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Roles of Na and Cl ions in Basal and Angiotesin II-Stimulated Aldosterone Secretion in Vitro by Bovine Adrenal Glomerulosa Cells

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The aim of this study was to observe basal and angiotensin II (AII)-stimulated aldosterone secretion in vitro by isolated bovine adrenal glomerulosa cells in the presence of varying concentrations of Na ion, and also to investigate the dependency of aldosterone secretion on extracellular Cl ion.

When Na in the incubation medium was replaced iso-osmotically by choline or N-methylglucamine, the absence of Na markedly inhibited basal and AII-stimulated aldosterone secretion, and concentrations of Na below 50mM impaired the ability of adrenal glomerulosa cells to respond to AII.

Basal aldosterone secretion was significantly higher at Na concentrations of 50 and 93mM in the choline substitution experiment, as compared with the normal concentration of Na, and the increment in aldosterone above basal levels by AII stimulation reached a maximum at a Na concentration of 93mM. The absence of Cl ion in the incubation medium didn't alter aldosterone secretion as Cl^- was replaced totally by NO_3^-

From these results, we conclude that extracellular Na ion appears to play an important role in the aldosterone secretion by isolated bovine adrenal glomerulosa cells.

Key Words: *Glomerulosa cell, Na^+, Cl^-, Basal, Angiotensin II (AII), Aldosterone*

INTRODUCTION

We have recently shown that changes in Na concentration between 102 and 168mM affect aldos terone secretion by a mechanism sensitive to the osmolality rather than the Na concentration.[1]

It is well recognized that Na ion is the main extracellular cation, and in addition to its role of maintaining extracellular osmolality, it plays many roles in cellular functions.[2-4] Several authors have postulated a specific role for extracellular Na ion in the regulation of aldosterone secretion,[5-6] but most studies were done in vivo and other factors may have been involved.

Saruta et al[7] and Lobo et al[8] reported that a decrease in Na concentration without changes in osmolality caused a small stimulation of aldosterone secretion in vitro by isolated adrenal tissue preparations. In contrast, Schiffrin et al[9] and Ball et al,[10] demonstrated that low extracellular Na concentrations inhibited aldosterone secretory response to AII. Thus, there is still some controversy in the role of extracellar Na ions in the regulation of in vitro aldosterone secretion.

In this study the direct effect of Na ion on basal and AII-stimulated aldosterone secretion in vitro by isolated bovine adrenal glomerulosa cells was examined by changing the Na ion concentration in the incubation medium. The effect on aldosterone secretion of total Cl ion removal in the incubation medium was also investigated.

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MATERIALS AND METHODS

1. Glomerulosa Cells Preparation

Glomerulosa cells were prepared from bovine adrenal glands by collagenase digestion and mechanical dispersion, according to Elliott's methods[11] with slight modification. In brief, fresh bovine adrenal glands were obtained in cold buffer solution at the slaughterhouse. This buffer contained 137mM NaCl, 3.6mM KCl, 1mM $MgSO_4$, 1.25mM $CaCl_2$, and 20mM Hepes (pH 7.4).

In the laboratory, each adrenal gland was freed of most of the surrounging tissues, and prepared by peeling off the capsule, bisecting and scraping away the medulla and zona fasciculata and reticularis. The tissue was cut into small pieces and placed into a vial containing 1.5ml of a modified Krebs-Ringer bicarbonate solution containing 0.1% bovine serum albumin (BSA), 10mM glucose, L-glutamin, and essential and non-essential amino acid mixtures with the potassium concentration adjusted to 3.5mM (KRBGA). Modified Krebs-Ringer bicarbonate solution contained 117mM NaCl, 2.5mM KCl, 1mM KH_2PO_4, 1.25mM $CaCl_2$, 1mM $MgSO_4$ and 24mM $NaHCO_3$. An 8.5ml aliquot of crude collagenase solution was added to each vial (total 10ml enzyme solution: 3.5mg collagenase/ml for two adrenal glands in each vial). The tissue in each vial was incubated at 37°C in a Dubnoff metabolic shaking incubator (100 rotations/min) for one hour under a stream of 95% O_2/5% CO_2 and the contents of each vial were mixed at 30 minute intervals by pipetting up and down 30-35 times through a wide-mouth 5ml pipette tip.

