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Aldosterone stimulates active Na^+ transport in rabbit urinary bladder by both genomic and non-genomic processes

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Abstract

The ability of aldosterone to stimulate Na^+ transport in a range of epithelial tissues has been known for many years. Early work suggested that aldosterone had a delayed action operating by transcriptional up-regulation of proteins such as the epithelial Na^+ channel. However more recent data has suggested that the hormone has a short-term non-genomic action. In this paper we investigate short and long-term actions of aldosterone on Na^+ transport in the rabbit urinary bladder. We have shown that aldosterone stimulates epithelial Na^+ channel activity, as measured by the amiloride-sensitive short-circuit current over a 3.75 h period and that this action is potentiated by cAMP. Using reverse transcriptase-polymerase chain reaction we have shown that aldosterone and forskolin in combination up-regulate mRNA synthesis for the β- and γ-subunits of the epithelial Na^+ channel. Using Western blotting we have shown in the case of the β-subunit that a corresponding increase in channel protein occurs. We have also demonstrated that aldosterone in the presence of inhibitors of phosphodiesterase can stimulate the short-circuit current across rabbit bladder epithelium over a 20 min period. An explanation for the synergistic interaction between aldosterone and cAMP is provided. We have shown that aldosterone can increase cAMP levels within urothelial cells over a 4 min period. We propose that this represents a non-genomic action of the steroid hormone.

Keywords: Urinary bladder; Epithelial Na^+ channel; Aldosterone

1. Introduction

Aldosterone has been known to increase Na^+ transport in amphibian urinary bladders since 1961 (Crabbe). This effect was demonstrated 24 h after injection of the hormone into the toad, *Bufo marinus*. Subsequent experiments with actinomycin D and puromycin (Edelman et al., 1963; Crabbe and de Weer, 1964) showed that the increase in Na^+ transport in response to the hormone was blocked if protein synthesis was inhibited. The ability of aldosterone to stimulate Na^+ transport in mammalian bladders was demonstrated by Wickham (1964) and Lewis and Wills (1983). The results of these experiments were in accord with the view that steroid hormones act physiologically as transcriptional regulators, and thereby induce the synthesis of new proteins. Although these early experiments identified transport of Na^+ through the apical membranes of urothelial cells as the rate limiting step for transmembrane sodium flux, the three subunits comprising the epithelial Na^+ channel (ENaC) which carry the Na^+ ions across the membrane were not identified until 1994 (Canessa et al.) in rat colon. These authors also confirmed the sensitivity of epithelial Na^+ channels to block by amiloride.

More recently Sheader et al. (2002) demonstrated that aldosterone increases cAMP levels in renal cortical collecting ducts over short time periods, thereby increasing Na^+ transport and demonstrating a non-genomic action of the hormone. The experiments reported in this paper investigate the actions of aldosterone on the rabbit bladder and reveal that these are both long and short term, and have the effect of enhancing active transepithelial Na^+ transport.

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2. Methods

2.1. Animals and reagents

The animals used were small Dutch rabbits (~1 kg) of either gender. They were cared for and killed according to Home Office guidelines. Reagents unless otherwise indicated were obtained from Sigma, Poole, UK.

2.2. Ussing chamber experiments

Urinary bladders were dissected free, washed in Krebs' solution and halved vertically. Each hemi-bladder was mounted separately between the two halves of an Ussing chamber, and clamped in position to act as a membrane, the electrical properties of which could be measured. The area of tissue exposed to the medium on each side was 0.2 cm^2. A DVC-1000 Dual Voltage Clamp (World Precision Instruments, FL, USA) was used to measure transepithelial voltage, and to clamp this potential difference to zero giving the short-circuit current. This has been demonstrated to correspond to the net transmembrane sodium flux in rabbit bladders (Lewis and Diamond, 1976). Transepithelial electrical potential difference was measured using KCl-agar bridges connected via Russell calomel electrodes (Russell, Auchtermuchty, Scotland) and the current passed via silver–silver chloride bridges. The bladders were oxygenated with 95% O_2 and 5% CO_2 and maintained at 37 °C. The composition of the Krebs' solution was (mM) NaCl 124, KCl 5, $MgCl_2$ 1.3, $NaHCO_3$ 26, $CaCl_2$ 1.1, KH_2PO_4 1.4 and glucose 10.

