# Cellular Responses to Steroids in the Enhancement of $Na^+$ Transport by Rat Collecting Duct Cells in Culture

## Differences between Glucocorticoid and Mineralocorticoid Hormones

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## Abstract

**It has recently been discovered that both mineralocorticoid (MC) and glucocorticoid (GC) hormones can stimulate electrogenic $Na^+$ absorption by mammalian collecting duct cells in culture. In primary cultures of rat inner medullary collecting duct (IMCD) cells, 24-h incubation with either MC or GC agonist stimulates $Na^+$ transport approximately threefold. We have now determined that the effects were not additive, but the time courses were different. As aldosterone is known to stimulate citrate synthase, $Na^+/K^+$ ATPase activity, and ouabain binding in cortical collecting duct principal cells, we determined the effects of steroids on these parameters in IMCD cells. MC and GC agonists both produced a small increase in citrate synthase activity. There was no increase in $Na^+/K^+$ ATPase activity but specific ouabain binding was increased more than twofold by either agonist. To determine the role of apical $Na^+$ entry in the steroid-induced effects, the $Na^+$ channel inhibitor, benzamil, was used. Benzamil did not alter the stimulation of citrate synthase activity by either steroid. In contrast, GC stimulation of ouabain binding was prevented by benzamil, whereas MC stimulation was not. We conclude that there are differences in the way that MC and GC hormones produce an increased $Na^+$ transport. Both appear to produce translocation (or activation) of pumps into the basolateral membrane. GC stimulation of pump translocation requires increased $Na^+$ entry whereas MC stimulation does not. (*J. Clin. Invest.* 1992. 90:1370–1378.) Key words: Na-K ATPase • citrate synthase • ouabain binding • cell culture • inner medullary collecting duct**

## Introduction

The adrenal steroid hormones are important regulatory substances for $Na^+$ and extracellular fluid volume homeostasis in mammals. Mineralocorticoid (MC)[1] hormones are well known to stimulate $Na^+$ reabsorption in the renal cortical and medullary collecting duct (1, 2). The mechanisms by which MC hormones are known to stimulate electrogenic $Na^+$ transport include augmentation of $Na^+$ entry across the apical (luminal) membrane through $Na^+$ channels; stimulation of metabolic enzyme activity; and activation of $Na^+/K^+$ ATPase, the enzymatic equivalent of the $Na^+$ pump located on the basolateral membrane (3). Several questions concerning the nature of the MC response, however, remain unresolved. The precise role of $Na^+$ entry in the induction of $Na^+/K^+$ ATPase in mammalian cells is incompletely understood. Neither is it clear whether stimulation of $Na^+$ transport is mediated solely by MC hormone action on MC receptors or whether "crossover" stimulation of glucocorticoid (GC) receptors (by MC hormone) is necessary to elicit a complete response (4). The recent demonstration that GC hormones (as well as MC hormones) can stimulate electrogenic $Na^+$ transport in mammalian collecting duct (5, 6) has raised additional questions regarding the cellular mechanisms whereby GC and MC hormones effect the increase in transport. It has recently been suggested, for example, that MC and GC hormones interact with their respective receptors and activate identical regulatory regions in the target genes (6, 7). The support for this assertion is simply the absence of data to indicate different mechanisms of action.

The certainty with which one can produce a "pure" GC or MC effect has been dramatically enhanced by the recent introduction of several synthetic steroid hormones that are specific agonists or antagonists of these receptors (8, 9). The availability of these compounds, together with the discovery of a cell culture system that demonstrates stimulation of electrogenic $Na^+$ transport by either GC or MC (5), has provided an excellent opportunity to examine some of these questions in greater detail and with greater precision. The present studies were therefore designed to further delineate the cellular mechanisms that mediate the effects of MC and GC hormones on $Na^+$ transport. Given that an increase in electrogenic $Na^+$ transport must be accompanied by an increase in apical membrane $Na^+$ permeability (3), we have concentrated on the other two well-established effects of adrenocorticoid hormones: the effects on metabolic capacity and the effects on $Na^+/K^+$ ATPase. We also address the role of $Na^+$ entry through the apical membrane $Na^+$ channel in the stimulation of the $Na^+$ pump. The results challenge existing notions regarding the changes that are required for stimulation of $Na^+$ transport and indicate some differences in the cellular pathways for MC and GC hormone action.

