

ORIGINAL ARTICLE

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Characterization of hormone-stimulated Na⁺ transport in a high-resistance clone of the MDCK cell line

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Abstract The Madin-Darby canine kidney (MDCK) cell line forms an epithelial monolayer which expresses many of the morphological and functional properties of the renal collecting duct. The C7 subclone of the parent line forms an epithelium which expresses many of the characteristics of principal cells. The MDCK-C7 subclone forms a high-resistance epithelium that is capable of vectorial ion transport. We have found that this epithelium responds to aldosterone, antidiuretic hormone (ADH) and insulin like growth factor 1 (IGF1) with increases in amiloride-sensitive Na⁺ transport. The responses to aldosterone and ADH follow time-courses that are consistent with the action of these hormones in vivo. This is the first demonstration of IGF1-induced Na⁺ reabsorption in a mammalian model system. Interestingly, a maximal response to any one of these natriuretic factors does not inhibit a subsequent response to another hormone. These studies indicate that the C7 subclone retains many of the natriuretic responses of the native principal cells and is an ideal model for studying hormonal modulation of Na⁺ transport.

Key words Aldosterone · Insulin · IGF1 · ADH · Vasopressin

Introduction

The polarized epithelial cells lining the distal portions of the nephron form an electrically “tight” cellular monolayer which constitutes a barrier capable of selec-

tive vectorial transport of ions and water. In contrast to more proximal portions of the nephron, distal transport processes are under hormonal control and provide fine-tuning mechanisms contributing to whole body salt and water balance. An understanding of various hormonal actions and interactions is the key to the elucidation of normal and abnormal regulation of salt and fluid homeostasis.

The complexity of the mammalian kidney makes it virtually impossible to obtain sufficient material from individual nephron segments for detailed structural, functional and biochemical studies. For this reason, well-characterized amphibian epithelia, such as the urinary bladder of the toad, *Bufo marinus*, and the A6 cell line derived from the kidney of the frog, *Xenopus laevis*, have been used as models of the distal nephron [1–7].

It is clear, as elucidation of the hormonal responses reaches a more refined level, that the amphibian model systems have several inherent limitations. For example, highly specific mammalian antibodies and molecular probes may not interact as readily with amphibian ligands. In addition, temperature-sensitive processes are not readily comparable between amphibian and mammalian systems and signal transduction pathways may show evolutionary divergence.

Cultured mammalian cell lines could replace the amphibian models. However, few such lines demonstrate all of the characteristics of the intact epithelia of the distal tubule and collecting duct. One well-characterized renal cell model is the Madin-Darby canine kidney (MDCK) line. This line has been used extensively as a model epithelium for studies examining vectorial sorting of proteins and lipids [8, 9] and, more recently, as an expression system for studying regulation of ion channels [10].

The MDCK line has many characteristics of the distal nephron [11, 12]. However the parent line is not homogeneous and several subtypes or strains have been identified [11–17]. Earlier biochemical, electrophysio-

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logical and morphological studies failed to demonstrate a consensus as to the identity of the cells in the heterogeneous epithelium. Richardson et al. [13], using biochemical and electrophysiological techniques, obtained data suggesting the presence of cells from both proximal and distal segments. Valentich [11] presented morphological data indicating that the cell types were identical to the principal and intercalated cells of the mammalian cortical collecting tubule. In more recent studies, Nakazato et al. [14] have obtained two MDCK subclones, one with similarities to the principal cells of the collecting ducts and one which resembles thick ascending limb cells or intercalated cells of the collecting ducts. Finally, Gekle et al. [15] have cloned two subtypes resembling principal and intercalated cells of the renal collecting duct. Wunsch et al. [17] found that the two subtypes differ phenotypically as well as genotypically. Despite the differences between the various studies, one consistent finding is the presence of cells resembling the principal cells, the cell type responsible for vectorial Na^+ transport [13–15].

We have used the recently isolated [15] principal cell subtype (C7) to delineate hormonal regulation of Na^+ reabsorption. We have characterized the natriuretic responses to aldosterone, antidiuretic hormone (ADH), insulin and insulin-like growth factor 1 (IGF1). Our results suggest that this subclone is a good cultured cell model for studying transport phenomena of the distal nephron.

