

# Rapid stimulation of cyclic AMP production by aldosterone in rat inner medullary collecting ducts

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## Abstract

Aldosterone stimulates sodium transport in the inner medullary collecting duct (IMCD) via the classic genomic pathway, but it is not known whether it also acts via a rapid, non-conventional pathway in this part of the nephron. The IMCD regulates the final sodium content of urine and expresses vasopressin receptors coupled to adenylate cyclase. The recently reported rapid, non-genomic actions of aldosterone have been associated mainly with an increase in intracellular  $\text{Ca}^{2+}$ ; however, it has also been shown to stimulate cAMP generation. Thus the aim of this study was to determine whether aldosterone stimulates rapid generation of cAMP in isolated IMCD segments. IMCD segments were microdissected from Sprague–Dawley rat kidneys and incubated at 37 °C for 4 min with aldosterone ( $10^{-12}$  to  $10^{-6}$  M), vasopressin ( $10^{-12}$  to  $10^{-6}$  M), or a combination of hormones

in the presence of a phosphodiesterase inhibitor. cAMP was measured by radioimmunoassay. While corticosterone and dexamethasone were ineffective, aldosterone stimulated a dose-dependent increase in cAMP within 4 min ( $P < 0.05$ ). This action of aldosterone was not inhibited by the MR antagonist spironolactone. Co-incubation of aldosterone with vasopressin resulted in a further increase in cAMP generation above that induced by the neurohypophysial hormone alone. Aldosterone-mediated cAMP generation was not inhibited by a vasopressin  $V_1$  or  $V_2$  receptor antagonist. These data support a novel and rapid, non-genomic effect of aldosterone in IMCD. Aldosterone does not apparently interact with the vasopressin receptor to stimulate cAMP generation.

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## Introduction

In recent years aldosterone has been shown to exert rapid, non-genomic effects in a number of tissues, including the kidney (Harvey & Higgins 2000), colon (Maguire *et al.* 1999) and vascular smooth muscle (Christ *et al.* 1995), as well as human mononuclear leukocytes (Wehling *et al.* 1987). These effects do not appear to be mediated via the classical type 1 mineralocorticoid receptor (MR), but rather by a distinct membrane-associated receptor whose identity has yet to be fully described. While the structure of this receptor remains obscure, there is a growing body of work describing the signal transduction pathways which link receptor activation to the rapid actions of aldosterone. To date, the pathway most commonly reported involves intracellular  $\text{Ca}^{2+}$ ; however, aldosterone has also been shown to stimulate rapid generation of cyclic AMP (cAMP) in porcine coronary vascular smooth muscle cells (Christ *et al.* 1999).

Aldosterone's classic target site is the principal cells of the cortical collecting duct; however, it has also been shown to stimulate sodium transport in the inner medullary collecting duct (IMCD) (Stokes 2000). The IMCD

comprises three morphologically distinct regions: the outer segment, IMCD<sub>1</sub>, which consists of principal and intercalated cells similar to those in the outer medullary collecting duct, the middle segment, IMCD<sub>2</sub>, which is a mixture of principal, intercalated and IMCD cells, and the inner segment, IMCD<sub>3</sub>, which consists solely of IMCD cells (Tisher & Madesen 1996). *In vivo* micropuncture studies have demonstrated that aldosterone is able to stimulate sodium reabsorption via the type 1 MR in IMCD (Uhlich *et al.* 1969), but it is not known whether it also exerts a rapid, non-genomic effect in this part of the nephron.

The major aim of this study was to determine whether aldosterone can stimulate rapid generation of cAMP in IMCD segments. We have also investigated the potential interaction between aldosterone and vasopressin in view of the sensitivity of IMCD to vasopressin via its  $V_2$  receptor coupled to adenylate cyclase (Wargent *et al.* 1999).

## Materials and Methods

Male Sprague–Dawley rats (200–250 g body weight; Charles Rivers Ltd, Margate, Kent, UK) were maintained

on a 12 h light:12 h darkness cycle and allowed free access to water and food (CRM; Labsure, Manea, Cambs, UK). Unless stated, all chemicals were obtained from Sigma Chemical Co (Poole, Dorset, UK).