## Methods

*Inner medullary collecting duct (IMCD) cell isolation and culture.* IMCD cells were isolated from the renal inner medulla of 100–150-g Wistar rats in preparation for primary culture using the hypotonic lysis

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1. *Abbreviations used in this paper:* CCD, cortical collecting duct; CoASH, reduced coenzyme A; GC, glucocorticoid; $IC_{50}$, concentration of an inhibitor required to produce 50% of its maximal effect; $I_{sc}$, short circuit current; IMCD, inner medullary collecting duct; MC, mineralocorticoid; $P_i$, inorganic phosphate; $R_T$, transmonolayer resistance.

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technique previously described (5, 10). Briefly, rats were anesthetized with methoxyfluorane and were decapitated. The kidneys were removed and rinsed in a PBS solution with the following composition (mM): 151 NaCl, 4.5 $KH_2PO_4$, and 2.5 NaOH, pH = 7.2. Amphotericin B (2 μg/ml), penicillin (500 U/ml), and streptomycin (500 μg/ml) were added to the rinse to reduce the risk of fungal or bacterial contamination. Renal inner medullae were then dissected, minced, and incubated in 0.1% collagenase in Kreb's buffer with the following composition (mM): 118 NaCl, 25 $NaHCO_3$, 4.7 KCl, 2.5 $CaCl_2$, 1.8 $MgSO_4$, 1.8 $KH_2PO_4$, and 14 glucose for 2–3 h at 37°C in a 5% $CO_2$ atmosphere. The solution was made hypotonic by the addition of 2 vol of sterile distilled water containing 10 μg/ml DNase, and after two centrifugation steps, the cells were seeded on filters. This entire process, using 6–12 kidneys, is one "isolation" and yields ~ 40–80 monolayers. For studies of freshly isolated cells we used an isotonic isolation procedure as previously described (11). Cells for primary culture were seeded on sterile collagen-coated polycarbonate filters, which were glued to the bottoms of winged plastic cylinders as previously described (5, 10). Seeding density was 20 μg DNA/$cm^2$ (~ 350,000 cells/$cm^2$). DNA was determined by the method of Labarca and Paigen (12). Cells were grown for 3 d in a medium composed of a 1:1 mixture of DME and Ham's F-12 supplemented with 50 μg/ml gentamicin, 5 pM triiodothyronine, 50 nM hydrocortisone, 5 μg/ml transferrin, 5 μg/ml bovine insulin, 10 nM sodium selenite, and 1% wt/vol bovine albumin. On the third day the medium was changed to one from which hydrocortisone and albumin were omitted for the purpose of steroid withdrawal. After 24 h in the steroid-free medium, the initial electrical measurements were made. Cell monolayers were confluent at this point, as demonstrated by a measurable transmonolayer resistance ($R_T$). After the electrical measurements, another medium change was performed, in which the appropriate steroid hormones and receptor antagonists or vehicle (ethanol) alone, were added. MC stimulation was obtained by the addition of 100 nM aldosterone plus the GC receptor blocker, RU 38486 (10 μM) (8); GC stimulation was obtained with 100 nM dexamethasone plus the MC receptor blockers, spironolactone (10 μM) or RU 28318 (10 μM) (9). A similar GC stimulation can be produced by the specific GC agonist RU 28362 (unpublished data).