Materials and methods

MDCK cell culture

The C7 subclone of the MDCK cell line was used for all the experiments. The details of the cloning and initial characterization have been reported earlier [15]. The cells were grown at 37°C in a humidified incubator gassed with 5% CO_2 in O_2 . Cells were initially grown in 75 cm^2 polystyrene tissue culture flasks in the presence of Minimum Medium with Earle's salts, non-essential amino acids and L-glutamine (MEM medium, Gibco/BRL, Grand Island, New York, USA). The MEM medium was supplemented with 10% fetal calf serum (CELlect Gold, ICN/Flow, Costa Mesa, Calif., USA) and 26 mmol/l NaHCO_3 and adjusted to pH 7.2. For electrophysiological studies, confluent monolayers were subcultured by trypsinization and the cells seeded ($5.4 \cdot 10^4$ cells/ cm^2) onto Nucleopore polycarbonate membranes forming the bottom of Transwell chambers (Costar, Cambridge, Mass., USA). The Transwells were placed in tissue culture cluster plates forming a two-compartment system and allowing media access to both apical and basolateral faces of the adhered cells. The medium was aspirated and renewed thrice weekly. Cells were used between passage 69 and 81.

Electrophysiological studies

Nucleopore filters (4.7 cm^2) containing confluent (day 17–24) MDCK-C7 cells were carefully removed from the Transwells and clamped between the halves of an Ussing chamber (World Precision Instruments, Sarasota, Fla., USA). The area of the epithelium in

the circular window of the chamber was 1 cm^2 . Each half of the chamber contained a tapered fluid compartment with ports for voltage-sensing electrodes (in close proximity to the epithelial membrane) and current electrodes (at opposite ends of the chamber). The fluid chamber was connected to a water-jacketed buffer reservoir which allowed temperature control (37°C). The buffer was circulated through the chamber using a 5% CO_2/O_2 gas lift. The electrodes were connected to a voltage clamp amplifier (World Precision Instruments) for measurement of transepithelial potential difference. After the initial measurements, net ion flux was monitored under short-circuit condition [1, 6]. The amiloride-sensitive portion of the short-circuit current (SCC) represented the net Na^+ flux. Transepithelial resistance was calculated from measured deflections in the current in response to an applied 2-mV pulse. Cultures were used only if the resistances before and during the experiments were $> 1000 \Omega \cdot \text{cm}^2$. Each experiment was performed using matched cultures which were grown in parallel.

Hormones and growth factors

Aldosterone and Arg-vasopressin (ADH) were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Porcine insulin was a gift from Eli Lilly (Indianapolis, Ind., USA) and IGF1 was kindly provided by Genentech, (San Francisco, Calif., USA). Stock solutions of the hormones were prepared at 1000x and added directly to the serosal bathing media.

Statistics

The data are presented as means \pm SE with *n* indicating the number of different experiments. Comparisons were performed using Student's one-tailed *t*-test for paired samples; values of *P* < 0.05 were considered significant.

Results

Several hormones act as positive effectors of Na^+ reabsorption in the renal distal nephron and collecting duct. While the mode of action of each of the hormones has been outlined in general terms, a detailed time-course over which the major hormones modulate Na^+ reabsorption has not been previously described for mammalian renal epithelia. We have examined the natriuretic effects of aldosterone, the major renal steroid hormone, as well as the responses to three peptide hormones, ADH, insulin and IGF1, which are known to modulate Na^+ reabsorption.

The aldosterone-induced increase in Na^+ transport (SCC) was delayed (Fig. 1), consistent with the known protein-synthesis-dependent mechanism of this steroid hormone. The transport rate in the aldosterone-treated tissues was significantly greater (*P* < 0.05) than matched controls after 3 h of hormone incubation. The transport response reaches a maximum after 4 h and this elevated level of Na^+ flux remained constant for at least 18 h (Fig. 2).

