

Effects of Cholesterol on (Na⁺,K⁺)-ATPase ATP Hydrolyzing Activity in Bovine Kidney[†]

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ABSTRACT: The (Na⁺,K⁺)-ATPase ATP hydrolyzing activity from rabbit kidney medulla basolateral membrane vesicles was studied as a function of the cholesterol content of the basolateral membranes. The cholesterol content of the membranes was modified by incubation with phospholipid vesicles. When the cholesterol content was increased above that found in the native membrane, the (Na⁺,K⁺)-ATPase ATP hydrolyzing activity was inhibited. When the cholesterol content was decreased from that found in the native membranes, the (Na⁺,K⁺)-ATPase ATP hydrolyzing activity was inhibited. Analogous effects were found with the K⁺-activated phosphatase activity of the same membrane vesicles. Therefore, at low cholesterol contents, cholesterol was stimulatory, and at high cholesterol contents, cholesterol was inhibitory. The structural specificity of this effect was tested by introducing lanosterol and ergosterol as 50% of the membrane sterol. Ergosterol was the least effective at supporting (Na⁺,K⁺)-ATPase ATP hydrolyzing activity, while lanosterol was more effective, but still not as effective as cholesterol.

The role of cholesterol in normal mammalian cell function has not yet been adequately defined. Much is known concerning the interaction of cholesterol with membrane lipids (Yeagle, 1985), but the connection between the interaction of cholesterol with membrane lipids and the effects of cholesterol on cell membrane function has not been clearly drawn.

More recently, information has appeared concerning effects of membrane cholesterol on mammalian cell membrane enzymes. One notable example is the (Na⁺,K⁺)-ATPase. Early reconstitution experiments noted an inhibition of the enzyme by cholesterol (Kimelberg & Papahadjopoulos, 1974). Subsequently, studies of (Na⁺,K⁺)-ATPase in human erythrocytes showed inhibition of activity by high levels of membrane cholesterol (Giraud et al., 1981; Yeagle, 1983).

The present study examined the effects of cholesterol on the ATP hydrolyzing activity of bovine kidney (Na⁺,K⁺)-ATPase at both high and low membrane cholesterol content in the basolateral membranes. At high cholesterol content, inhibition of ouabain-sensitive activity by cholesterol was observed, in good agreement with previous studies on the (Na⁺,K⁺)-ATPase found in other tissues. The effects of cholesterol on this enzyme at low membrane cholesterol content had not been previously reported from any tissue. In the study described here, the data suggested that at low membrane cholesterol content, cholesterol apparently stimulated the activity of the (Na⁺,K⁺)-ATPase.

MATERIALS AND METHODS

Egg phosphatidylcholine was obtained from Avanti Polar Lipids, Inc., and produced a single spot with two-dimensional thin-layer chromatography: (a) chloroform/methanol/ammonia (65:25:5) and (b) chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1). The plates were developed by sulfuric acid charring. Lanosterol was obtained from Steraloids, Inc. Cholesterol, ergosterol, ATP, Percoll, EDTA, triethanolamine hydrochloride, histidine, ouabain, deoxycholic acid, Fiske-SubbaRow reducer, and nitrophenyl phosphate were obtained

from Sigma Chemical Co. Whatman Linear K-Silica Gel thin-layer chromatography (TLC)¹ plates were used for thin-layer chromatography experiments. Biorad protein assay dye reagent concentrate was obtained from Bio-Rad Laboratories. The sterols produced a single spot when analyzed on thin-layer chromatography in petroleum ether/diethyl ether/glacial acetic acid (90:10:1 v/v) visualized by sulfuric acid charring of 0.1 mg of sterol.

Membrane Preparation. All preparations were done at 4 °C on ice, using a modified method similar to Jorgensen and Skou (1971). A fresh bovine kidney was obtained from a local slaughterhouse and placed on ice until ready for preparation. The cortex and medulla were removed from the kidney and passed through a meat grinder. The resulting tissue was placed in a blender with homogenization buffer consisting of 250 mM sucrose and 10 mM triethanolamine hydrochloride (pH 7.6) with 2.6 mg of PMSF/L of buffer. The homogenization buffer was added to the tissue in a 10% (w/v) ratio of tissue to buffer. The resulting mixture was blended for 10 1-min intervals with 3-min cooling periods between blendings. The resulting mixture was centrifuged at 2750 rpm, for 10 min, in a GSA rotor, in a Sorvall superspeed centrifuge. The supernatant was removed and placed on ice. The pellets were resuspended in an equal volume of homogenization buffer and blended again for 10 1-min intervals with cooling periods between blendings. The mixture was centrifuged as before, and the supernatants were pooled together. The pooled supernatants were centrifuged at 4750 rpm for 15 min in a GSA rotor. The supernatant and the lighter, fluffy pellet was removed by gentle swirling, and the lower dark pellet was discarded. The supernatant and fluffy pellet were homogenized with five strokes in a Teflon glass homogenizer (Caframo). The resulting homogenate was centrifuged at 14 500 rpm, for 30 min, in a GSA rotor. The supernatant and lower dark pellet were removed, and the fluffy pellet was resuspended in a small amount of homogenization buffer. The pellet was centrifuged at 45 000 rpm, for 20 min, in a 50 TI rotor in a Beckman ultracentrifuge. The resulting pellet was resuspended in homogenization buffer,