*Measurement of electrical parameters.* Electrical measurements across the monolayers were made as previously described (5, 10). Briefly, the filter-bottom cylinders were inserted in a modified Ussing chamber in which the apical and basolateral surfaces were bathed in media (without additives), and $R_T$ and short circuit current ($I_{sc}$) were measured (University of Iowa voltage clamp) under sterile conditions. The monolayers were measured and assigned to treatment groups using a Latin square randomization based on $I_{sc}$, so that the initial mean $I_{sc}$ of the groups was equal. Electrical measurements were repeated after the appropriate experimental incubation period. The initial set of measurements (on day 4) were recorded 30 s after insertion into the chamber; the final set of measurements were recorded at the time of peak $I_{sc}$ (2–3 min). The treatment groups for the time course experiments were assigned so that each monolayer was only measured twice and the possibility of damage from repeated manipulation was minimized.

*$Na^+/K^+$ ATPase assay.* $Na^+/K^+$ ATPase activity was measured using a previously reported assay (13) that was modified for use in cells grown on filters. The ouabain-inhibitable fraction of inorganic phosphate ($P_i$) generation (from ATP hydrolysis) was measured as an index of activity. Electrical parameters were measured before the assay to be sure that the steroid effects on $Na^+$ transport had occurred. Filters were then removed from the plastic cylinders and cut in half with a scalpel blade. Filter halves were rinsed in a phosphate-free Hepes solution with the following composition (mM): 1.8 $CaCl_2 \cdot 2H_2O$, 5.4 KCl, 0.8 $MgSO_4 \cdot 7H_2O$, 10 $Na^+$ · Hepes, 10 mM H · Hepes, 115.2 NaCl, and 7.8 glucose, pH = 7.5, and the excess Hepes was removed by blotting the cell-free side of the filter on absorbent paper. Each filter half was placed in a tube to which 100 μl of phosphate-free Hepes solution was added. Samples were then frozen at −70°C and stored for up to 24 h. They were thawed, frozen, and thawed immediately before use to disrupt cell membranes. $P_i$ solutions of known concentrations (0, 0.2, 1.0, and 2.0 mM) were assayed in parallel with the cell samples and were used to define a standard curve relating $P_i$ concentration to absorption at 705 nm. To initiate the assay reaction, 0.5 ml assay medium was added to each sample so that final concentrations were as follows (mM): 120 NaCl, 25 KCl, 4.0 $MgCl_2 \cdot 6H_2O$, 60 Tris, 1.0 $(Na)_3$ EDTA, and 4.16 $Na_2ATP$, pH = 7.5. The medium bathing one of the halves of each filter also contained ouabain (5.0 mM), so that each filter served as its own control. The samples were then agitated in a water bath at 37°C for 10 min. To stop the reaction and to solubilize cell protein, 100 μl 0.7% SDS was added to the reaction mixture (for a final SDS concentration of 0.1%), samples were vortexed and allowed to stand at 25°C for a total of 10 min. 5 min after the addition of SDS, each sample was vortexed again, and 100 μl was removed to be used for protein determination. The remainder of the assay was performed using the technique of Baginski et al. (14) for the determination of $P_i$. The $P_i$ concentration was linearly related to absorbance at 705 nm throughout the range of concentrations measured.

In preliminary experiments, we tried to maximize enzyme activity using the detergent activation method previously described (13, 15). Activation curves obtained after pretreatment with varying SDS concentrations in the presence of bovine serum albumin demonstrated no increase in enzyme activity in the IMCD cells, although we were able to demonstrate typical activation curves in a microsomal fraction of rabbit medulla (data not shown). For this reason, we did not use detergent pretreatment in the present studies. Protein was measured by a technique that uses the intrinsic fluorescence of tryptophan, as previously described (11, 16).

$Na^+/K^+$ ATPase activity from freshly isolated IMCD cells was measured by the same procedure except that cells were obtained using the isotonic isolation method (11). The freshly isolated cells were incubated in experimental media for 6 h before assay.

*Citrate synthase assay.* Citrate synthase activity was measured using a modification of the method of Srere et al. (17) in which the rate of generation of reduced coenzyme A (CoA-SH) from acetyl-Co A and oxaloacetate can be monitored as an index of enzyme activity. Preliminary experiments using variable quantities of IMCD cell protein showed that we were able to reproducibly measure rates of citrate synthase activity at least within the range of 0.5–3 times the mean activity of IMCD cells.