2.3. RNA extraction and reverse transcriptase-polymerase chain reaction

RNA was isolated from urothelial cells firstly by homogenising them in Tripure isolation reagent (Boehringer, Mannheim, Germany). This solution was extracted with chloroform, then propanol. Samples were centrifuged at 16,000×*g* for 15 min at 4 °C and the pellet taken up in ethanol and re-centrifuged. The final pellet was taken up in 20 ml sterile water and dissolved at 60 °C for 5 min. Samples were treated with a DNA-free kit (Ambion, TX, USA) to remove remaining DNA, and the RNA quantified by absorbance at 260 nM. Purity and integrity of the resulting RNA was assessed on agarose gel electrophoresis which demonstrated 28S and 18S rRNA as well defined bands.

Reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out using kits from HTBiotechnology Ltd. (Cambridge, UK) using the primers listed in Table 1.

Magnesium concentrations and cycling conditions were optimised for each pair of primers. These are outlined as follows. The optimal magnesium concentration was 2.0 mM for the β-subunit of the epithelial Na^+ channel, 2.5 mM for γ-subunit and β-actin, and 3.0 mM for α-subunit. Optimal parameters for the three stage cycling reaction were as follows: 30 s at 94 °C (denaturation); 30 s at 58 °C (54 °C in the case of γ-subunit) (primer annealing); and 30 s (1 min in the case of β-subunit) at 72 °C (primer extension). The number of denaturation-annealing-extension cycles required varied for each primer pair.

Table 1
Primer sequences for RT-PCR amplification of β-actin and rabbit epithelial Na^+ channel (rbENaC) subunits

Gene	Primer	Sequence	Product length (bp)
α-rbENaC	Forward	5′-tgg cga gga aag act gg-3′	417
	Reverse	5′-tca tcc tgt ccg tgc ac-3′	
β-rbENaC	Forward	5′-ctg aag ctg atc ctg gac-3′	753
	Reverse	5′-caa tga tga tct cgg caa ac-3′	
γ-rbENaC	Forward	5′-cga gat gct tct gtc cca at-3′	234
	Reverse	5′-cag gtc gtc gtc tat ctc-3′	
β-actin	Forward	5′-gct acg agc tgc ctg acg g-3′	328
	Reverse	5′-gag gcc agg atg gag cc-3′	

The expected size of product amplified by the four pairs of primers is given, based on the published mRNA sequences that code for these proteins. Accession numbers for the mRNA sequences of α-, β- and γ-subunits of the rabbit epithelial Na^+ channel are, respectively, AJ132108, AJ132109 and AJ132110.

PCR products were visualised under UV light and quantified using a UVI band software programme (UVItec Ltd., Cambridge, U.K). Restriction enzyme treatment of PCR products of all three subunits yielded the expected products.

2.4. Western blotting

Western blotting was used to investigate the effects of aldosterone on the expression of epithelial Na^+ channel subunit proteins. Rabbit urinary bladder epithelium (~100 mg of tissue) was suspended in 5 ml of hypotonic cell lysis buffer containing 100 mM Tris–HCl, 1 mM EDTA, 1 mM benzamidine hydrochloride and 1 mM AEBSF (4-(2-aminoethyl)benzenesulphonylfluoride). The tissue was homogenised to a cloudy suspension using a Polytron® PCU-2. The protein concentration of each sample was determined using a BCA protein assay (Pierce, IL, USA) containing bicinchoninic acid.

Polyacrylamide gels were cast 1.0 mm in thickness. The composition of the lower separating gel was10% (w/v) acrylamide, 375 mM Tris–HCl (pH=8.8), 0.1% (w/v) sodium dodecyl sulphate (SDS), 0.05% (w/v) ammonium persulphate and 0.05% (v/v) *N*,*N*,*N*′,*N*′-tetra-methyl-ethylenediamine (TEMED). The composition of the upper stacking gel was 4.2% (w/v) acrylamide, 125 mM Tris–HCl (pH=6.8), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate and 0.08% (v/v) TEMED.

Protein samples were mixed with sample buffer in the volumetric ratio 4 protein: 1 buffer. Sample buffer had the composition 250 mM Tris base, 16% (w/v) SDS, 40% (v/v) glycerol, 20% (v/v) β-mercaptoethanol and 0.04 mg/ml bromophenol blue (pH=8.7). The volume of each sample loaded was adjusted on the basis of the protein concentration to achieve approximately equal amounts of protein per well.