985) as well as the binding site for the cardiac glycoside ouabain (Rossi et al., 1982; Shull et al., 1985). The beta subunit is a sialoglycoprotein which may function during transport and membrane assembly of the alpha subunit (Sweedner and Goldin, 1980; Freytag, 1983; McDonough et al., 1990). The function of the gamma subunit is presently unknown (Mercer et al., 1993).

The Na⁺K⁺-ATPase undergoes a series of partial reactions that correlate with the binding and transport of monovalent cations (reviewed by Jorgensen, 1982). The Na⁺K⁺-ATPase exists in two conformations which have been identified by tryptic mapping and fluorescence labeling (Jorgensen and Peterson, 1982). Stabilized in the presence of Na⁺, the E1 conformation is phosphorylated in the presence of Mg²⁺ and ATP to yield E1 ~ P (Avruch and Fairbanks, 1972; Post et al., 1972, 1973). A conformational shift to E2 ~ P can be selectively blocked by addition of N-ethylmaleimide or oligomycin (Wallick et al., 1978). In the presence of K⁺, E2 ~ P is rapidly dephosphorylated (Glynn and Karlish, 1975). This step is ouabain-sensitive (Avruch and Fairbanks, 1972). Dephosphorylated E2 will revert to E1 in the presence of Na⁺ in a vanadate-sensitive conformational shift (Cantley, 1978; Karlish et al., 1979).

This investigation was initiated to define the mechanism by which Sindbis virus infection of avian cells results in diminished Na⁺K⁺-ATPase activity. We demonstrate a reduction in enzyme activity in membrane preparations derived from Sindbis virus-infected cells, indicating that limitation of cellular factors is not responsible for inhibition of this activity. Although the Na⁺K⁺-ATPase was phosphorylated to the same extent in membranes preparations derived from uninfected and virus-infected cells, Na⁺ pump phosphorylated intermediates prepared from virus-infected cells were comparatively unstable. A possible consequence of this lesion is the loss of cation selectivity during Na⁺K⁺-ATPase-mediated cation transport in virus-infected cells.

**MATERIALS AND METHODS**

**Cells, viruses, and media**

Primary cultures of chick embryo cells were plated 36 to 48 hr prior to experimentation at a density of 4 × 10⁵ per 35-mm 6-well or 2.4 × 10⁶ per 100-mm plastic culture dish. Cultures were propagated in minimal essential medium (MEM) containing Earle's salts and supplemented with 5% newborn calf serum (NCS) in a humidified 5% CO₂ atmosphere at 37°C. Cells were mock-infected by the addition of MEM or infected with the HR strain of Sindbis virus (Burge and Pfefferkorn, 1966) in MEM at a multiplicity of approximately 20. After adsorption for 1 hr at 37°C (0 hr postinfection), cultures were rinsed and incubated in MEM containing 2% NCS to initiate the experiments.

**Plasma membrane isolation**

The enrichment of plasma membranes from approximately 1 × 10⁸ cells was performed according to the procedure of Erwin and Brown (1980) with slight modifications. Infected or mock infected cultures were washed twice in calcium and magnesium-free PBS and twice in ET buffer (1 mM EDTA, 10 mM Tris/HCl, pH 8.0). This and future steps were performed at 4°C. Cells were allowed to swell for 10 min, after which they were triturated from the dishes. Fifteen strokes of a tight-fitting dounce homogenizer was sufficient to rupture the cells, as monitored by phase contrast microscopy. Nuclei were removed by centrifugation at 1,000 g for 5 min in a swinging bucket rotor. The postnuclear supernatant was centrifuged in a Beckman Type 40 rotor at 30,000 RPM for 1 hr. The membrane-containing pellet was suspended in 0.5 ml ET buffer and mixed with 3 vol 65% w/w sucrose in ET. This was placed in a SW41 centrifuge tube and sequentially overlaid with 44, 39, 34, and 24% w/w sucrose. This was placed in a SW41 centrifuge tube and sequentially overlaid with 44, 39, 34, and 24% w/w sucrose in ET. Tubes were centrifuged 12–18 hr at 30,000 RPM in the SW41 rotor. Samples were removed by puncturing the bottom of the tubes and interface fractions, identified by A₂₈₀ profiles, were collected. Membrane preparations were diluted with ET buffer and collected by centrifugation in a Type 40 rotor at 30,000 RPM for 1 hr. Pellets were suspended to a final protein concentration of approximately 0.5 mg/ml (Bradford, 1976) in 0.1 M imidazole (pH 7.4) and stored on ice for no longer than 1 week. Both Na⁺K⁺-ATPase activity and membrane fractions derived from ¹²⁵I-labeled cells concentrated at the 29–34% sucrose interface, and these fractions were therefore used for the enzyme assays reported here.