At the end of one hour, the digested tissue was filtered through nylon mesh into a cornical tube and centrifuged at 900 r.p.m. for ten minues, the supernatant was discarded and the pellet was washed twice with a sufficient volume of KRBGA.

Cells were counted in a hemocytometer and cell viability was monitored by observing the exclusion of trypan blue dye. Over 90% of glomerulosa cells excluded trypan blue in this cell preparation.

2. Experimental Procedures

1) Na Effect

Glomerulosa cells were incubated under one of the following media of different Na concentrations in which NaCl was replaced iso-osmotically by choline Cl or N-methylglucamine Cl: 1) O-Na medium; 2) 25mM-Na medium (N-methylglucamine Cl only); 3) 50mM-Na medium; 4) 93mM-Na medium; and 5) 135mM-Na medium.

In this experiment, a 135mM concentration of Na was arbitarily decided as the normal Na concentration. Changes in Na concentration between 135 and 145mM without changes in osmolality didn't cause any significant effect on aldosterone secretion by our cell preparations (data not shown).

These media were prepared by mixing the appropriate portion of 135mM-Na solution with that of 135mM choline Cl or N-methylglucamine Cl solution (O-Na solution). The 135mM-Na solution contained 135mM MaCl, 2.5mM KCl, 1mM KH_2PO_4, 1.25mM $CaCl_2$, 1mM $MgSO_4$, 10mM glucose, 0.1% BSA and 20mM Hepes-Tris (pH 7.4 at 37C). The osmolality of this solution was between 290 and 295mOsm. The 135mM choline Cl or N-methylglucamine Cl solution contained the same concentrations of all constituents as 135mM-Na solution, except that it contained 135mM choline Cl or N- methylglucamine Cl instead of 135mM NaCl.

The osmolality of these solution was similar to that of the 135mM-Na solution. This method of preparation ensured that the concentrations of all constituents except Na, choline or N-methylglucamine were the same in all media.

Angiotensin II (5-isoleucine AII, Bechman Inc.) solution was prepared daily by diluting aliquots of frozen stock solution with O-Na solution for O-Na medium, 25mM-Na solution for 25mM-Na medium and 135mM-Na solution for 50, 93 and 135mM-Na media respectively, and the same volume of each solution was added to the incubation tubes for basal aldosterone secretion.

After the glomerulosa cells were prepared, the cell suspension was preincubated in KRBGA for one hour at 37C under a stream of 95% O_2/5% CO_2. The tubes were again centrifuged and the pellets were washed once with KRBGA. The final cell suspension was divided into 4 (choline Cl substitution) or 5 aliquots (N-methylglucamine Cl substitution) and each aliquot was washed twice with each medium of different Na concentration. The final cell suspension was made by mixing cell pellets with the desired volume of each medium of different Na concentration.

A cell suspension of 0.45ml was pipetted into tubes containing 0.05ml of AII solution or each medium of different Na concentration (total incubation volume = 0.5ml).

The tubes were stoppered and incubated at 37C in a Dubnoff metabolic shaking incubator for 90 minutes. At the end of incubation the tubes were cen-

trifuged and the supernatants were stored at -20C until assayed for aldosterone.

2) Cl Effect

Glomerulosa cells were incubated under one of the following two media: 1) normal KRBGA (Cl^- medium) and 2) NO_3^- medium. The NO_3^- medium contained the same concentration of all constituents as the KRBGA except that it contained NO_3^- instead of Cl^-.

Angiotensin II (5-isoleucine AII, Bechman Inc.) solution was prepared daily by diluting aliquots of frozen stock solution with each medium and the same volume of each medium was added to the incubation tubes for basal aldosterone secretion.

After preincubating the cells as in the previous experiment, the final cell suspension was divided into two aliquots and each aliquot was washed twice with each medium.

