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ALTERATIONS IN LACTOPEROXIDASE-CATALYZED RADIO-IODINATION OF MEMBRANE PROTEINS ASSOCIATED WITH VASOPRESSIN-INDUCED CHANGES IN TISSUE PERMEABILITY TO WATER

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SUMMARY

Lactoperoxidase-catalyzed radio-iodination was used to study the effects of vasopressin upon the luminal membrane of the toad's urinary bladder. Iodination of several proteins of this membrane, which represents the primary barrier to the flux of water, was increased by exposing the tissue to vasopressin and cyclic AMP. This effect was inhibited by colchicine, which reduces the effects of vasopressin upon water but not sodium flux; aldosterone, which stimulates sodium transport but not water flux, had no effect on tissue labeling under these conditions. The results provide evidence of hormone-induced alterations in the molecular conformation of membrane proteins related to hormone-regulated membrane permability to water and indicate this technique may be used to identify and isolate those proteins mediating the hormone response.

The distribution and conformation of cell membrane proteins are important determinants of membrane function. In the toad urinary bladder, which bears many similarities to the mammalian distal nephron, vasopressin causes large increases in both water permeability (1) and sodium transport (2). It has been suggested that these increases in tissue permability and transport, believed to be mediated by cyclic AMP (3,4), are the result of increased permeability of the luminal membrane (5,6). Hormone-induced alterations in the characteristics of this membrane would presumably be associated with conformational changes in the membrane proteins and with alterations in the accessibility of certain of these proteins to the luminal field.

The enzymatic radio-iodination of membrane proteins has recently been introduced as a technique for examining the molecular organization of membranes (7). Because lactoperoxidase (LPO), which catalyzes the iodination of tyrosine residues, does not penetrate the cell, it is possible to radioactively label and identify those membrane proteins exposed to the external milieu

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and to detect changes in the accessibility of these proteins to the catalytic mixture (8-11). Strum and Edelman showed this technique may be used to label the mucosal cells of the toad's urinary bladder without detectable changes in the function of the tissue as determined by electrophysiological parameters (12). However, attempts to use this technique to demonstrate vasopressin-induced changes in membrane structure were unsuccessful (13). We have devised a procedure to use the (^{125}I)-lactoperoxidase system as a probe for vasopressin-induced changes in the accessibility of toad bladder membrane proteins to the external milieu. Our data provide evidence for conformational changes in proteins of the apical membrane of the mucosal cells following the addition of either vasopressin or cyclic AMP to the tissue.

METHODS

Preparation of Toad Bladders. Toads of Dominical origin were partially immersed in tap water for 3-10 days prior to use. Paired urinary hemibladders, mounted as sacs on Luer-Lock syringes, were incubated at room temperature in a Ringers solution: NaCl, 85 mM; KCl, 4 mM; $NaHCO_3$, 17.5 mM; KH_2PO_4, 0.8 mM; $MgSO_4$, 0.8 mM; $CaCl_2$, 1.5 mM; dextrose, 10 mM; and pH 7.2-7.6.

Lactoperoxidase Labeling of Mucosal Cells. The hemibladders were filled with 3 ml of Ringers solution containing glucose oxidase, 0.03 mg/ml; lactoperoxidase (Sigma Chemical Co.), 0.09 mg/ml; and Na (^{125}I) (New England Nuclear, 17 Ci/mg), 1 x 10^{-7}M. After 15 minutes the luminal solution was removed, the bladders were rinsed with Ringers solution containing unlabeled NaI (2 mM), and the bladders were filled with and immersed in calcium-free Ringers solution containing 1.5 mM Na_2 EDTA. After 45 minutes the disaggregated mucosal cells were collected and sedimented (2000 x g for 10 minutes). In specified experiments, the luminal catalytic mixture was prepared in dilute (1/5) Ringers solution.

Separation of Mitochondria-Rich and Granular Mucosal Cells. The disaggregated mucosal cells were layered over discontinuous gradients of Ficoll (Pharmacia, Piscataway, New Jersey), and centrifuged at 27,000 rpm for 45 minutes in a Beckman SW-27 rotor at 4°C (14). The bands of material containing mitochondria-rich (MR) and granular (G) cells were removed separately, diluted with EDTA-Ringers solution and sedimented (15,000 rpm for 10 minutes).

Preparation of Total Membrane Fraction. The separated cells were resuspended in 8 ml of normal Ringers and disrupted by cavitation following equilibration with nitrogen in a Parr bomb for 30 minutes at 100 atm. These preparations were sedimented at 42,000 rpm for 60 minutes in a Beckman 42.1 rotor.

SDS-Acrylamide Electrophoresis. The pellets were dissolved in a solution of 2% SDS, 2% mercaptoethanol, and 8M urea by heating to 100°C for

5 minutes. 8% acrylamide gels containing 0.1% SDS were prepared by the method of Zahler (15) and pre-electrophoresed for 30 minutes at 5 mA per tube. Samples containing equal amounts of protein were layered over the gels, electrophoresed for 6 hours, and stained with Amido Black in 10% acetic acid-50% methanol. The gels were scanned in a Gilford Model 2320 gel scanner. Duplicate gels were sliced (1 mm thick) and immersed in 0.5 ml NCS (Amersham-Searle) for 12-18 hours. Ten ml of toluene scintillation fluid (PPO, 6g/L; POPOP, 75 mg/L) were added and the samples were counted in a Beckman LS-230 liquid scintillation counter.