**In vitro Na⁺K⁺-ATPase assay**

A modification of the procedure of Wallach and Kamat (1964) was used to measure Na⁺K⁺-ATPase activity in plasma membrane preparations. Final assay reaction mixtures (0.5 ml) contained 5–20 μg protein, 25 mM imidazole (pH 7.4), 1 mM MgCl₂, 100 mM NaCl, 5 mM KCl, and 3 mM ATP (Tris salt) to which 1 μCi [γ⁻³²P]ATP (NEN, 3000 Ci/mmol) had been added. Background measurements were made by using the same reaction mixture lacking KCl. Na⁺K⁺-ATPase activity was defined as the difference in ³²P, released from [γ⁻³²P]ATP when membranes were incubated in the presence and absence of 5 mM KCl. After incubation at 37°C for 30 min, 0.1 ml 50% (w/w) trichloroacetic acid (TCA) was added to terminate
the reactions. Inorganic phosphate was extracted by the isobutanol–benzene procedure of Penniall (1966) and aliquots of the organic phases were quantitated by Cerenkov counting in a beta counter. Release of inorganic phosphate from [γ-32P]ATP was linear for approximately 60 min under these conditions.

Formation of Na⁺K⁺ATPase phosphorylated intermediates

Phosphoenzyme intermediates of the Na⁺K⁺ATPase were prepared in vitro by using two procedures. The first involved direct transfer of [32P]orthophosphate to the protein in a Mg²⁺ and ouabain dependent reaction (Resh, 1982). Membranes (5–20 μg protein) were incubated in a total volume of 0.1 ml containing 25 mMimidazole (pH 7.4), 0.1 mM ouabain, 5 mM MgCl₂, and 10 μCi [32P]-orthophosphate (Amershams, carrier free) which was diluted with H₂PO₄ to give the indicated phosphate concentrations. After 15 min at 23°, reactions were terminated by addition of ice-cold TCA (10% TCA w/v, 1 M H₃PO₄). TCA precipitates were pelleted and washed four times with TCA in a refrigerated tabletop centrifuge. Background binding, determined by omission of MgCl₂ and ouabain from the reaction mixture, accounted for approximately 5–15% of the total counts bound in their presence. TCA precipitates were either dissolved in 1 N NaOH for scintillation counting or suspended in HPC sample buffer (see below) for gel electrophoresis.

Phosphorylation of plasma membrane fragments by [γ-32P]ATP was performed essentially as described (Avruch and Fairbanks, 1972). Membranes (1–5 μg protein) were incubated in a total volume of 0.1 ml containing 100 mM NaCl, 5 mM MgCl₂, and 10 μCi [γ-32P]ATP (NEN, 3000 Ci/mmol). After 1 min at 4°, reactions were terminated by addition of ice-cold TCA and processed as described above. Background values for ATP-dependent phosphorylation reactions were performed with 100 mM KCl substituted for NaCl. Under these conditions, the catalytic subunit of the Na⁺ pump was not phosphorylated and no difference in the densitometry scannings of other phosphorylated polypeptides could be detected. When indicated, membranes were incubated with 2 μg/ml oligomycin (Sigma) which was added from a 500× stock solution in 95% ethanol. Ethanol did not affect the phosphorylation reactions at the concentrations used.

To determine the rate of enzyme dephosphorylation, membranes (1–5 μg) were incubated in a total volume of 0.1 ml containing 25 mM NaCl, 3 mM MgCl₂, and 10 μCi [γ-32P]ATP (NEN, 3000 Ci/mmol). After 30 sec at 4°, 0.1 nmol (in 10 μl) unlabeled ATP was added to the phosphorylation reactions in the presence or absence of KCl. Dephosphorylation reactions were terminated by addition of TCA and processed as described above. Background values for the dephosphorylation reactions were performed by using 25 mM KCl substituted for NaCl. Polyacrylamide gel electrophoresis at acid pH

The acylphosphate linkage of the phosphorylated intermediate of the Na⁺K⁺ATPase is labile at neutral and alkaline pH and, therefore, polyacrylamide gel electrophoresis at acid pH was employed to analyze phosphorylated products (Amory et al., 1980). The resolving gel contained 8% (w/v) acrylamide, 0.2% (w/v) N,N-methylene-bisacrylamide, 0.023% FeSO₄·7H₂O, and 0.077% ascorbic acid in 0.75 M KH₂PO₄ (pH 2.0 with H₃PO₄). The stacking gel contained 8% (w/v) acrylamide, 0.63% (w/v) N,N-methylene-bisacrylamide, 0.077% ascorbic acid in 0.15 M KH₂PO₄ (pH 4.0 with H₃PO₄). Polymerization of resolving or stacking gels was initiated by addition of 0.03% H₂O₂. Samples for electrophoresis were suspended in 0.25 M sucrose, 0.035 M 1-hexadecylpyridinium chloride (HPC), 10 μg/ml pyronin Y, and 0.128 M 2-mercaptoethanol in 0.1 M KH₂PO₄ (pH 4.0 with H₃PO₄). Gels were run in 0.124% (w/v) HPC, 0.075 M glycine (pH 2.9 with H₃PO₄) for 6–8 hr at 30 mA constant current. Following electrophoresis, gels were briefly fixed in 10% (v/v) acetic acid/10% (v/v) isopropyl alcohol, dried, and exposed to Kodak XAR-2 film with an intensifying screen at −70°.