After measurement of electrical parameters, filters containing cell monolayers were removed from their plastic cylinders with a scalpel blade, placed in 0.5 ml of 0.2 M Tris (pH adjusted to 8.1 with concentrated HCl), and frozen at −70°C for 18–48 h. The samples were then thawed, sonicated in an ultrasonic bath for 2 min, and the resulting suspensions were pipetted into cuvettes, leaving the filters behind. The reaction was initiated by the addition of 0.5 ml reaction medium consisting of the following (mM): 0.2 acetyl coenzyme A, 0.4 sodium oxaloacetate, and 0.5 5,5′-dithio-bis-2-nitrobenzoic acid) and the change in absorbance at 412 nm was followed spectrophotometrically at 25°C. The extinction coefficient of the reaction product 2-nitro-5-mercaptide benzoate, which is generated in equimolar amounts with CoA-SH was used to convert absorbance units into molar quantities (17). Enzyme activity was factored for cell monolayer surface area, as protein concentration per area was demonstrated to be constant in a separate series of experiments.

*Ouabain-binding assay.* The ouabain-binding assay was performed using a modification of the methods previously described by El Mernissi and Doucet (18) for isolated nephron segments. After electrical measurements were completed, the intact filter-cylinder units containing the cell monolayers were incubated for 1 h at 37°C in one of two $^3$H-ouabain–containing Hepes solutions. The first solution was $K^+$ free to determine total ouabain binding and contained the following (mM): 1.8 $CaCl_2$, 0.8 $MgSO_4$, 1.0 $NaH_2PO_4$, 10.0 $Na^+$ · Hepes, 10.0 H · Hepes, 7.8 glucose, 1.0 $Na^+$ pyruvate, 120.6 NaCl. The second solution contained both $K^+$ and unlabeled ouabain to determine nonspecific binding and contained the following (mM): 1.8 $CaCl_2$, 0.8 $MgSO_4$, 1.0 $NaH_2PO_4$, 10.0 $Na^+$ · Hepes, 10.0 H · Hepes, 7.8 glucose,

1.0 $Na^+$ pyruvate, 30 KCl, 90.6 NaCl, and 2.0 (unlabeled) ouabain. Both solutions contained 100 $\mu$Ci/ml $^3$H-ouabain ($\sim$ 6.5 $\mu$M). A 1-h incubation period was chosen because preliminary experiments demonstrated that binding was maximal within that time period. $^3$H-ouabain did not bind to blank filters.

After the initial incubation, the monolayers were rinsed with ice-cold $K^+$-free Hepes and incubated at 0°C for 1 h, also in $K^+$-free Hepes. It has been demonstrated that specific binding remains, while nonspecific binding is reduced under these conditions (18). After a second rinse, the filters were removed from their plastic cylinders with a scalpel blade and incubated in 0.2% SDS overnight to solubilize cell protein. An aliquot of the sample was then removed for protein determination and the radioactivity of the remainder was determined in a beta counter.

*Statistics.* Values are reported as mean±SEM. Statistical analysis was performed using *t* test or analysis of variance as appropriate. In cases where significant inhomogeneity of group variance was detected, statistical analysis was completed with log-transformed data. Significance was concluded when $P < 0.05$.

*Materials.* Pathogen-free Wistar rats (100–150 g) were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Aldosterone, dexamethasone, spironolactone, amphotericin B, hyaluronidase, acetylsalicylic acid, and nordihidroguaiaretic acid were obtained from Sigma Chemical Co. (St. Louis, MO); RU 38486 and RU 28318 were generous gifts of Roussel Uclaf, Romainville, France. Benzamil was obtained from Merck Sharp & Dohme (West Point, PA); SDS from Bio-Rad Laboratories (Richmond, CA); collagenase was obtained from Boehringer Mannheim Diagnostics (Indianapolis, IN); and albumin was obtained from Intergen Company (Purchase, NY). Penicillin, gentamycin, streptomycin, and tissue culture media were obtained from the University of Iowa Cancer Center (Iowa City, IA). Polycarbonate filters (13-mm, 0.8-mm pore) were purchased from either Nuclepore Corp. (Pleasanton, CA) or Poretics (Livermore, CA); winged plastic cylinders (PC-2) from ADAPS Inc. (Dedham, MA).