RESULTS AND DISCUSSION

Exposure of the luminal surface of the intact bladder to the (^{125}I)-LPO mixture resulted in the incorporation of significant amounts of radioiodine into several membrane proteins. Deletion of either LPO, glucose oxidase, or glucose from the incubation mixture completely abolished the labeling.

To determine whether vasopressin altered the accessibility of membrane proteins to the fluid bathing the luminal surface, the serosal (or nutrient) surface of one set of hemibladders was bathed for 20 minutes in Ringers solution containing vasopressin (10^{-8}M). These bladders, and a paired set of control tissues, were then filled with the (^{125}I)-LPO labeling mixture for 15 minutes. In Figure 1, which is representative of sixteen such experiments, the proteins of G cell membranes prepared from the hormone-treated bladders contained significantly ($p<0.01$) more (^{125}I) than the control tissues. Exogenous cyclic AMP caused similar changes in the incorporation of (^{125}I) into G cell membranes. The amount of label incorporated into the membranes of MR cells was much less than that of the G cells and was not consistently altered by vasopressin. This observation may be related to the relatively small surface area of the apical membrane of the MR cell and is consistent with previous autoradiographic studies in which labeling of MR cell membranes with ^{125}I-LPO was not detected (12).

Since our initial labeling experiments with vasopressin (and cyclic AMP) were carried out in the presence of osmotic gradients, and under these conditions vasopressin causes swelling of the mucosal cells (16), the

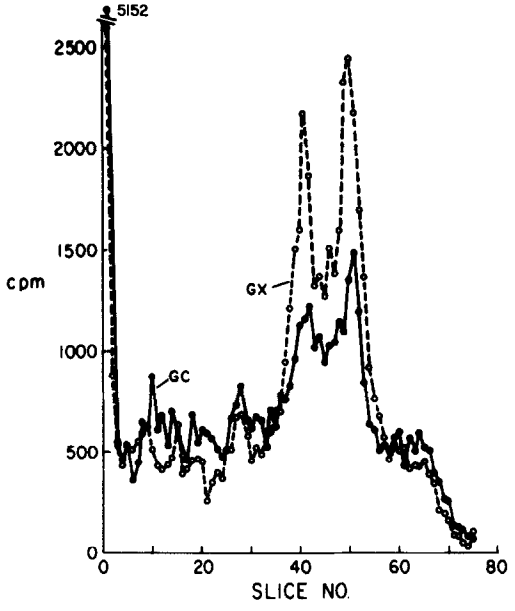

Figure 1. A typical experiment showing the effect of vasopressin upon LPO-catalyzed labeling of G cell membranes. Experimental tissues were incubated in Ringers containing vasopressin (2 X 10^{-8}M) for 20 minutes. These bladders, and the control tissues, were then filled with the LPO-(^{125}I) solution for 30 minutes. Equal amounts of membrane protein from the control (GC) and the vasopressin-treated (GX) tissues, as determined spectroscopically, were analyzed by SDS-acrylamide electrophoresis.

possibility existed that the observed changes in labeling were secondary only to this change in the size and shape of the cells. To rule out this possibility, we induced swelling of the mucosal cells by imposing a "reverse" osmotic gradient across the tissue (17), and compared the (^{125}I)-LPO labeling with that of control tissues. As shown in Figure 2, swelling the mucosal cells by means of the "reverse" gradient did not cause a significant alteration in the pattern of the labeling, indicating that the vasopressin-induced change in morphology is not solely responsible for the effects of the hormone upon labeling. Furthermore, vasopressin caused the same alterations in ^{125}I-labeling of proteins when the luminal and serosal solutions were undiluted Ringers, i.e., in the absence of an osmotic gradient.

Vasopressin (and cyclic AMP) might increase the labeling of membrane proteins by inhibiting the rate of hydrolysis or turnover of labeled mem-

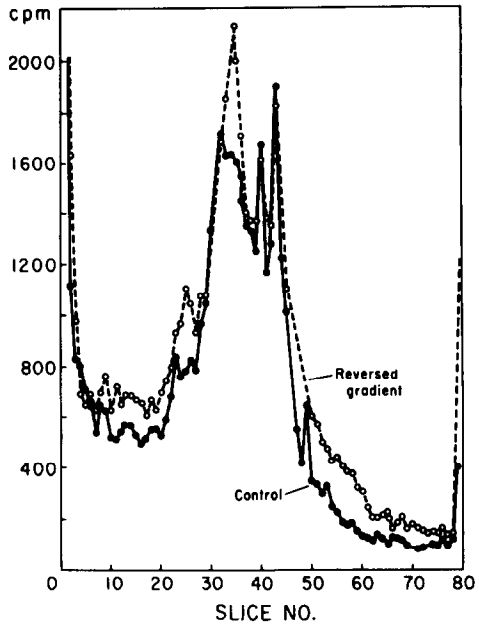

Figure 2. Effects of a "reversed" osmotic gradient upon LPO-catalyzed iodination of G cell membrane proteins. Control tissues were incubated in normal Ringers prior to and during labeling. "Reversed gradient" tissues were filled with Ringers and immersed in 1/5 strength Ringers for 20 minutes prior to and during radio-iodination.

brane protein. To examine this possibility, two sets of bladders were labeled with (^{125}I) in the absence of hormone, the bladders were rinsed and filled with fresh Ringers solution, and vasopressin was added to one set for 20 minutes. The pattern of labeling was identical in the two sets of tissues suggesting that an effect of vasopressin on the turnover of membrane protein is not responsible for our results.