Measurement of ⁸⁶Rb⁺ and ²²Na⁺ uptake

Monovalent cation uptake was monitored by measuring the influx of the K⁺ tracer ⁸⁶Rb⁺ (New England Nuclear; 12.5 Ci/mg) or ²²Na⁺ (New England Nuclear; 1.8 Ci/mg) as described previously (Ulug and Bose, 1985). Briefly, triplicate cultures of mock-infected or Sindbis-infected cells (4 hr p.i.) were incubated in phosphate-buffered saline containing tracer amounts of either ⁸⁶RbCl for 20 min or ²²NaCl for 2 min in the presence or absence of 100 μM ouabain at 37°. Cells were then rapidly rinsed with ice-cold PBS, air dried, and dissolved in NaOH (0.1 N). Under these conditions, ⁸⁶Rb⁺ transport into cells was linear for at least 30 min and ²²Na⁺ transport into cells was linear for at least 3 min. Protein content was determined by using the method of Bradford (1976) after neutralization of cell extracts with HCl.

RESULTS

Na⁺K⁺ATPase activity is reduced in membranes of Sindbis virus-infected cells

Previous studies revealed a reduction in Na⁺K⁺ATPase-mediated uptake of the potassium tracer ⁸⁶Rb⁺ following infection of cells with Sindbis virus (Ulug et al., 1984; Ulug and Bose, 1985). Reduced intracellular levels of ATP have been detected in alphavirus infected cells (Cassells and Burke, 1973; Whitehead et al., 1981) and could account for the reduction in ouabain-sensitive ion transport. To test whether the reduction in Na⁺ pump activity detected in Sindbis virus-infected cells is direct, Na⁺K⁺ATPase activity
hydrolysis in the presence of Na\textsuperscript{+}et al., reported previously (Ulug
infection (not shown). NaCl and 5 m Na\textsuperscript{+}triplicate determinations. Error between replicate samples did not exceed 10%.

The data plotted represent the average values from Incubation of uninfected chick cell membranes with \[^{32}P\]orthophosphate in the presence of Mg\textsuperscript{2+} and oua-
K\textsuperscript{+}ATPase activity was inhibited by the presence of Na\textsuperscript{+}or 100 m
g to regulate Na\textsuperscript{+}pump activity in intact cells (Rossier et al., 1987).

Phosphorylation of the Na\textsuperscript{+}K\textsuperscript{+}ATPase in membranes of Sindbis virus-infected cells

To determine whether qualitative or quantitative changes in the Na\textsuperscript{+}K\textsuperscript{+}ATPase occur as a consequence of Sindbis virus infection of chick cells, phosphorylated intermediates of the alpha subunit were examined by gel electrophoresis. The catalytic subunit of the Na\textsuperscript{+}K\textsuperscript{+}ATPase was phosphorylated in vitro by using two procedures. The first involved direct transfer of \[^{32}P\]orthophosphate to the alpha subunit of the Na\textsuperscript{+}K\textsuperscript{+}ATPase in a ouabain and Mg\textsuperscript{2+}-dependent reaction (Resh, 1982). The second reaction involved transfer of the terminal phosphate of [\(^{\gamma-32}P\)]ATP to the alpha subunit in a Na\textsuperscript{+}and Mg\textsuperscript{2+}-dependent reaction (Avruch and Fairbanks, 1972). Phosphorylated intermediates formed using both reactions are labile at alkaline or neutral pH and, therefore, were examined by electrophoresis under acidic conditions.

Incubation of uninfected chick cell membranes with \[^{32}P\]orthophosphate in the presence of MgCl\textsubscript{2} and ouabain resulted in the phosphorylation of a single polypeptide species (M\textsubscript{r} = 95 kDa) that was resolved by acidic gel electrophoresis in the presence of the anionic detergent hexadecylpyridinium chloride (Fig. 2, lane 1). Phosphorylation of this protein was dependent upon the presence of Mg\textsuperscript{2+} and ouabain in the reaction mixture and was inhibited by the presence of Na\textsuperscript{+} and K\textsuperscript{+} (data not