#### Microdissection and isolation of IMCD segments

On the day of experimentation, rats were anaesthetized with 60 mg/kg Sagatal (pentobarbitone sodium; Rhone Merieux Ltd, Harlow, Essex, UK). The abdominal aorta was cannulated and, on occlusion of the aorta immediately above the left renal artery, the left kidney was perfused with 5 ml ice-cold Hanks-HEPES medium (137 mM NaCl, 5 mM KCl, 0.4 mM  $\text{NaH}_2\text{PO}_4$ , 0.8 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 4 mM  $\text{NaHCO}_3$ , 0.44 mM  $\text{KH}_2\text{PO}_4$ , 1 mM  $\text{MgCl}_2$ , 2 mM  $\text{CaCl}_2$ , 5 mM D-glucose, 10 mM  $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ , 20 mM N-2 hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES), 6% dextran (~87 000 kDa) and 0.1% bovine serum albumin (BSA; fraction V, fatty acid free), pH 7.4) followed by 4 ml ice-cold 550 U/ml collagenase (Worthington Biochemical Corp., Lakewood, NJ, USA) in Hanks-HEPES. The left kidney was removed, decapsulated and a 1–2 mm thick slice taken through the cortico-medullary axis. 'Pyramids' of tissue (1–2 mm wide) were peeled from this slice and incubated for 20 min in an aerated (95%  $\text{O}_2$ /5%  $\text{CO}_2$ ) solution of 110 U/ml collagenase in Hanks-HEPES. After repeated rinsing of the tissue in ice-cold Hanks-HEPES, IMCD segments (0.3–1 mm in length) were dissected out, on ice, under an inverted microscope and transferred singly in 2  $\mu\text{l}$  Hanks-HEPES onto cavity slides pretreated with Sigmacote (Sigma) followed by 0.5% BSA and dried between both applications. Another cavity slide was inverted over the first and the two sealed with petroleum jelly. Individual tubules were photographed using an image-capturing system (VGACAM; Electrim Corporation, Princeton, NJ, USA) to enable measurement of tubule length.

#### Experimental protocol

The slides were preincubated for 10 min at 35 °C. The incubation period was initiated by the addition of 2  $\mu\text{l}$  incubation medium containing the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), made up in dimethylsulphoxide (DMSO) and added to Hanks-HEPES so that the final concentration of IBMX was 1 mM and the final concentration of DMSO was 0.1% (v/v), and 1 mg/ml bacitracin. To establish hormone-stimulated cAMP production, the incubation medium also contained aldosterone, corticosterone, dexamethasone or arginine vasopressin (AVP) in a concentration range of  $10^{-12}$  to  $10^{-6}$  M. Co-incubations of aldosterone and AVP were performed at an AVP concentration range of  $10^{-12}$  to  $10^{-6}$  M with either  $10^{-10}$  or  $10^{-6}$  M aldo-

sterone. Steroids were solubilised in ethanol, the final concentration in the incubation medium being 0.05% (v/v).

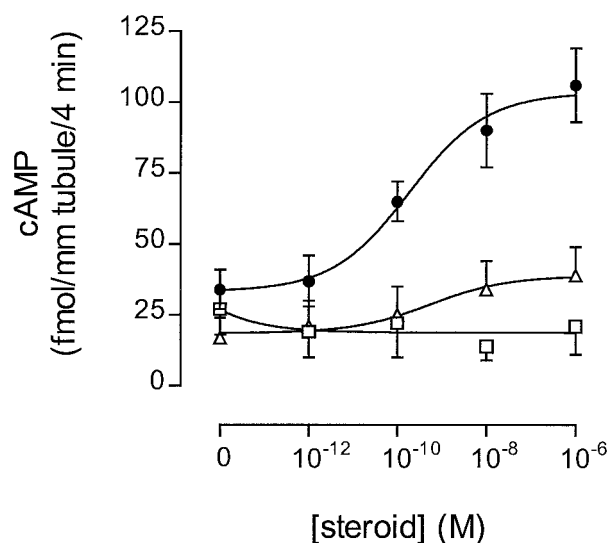
To investigate the potential involvement of the conventional MR in this rapid aldosterone-induced cAMP accumulation, tubules were preincubated with 1  $\mu\text{l}$  incubation medium containing 1 mM spironolactone (MR antagonist) for 10 min at 35 °C prior to incubation with 1  $\mu\text{l}$  aldosterone in incubation medium. In this experiment, spironolactone and aldosterone were solubilised in propan-1,2-diol, the final concentration at the incubation step being 0.1%. The slides were resealed and incubated for 4 min at 37 °C. In order to determine whether aldosterone was interacting with the vasopressin receptor, a further experiment was performed in which tubules were co-incubated with aldosterone ( $10^{-6}$  M) and either the AVP  $V_1$  antagonist (d(CH<sub>2</sub>)<sub>5</sub><sup>1</sup>, Tyr(Me)<sup>2</sup>, Arg<sup>8</sup>)-vasopressin ( $10^{-4}$  M) (Kruszynski *et al.* 1980; Bachem (UK) Ltd, Saffron Walden, Essex, UK) or the AVP  $V_2$  antagonist (d(CH<sub>2</sub>)<sub>5</sub><sup>1</sup>, D-Ile<sup>2</sup>, Ile<sup>4</sup>, Arg<sup>8</sup>, Ala-NH<sub>2</sub><sup>9</sup>)-vasopressin ( $10^{-4}$  M) (Sawyer *et al.* 1988; Bachem).