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¹ Abbreviations: BLM, basolateral membrane(s); PC, phosphatidylcholine; SUV, small unilamellar vesicle(s); TLC, thin-layer chromatography.

sealed with argon, and frozen at -70°C for later use.

Basolateral Membrane Preparation. Membranes obtained from the above procedure were thawed, and 3 mL of membranes was resuspended in 23.5 mL of homogenization buffer with five strokes in the Teflon glass homogenizer. Then 3.5 mL of Percoll was added to the homogenate and mixed well. The mixture was then centrifuged at 16 500 rpm, for 45 min, in an SS-34 rotor. The resulting gradient was pumped off through a UV monitor into a fraction collector. Each fraction from the gradient was assayed for protein and $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ activity. The gradient fractions containing the peak of the $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ activity (usually 7–15 mL from the top of the gradient) were collected as the basolateral membranes and centrifuged at 40 000 rpm, for 1 h, to remove the Percoll. The resulting pellet was resuspended in 100 mM NaCl, 10 mM histidine, and 1 mM EDTA (pH 7.0) and washed twice by centrifuging at 45 000 rpm, for 20 min, in a 50 Ti rotor. The basolateral membranes were resuspended in the same NaCl, histidine, and EDTA buffer for incubation with small unilamellar vesicles. The sidedness of this preparation of basolateral membrane vesicles was determined previously (Goldinger et al., 1984) to be a mixture of rightside-out, inside-out, and leaky vesicles. This membrane preparation has been characterized previously (Goldinger et al., 1984). The bovine kidney preparation used here exhibited the same characteristics with respect to the separation of basolateral from brush border membranes on the Percoll gradient as did the previously described rabbit kidney preparation (Goldinger et al., 1984).

Preparation of Small Unilamellar Vesicles. Small unilamellar vesicles (SUV) were made according to the procedure previously described by Yeagle and Young (1986). When cholesterol, ergosterol, or lanosterol was added to small unilamellar vesicles, the sterols were then added in a 1:1 weight ratio with egg phosphatidylcholine (PC) or at the normal physiological level found in membranes of 40 mol % cholesterol. The lipid was suspended at a concentration of 8 mg/mL and sonicated and centrifuged as described.

Membrane Sterol Modification. The following procedure was used to incorporate cholesterol, ergosterol, and lanosterol into basolateral membranes or delete cholesterol from the membranes. The incubation mixture consisted of 6 mL of small unilamellar vesicles (8 mg/mL phospholipid) and 2 mL of washed basolateral membranes (10 mg/mL protein). The mixture was incubated at 37°C , for 18 h, under nitrogen, in a shaking water bath. The membranes and small unilamellar vesicles were separated by centrifugation at 45 000 rpm, for 15 min, in a 50 Ti rotor. The supernatant, containing the small unilamellar vesicles, was discarded, and the basolateral membranes were stored at 4°C until ready for assays.

Assays. Phospholipid content was determined as inorganic phosphate by the method of Barlett (1959). Cholesterol content was determined by the method described by Allain et al. (1974). Protein concentration, for the $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ assay, was determined by the method of Bradford (1976), using a commercial dye reagent (Biorad Laboratories). Protein determination for K^+ -dependent phosphatase assay was determined by the method of Lowry et al. (1951). K^+ -dependent phosphatase activity was determined by the assay described by Yoshida et al. (1969), using protein concentrations of 0.100 mg of protein and a 2 mM KCl concentration to maximize phosphatase activity. $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ activity was determined by the method of Jorgensen and Skou (1969), coupled to the Fiske and SubbaRow method (Sigma) for the determination of inorganic phosphate. Membranes were preincu-