[FIG. 1. Time course of inhibition of Na\textsuperscript{+}K\textsuperscript{+}ATPase activity in membranes isolated from Sindbis virus-infected cells. Na\textsuperscript{+}K\textsuperscript{+}ATPase activity was measured in plasma membranes prepared from Sindbis virus-infected cells (closed symbols) or uninfected cultures (open symbols). Na\textsuperscript{+}K\textsuperscript{+}ATPase activity represents the difference in release of \[^{32}P\]Pi from \[^{32}P\]orthophosphate in the presence of Mg\textsuperscript{2+} and ouabain resulted in the phosphorylation of a single polypeptide (not shown). ATPase activity detected in plasma membranes prepared from Sindbis virus-infected cells (closed symbols) or uninfected cultures (open symbols) was measured by monitoring the rate of \[^{32}P\]Pi release from \[^{32}P\]orthophosphate in the presence of Mg\textsuperscript{2+} and ouabain resulted in the phosphorylation of a single polypeptide (not shown). ATPase activity detected in plasma membranes prepared from Sindbis virus-infected cells (closed symbols) or uninfected cultures (open symbols) was measured by monitoring the rate of \[^{32}P\]Pi release from \[^{32}P\]orthophosphate in the presence of Mg\textsuperscript{2+} and ouabain resulted in the phosphorylation of a single polypeptide (not shown). ATPase activity detected in plasma membranes prepared from Sindbis virus-infected cells (closed symbols) or uninfected cultures (open symbols) was measured by monitoring the rate of \[^{32}P\]Pi release from \[^{32}P\]orthophosphate in the presence of Mg\textsuperscript{2+} and ouabain resulted in the phosphorylation of a single polypeptide (not shown).

[FIG. 2. HPC-gel electrophoresis of phosphorylated membrane preparations from uninfected and Sindbis virus-infected cells. Membranes were incubated with \[^{32}P\]orthophosphate in the presence of ouabain and MgCl\textsubscript{2} (lanes 1 and 2) or incubated with \[^{\gamma-32}P\]]ATP and MgCl\textsubscript{2} in the presence of 100 mM NaCl (lanes 3 and 4), 100 mM NaCl and 5 mM KCl (lanes 5 and 6), 100 mM KCl (lanes 7 and 8), or 100 mM NaCl and 2 \(\mu\text{g} / \text{ml}\) oligomycin (lanes 9 and 10). Lanes 1, 3, 5, 7, 9 represent membrane preparations derived from uninfected cells and lanes 2, 4, 6, 8, 10 represent membrane preparations derived from cells infected 5 hr with Sindbis virus. Lanes 3–10 in this autoradiogram were exposed for 20 min and lanes 1 and 2 were exposed for 24 hr at ~70° using Kodak XAR-2 film with an enhancing screen.]

86Rb\textsuperscript{+} uptake into Sindbis virus-infected cells reported previously (Ulugh et al., 1984). These results indicate that the reduction in ion transport by cells

was measured in membrane fractions derived from virus-infected and uninfected cells.

Plasma membranes were isolated by discontinuous gradient centrifugation from uninfected cells and from cells infected with Sindbis virus for various periods of time. Na\textsuperscript{+}K\textsuperscript{+}ATPase activity in the membrane preparations was measured by monitoring the rate of \[^{\gamma-32}P\]]ATP hydrolysis in the presence of Na\textsuperscript{+}, Mg\textsuperscript{2+}, and K\textsuperscript{+} or in the presence of Na\textsuperscript{+} and Mg\textsuperscript{2+} alone. K\textsuperscript{+}-stimulated ATPase activity measured using this procedure was used to define Na\textsuperscript{+}K\textsuperscript{+}ATPase activity. As illustrated in Fig. 1, membranes derived from cells infected with Sindbis virus for 1.5 hr exhibited a significant decrease (P < 0.05) in Na\textsuperscript{+}K\textsuperscript{+}ATPase activity, relative to mock-infected control samples. By 3.5 hr after infection, Na\textsuperscript{+}K\textsuperscript{+}ATPase activity was reduced approximately 66% in membranes of virus-infected cells, relative to control preparations. Na\textsuperscript{+}K\textsuperscript{+}ATPase activity measured using this assay was sensitive to the Na\textsuperscript{+}\textsuperscript{+}ATPase inhibitor ouabain (not shown). ATPase activity measured in the presence of Na\textsuperscript{+} and Mg\textsuperscript{2+}, but in the absence of K\textsuperscript{+}, accounted for approximately 65% of the total ATPase activity in uninfected cell membranes and was unaltered as a consequence of Sindbis virus infection (not shown).

The reduction in Na\textsuperscript{+}K\textsuperscript{+}ATPase activity detected in membrane preparations derived from virus-infected cells temporally correlated with the reduction in oua-

virus-infected cells (Rossier et al., 1987).