## Results

*Steroid effects on $Na^+$ transport.* The effects of 24-h incubations with pure MC and GC agonists on $I_{sc}$ are shown in Fig. 1. Both GC and MC stimulated $I_{sc}$ approximately threefold; there was no additive stimulation when GC and MC were applied together (in the absence of receptor antagonists). We have previously demonstrated that the $I_{sc}$ results from electrogenic $Na^+$ transport across the monolayers in an apical to basolateral direction (5). The lack of additive effects by the two steroid classes indicates that the mechanisms of stimulation of $Na^+$ transport are not completely separate.

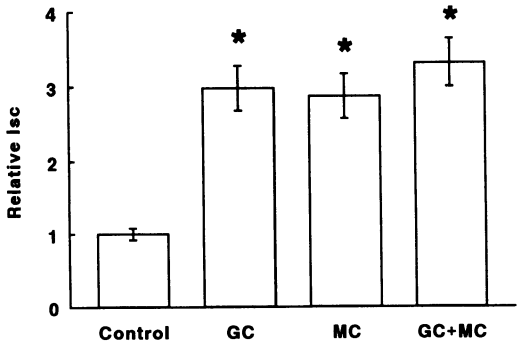


*Figure 1.* Effects of 24-h incubation with GC and MC on $I_{sc}$ of IMCD cell monolayers. The GC effect was obtained with 100 nM dexamethasone plus 10 $\mu$M spironolactone; MC effect was obtained with 100 nM aldosterone plus 10 $\mu$M RU 38486; GC + MC effect was obtained with 100 nM dexamethasone and 100 nM aldosterone. Relative $I_{sc}$ is the $I_{sc}$ of the stimulated monolayers normalized to the control $I_{sc}$ for that isolation. $n$ = 27 monolayers from three separate isolations. *$P < 0.05$ by analysis of variance (ANOVA).

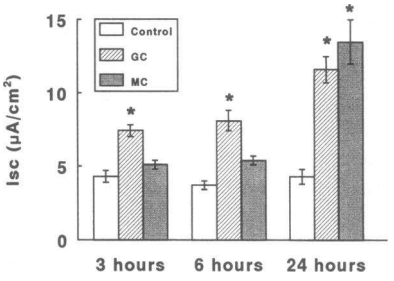


*Figure 2.* Time course of steroid effects on $I_{sc}$. GC stimulation of $Na^+$ transport occurred earlier than that of MC. $n$ = 23 monolayers from five isolations for the 3-h group, and $n$ = 27 monolayers from six isolations for both the 6- and 24-h groups. *$P < 0.05$ by ANOVA.

*Time course of steroid effects.* We performed the next series of experiments to determine whether the time courses of the steroid stimulation of sodium transport might provide some evidence of different modes of action of MC and GC hormones. Measurements of $I_{sc}$ were made after 3, 6, and 24 h of incubation with each steroid. The results are shown in Fig. 2. $I_{sc}$ remained constant in the control group. GC stimulated $I_{sc}$ significantly at 3 and 6 h; still further stimulation occurred at 24 h. MC did not stimulate $I_{sc}$ at 3 or 6 h; at 24 h, however, stimulation of $I_{sc}$ was equal to that of GC. The simplest interpretation of the difference in time courses is that GC and MC initially activate different cellular events.