In all experiments, the 4-min incubations were terminated by aspirating the tubules in 1  $\mu\text{l}$  aspiration medium (1 mg/ml BSA and 1 mM IBMX in deionised water) and 1  $\mu\text{l}$  incubation medium into Durham soda glass tubes (35 × 8 mm) containing 20  $\mu\text{l}$  formic acid in ethanol (5%, v/v), which were then dried overnight at 40 °C and stored at –20 °C until radioimmunoassay for cAMP content.

#### cAMP measurement

cAMP levels were determined by radioimmunoassay using the protocol developed by Harper & Brooker (1975) and incorporating acetylation of cAMP. Samples and standards were allowed to equilibrate to room temperature and then acetylated by addition of 1  $\mu\text{l}$  of a 2:1 mix of triethylamine: acetic anhydride and tubes allowed to stand for 10 min before being transferred to ice. After acetylation, 20  $\mu\text{l}$  [<sup>125</sup>I]cAMP (adenosine 3',5'-cyclic phosphoric acid 2'-O-succinyl 3-[<sup>125</sup>I]iodotyrosine methyl ester; specific activity 2000 Ci/mmol, 3000 c.p.m./20  $\mu\text{l}$ ; Amersham International plc, Amersham, Bucks, UK) in phosphate buffer (39 mM  $\text{KH}_2\text{PO}_4$ , 48 mM  $\text{K}_2\text{HPO}_4$  and 7.7 mM  $\text{NaN}_3$ , pH 6.2) and 20  $\mu\text{l}$  anti-cAMP (Calbiochem, Beeston, Nottingham, UK) at a final dilution of 1:3000 in phosphate buffer, were added and all tubes vortexed and incubated for 24 h at 4 °C.

The antigen-antibody complex was precipitated by the addition of 50  $\mu\text{l}$  0.5% bovine  $\gamma$ -globulin (fractions II and III) and 250  $\mu\text{l}$  16% polyethylene glycol (molecular weight 3350), each made up in phosphate buffer, pH 6.8. After 15 min, the tubes were centrifuged at 2000 g and 5 °C for 20 min (Mistral 3000; MSE Scientific Instruments, Sanyo Gallenkamp plc, Loughborough, UK). The supernatant was then aspirated and the precipitate (bound fraction) counted for 10 min (Cobra II gamma counter; Canberra



**Figure 1** cAMP accumulation in IMCD segments incubated for 4 min with aldosterone (●), corticosterone (□) or dexamethasone (△). Each point represents the mean  $\pm$  S.E.M. of four to ten IMCD segments from three rats.

Packard, Pangbourne, Berkshire, UK). The lower limit of detection was 2.5 fmol cAMP/tube. Inter- and intra-assay variabilities were 15.7% and 12.6% respectively.