Incubation with insulin for up to 3 h did not produce an increase in Na^+ transport (data not shown). In contrast, addition of IGF1 to the serosal bathing

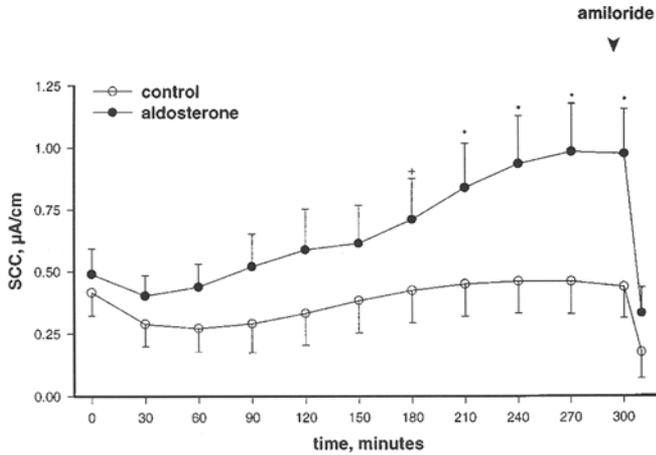


Fig. 1 Effect of aldosterone on Na^+ transport in the MDCK-C7 cell line. For each experiment, aldosterone (10^{-6} M) was added to the serosal bathing medium of one of two matched cultures at time zero. After 300 min, amiloride (10^{-5} M) was added to the apical bathing media. Symbols denote the means \pm SEM for 8 pairs. The mean of the aldosterone-treated cultures was significantly different from control (+, $P < 0.05$) at 180 min and (*, $P < 0.02$) at later time points. (SCC short-circuit current)

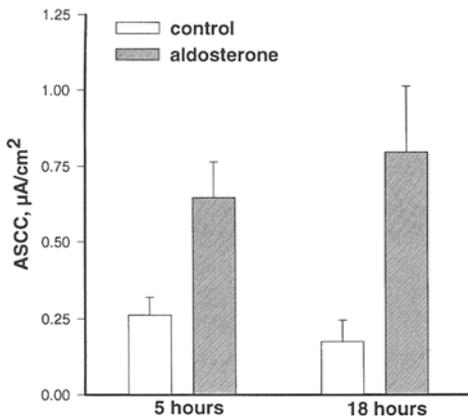


Fig. 2 Comparison of short- and long-term effects of aldosterone on Na^+ transport in MDCK-C7 cell line. The bars denote the amiloride-sensitive current in basal and aldosterone-treated (10^{-6} M) cultures 5 and 18 h after addition of the hormone. Lines denote SEM for 8 matched pairs at each time point. Sodium transport in the aldosterone-treated cultures was significantly ($P < 0.005$) higher than the corresponding controls at both time points. There was no difference between the aldosterone-induced ASCC at 5 h and 18 h; there was no difference between the controls at 5 h and 18 h. (ASCC Amiloride-sensitive short-circuit current)

media stimulated an increase in amiloride-sensitive transport (Fig. 3). The rate of transport increased ≈ 5 min after the addition of the growth factor, reached a maximum after 30 min and remained elevated for at least 2 h.

ADH also stimulated Na^+ transport in the MDCK cell line. The response to the peptide was rapid and transient (Fig. 4). One h after hormonal stimulation, the remaining increase in ADH-stimulated ion flux was

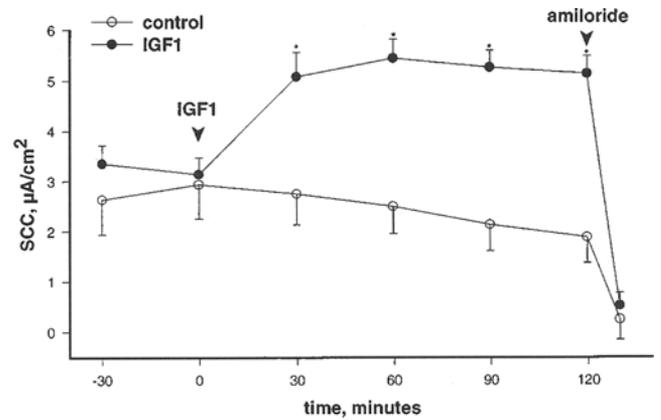


Fig. 3 Effect of insulin-like growth factor 1 (IGF1) on Na^+ transport in the MDCK-C7 cell line. For each experiment IGF1 (100 nM) was added to the serosal bathing media of one of two matched cultures at time zero. After 120 min amiloride (10^{-5} M) was added to the apical bathing media. Symbols denote the means \pm SEM for 6 pairs. The IGF1-treated samples have a statistically larger SCC at each time point after the addition of the hormone (*, $P < 0.02$)