*Inhibition of arachidonic acid metabolism.* The rationale for this series of experiments was as follows: IMCD cells synthesize $PGE_2$, a known inhibitor of $Na^+$ reabsorption by the collecting duct (19–21). GCs can inhibit PG synthesis (22, 23). Therefore, GC may stimulate $Na^+$ transport by interrupting a tonic inhibition by $PGE_2$. The results are shown in Table I. Neither ibuprofen (1 $\mu$M) nor acetylsalicylic acid (500 $\mu$M) altered control or steroid-stimulated $I_{sc}$. The leukotriene synthesis inhibitor, nordihydroguaiaretic acid (10 $\mu$M) (24), also had no effect on stimulated $Na^+$ transport. Evidently, endogenously synthesized PGs and leukotrienes have no effect on $Na^+$ transport by IMCD cells under these conditions.

*Protein synthesis and steroid effects.* Because steroid effects are usually dependent on protein synthesis we studied the effects of the protein synthesis inhibitor, cycloheximide, on steroid-induced $Na^+$ transport. Addition of 1 $\mu$M cycloheximide to the incubation medium for 24 h completely prevented stimulation of $I_{sc}$ by either steroid. Cycloheximide-treated monolayers maintained their transepithelial resistance, indicating that cellular integrity was preserved (Table II).

To determine whether the steroids caused cell hypertrophy, cell monolayer protein determinations were made. Total protein content was not different after 24-h incubations in control, GC-, or MC-treated monolayers (202±6, 214±10, and 195±7 $\mu$g/cm$^2$, respectively; $n$ = 6 isolations including 40, 41, and 39 monolayers).

*Inhibition of cytoskeletal function.* The cytoskeleton is essential for transport of newly synthesized proteins to the cell surface and insertion into the plasma membrane. Colchicine (20 $\mu$M), an inhibitor of microtubule function (25), inhibited basal $Na^+$ transport and prevented stimulation by either GC or MC, suggesting that effective sorting, delivery, and membrane insertion of transport proteins are necessary for $Na^+$ transport to occur (Table II).

*Table I. Effect of Inhibitors of Arachidonic Acid Metabolism on the Stimulation of $I_{SC}$ by Steroids*

| | No steroid | | | | Glucocorticoid | | | | Mineralocorticoid | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *n* | | | | *n* | | | | *n* | | | |
| | Filters | Isolations | $I_{SC}$ | Resistance | Filters | Isolations | $I_{SC}$ | Resistance | Filters | Isolations | $I_{SC}$ | Resistance |
| | | | $\mu A/cm^2$ | $\Omega \cdot cm^2$ | | | $\mu A/cm^2$ | $\Omega \cdot cm^2$ | | | $\mu A/cm^2$ | $\Omega \cdot cm^2$ |
| Control | 25 | 4 | 5.1±0.9 | 180±17 | 26 | 4 | 17.1±2.4 | 186±14 | 26 | 4 | 12.6±2.1 | 162±18 |
| Ibuprofen | 26 | 4 | 4.4±0.6 | 204±20 | 26 | 4 | 15.6±2.7 | 180±16 | 26 | 4 | 10.9±1.5 | 182±17 |
| Control | 13 | 2 | 2.2±0.2 | 99±20 | 13 | 2 | 7.3±0.7 | 161±16 | 13 | 2 | 5.5±0.8 | 134±22 |
| ASA | 13 | 2 | 2.4±0.4 | 121±19 | 13 | 2 | 6.9±0.8 | 143±15 | 13 | 2 | 4.6±0.5 | 112±114 |
| Control | 6 | 1 | 2.5±0.3 | 70±18 | 6 | 1 | 8.8±0.6 | 144±13 | 6 | 1 | 8.0±0.7 | 121±19 |
| NDGA | 6 | 1 | 3.3±0.4 | 120±25 | 6 | 1 | 9.1±1.1 | 126±13 | 6 | 1 | 6.2±0.8 | 170±41 |

Ibuprofen (1 $\mu$M), acetylsalicylic acid (ASA, 500 $\mu$M), nordihydroguaiaretic acid (NDGA, 10 $\mu$M), and steroids were added to the cell culture media 24 h before electrical measurements were made. Values are means±SE. There are no differences between control values and monolayers treated with inhibitors.