Aliquots of the cell suspension were placed in small tubes containing AII solution or the same volume of each medium and incubated at 37°C for 90 minutes under a stream of 95% O_2/5% CO_2 (total incubation volume = 0.5ml).

The supernatants were stored at −20°C until assayed for aldosterone.

3. Analytic Methods

The sodium and potassium concentrations were measured by flame photometry, osmolality was measured by freezing point depression, and the aldosterone level was measured in duplicate directly, using a commercial RIA Kit (Diagnostic products, Los Angeles CA).

4. Data Analysis

Statistical tests were performed using the paired student t-test. A p-value below 0.05 was regarded as significant. Interexperiment variation was minimized by determining absolute levels (ng/10^6 cells), dividing absolute levels by AII-stimulated levels in 135mM-Na medium (Na effect) or KRBGA (Cl effect) and multiplying by 100 to obtain % of AII-stimulated levels in 135mM- Na medium or in KRBGA, e.g.

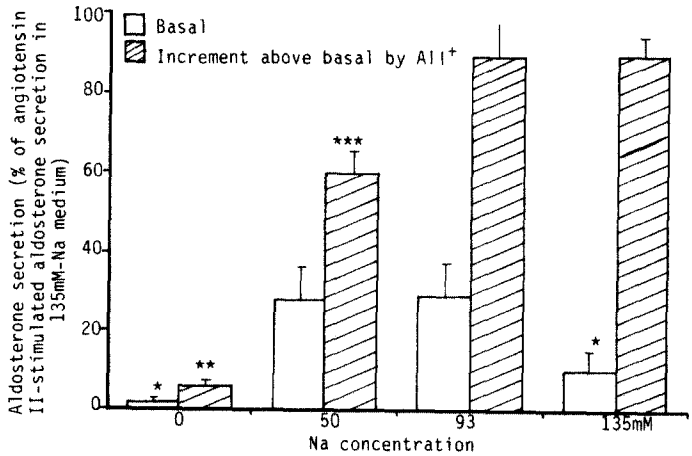

Fig. 1. *Effect of Na concentration on basal and angiotensin II-stimulated aldosterone secretion. Na was replaced iso-osmotically by choline. Each bar represents the Mean ± 1 S.E.M. of 5 experiments. All data are converted to % of angiotensin II-stimulated aldosterone secretion in the 135mM-Na Medium (angiotensin II-stimulated aldosterone secretion in the 135mM-Na Medium = 100%).*

† Increment in aldosterone above basal levels is determined by substracting the basal level from the angiotensin II-stimulated aldosterone level.

** $p<0.05$ vs. 50 and 93mM-Na media*

*** $p<0.001$ vs. 50, 93 and 135mM-Na media*

**** $p<0.05$ vs. 93mM-Na medium, $p<0.01$ vs. 135mM-Na medium.*

$$\frac{\text{basal level or increment in aldosterone above basal levels by AII-stimulation in other media}}{\text{AII-stimulated level in 135mM-Na medium or in KRBGA}} \times 100$$

= % of AII-stimulated level in 135mM-Na medium or in KRBGA.

The increment in aldosterone above basal levels is determined by substracting the basal level from the angiotensin II-stimulated aldosterone level.

RESULTS

In this experiment, a 10^{-7}M concentration of AII was used for stimulation of aldosterone secretion. Aldosterone secretion by isolated bovine adrenal glomerulosa cells in KRBGA was maximally stimulated as the concentration of AII reached 10^{-7}M (data not shown).

The effect of different Na concentrations in the incubation media on basal and AII-stimulated aldosterone secretion is shown in Figure 1. NaCl in the incubation media was replaced iso- osmotically by choline Cl.

A marked reduction in both basal above basal level and increment in aldosterone levels by AII stimulation was found when Na was not present in the incubation medium (O-Na medium). Basal aldosterone secretion was significantly higher in 50 and 93mM-Na media than in 135mM-Na medium. The increment in aldosterone above basal levels by AII stimulation was impaired at 50mM-Na medium and reached a maximum at 93mM-Na medium.