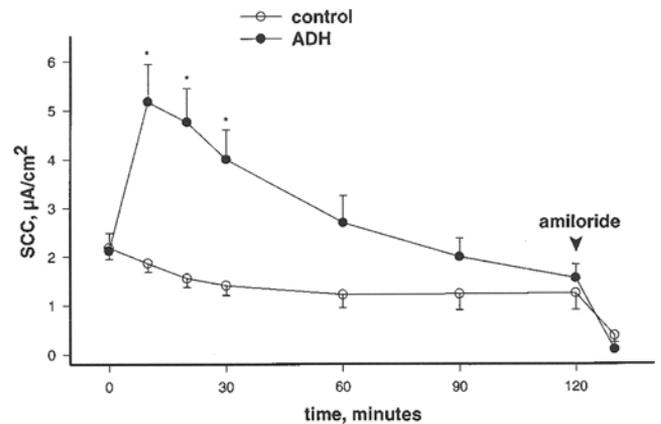


Fig. 4 Effect of antidiuretic hormone (ADH) on Na^+ transport in the MDCK-C7 cell line. For each experiment, ADH (100 mU/ml) was added to the serosal bathing media of one of two matched cultures at time zero. After 120 min amiloride (10^{-5} M) was added to the apical bathing media. Symbols denote the means \pm SEM for 9 pairs. The mean of the ADH-treated cultures was significantly different from control (*, $P < 0.02$) at the indicated time points

sensitive to amiloride (data not shown). Two h after the addition of ADH, the SCC returned to base-line and the current was amiloride sensitive.

In vivo the epithelial cells of the distal nephron are exposed to a variety of hormones and growth factors. It is important, therefore, to delineate the interactions between the various stimulatory effectors. We examined the Na^+ transport response to maximal doses of individual hormones followed, at peak response, by a maximal dose of a second factor.

ADH or IGF1 were added to cultures that had been pretreated with aldosterone for 4 h (Fig. 5 and 6, respectively). The natriuretic response to ADH appeared to be additive with the response to aldosterone. In

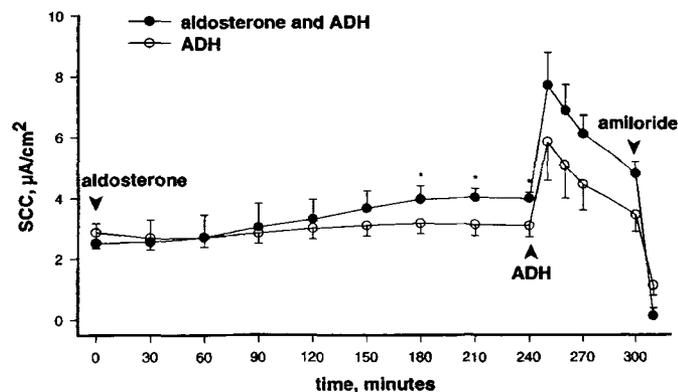


Fig. 5 Effects of ADH on aldosterone-stimulated Na^+ transport in the MDCK-C7 cell line. For each experiment, aldosterone was added to one of two matched cultures at time zero. After 240 min of incubation, ADH (100 mU/ml) was added to both cultures. After 300 min amiloride (10^{-5} M) was added to the apical bathing media. Symbols denote the means \pm SEM for 3 pairs. The mean of the aldosterone-treated cultures was significantly different from control (*, $P < 0.02$) at 180 min. Aldosterone pretreatment did not have an effect on the magnitude of the ADH response