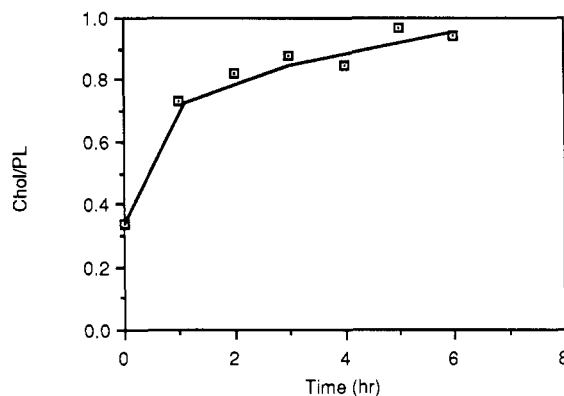


FIGURE 1: Effect of the cholesterol:phospholipid mole ratio in bovine kidney basolateral membranes due to incubation with an excess of PC SUV containing cholesterol at a 1:1 mole ratio with phospholipids. The data show an increase in the cholesterol content of the native membranes with time. Each point represents the average of data in triplicate.

bated in 25 mM imidazole, pH 7, and 2 mM EDTA with 0.07% deoxycholate to permeabilize the membrane vesicles for entrance of the substrate MgATP. The ATPase assays were performed in 30 mM histidine, pH 7.5, with 130 mM NaCl and 20 mM KCl in the total ATPase assay and 150 mM NaCl in the ouabain ATPase assay. The difference in activity between the total ATPase activity and the activity measured in the presence of 1 mM ouabain (and the absence of potassium) was assigned to the $(\text{Na}^+, \text{K}^+)\text{-ATPase}$.

Thin-Layer Chromatography. Incubated samples of basolateral membranes were extracted by the method of Folch et al. (1957). Cholesterol, ergosterol, and lanosterol standards of a variety of concentrations, as well as extracted membrane samples, were run on thin-layer chromatography (TLC) plates using the solvent system of petroleum ether, diethyl ether, and acetic acid (90:10:1), as described by Lange and Steck (1985). The plates were developed with sulfuric acid/methanol spray (10:90) and charring.

RESULTS

Modulation of Cholesterol Content of Basolateral Membranes. The cholesterol content of the basolateral membranes of bovine kidney was modified by using procedures analogous to those described previously by Yeagle and Young (1986). Membrane vesicles were incubated with sonicated egg phosphatidylcholine vesicles for cholesterol transfer. Net cholesterol movement occurred if a significant difference existed in the cholesterol content of the egg PC SUV and the BLM vesicles. In those cases where the cholesterol content of the egg PC SUV was initially higher than the BLM vesicles, net cholesterol movement occurred to increase the cholesterol content of the BLM vesicles. When the egg PC SUV were initially lower in cholesterol content than the BLM vesicles, net cholesterol movement occurred to decrease the cholesterol content of the BLM vesicles.

An example of this is shown in Figure 1. Egg PC vesicles containing cholesterol at a 1:1 mole ratio with the phospholipids were incubated with the BLM vesicles. At the indicated times, aliquots were removed, and the BLM were separated from the egg phosphatidylcholine SUV by differential centrifugation. These isolated fractions were analyzed for their cholesterol and phospholipid content. In Figure 1, the cholesterol:phospholipid mole ratio of the BLM membranes is plotted as a function of incubation time. The data show evidence for an increase in cholesterol/phospholipid content with increase in incubation time. Analogous results were obtained

for cholesterol depletion of the BLM vesicles, using egg PC SUV which contained no cholesterol. Intermediate results were obtained when egg PC SUV with intermediate cholesterol content were employed in the incubation (data not shown). By use of these methods, the cholesterol content of the BLM could be varied from a cholesterol to phospholipid mole ratio of 0.05 to 1.1.

One of the potential problems with such incubations is incidental fusion between the SUV and BLM vesicles. Such fusion would be manifest as an increase in the phospholipid content of the BLM membranes. Therefore, the phospholipid:protein ratio of the BLM membranes was determined during these incubations. In most cases, no significant change occurred in the phospholipid:protein ratio. In those cases where a significant change occurred, suggesting a fusion problem, the subsequent data on $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ activity were not used.

Effects of Incubation of BLM Vesicles with Egg PC SUV on $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ Activity. The purpose of these experiments was to examine the effects of varying membrane cholesterol content on the activity of the $(\text{Na}^+, \text{K}^+)\text{-ATPase}$. Since the method available for modifying the cholesterol content of the BLM membranes involved incubation with egg PC SUV, it was necessary to determine the effects of such incubation on $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ activity in the absence of a change in membrane cholesterol content. Activity was measured before and after typical incubations in the absence of egg PC SUV. Changes in activity from 0% to 20% were observed, depending upon the preparation and the experiment. Therefore, in each experiment in which cholesterol content was modified by incubation, a companion control experiment (with no net change in membrane cholesterol content) was performed to account for any changes in activity due solely to the incubation. This involved incubation of BLM with egg PC SUV with a cholesterol content matching that of BLM. The activity of this control was then assigned the value of 100% activity for that particular experiment. In this manner, changes due to the incubation and variations from preparation to preparation could be controlled for in these experiments.