Phosphorylation of the Na\textsuperscript{+}K\textsuperscript{+}ATPase in membranes of Sindbis virus-infected cells

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Incubation of uninfected chick cell membranes with \[^{32}P\]orthophosphate in the presence of MgCl\textsubscript{2} and ouabain resulted in the phosphorylation of a single polypeptide species (M\textsubscript{r} = 95 kDa) that was resolved by acidic gel electrophoresis in the presence of the anionic detergent hexadecylpyridinium chloride (Fig. 2, lane 1). Phosphorylation of this protein was dependent upon the presence of Mg\textsuperscript{2+} and ouabain in the reaction mixture and was inhibited by the presence of Na\textsuperscript{+} and K\textsuperscript{+} (data not
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shown), confirming its identity as the Na⁺⁻K⁺⁻ATPase catalytic subunit. When uninfected and Sindbis virus-infected cell membranes were analyzed under these conditions, equivalent amounts of the 95 kDa phosphoprotein product were generated (compare intensities of bands in lanes 1 and 2).

Identification of the 95-kDa protein as the catalytic subunit of the Na⁺⁻K⁺⁻ATPase was confirmed by phosphorylation of chick cell membrane proteins with [γ-³²P]ATP in the presence of NaCl and MgCl₂. Under conditions of this assay, acid-stable phosphorylation of two prominent polypeptide species was observed. As illustrated in lanes 3 and 4, one had an apparent molecular weight of 95 kDa and comigrated with the protein phosphorylated in the presence of [³²P]orthophosphate, MgCl₂, and ouabain (lanes 1 and 2). Phosphorylation of the 95-kDa protein was not observed when KCl was substituted for NaCl in the reaction mixture (lanes 7 and 8). In the presence of 100 mM NaCl, addition of KCl (to 5 mM) resulted in a significant reduction in recovery of the 95-kDa phosphoprotein (lanes 5 and 6). Finally, incubation of membranes in the presence of oligomycin resulted in a 5- to 10-fold increase in phosphorylation of the 95-kDa product (lanes 9 and 10). These results confirm the identity of the 95-kDa protein as the catalytic subunit of the Na⁺⁻K⁺⁻ATPase and indicate that infection of cells with Sindbis virus did not affect either the formation or electrophoretic mobility of this product. No consistent difference in phosphorylation of the 95-kDa protein was detected under any of these conditions when assays were performed using membranes derived from uninfected and virus-infected cells (compare intensities of even and odd lanes in Fig. 2). The identity of the larger, 130-kDa protein is not known, but was apparently unaffected by the conditions which affect formation of Na⁺⁻ pump phosphorylated intermediates.

To confirm that the reduction in Na⁺⁻K⁺⁻ATPase activity detected in membranes derived from Sindbis virus-infected cells was not due to a loss of functional Na⁺⁻K⁺⁻ATPase molecules, the maximal phosphate binding capacity of membranes isolated from uninfected and Sindbis virus-infected cells was determined. Membrane preparations were exposed to various concentrations of [³²P]orthophosphate in the presence of MgCl₂ and ouabain for 15 min (a time resulting in maximal phosphorylation), then precipitated and washed extensively with TCA. Background binding was measured in parallel reaction mixtures lacking ouabain and MgCl₂. As illustrated in Fig. 3A, incorporation of [³²P]orthophosphate increased as a function of the phosphate concentration when membranes of both uninfected and virus-infected cultures were incubated in the presence of MgCl₂ and ouabain (circles). Baseline incorporation of [³²P]orthophosphate into TCA precipitates was observed when assays were performed in the absence of MgCl₂ and ouabain (squares). No difference in phosphate binding was observed when membranes derived from uninfected and Sindbis virus-infected cultures were compared.

To quantitate the maximal phosphorylation capacity of membranes derived from uninfected and Sindbis virus-infected cells, data in Fig. 3A were plotted as a double-reciprocal function in Fig. 3B. No significant difference in the calculated dissociation constant K₀ (–1/x intercept) for the phosphoenzyme was observed in membranes derived from uninfected cells (21.1 μM) and Sindbis virus-infected cells (22.4 μM). Similarly, the maximal phosphorylation capacity (calculated as 1/y inter-
cept) was not significantly altered in membranes of uninfected (16.1 pmol/mg protein) and Sindbis virus-infected cells (16.7 pmol/mg protein). The reduced Na\(^+\)K\(^+\)ATPase activity measured in membrane preparations derived from virus-infected cells, therefore, reflects a reduction in the specific activity of this enzyme and not a reduction in the number of functional enzyme molecules present in the membrane preparations.

A comparison of the specific activity of the Na\(^+\)K\(^+\)ATPase in Sindbis virus-infected cells and in membranes derived from uninfected cells is presented in Table 1. The turnover rate of uninfected and Sindbis virus-infected cells represents the maximum ouabain binding capacity of uninfected and Sindbis virus-infected cells (Ulug et al., 1984). The rate of ATP hydrolysis in uninfected and Sindbis virus-infected cell membranes under V\(_{\text{max}}\) conditions is derived from Fig. 1. The turnover rate of uninfected and Sindbis virus-infected CEF (5 hr p.i.) is presented in Table 1. The turnover rate of uninfected and Sindbis virus-infected cell membranes under V\(_{\text{max}}\) conditions is derived from Fig. 1. The ratio of enzymatic turnover in cells and membrane preparations has been utilized to calculate the efficiency of Na\(^+\) pump activity in cells (Resh, 1982). Assuming a 2:1 stoichiometry for K\(^+\) transport and ATP hydrolysis, the efficiencies for Na\(^+\) pump activity in uninfected and Sindbis virus-infected cells were 0.45 and 0.50, respectively. The efficiency of Na\(^+\) pump activity was not significantly altered as a consequence of Sindbis virus infection.