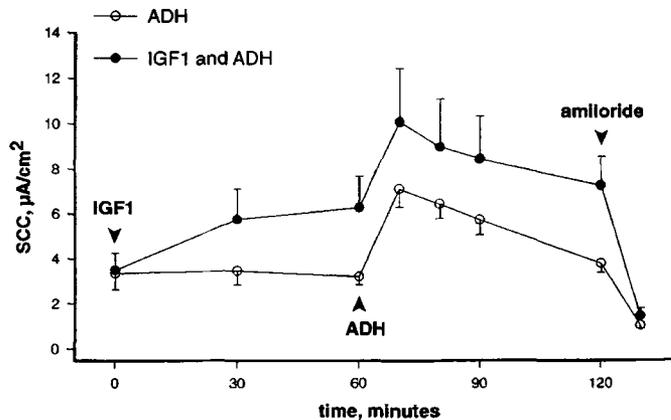


Fig. 7 Effects of ADH on IGF1-stimulated Na^+ transport in MDCK-C7 cells. For each experiment, IGF1 (100 nM) was added to one of two matched cultures at time zero. After 60 min, ADH (100 mU/ml) was added to both cultures. After 120 min amiloride (10^{-5} M) was added to the apical bathing media. Symbols denote the means \pm SEM for 3 pairs. The magnitude of the current induced by ADH alone was not significantly different than the magnitude of the current induced by ADH in IGF1-treated cells

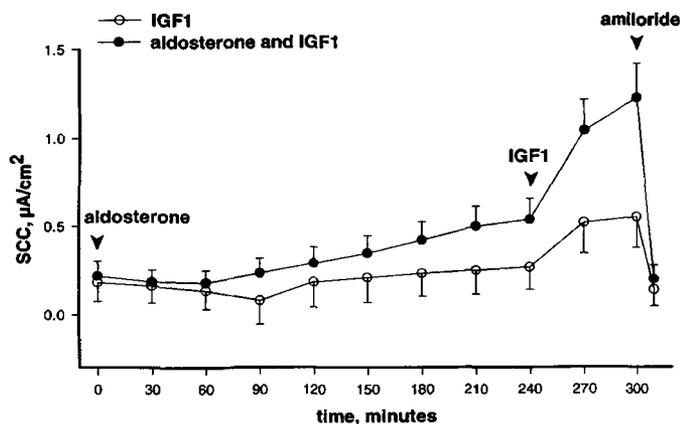


Fig. 6 Effects of IGF1 on aldosterone-stimulated Na^+ transport in the MDCK-C7 cell line. For each experiment, aldosterone was added to one of two matched cultures at time zero. After 240 min of incubation, IGF1 (100 nM) was added to both cultures. After 300 min amiloride (10^{-5} M) was added to the apical bathing media. Symbols denote the means \pm SEM for 9 pairs. The magnitude of the current induced by IGF1 and aldosterone was significantly higher than the current in response to IGF1 alone ($P = 0.012$ at 270 min; $P = 0.025$ at 300 min)

contrast, the response to IGF1 after 4 h of aldosterone was statistically greater than the response to IGF1 in non-aldosterone-treated cultures. Thus, there appears to be some synergistic interaction between aldosterone and IGF1.

The sequential addition of the two peptide factors was also examined. In Fig. 7, cells were treated with IGF1 for 1 h and subsequently stimulated with ADH. The SCC responses to IGF1 and ADH were additive.

Discussion

The MDCK cell line has been extensively used as a model polarized epithelium in which to study protein synthesis, protein trafficking and membrane recycling [8, 9]. In addition, it can serve as a useful model to investigate a variety of transport mechanisms located in the distal nephron. The wild-type MDCK cells as obtained from the American Type Culture Collection (ATCC) is composed of at least two different cell types. They resemble morphological and functional properties of the renal collecting duct [18–21]. Culture conditions can cause the wild-type epithelium to differentiate either into an intercalated- or into a principal-cell-type monolayer [16]. When MDCK wild-type cells were cloned, two stable populations emerged, one resembling the functional and morphological properties of principal cells while the other resembled the intercalated cell type [15]. Interestingly, recent studies revealed that besides clear morphological differences, the two cell clones named C7 (principal cells) and C11 (intercalated cells) were also genetically different. The karyotypes of the two clones significantly vary in the number of chromosomes [17].