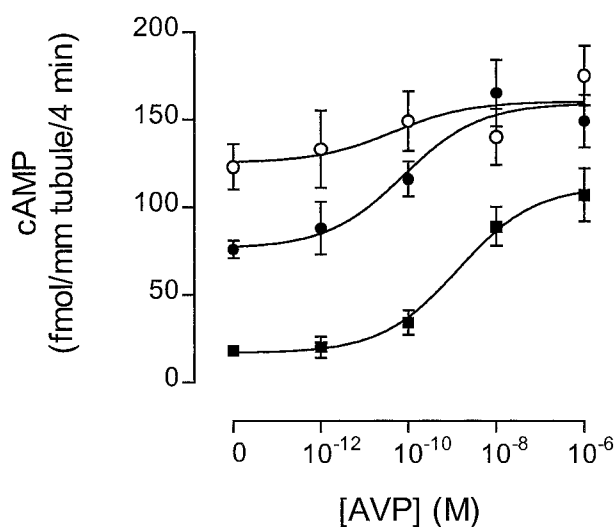
#### Data presentation and statistical analysis

cAMP generation is expressed per 4-min incubation period per mm tubule. For each treatment, results are expressed as means  $\pm$  S.E.M. for the number of tubules ( $n$ ). Data were analysed by ANOVA followed by Scheffé's test; significance was ascribed at the 5% level (SPSS for Windows, Ver. 10.1.0; SPSS UK Ltd, Woking, Surrey, UK).

## Results

Basal cAMP accumulation during the 4-min incubation of IMCD was approximately 25 fmol/mm tubule. As indicated in Fig. 1 addition of corticosterone to the incubation in the range  $10^{-12}$  M to  $10^{-6}$  M had no effect on cAMP production. Addition of the glucocorticoid receptor agonist, dexamethasone, over the same concentration range was similarly without significant effect on the cAMP content of tubules. By contrast, the presence of aldosterone during the 4-min incubation produced a dose-dependent increase ( $P < 0.05$ ) in cAMP accumulation ( $EC_{50}$   $1.2 \times 10^{-10}$  M). The highest concentration of aldosterone ( $10^{-6}$  M) produced a fourfold increase to approximately 100 fmol cAMP/mm per 4 min.

When the lowest concentration of aldosterone to produce a significant increase in cAMP ( $10^{-10}$  M) was



**Figure 2** cAMP accumulation in IMCD segments incubated for 4 min with AVP alone (■), AVP and aldosterone ( $10^{-10}$  M) (●) or AVP and aldosterone ( $10^{-6}$  M) (○). Each point represents the mean  $\pm$  S.E.M. of four to ten IMCD segments from three rats.

co-incubated with the type 1 MR antagonist, spiranolactone ( $10^{-3}$  M), there was no effect on the resulting aldosterone-stimulated accumulation of cAMP ( $65 \pm 7$  vs  $76 \pm 7$  fmol cAMP/mm tubule per 4 min).

Incubation of IMCD segments with AVP (Fig. 2) produced a dose-dependent stimulation ( $P < 0.05$ ) of cAMP similar to that observed with aldosterone, with maximal stimulated levels of 100 fmol/mm per 4 min. As shown in Fig. 2 the addition of  $10^{-10}$  M and  $10^{-6}$  M aldosterone alone (no AVP) again produced an increase in cAMP accumulation comparable with measures shown in Fig. 1. Co-incubation of tubules with aldosterone ( $10^{-10}$  M or  $10^{-6}$  M) with increasing concentrations of AVP resulted in further increases in cAMP accumulation ( $EC_{50}$   $1.4 \times 10^{-10}$  M and  $1.4 \times 10^{-6}$  M respectively). This was additive in nature, at least at the lower doses of vasopressin, with an apparent maximum stimulated cAMP level of approximately 150 fmol/mm per 4 min with the highest combined doses. This apparent higher upper limit of stimulated cAMP was independent of the concentration of aldosterone employed.

Aldosterone ( $10^{-6}$  M)-stimulated cAMP production ( $138 \pm 9$ ,  $n=23$ ) in IMCD was not inhibited by co-incubation with either  $10^{-4}$  M AVP  $V_1$  receptor antagonist ( $181 \pm 20$ ,  $n=19$ ) or  $10^{-4}$  M AVP  $V_2$  receptor antagonist ( $169 \pm 19$  fmol cAMP/mm tubule per 4 min,  $n=21$ ).