Effects of Varying Membrane Cholesterol Content on $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ Activity. The cholesterol content of BLM membranes were altered by using the incubation methods described above, and the ouabain-sensitive ATP hydrolyzing activity was measured. Figure 2 presents the results. Maximal activity is noted at the native membrane cholesterol content. As the membrane cholesterol content is increased beyond that found in the native membrane, the ouabain-sensitive ATP hydrolyzing activity decreases. This is quantitatively analogous to previously reported results on the human erythrocyte $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ (Giraud et al., 1981; Yeagle, 1983). Some of these previous results are therefore presented on the same graph for comparison.

Interestingly, activity also decreases with a decrease in membrane cholesterol content from that found in the native membrane. This effect had not previously been reported for the $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ because experiments had not previously been performed at cholesterol contents significantly lower than found in the native membrane. These results suggest that very little, or perhaps no, $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ activity is found in the absence of membrane cholesterol.

Total ATPase activity of these preparations showed no significant change with changes in the membrane cholesterol content.

Restoration of $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ Activity. Activity lost due to loss of cholesterol could be mostly restored by reincu-

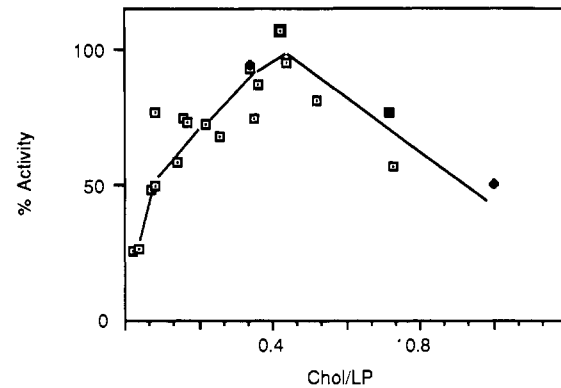


FIGURE 2: Relationship between the ouabain-sensitive ATP hydrolyzing activity of bovine kidney basolateral membranes and the membrane cholesterol content of these membranes, expressed as the cholesterol:phospholipid mole ratio. (□) Data from this work on kidney basolateral membranes; (●) data from previous work on erythrocyte $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ (Yeagle, 1983). Each point represents the average of data in triplicate.

bation with cholesterol-containing vesicles to add back membrane cholesterol. Total recovery of activity was not always achieved, presumably due to membrane damage resulting from two long, back-to-back incubations.

Effects of Other Sterols on $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ ATP Hydrolyzing Activity. Experiments were performed to determine whether other sterols could substitute for cholesterol in supporting $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ ATP hydrolyzing activity. BLM vesicles were incubated with egg PC SUV containing either lanosterol or ergosterol. The incubations were followed over time, analyzing the BLM for sterol content. In these incubations, a net flux of cholesterol was observed from the BLM into the SUV. Also, a net flux of the other sterol was observed from the SUV to the BLM. Conditions were established where 50% of the membrane cholesterol was removed and quantitatively replaced with either lanosterol or ergosterol. The ouabain-sensitive ATP hydrolyzing activity was then determined and compared with controls which had been incubated similarly, but had experienced no loss of membrane cholesterol and no incorporation of exogenous sterol. A membrane with half of its sterol cholesterol and the other half lanosterol supported $78 \pm 8\%$ of the activity seen with the 100% cholesterol sample. BLM with half of its sterol cholesterol and the other half ergosterol supported $52 \pm 8\%$ of the activity seen with the 100% cholesterol sample. Apparently, cholesterol is better than either lanosterol or ergosterol at supporting $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ ATP hydrolyzing activity. Lanosterol, a precursor to cholesterol in the biosynthesis of cholesterol, supported higher activity than did the nonmammalian sterol ergosterol. It did not prove experimentally feasible to achieve a higher replacement of native membrane cholesterol by exogenous sterols other than cholesterol.

Effects of Varying Membrane Cholesterol Content on Potassium-Activated Phosphatase Activity in Basolateral Membranes. The phosphatase activity was measured as a function of membrane cholesterol in the basolateral membranes, following similar protocols to those described above. The results are presented in Figure 3. As in the case of the ATPase activity, increasing cholesterol from low levels results in a stimulation of the activity, and at high cholesterol levels, there is a corresponding decrease in activity. Maximal activity occurs in the region of membrane cholesterol content corresponding to the native BLM.