*Steroid effects on citrate synthase activity.* One of the most consistent effects of aldosterone in MC-responsive tissues is an increase in activity of the mitochondrial enzyme, citrate synthase (3). This increase in metabolic capacity may play a role in supplying ATP to support the energy requirements of increased $Na^+$ transport. The effects of a 24-h incubation with steroids on citrate synthase activity is shown in Table III. Both GC and MC caused small but significant increases in enzyme activity. $I_{sc}$, measured in the same monolayers before enzyme assay, demonstrated that the characteristic stimulation by both GC and MC was present.

*Steroid effects on $Na^+/K^+$ ATPase activity.* We measured $Na^+/K^+$ ATPase activity in cell monolayers after 24-h incubations with MC and GC. The results are shown in Fig. 3 and Table IV. $I_{sc}$ was stimulated three to four fold by both GC and MC. $Na^+/K^+$ ATPase activity in the same monolayers was not stimulated by either steroid. This result was not influenced by expression of activity by area or by protein content as protein content was similar in all groups. These data suggest that steroids do not increase the total number of functioning pumps in the cell, even when $Na^+$ transport is stimulated.

We also performed the $Na^+/K^+$ ATPase assay on freshly isolated rat IMCD cells. There was no stimulation of activity after 6 h of incubation in the culture media with GC or MC (control, 1.41±0.17; GC, 1.20±0.17; MC, 1.41±0.21 $\mu$mol $P_i$/mg protein per hour, $n$ = 12 samples/group from two isolations), suggesting that the lack of increase in enzyme activity was not limited to the cultured cells.

*Steroid effects on ouabain binding.* We next studied radiolabeled ouabain binding to the monolayers. As shown in Fig. 4 and Table V, the $I_{sc}$ increased the usual three to four fold after 24-h incubations with GC and MC. Nonspecific ouabain binding increased slightly with steroid treatment. Specific ouabain binding in the same monolayers was increased more than two-fold by both GC or MC.

There are two possible explanations for this result: the steroids could have induced an increase in the number of active pumps present in the basolateral membrane or there was an increase in the affinity of the $Na^+/K^+$ ATPase for ouabain. It has been clearly demonstrated in rat tissue that the affinity of the $Na^+/K^+$ ATPase for ouabain is dependent upon which isozyme of the $\alpha$ subunit is present in a given tissue. The $\alpha_2$ isoform has a greater affinity for ouabain than $\alpha_1$ (26). It has also been shown that differential expression of the $\alpha$ subunit can be hormonally regulated (27, 28).

To determine whether the steroids altered the affinity of the pump for ouabain, we performed a dose response of the inhibition of $I_{sc}$ by ouabain. As shown in Fig. 5, the $IC_{50}$ for ouabain

*Table II. Effect of Inhibitors of Protein Synthesis and Cytoskeletal Function on the Stimulation of $I_{SC}$ by Steroids*

| | No steroid | | | | Glucocorticoid | | | | Mineralocorticoid | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *n* | | | | *n* | | | | *n* | | | |
| | Filter | Isolations | $I_{SC}$ | Resistance | Filter | Isolations | $I_{SC}$ | Resistance | Filter | Isolations | $I_{SC}$ | Resistance |
| | | | $\mu A/cm^2$ | $\Omega \cdot cm^2$ | | | $\mu A/cm^2$ | $\Omega \cdot cm^2$ | | | $\mu A/cm^2$ | $\Omega \cdot cm^2$ |
| Control | 7 | 1 | 2.0±0.3 | 125±32 | 19 | 2 | 11.1±1.5 | 179±11 | 12 | 1 | 20.5±2.2 | 174±15 |
| Cycloheximide | 11 | 2 | 1.0±0.1 | 194±25 | 12 | 1 | 0.9±0.1* | 158±28 | 7 | 1 | 1.0±0.2* | 195±28 |
| Control | 12 | 2 | 6.2±1.3 | 348±21 | 12 | 2 | 18.9±4.0 | 325±37 | 12 | 2 | 18.8±4.7 | 260±35 |
| Colchicine | 12 | 2 | 1.0±0.3* | 102±30‡ | 12 | 2 | 1.2±0.2* | 177±43‡ | 12 | 12 | 0.5±0.3* | 116±39‡ |

Cycloheximide (1 $\mu$M), colchicine (20 $\mu$M), and steroids were added to the cell culture media 24 h before electrical measurements were made. * Values less than controls from same isolations; $P < 0.05$ by ANOVA. ‡ Colchicine significantly decreased transepithelial resistance while cycloheximide did not, $P < 0.05$ by two-way ANOVA.