In this study we have used the C7 subclone which exhibits characteristics of principal cells. In previous work, the C7 subclone was shown to form a high-resistance epithelium that secreted K^+ in response to aldosterone, was peanut-lectin-negative and exhibited an intracellular pH of 7.4 [15]. In the current work we have characterized the magnitude and time-course of natriuretic responses of the subclone to the major hormones that are positive effectors of renal Na^+ reabsorption.

The initial time-course of the responses to aldosterone is remarkably similar to what we have observed in amphibian kidney (A6 cell line) and urinary bladder [7]. There is a delay of 1–3 h before the initial increase in Na^+ flux and the hormone-stimulated transport reaches a maximal level in 4–5 h. However, in contrast to the amphibian model system, there was no evidence for multiple “phases” in the response to aldosterone [22]. The maximal natriuretic response measured at 5 h was identical to the level of stimulation after 18 h of hormone incubation (Figs. 1 and 2).

The natriuretic effect of insulin has been well documented in clinical trials involving diabetic and/or obese patients [23, 24], in normal humans [25] and in dogs [26]. Collectively these studies have shown that one site of insulin's effects on Na^+ transport is the distal nephron. In addition, most of the amphibian models of the distal nephron also demonstrate a natriuretic response to insulin [1, 2, 4, 5].

The MDCK-C7 clone did not respond to pharmacological (20 nM) concentrations of insulin. This is in agreement with previous studies in the MDCK parent line demonstrating a lack of insulin receptors [27, 28]. We can only speculate why this cell line does not contain insulin receptors. It is possible that the original principal cells possessed functional insulin receptors and these were lost in the process of immortalization. Alternatively, the original cells which gave rise to this cell line may not have had insulin receptors. While the natriuretic effects of insulin have been localized to the distal portions of the nephron, *in vivo* studies cannot identify the exact segment responsible for insulin-stimulated reabsorption.

IGF1 is one of a family of endogenous polypeptides that modulate the growth of many tissues including renal epithelia [29, 30]. Specific IGF1 receptors are present in canine proximal tubular membranes [31] and rat mesangial cells [32]. Within the rat kidney collecting duct, immunostainable IGF1 was present in the principal cells and not in the intercalated cells [33]. Although changes in ion transport have been linked to growth [34, 35], IGF1 has yet to be demonstrated to be natriuretic *in vivo*. However, we have previously shown that IGF1, like insulin, stimulates transcellular Na^+ transport in an amphibian model “high-resistance” renal epithelia via IGF1 receptors [4]. Although mediated by different receptors, the natriuretic effects of insulin and IGF1 appear to share overlapping pathways; a response to a maximal concentration of one peptide inhibits a subsequent response to the alternate peptide.

Interestingly, we have found that IGF1 stimulates Na^+ reabsorption in the MDCK cell line (Fig. 3). Whether this is an integral component of the mitogenic process or an effect of the peptide that is unrelated to its classic growth factor effects is unknown. More whole animal and clinical studies will be necessary to address these important issues.

The major effect of ADH is control of water permeability of the epithelial cells lining the distal tubule and collecting duct. However, in amphibian and mammalian renal epithelia, ADH also stimulates Na^+ reabsorption. It is well established that both the hydraulic and natriuretic effects of ADH are mediated via the adenylate cyclase signalling cascade. In mammals the natriuretic effect of ADH may be species-dependent — the rabbit distal tubule and cortical collecting epithelia appear to be less sensitive than those lining the corresponding segments of the rat [36]. However, even in ADH-responsive species like the rat, chronic steroid treatment may be necessary to elicit an ADH-mediated increase in Na^+ flux [36, 37].

To provide a more controlled system for examining the hormonal effects of ADH, Canessa and Schafer immunodissected rabbit cortical collecting duct epithelial cells [38]. The resulting high-resistance epithelia responded to ADH stimulation with a rapid and stable increase in Na^+ flux even in the absence of mineralocorticoid treatment [38].

The response to ADH in the MDCK-C7 cells is rapid but transient. The stimulation of amiloride-sensitive current has completely dissipated after 2 h (Fig. 4). The transient nature of the time-course is in contrast to the primary rabbit cultures but is very similar to observations in perfused collecting ducts [36]. Thus the MDCK-C7 cell line exhibits a natriuretic response to ADH which closely resembles that seen in the mammalian perfused tubule model.