Taylor et al. demonstrated that colchicine and related agents that block the polymerization of microtubules inhibit by 65-80% the vasopressin-induced increase in water permability without reducing its stimulation of sodium transport (18). In four experiments we found that pre-incubation of tissues with colchicine causes a large decrease in the labeling of G cell membrane proteins associated with vasopressin (Fig. 3). Lumicolchicine, a biologically inactive isomer of colchicine, had no effect on the LPO-catalyzed radio-labeling of the tissue. Aldosterone, which stimulates

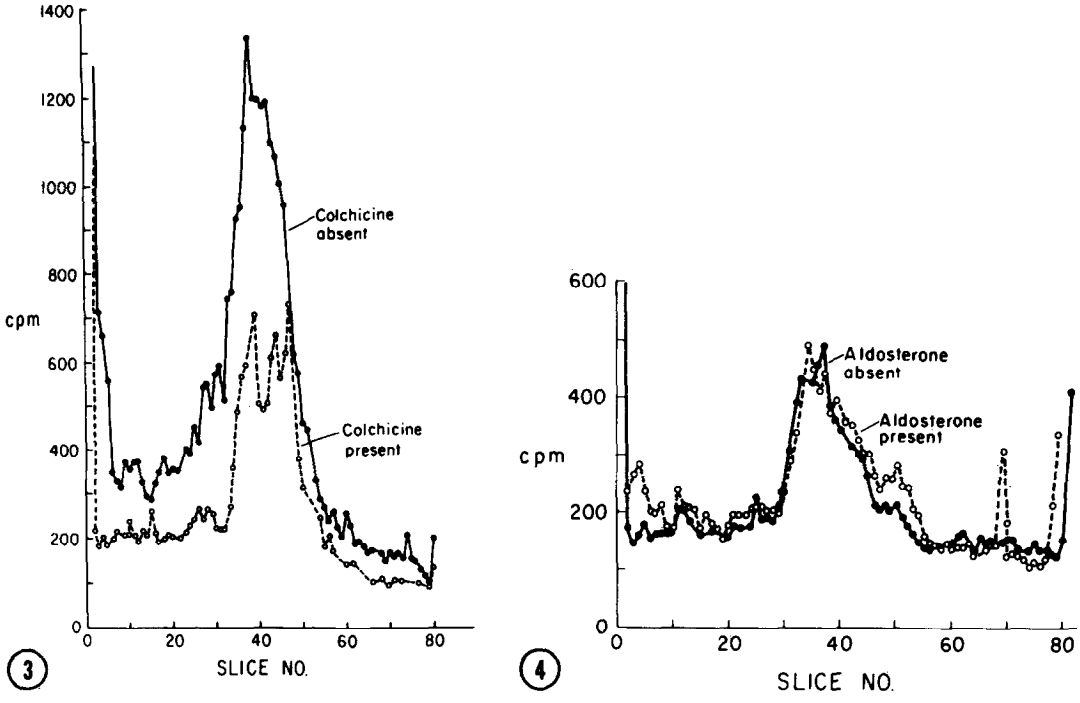

Figure 3. The effects of colchicine on LPO-catalyzed iodination of G cell membranes. Hemibladders were incubated in Ringers containing 10^{-4}M colchicine for 4 hours and the paired control tissues were incubated in Ringers alone. Both sets of tissues were exposed to vasopressin (2 X 10^{-8}M) for 20 minutes and then filled with the LPO-(^{125}I) mixture for 15 minutes.

Figure 4. The effect of aldosterone on LPO-catalyzed iodination of G cell membranes. Experimental hemibladders were pre-incubated with 10^{-7}M aldosterone for 4 hours, while the paired controls were incubated in diluent. Both sets of tissues were then labeled with LPO-(^{125}I) for 15 minutes.

sodium transport without increasing water permability had no effect on the LPO-catalyzed labeling of G cell proteins (Fig. 4). These results suggest that the vasopressin-induced changes in labeling are associated specifically with the effects of the hormone upon water flux rather than with its effects upon sodium transport.

Both scanning electron microscopy (19) and freeze-fracture techniques (20,21) have illustrated changes in the fine structure of the G cell membrane in association with vasopressin-induced increases in water permability. The technique described herein extends the structural study of this membrane from the microscopical to the molecular level. It also pro-

vides an opportunity to covalently label those proteins mediating the tissues response to this hormone so that they may be isolated and characterized.

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