Fig. 2 depicts the effect of different Na concentrations on aldosterone secretion as NaCl in the media was replaced iso-osmotically by N-methylglucamine Cl. Aldosterone secretion was markedly inhibited at O-Na medium and concentrations of Na below 50mM impaired the ability of the adrenal cells to respond to AII. Increment in aldosterone above basal levels by AII stimulation also reached a maximum at 93mM-Na medium. In contrast to choline Cl replacement, basal aldosterone secretion was not different between 25, 50, 93 and 135mM-Na media.

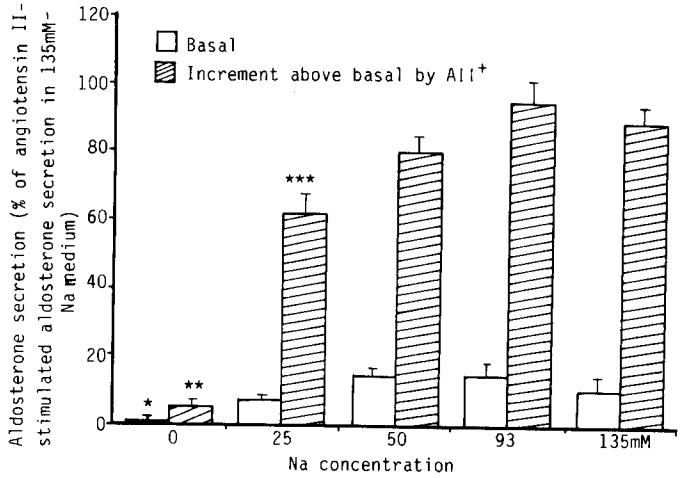

Fig. 2. *Effect of Na concentration on basal and angiotensin II-stimulated aldosterone secretion. Na was replaced iso-osmotically by N-methylglucamine. Each bar represents the Mean ± 1 S.E.M. of 5 experiments (n = 3 in 25mM-Na medium). All data are converted to % of angiotensin II-stimulated aldosterone secretion in the 135mM-Na medium (angiotensin II-stimulated aldosterone secretion in the 135mM-Na medium = 100%)*

† see legend in Fig. 1.

** $p<0.05$ vs. 50 and 93mM-Na media*

*** $p<0.001$ vs. 50, 93 and 135mM-Na media, $p<0.01$ vs. 25mM-Na medium*

**** $p<0.05$ vs. 50 and 135mM Na media.*

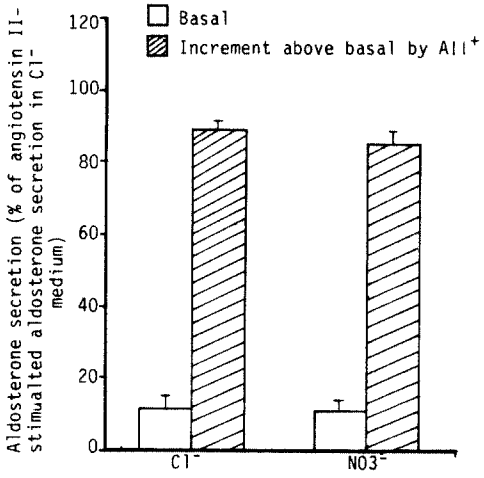

Fig. 3. *Effect on basal and angiotensin II-stimulated aldosterone secretion of replacing Cl ion of the incubation medium totally with NO_3 ion. Each bar represents the Mean ± 1 S.E.M. of 5 experiments. All data are converted to % of angiotensin II-stimulated aldosterone secretion in Cl medium (angiotensin II-stimulated aldosterone secretion in Cl medium = 100 %). † see legend in Fig. 1.*

When Cl ion in the incubation medium was replaced totally by NO_3 ion, basal and AII-stimulated aldosterone secretions were not affected, as shown in Fig. 3.

DISCUSSION

The present study shows that extracellular Na ion appears to be important for aldosterone secretion by isolated bovine glomerulosa cells, and the effect of Na ion is not due to changes in osmolality of the incubation media because the osmolality of the incubation medium was kept constant to reach a value of 290-295mOsm.