In vivo, the various hormones and growth factors are all present in the serum on a continuous basis, therefore these epithelial cells are routinely exposed to concurrent stimuli. Very little is known regarding potential hormonal interactions in mammalian epithelia. In the model systems, aldosterone, ADH and insulin exert natriuretic actions that have been shown to be additive or synergistic. Understanding the functional interactions between the various effectors is necessary for understanding normal and abnormal regulation under physiological conditions.

In rats, steroid pretreatment resulted in a synergistic effect on ADH responsiveness of the isolated tubules [36]. The mechanism of action of long-term steroid treatment is unclear and this synergism could represent, in part, an adaptation to chronic pharmacological doses of steroid. Immunodissected epithelial cells of rabbit cortical collecting duct cells placed in primary culture respond to both aldosterone and ADH with an increase in amiloride-sensitive ion flux [38]. The responses to sequential stimulation by the two hormones resulted in an additive effect on Na^+ transport. Furthermore, using intracellular microelectrodes, Schafer and Hawk [36] have shown that both ADH and deoxycorticosterone decrease the fractional resistance of the apical membrane of rat cortical collecting duct principal cells and these apical membrane effects appear to be additive.

In the current experiments, maximal concentrations of all the factors were used in order to determine if a maximal response to any one of the natriuretic hormones would modulate a subsequent response to a second type of stimulus. Using this protocol, we found that the acute effects of aldosterone and ADH are additive (Fig. 5). These results are in agreement with the effects of ADH and steroids on primary cultures [36].

The addition of IGF1 to cultures that have been maximally stimulated with aldosterone results in a synergistic effect on Na^+ transport when compared to stimulation by either effector alone (Fig. 6). This is the first demonstration of interactions between IGF1 and any other natriuretic hormone in mammalian systems. The results suggest that the natriuretic pathways stimulated by aldosterone and IGF1 may interact. Given the uncertainty regarding the mechanism of action of any of these effectors, we feel it is somewhat premature to speculate what, if any, biochemical steps may be shared in the two pathways.

In the present study we have also found that the responses to IGF1 and ADH are additive (Fig. 7). It is very well documented that ADH exerts its cellular actions on salt and water permeability via an adenylate cyclase pathway. The responses are preceded by an increase in cAMP and an increase in the activity of protein kinase A [36]. The signal transduction pathways activated in response to IGF1 are unknown. However, based on the additive nature of the responses, it is unlikely that the growth factor pathway involves a cAMP-mediated mechanism.

An unanticipated finding from these studies has been that maximal concentrations of each of the hormones exert relatively independent effects on Na^+ transport. The interactions between aldosterone and IGF1 are synergistic; the interactions between the other hormone pairs are additive. This implies, in the simplest theoretical model, that the pathways stimulated by each hormone are discrete and separate. Thus far, all natriuretic effectors in amphibian and mammalian renal high-resistance epithelia have direct actions on the amiloride-sensitive Na^+ channel. A logical extension of our findings is that each hormone is exerting separate effects on amiloride-sensitive Na^+ channels or, alternatively, that there are separate pools of channels responding to each hormone.

With regard to the first postulate, it is possible that different hormones activate different members of the Na^+ channel family. While channels having various conductances, selectivity and amiloride sensitivities have been described in purified and reconstituted preparations [39], very little data exist suggesting that there are multiple types of amiloride-sensitive Na^+ channels in native mammalian epithelia. With regard to other possibilities of regulatory mechanisms, we have no data to support or exclude other hypotheses. It is clear, however, that Na^+ transport is regulated by exquisite control mechanisms which allow the tissue

to respond to various hormones individually or in concert.

High-resistance clones of the MDCK line can provide an ideal model for the study of hormonal regulation of Na^+ transport in the mammalian renal principal cells. Characterization of hormonal responses is a crucial initial step for more detailed studies of the signal transduction pathways that link receptor binding to the final effector. This characterization also provides an important framework for transfection studies where exogenous proteins are added to an existing endogenous pathway.

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