**Kinetics of Na\(^+\)K\(^+\)ATPase dephosphorylation**

A reduction in the specific activity of the Na\(^+\)K\(^+\)ATPase would predict that one or more partial reactions of this enzyme is altered during the course of virus infection. The phosphorylation studies (Figs. 2 and 3) indicated that events leading to formation of phosphoenzyme intermediates could not account for the reduction in Na\(^+\)K\(^+\)ATPase activity in Sindbis virus-infected cells. We therefore examined whether the stability of the Na\(^+\)K\(^+\)ATPase phosphorylated intermediates might be altered as a consequence of Sindbis virus infection. To test this, plasma membranes were incubated in the presence of Na\(^+\), Mg\(^{2+}\), and [\(\gamma\)-\(\text{P}\)]ATP to generate Na\(^+\)K\(^+\)ATPase phosphorylated intermediates. The stability of these intermediates was then analyzed by addition of 1000-fold excess unlabeled ATP (resulting in a final concentration of approximately 1 \(\mu\)M ATP) in the presence and absence of K\(^+\) for various periods of time. Reactions were terminated by addition of TCA and the amount of radioactivity associated with the membranes was determined. To assess phosphorylation of proteins other than the Na\(^+\)K\(^+\)ATPase, parallel reactions were run in the presence of KCl and MgCl\(_2\).

The kinetics of Na\(^+\)K\(^+\)ATPase dephosphorylation differed markedly when phosphoenzymes prepared from uninfected and Sindbis virus-infected cell membranes were analyzed (Fig. 4). Extrapolation of results from this experiment revealed that the phosphoenzyme prepared from membranes of uninfected cells exhibited an apparent half life (T\(_{1/2}\)) of approximately 15 sec. Phosphoenzyme prepared from Sindbis virus-infected cell membranes was less stable, having a T\(_{1/2}\) of 7.2 sec. Preliminary experiments demonstrated that both untreated and oligomycin-treated enzymes were half maximally inhibited by addition of 50 \(\mu\)M K\(^+\) in the phosphorylation reaction mixture (data not shown). Therefore, to test whether K\(^+\) stimulated dephosphorylation of the Na\(^+\)K\(^+\)ATPase, KCl was included with unlabeled ATP in the dephosphorylation reaction mixture. Addition of 50 \(\mu\)M KCl stimulated dephosphorylation of the phosphoenzyme in both uninfected and virus-infected cell membranes. However, the rate of K\(^+\)-stimulated enzyme dephosphorylation (the difference in rates in the presence and absence of KCl)
Sindbis virus stimulates ouabain-sensitive Na⁺ uptake

K⁺-stimulated dephosphorylation of the Na⁺K⁺ATPase in vitro has been correlated with the binding and transport of K⁺ ions (Wallick et al., 1979; Kyte, 1981; Jorgensen, 1982; Cantley et al., 1983). The enhanced rate of K⁺-independent dephosphorylation of the Na⁺ pump in vitro suggested that enzyme dephosphorylation was not coupled to K⁺ transport in Sindbis virus-infected cells. To test this, we compared the relative efficiencies by which Na⁺ and K⁺ were transported into uninfected and Sindbis virus-infected cells.

Uninfected cells and cells infected with Sindbis virus for 4 hr were pulsed with ⁸⁶Rb⁺, a K⁺ tracer, or with ³²Na⁺ in the presence and absence of ouabain (Table 2). As demonstrated previously (Ulug et al., 1984), approximately 60% of total ⁸⁶Rb⁺ uptake into uninfected chick cells could be blocked by the Na⁺K⁺ATPase inhibitor ouabain and cells infected by Sindbis virus exhibited a 60% reduction in the rate of ouabain-sensitive ⁸⁶Rb⁺ uptake, relative to uninfected cultures.