DISCUSSION

These data indicate an inhibition of ouabain-sensitive ATP

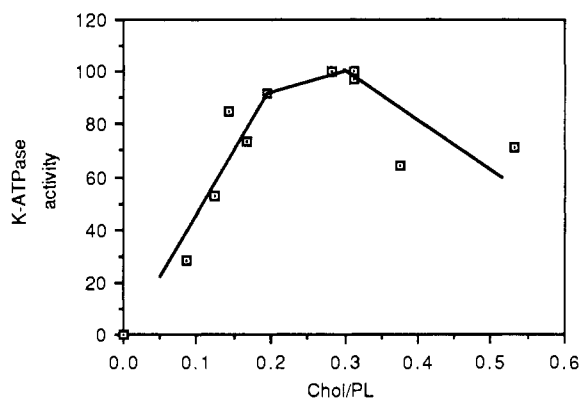


FIGURE 3: Relationship between K^+ -activated phosphatase activity of basolateral kidney basolateral membranes and the membrane cholesterol content of these membranes, expressed as the cholesterol:phospholipid mole ratio. Each point represents the average of data in triplicate.

hydrolyzing activity in bovine kidney basolateral membrane vesicles by membrane cholesterol at elevated (over normal) cholesterol:phospholipid mole ratios. These data also indicate that at low cholesterol levels (less than found in the native membranes) cholesterol stimulated ouabain-sensitive ATP hydrolyzing activity in the basolateral membrane vesicles. Interestingly, the cholesterol content that is found to support maximal activity in these bovine kidney basolateral membranes appears to be the level of cholesterol found in the native membrane.

Although the mechanisms of these effects cannot be described without ambiguity, some hypotheses can be advanced that are consistent with the observed data. High levels of cholesterol in the membrane lead to an increase in motional order in the membrane (Yeagle, 1985). This increase is linear with increases in cholesterol content. In human erythrocyte membranes (Giraud et al., 1981; Yeagle, 1983), rabbit erythrocyte membranes (Kamber & Kopeikina-Tsiboukidou, 1986; Uysal, 1986), guinea pig erythrocyte membranes (Kroes & Ostwald, 1971), rat liver membranes (Field et al., 1986), and in the basolateral membranes studied here, high cholesterol levels (above the levels found in the native membranes) inhibit the ouabain-sensitive ATP hydrolyzing activity. In this range of basolateral membrane cholesterol content, the decrease in ouabain-sensitive ATP hydrolyzing activity appears to be linear with increases in cholesterol content. Thus, there is a direct correlation between increases in motional order of the lipid hydrocarbon chains, induced by cholesterol, and decreases in enzyme activity, induced by cholesterol. This correlation might result from a decrease in conformational flexibility of the enzyme, limited by the increase in motional order of the lipid hydrocarbon chains in the membrane.

Even more interesting is the stimulation of both ouabain-sensitive ATPase activity and K^+ -activated phosphatase activity by increases in cholesterol content at low levels of basolateral membrane cholesterol. This stimulation may suggest an essential role for cholesterol in the activity of the (Na^+, K^+) -ATPase, to the extent to which the (Na^+, K^+) -ATPase is reflected in these two assays. Without cholesterol, these activities would appear to be low. The enzyme might even be inactive without cholesterol, though this cannot be unambiguously concluded from these data.

These results may provide a clue to the essential role of cholesterol in mammalian cells; that is, cholesterol is required for certain important membrane functions. This essential role of cholesterol has been found at the cellular level (Dahl et al., 1981). For example, cells with an obligatory requirement for

exogenous cholesterol require small amounts of cholesterol to exhibit growth. Removal of the sterol from the media inhibits growth. Other sterols cannot substitute for this obligatory requirement at low sterol contents.

The mechanism of the stimulatory effect of cholesterol would appear to be unrelated to the influence of cholesterol on the motional order of the membrane. Another possibility is that cholesterol may interact directly with the enzyme as an essential positive effector. Such a possibility has been raised previously (Giraud et al., 1981; Yeagle, 1983), although no firm evidence has yet been provided for this hypothesis. Such a direct interaction with the enzyme would likely have structural specificities for the sterol to be effective. The data presented here for lanosterol and ergosterol suggest that changes in the structure of cholesterol may render the resulting sterol less capable in its putative role of positive effector of the enzyme activity.

These data may represent a requirement for membrane cholesterol to maintain maximal activity of an important plasma membrane enzyme.

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Registry No. ATPase, 9000-83-3; cholesterol, 57-88-5; lanosterol, 79-63-0; ergosterol, 57-87-4.

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