Saruta et al,[7] reported that decreased Na concentrations stimulated aldosterone secretion of outer bovine adrenal slices. A decrease of 40mM Na was necessary in order to produce any stimulatory effect. Lovo et al,[8] reported that a change as small as 10mM Na had a significant effect on basal aldosterone secretion and this effect was due to Na concentration per se. The effects of Na concentration were examined only at concentrations between 160 and 120mM in their study.

These results cited above are consistent with those of the present study that basal aldosterone secretion was increased in media of subnormal Na concentrations (Fig. 1).

We have confirmed the observations of Schiffrin et al,[9] and Ball et al,[10] that a very low extracellular Na concentration (below 50mM in our experiment) inhibits basal and AII-stimulated aldosterone secretion (Fig. 1,2) However in the present study, basal aldosterone secretion was increased at 50 and 93mM concentrations of Na in choline Cl substitution, and the increment in aldosterone above basal levels by AII-stimulation reached a maximum at a Na concentration of 93mM. These results were inconsistent with those of Schiffrin et al,[9] that an isoosmotic reduction in Na concentration depressed both basal and AII-stimulated aldosterone secretion, and basal and AII- stimulated aldosterone secretions were maximal at normal Na concentration.

The reason for this discrepancy is not readily apparent but may be related to differences in species and isolation procedures, and consequently to the responsiveness of glomerulosa cells (rat vs, calf), or to differences in substitution solutes (Li, sucrose vs. choline).

One possible explanation that may be considered is that choline itself may stimulate aldosterone secretion in the presence of adequate Na ions. Extracellular choline is a constituent of acetylcholine and may act as an acetylcholine analogue which has been found to stimulate aldosterone secretion by isolated bovine glomerulosa cells through action similar to that of AII on glomerulosa cells.[12] Our findings that basal aldosterone secretion was not increased in N-methylglucamine cation substitution support this possibility (Fig. 2). In addition, Li used for replacing Na in the study of Schiffrin et al,[9] is known to inhibit aldosterone secretion through an inhibitory effect on phosphatidylinositol turnover,[13] which is regarded as the main membrane signal transduction mechanism utilized by AII in stimulating aldosterone secretion.[14]

Our findings that, despite differences in basal aldosterone secretion, the increments in aldosterone above basal levels by AII stimulation were similar between media of rather higher Na concentration (above 50mM) support the contention that adequate Na ions are also necessary for AII-stimulated aldosterone secretion.

The mechanism responsible for the effects of Na ion on aldosterone secretion was not addressed in this study.

Many investigators have reported that extracellular sodium ion is also required for other hormone secretion[15-18] in addition to aldosterone

secretion[8,8,10,19], and have presented possible mechanisms of the role for extracellular Na ion on hormone secretion, such as activation of Na^- channel or Na^+- Ca^{+2} exchange, etc. However no definitive conclusion has been made.

The well recognized effects of extracellular Na ion on cellular function,[2-4] indicate that mechanisms for the effects of extracellular Na ion on hormone secretion may be different, according to endocrine cell types. Further stuies evaluating mechanisms for the effects of extracellular Na ion on aldosterone secretion will be needed.

In the present study, total replacement of Cl ion by NO_3 ion didn't affect aldosterone secretion (Fig. 3)

Quinn et al,[20] in his electrophysiology study demonstrated that removal of Cl ion with acetate ion produced small (less than 10mV), transient hyperpolarization of the membrane and didn't alter the relationship between membrane potential and external K ion concentration, and concluded that Cl ion is passively distributed across the cell membrane and has a relatively low permeability in glomerulosa cells. These results suggest that Cl ion may play a minor role in aldosterone secretion.

Consequently, the results of the present and past studies[1,9,10,21-23] indicate that in media with Na concentrations above 100mM, changes in Na concentration affect aldosterone secretion by a mechouism sensitive to osmolality, changes in Na osmolality, but in Na concentrations below 100mM, Na ion per se appears to play an important role in aldosterone secretion.

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