Treatment of uninfected chick cells with ouabain resulted in an enhanced rate of ²²Na⁺ influx (Table 2). The difference in ²²Na⁺ uptake measured in the presence and absence of ouabain, therefore, defines a net negative (i.e., outward) flux rate for Na⁺ directed by the Na⁺K⁺ATPase in uninfected chick cells. This result is expected, since Na⁺ is actively transported out of cells by the Na⁺K⁺ATPase. Uptake of ³²Na⁺ into Sindbis virus-infected cells was elevated approximately 80%, relative to uninfected chick cells, in the absence of ouabain. This is consistent with earlier observations that demonstrated elevated levels of Na⁺ accumulation in alphavirus-infected cells (Garry et al., 1979; Ulug et al., 1989). Surprisingly, treatment of Sindbis virus-infected cultures with ouabain resulted in a significant reduction in the rate of ²²Na⁺ uptake. Therefore, in contrast to its mode of action in uninfected cells, the Na⁺K⁺ATPase directed a net positive (i.e., inward) flux of ²²Na⁺ in Sindbis virus-infected cultures. This result suggests that an alteration in the ionic selectivity of the Na⁺K⁺ATPase contributes to enhanced accumulation of Na⁺ by Sindbis virus-infected cells. Furthermore, since the rate of ouabain-resistant ²²Na⁺ influx was not significantly altered during the course of infection of cells with Sindbis virus, the enhanced uptake of Na⁺ by Sindbis virus-infected cells can be attributed to a specific effect on the Na⁺K⁺ATPase and not to other transport systems.

DISCUSSION

Sindbis virus infection of avian cells results in an increase in the intracellular concentration of Na⁺ and a

![Diagram](image-url)

FIG. 4. Dephosphorylation kinetics of membranes from Sindbis virus-infected cells. Plasma membranes from uninfected (open symbols) and Sindbis virus-infected cells (closed symbols) were phosphorylated with [γ-³²P]ATP in the presence of 25 mM NaCl and 3 mM MgCl₂, for 30 sec at 4°C. Dephosphorylation was initiated by the addition of 1000-fold excess of unlabeled ATP in the presence (squares) and absence (circles) of 50 μM KCl. Background phosphorylation, monitored under identical conditions in reaction mixtures containing 25 mM KCl and 3 mM MgCl₂, was subtracted to obtain these results. Each datum point represents the means of triplicate determinations.

| Uptake of ⁸⁶Rb⁺ and ²²Na⁺ was quantitated 4 hr after Sindbis virus infection |
| Data are expressed as nmol/mg protein/min and represent the mean ± SEM of triplicate measurements. |
reduction in the intracellular concentration of K⁺ (Garry et al., 1979; Ulug et al., 1989). A reduction in the activity of the ouabain-sensitive Na⁺ pump occurs early after infection of cells with Sindbis virus and can account for the drop in cellular K⁺ levels (Ulugu et al., 1984). Results presented here (Table 2) suggest that elevated intracellular Na⁺ concentrations in Sindbis virus-infected cells also result from a selective inhibition of Na⁺K⁺ ATPase activity. Although Sindbis virus-infected cultures exhibited an elevated rate of Na⁺ uptake relative to uninfected controls in the absence of added drugs, the rate of ouabain-resistant 22Na⁺ uptake was unaffected. This indicates that elevated levels of Na⁺ in Sindbis virus-infected cells also result from selective inhibition of Na⁺K⁺ ATPase activity and not from increased nonspecific permeability (“leakage”) of the cell membrane or from an effect on other ion transport systems. These findings support the suggestion (Ulugu et al., 1987) that alphavirus-infected cells do not exhibit enhanced permeability to small molecules, as reported for cells infected with other RNA viruses (Carrascco, 1978; Impraim et al., 1980).

Results presented here demonstrate a reduction in the specific activity of the Na⁺K⁺ ATPase in membranes derived from Sindbis virus-infected cells. The reduction in enzymatic activity measured in vitro (Fig. 1) was not due to a loss of the enzyme during purification, since the Na⁺K⁺ ATPase was phosphorylated to the same extent in membranes derived from uninfected and virus-infected cells (Figs. 2 and 3). Furthermore, gel electrophoresis of Na⁺K⁺ ATPase phosphorylated intermediates revealed no qualitative difference in the mobility of the alpha subunit, suggesting that gross modification of this protein did not occur in virus-infected cells. These results indicate that loss of Na⁺ pump activity in cells infected with Sindbis virus does not occur as a consequence of substrate limitation or regulation by other soluble components. This is consistent with previous findings that correlated inhibition of Na⁺ pump activity with the appearance of Sindbis virus envelope proteins on the cell surface (Ulugu and Bose, 1985).

