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Phospholipase activation, free fatty acids and the proton permeability of a biological membrane

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The rate of collapse of a proton gradient across the apical membrane of rat kidney proximal tubule increases upon treatment with calcium, mercuric chloride and mellitin, substances which activate phospholipase A_2. Treatment with phospholipase A_2 or oleic acid also enhances the rate of proton gradient dissipation. Membrane water permeability is not affected. This phenomenon may have implications in pathological states arising from ischemia or toxic exposure.

Proton transport; Fatty acid; Phospholipase; Plasma membrane vesicle; Mercuric chloride; Ca^{2+}

1. INTRODUCTION

Maintenance of proton gradients in cellular systems is essential for normal cellular homeostasis. For example, if the proton gradient in the mitochondria is dissipated, the cell cannot produce ATP and the cell will eventually die. Consequently, factors which increase proton permeability in biological membranes may also be factors which lead to disintegration of cellular homeostasis via disruption of cellular energy transduction.

We have used the isolated biological membrane system, brush border membrane vesicles (BBMVs) obtained from the proximal tubule of rat kidney, to show that intravesicular calcium ion, intravesicular mercuric ion, fatty acids and exogenous phospholipase A_2 all increase proton permeability of the plasma membrane. These factors may be significant in mechanisms of necrotic cell death.

2. MATERIALS AND METHODS

BBMVs were obtained from rat kidneys and isolated by differential centrifugation using the magnesium-precipitation method [1] to prevent inadvertent activation of phospholipases by calcium [2]. Vesicles were used within 4 h of preparation, and not frozen in order to avoid freeze-thaw activation of endogenous phospholipase [3].

Proton transport was monitored by acridine orange fluorescence [4]. Water transport was measured as described previously [5].

Membrane fatty acids were extracted from BBMVs with methanol/hexane/1 M phosphoric acid (3:8:1 by vol.). The hexane phase was washed twice with 0.1 M phosphoric acid. The fatty acids were back extracted into 0.01 volume of 0.5 M KOH, esterified with dimethylacetamide and methyl iodide, and analyzed by gas chromatography using pentadecanoic acid as an internal standard.

3. RESULTS

Addition of 1.8 mM calcium to the extravesicular solution did not change the rate of dissipation of the proton gradient in BBMVs (fig.1). However, in the presence of the calcium ionophore, A23187, calcium increased the rate substantially. By comparison, A23187 alone had only a slight protonophoric effect. Since BBMV membrane has a low baseline permeability to calcium ion, and addition of A23187 will equalize the intravesicular and extravesicular calcium concentrations [6], these experiments demonstrate that intravesicular calcium increases the proton permeability of the BBMV membrane.

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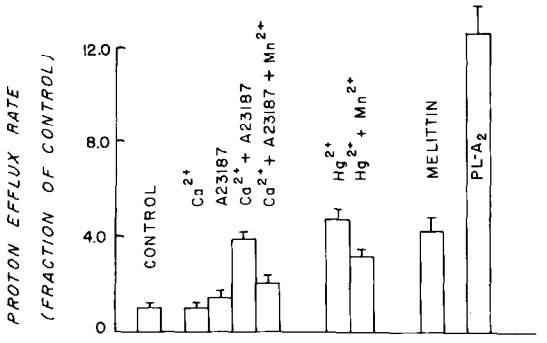

Fig.1. Rates of collapse of the proton gradient. BBMVs were suspended to 0.3 mg protein/ml in 190 mM mannitol, 50 mM choline chloride, 10 mM Hepes, 6 μM acridine orange, pH 6.0, then mixed in a stopped-flow photometer with an equal volume of an identical buffer but at pH 9.0, producing a final extravesicular pH of 7.5. The acridine orange fluorescence 100 ms after mixing was described well by a single exponential function; the exponential rate constant, obtained by non-linear least squares analysis, is expressed relative to control. Treatments were 1.8 mM $CaCl_2$, 10 μM A23187, 100 μM $MnCl_2$, 5 μM $HgCl_2$, 0.5 μg/ml melittin and 0.1 μg/ml porcine pancreatic phospholipase A_2 (PLA_2) for 2 h at 25°C.