## Discussion

This study has clearly shown for the first time that aldosterone is able to induce a rapid increase in cAMP in

IMCD segments *in vitro*. This effect appears specific to aldosterone, as neither corticosterone nor the glucocorticoid receptor agonist, dexamethasone, affected cAMP generation. Potential effects of corticosterone could have been inhibited by any 11 $\beta$ -hydroxysteroid dehydrogenase type 2 (11 $\beta$ -HSD2) activity in the tubule preparation. However, the absence of an effect of dexamethasone, which is resistant to metabolism by 11 $\beta$ -HSD2 (Stewart *et al.* 1995), implies that activation of the conventional MR is not responsible for the increase in cAMP induced by aldosterone. This contention is further supported by the lack of effect of 10<sup>-3</sup> M spironolactone, the specific type 1 MR antagonist, on the aldosterone-induced increase in cAMP. The rapid action of aldosterone (within 4 min) is also consistent with a non-genomic mechanism. Furthermore, the half maximal effect was within the physiological range of free aldosterone in the rat (0.2 nM/l) (Menachery *et al.* 1991, Musabayane *et al.* 1994) and was comparable with that previously reported for rapid actions of aldosterone in other tissues (0.1–0.2 nM/l) (Wehling *et al.* 1987, Christ *et al.* 1995). These characteristics are typical of the reported non-genomic actions of aldosterone in other tissues, strongly suggesting that the IMCD is also a target site for a novel, rapid action of aldosterone.

The molecular identity of the receptor which mediates aldosterone's rapid effects is unknown; however, binding studies have identified high-affinity binding sites for aldosterone in membranes from various cells, which probably represent a membrane-bound receptor. It is unlikely that this receptor is a modified form of the classical MR, which shows similar affinity for aldosterone and cortisol/corticosterone, as attempts to characterise the receptor in porcine coronary artery smooth muscle indicate high selectivity for aldosterone over other steroids (Christ *et al.* 1999). It is also noteworthy that skin cells from MR knockout mice continue to exhibit a rapid increase in both cAMP and Ca<sup>2+</sup> in response to aldosterone (Haseroth *et al.* 1999).

In the present study, we have investigated the possibility that aldosterone may interact with vasopressin receptors localised in the IMCD and/or activate a common pool of adenylate cyclase. Neither the AVP V<sub>1</sub> nor the V<sub>2</sub> receptor antagonists employed was able to block the effect of aldosterone, suggesting that aldosterone does not interact with either subtype of AVP receptor in the IMCD. The maximum cAMP levels achieved when AVP or aldosterone were administered separately were similar (approximately 100 fmol/mm tubule per 4 min). However, when the two hormones were incubated in combination, although there was an increased response, the maximum levels of cAMP achieved (approximately 150 fmol/mm tubule per 4 min) were less than the simple addition of the two separate responses. This would be consistent with both hormones acting upon common target cells, in which a common pool of ATP provided a limit to maximal cAMP generation. However,

the potential heterogeneity of cell types present in the mixed population of IMCD segments used in these studies does not preclude the two hormones acting on separate cell types.

The physiological consequences of aldosterone-induced cAMP generation in IMCD cells remains to be established, though one possible target protein is the Na<sup>+</sup>/H<sup>+</sup> exchanger (NHE) in this segment (Sun *et al.* 1998). Activity of NHE1 is stimulated by vasopressin acting through cAMP in the medullary thick ascending limb (Sun *et al.* 1990) and NHE1 has also been shown to be activated by cAMP in osteoblasts (Azarani *et al.* 1995). Aldosterone is known to stimulate Na<sup>+</sup>/H<sup>+</sup> exchange via a rapid, non-genomic mechanism in human mononuclear leukocytes (Wehling *et al.* 1987), rat vascular smooth muscle cells (Christ *et al.* 1995), amphibian distal nephron (Oberleithner *et al.* 1987) and renal Madin-Darby canine kidney (MDCK) cells (Gekle *et al.* 1996). This raises the possibility that aldosterone may also stimulate cAMP leading to activation of NHE in the IMCD. Nonetheless, increased cAMP in the IMCD has also been shown to influence anion and water transport and urea permeability.

In summary, we have shown for the first time that aldosterone induces a rapid, dose-dependent increase in cAMP in IMCD segments *in vitro*. This was not blocked by the type 1 MR antagonist spironolactone and could not be mimicked by corticosterone or dexamethasone over the same concentration range, strongly suggesting that aldosterone was acting via a specific non-genomic mechanism. There appeared to be no interaction with the vasopressin receptor but the aldosterone response may involve a common adenylate cyclase and/or ATP pool.

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