A reduction in the specific activity of the Na⁺K⁺ ATPase would indicate that one or more partial reactions of this enzyme is altered during the course of Sindbis virus infection. According to current models regarding Na⁺ pump function, phosphorylated intermediates of the Na⁺K⁺ ATPase undergo a conformational change which is necessary for binding of extracellular K⁺ with high affinity. Enzyme dephosphorylation is correlated with the transport and dissociation of the bound ion. In experiments presented here, in vitro phosphorylation of the Na⁺K⁺ ATPase by [32P]orthophosphate or [γ-32P]ATP was unaltered in membranes of Sindbis virus-infected cells, suggesting that formation of the Na⁺K⁺ ATPase phosphoenzyme was unaffected by the viral infection. Dephosphorylation of phosphoenzyme intermediates present in membranes of both uninfected and virus-infected cells was enhanced by low concentrations of potassium, albeit to a lesser extent in membranes of the virus-infected cells. However, phosphorylated intermediates of the Na⁺K⁺ ATPase prepared using membranes of virus-infected cells exhibited an elevated rate of dephosphorylation in the absence of added potassium. An enhanced rate of enzyme dephosphorylation in the absence of K⁺ indicates that these phosphoenzyme intermediates are either inherently unstable, or that their enzymatic turnover occurs in a K⁺-independent fashion. As discussed below, the 22Na⁺ transport studies presented in this report suggest that the Na⁺K⁺ ATPase indeed functions in a K⁺-independent mode in Sindbis virus-infected cells and raise the interesting possibility that Na⁺ may act as a substitute for K⁺, thereby accelerating the rate of the Na⁺K⁺ ATPase dephosphorylation in the absence of K⁺.

22Na⁺ transport studies presented in Table 2 suggest that the ability of the Na⁺ pump to discriminate between binding of extracellular Na⁺ and K⁺ is specifically compromised during the course of infection by Sindbis virus. Treatment of Sindbis virus-infected cells with ouabain resulted in a reduction in the rate of 22Na⁺ uptake. Therefore, in contrast to its mode of action in uninfected cells, the Na⁺K⁺ ATPase directed a net positive (i.e., inward) flux of 22Na⁺ in Sindbis virus-infected cultures. This suggests that the Na⁺ pump actively transports extracellular Na⁺ (instead of K⁺) into Sindbis virus-infected cells. Similar Na⁺-K⁺ exchange reactions catalyzed by the Na⁺K⁺ ATPase have been observed in erythrocyte membranes under certain experimental conditions (Blostein, 1983; Morgan et al., 1986). Future experiments will determine whether related mechanisms can account for the altered transport of Na⁺ by Sindbis virus-infected cells.

The notion that the Na⁺K⁺ ATPase in Sindbis virus-infected cells lacks selectivity for binding of K⁺ is in agreement with earlier findings that demonstrated a reduced affinity of Sindbis virus-infected cells for the cardiac glycoside ouabain (Ulugu et al., 1984). Although uninfected and Sindbis virus-infected cultures exhibited an equivalent maximal ouabain binding capacity, the virus-infected cultures exhibited a 3.5-fold weaker affinity for ouabain binding. K⁺ competes with ouabain for binding to the Na⁺K⁺ ATPase alpha subunit (Baker and Willis, 1972) and it has been suggested that ouabain binds to the enzyme conformation involved in K⁺ transport (Avruch and Fairbanks, 1972). An alteration in the conformation of the alpha subunit (or another change in the cell membrane that results from alphavirus infections) that reduces the ability of the Na⁺K⁺ ATPase to bind ouabain might also be expected to result in reduced selectivity for binding of extracellular monovalent ions.

Alphavirus-induced alterations in monovalent cation transport may serve to facilitate efficient maturation and release of progeny virions. It has been known for many years that release of Sindbis virus can be reversibly blocked by treatment of cultures with medium containing lowered NaCl concentrations (Waite and Pfefferkorn, 1984). Although lowering NaCl concentrations in the culture medium reduced the rate of 22Na⁺ uptake into uninfected cells, the 22Na⁺ uptake was unaffected. This indicates that elevated levels of Na⁺ in Sindbis virus-infected cells may act as a substitute for K⁺, thereby accelerating the rate of the Na⁺K⁺ ATPase dephosphorylation in the absence of K⁺.
VIRAL INHIBITION OF Na⁺ PUMP ACTIVITY

1970). Furthermore, ouabain enhances release of virus from hypotonically arrested cultures upon transfer into isotonic conditions, suggesting that inhibition of Na⁺-K⁺-ATPase activity by Sindbis virus may elevate the overall intracellular ion content, thereby favoring virus release (Ulug et al., 1989). Recently, Sindbis virus mutants have been isolated by selection for growth in hypotonic medium (Li and Stollar, 1995). Mutations that facilitate release of Sindbis virus under hypotonic conditions were mapped to the envelope protein E2. It will be valuable to determine whether infection of cells with these virus strains results in alterations in monovalent cation transport. Such studies with Sindbis virus mutants may define whether alteration of ion transport, hypotonic growth arrest, and induction of cytopathology are distinct viral functions.

ACKNOWLEDGMENTS

This investigation was supported by PHS Grants CA58291 to E.T.U.; AI22720, AI25909, Al34754, Al38844, and DE10862 to R.F.G.; and CA33192 to H.R.B. and by support to E.T.U. from the Kansas Agriculture Experiment Station. This is contribution 96-102-j of the Kansas Agriculture Experiment Station, Manhattan, KS.

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