A23187 is known to catalyze Ca^{2+}-H^+ exchange directly [7]; however, this exchange cannot account for the observed increase in proton permeability. The proton efflux experiments were done such that there was an outwardly-directed calcium gradient. If A23187-catalyzed Ca^{2+}-H^+ exchange was operative in our system, then A23187 would enhance proton influx, leading to a decrease in the rate of proton efflux. We observed an increase in proton efflux rate; therefore, if anything, our data may represent an underestimate of the true effect of intravesicular calcium.

Calcium is known to activate phospholipases [8]; we investigated the relation between an increase in intracellular calcium and enhanced phospholipase activity by measuring the effect of intravesicular divalent manganese on the calcium-induced enhancement of the proton permeability. Calcium-stimulated phospholipase activity can be inhibited by manganese [8]. Fig.1 shows that intravesicular manganese also inhibits the enhancement in proton permeability.

Divalent mercury, a calcium-mimetic activator of phospholipase [9], increased the proton permeability of BBMVs by 5.7-fold after a 2 h treatment (fig.1). Intravesicular manganese resulted in an inhibition of the mercury-induced enhancement. Similar effects of mercury and manganese on phospholipase activity have been reported in a cultured mouse fibroblast system [9].

That calcium and mercuric ions do indeed activate phospholipase in BBMVs was demonstrated by measuring the fatty acid content of the membrane subsequent to treatment. In two experiments, treatment with 2 mM Ca^{2+}, under conditions producing the increases in proton permeability seen in fig.1, increased the amount of oleic and arachidonic acids by factors of 2.9 and 5.6, respectively, over control values. Treatment with 5 μM Hg^{2+} increased the amount of oleic acid by 2.7 over control, while the concentration of arachidonic acid was relatively unaffected.

Melittin, isolated from bee venom, is a potent phospholipase activator [10]. Vesicles treated with melittin had a passive proton permeability four times greater than control. Treatment of the vesicles with exogenous pancreatic phospholipase A_2, which catalyzes the hydrolysis of phospholipids into free fatty acids and lysophosphatides, enhanced the dissipation rate of a proton gradient by a factor of 13 (fig.1).

These experiments suggest that enhanced phospholipase activity results in an increase in the proton permeability of BBMVs. A biochemically important product of phospholipase activity is fatty acids, since pharmacological effects of

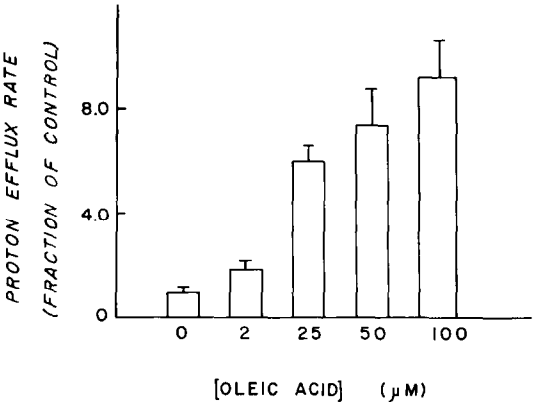

Fig.2. Oleic acid addition enhances proton permeability. 50 μl of a stock solution of oleic acid in ethanol were added to 50 ml of 0.3 mg/ml BBMV suspension and homogenized with a glass-teflon homogenizer. The rate of dissipation of a proton gradient was measured as described in the legend to fig.1. Treatment with ethanol alone had no effect on the dissipation rate.

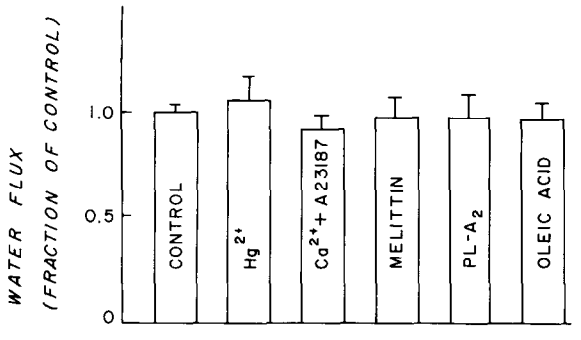

Fig.3. Osmotic water permeability of BBMVs is not affected by treatments affecting proton permeability.

phospholipase A_2 resemble those produced by high concentrations of fatty acids [11]. The effect of fatty acids on the rate of dissipation of proton gradients was examined by adding oleic acid directly to the BBMV suspension. A dose-dependent increase in the proton permeability was observed (fig.2).

The possibility that the increase in proton permeability seen with calcium, mercury, melittin, phospholipase A_2 and oleic acid was a result of a general disruption of the BBMV membrane rather than a result of protonophoric activity mediated by fatty acids was tested by measuring the effect of these agents on the osmotic water permeability of the vesicles. The data (fig.3) indicate that no significant differences exist between the experimental groups and the control group during the time that the same treatment produced marked enhancements in proton permeability. These experiments indicate that proton transport is enhanced before the membranes become disrupted or lysed. For instance, melittin at high concentrations has been reported to disrupt phospholipid bilayer membranes [12], presumably causing a large increase in water permeability. Our experiments indicate that melittin-treated BBMV membranes had a water permeability identical to control, but with much higher proton permeability, suggesting that phospholipase activation is the predominant effect of melittin at the dose employed.

4. DISCUSSION

Taken together, our results indicate that the rate of dissipation of a proton gradient in BBMVs can be enhanced by the incorporation of fatty acids into the membrane, either by activation of phospholipases or by direct addition of fatty acids. Because of the basic structural similarity of biological membranes, our results suggest that proton gradients in other biological membranes can also be dissipated by these agents. Our results do not give direct insights into the mechanism for the baseline proton permeability of biological membranes, but indicate that the permeability can be enhanced above this level by incorporation of fatty acids into the membrane.

Several recent reports in the literature indicate that the factors we have found which increase fatty acid concentration in BBMVs have similar effects in other membrane systems. An increase in the intracellular calcium ion concentration results in an increase in free fatty acids in a cultured fibroblast system [8,13]. Exposure of cultured LLC-PK_1 cells to 50 μM $HgCl_2$ for 30 min results in an increase of lysophosphatides and free fatty acids and a concomitant decrease in phospholipid, presumably by mercuric activation of phospholipase A [14,15]. Our data indicate that an increase in fatty acid concentration leads to an increase in BBMV proton permeability; addition of fatty acids also increases membrane proton permeability in lipid bilayers [16], presumably by the carrier mechanism proposed for protonophoric effects of other weak acids [17–19].

Fatty acids are known uncouplers of oxidative phosphorylation in mitochondria [20,21]. The uncoupling effect at high fatty acid concentration may be due to the increase in the proton permeability of the inner mitochondrial membrane, leading to dissipation of proton gradients and inability to drive ATP synthesis according to the chemiosmotic hypothesis [22]. Therefore, the distinct possibility exists that any insult to a living cell that would increase the fatty acid content of mitochondrial membranes would have severe pathological consequences.

Ischemia may be such an insult. In a rat liver system, mitochondrial free fatty acid concentration increases soon after the onset of ischemia [23]. Addition of bovine serum albumin, a fatty-acid scavenger, protects against the loss of phosphorylation capability. In rat kidney, the ischemia-induced increase in mitochondrial fatty acid content is correlated with a progressive loss of

oxidative phosphorylation capability [24]. Similar results correlating activation of phospholipase or elevated levels of fatty acid with ischemic insult have been obtained with perfused rat hearts [25], cultured myocardial cells [26], rat livers in vivo [27], dog hearts in vivo [28,29], and perfused pig kidneys [30].

The key irreversible step in cell death has not been specified on a molecular level. Our data and various reports in the literature suggest that the key step may be an accumulation of fatty acids in the mitochondrial membrane which dissipate the proton gradients necessary for ATP synthesis. In ischemia, the fatty acid concentration would increase via activation of phospholipases triggered by the rise in intracellular calcium concentration seen in dead and dying cells [13,31,32]; in mercury toxicity, the phospholipases would be activated directly by intracellular mercuric ion [9]. Fatty acids may have a dual pathogenic role in ischemia because of their ability to increase the calcium permeability of biological membranes [33,34].

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