*Table III. Steroid Effects on Citrate Synthese Activity*

| | n | | | |
|---|---|---|---|---|
| | Filters | Isolations | $I_{sc}$ | CSA |
| | | | $\mu A/cm^2$ | $\mu mol/min$ per $cm^2$ |
| Control | 30 | 5 | 4.2±0.4 | 3.88±0.17 |
| Glucocorticoid | 30 | 5 | 14.4±1.4* | 4.36±0.15* |
| Mineralocorticoid | 30 | 5 | 16.3±1.7* | 4.56±0.19* |

Citrate synthase activity (CSA) was assayed in the same cell monolayers in which $I_{sc}$ was measured. Both measurements followed a 24-h incubation with steroids. * Values greater than controls from the same isolations, $P < 0.05$ by ANOVA.

was not significantly different between control, MC-, or GC-treated monolayers. Assuming that $IC_{50}$ reflects the ouabain affinity of the pump, the changes in ouabain binding produced by the steroids can not be attributed to changes in affinity for ouabain. Thus, it appears that the increased ouabain binding reflects an increased number of functioning pumps.

*Effect of inhibition of apical $Na^+$ entry on citrate synthase activity and ouabain binding.* There has been a longstanding debate regarding which effects of aldosterone are primary (or directly receptor mediated) and which effects are secondary to an increase in $Na^+$ entry across the apical membrane. To evaluate the role of $Na^+$ entry in our cell system, we performed a series of studies using the amiloride analogue, benzamil. The advantage of benzamil over amiloride is its increased specificity for the $Na^+$ channel as opposed to other $Na^+$ transporters such as the Na/H and the Na/Ca exchanger (29). As expected, 24-h incubations with benzamil (in combination with steroid) prevented the increase in $I_{sc}$ by both MC and GC (Fig. 6 *A*). The steroid-induced increase in citrate synthase activity, however, was not changed by incubation with benzamil (Fig. 6 *B*).

We next evaluated the effect of inhibiting $Na^+$ entry on ouabain binding in steroid-stimulated monolayers. Preliminary experiments showed that the presence of benzamil during the binding assay did not alter ouabain binding. Measurement of $I_{sc}$ demonstrated that 1 $\mu$M benzamil inhibited $Na^+$ transport in both control and steroid-treated cells (data not shown).

Inhibition of apical $Na^+$ entry by the addition of 1 $\mu$M benzamil to the incubation media for 24 h uncovered a difference between the GC and MC effects. Benzamil had no effect on specific ouabain binding in the MC-treated cells. In contrast, benzamil prevented the increase in specific ouabain binding in the GC-treated cells (Fig. 7).

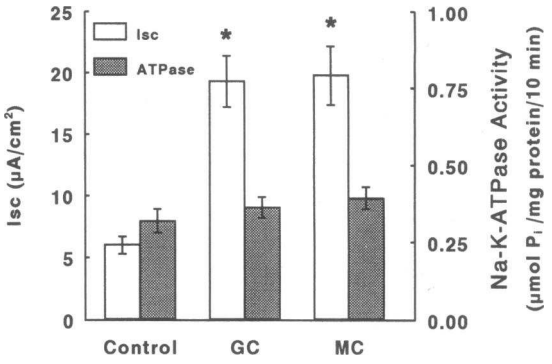


*Figure 3.* Effect of steroids on $I_{sc}$ and $Na^+/K^+$ ATPase activity measured in the same monolayers. No increase in $Na^+/K^+$ ATPase activity was present despite clear stimulation of $I_{sc}$ by both GC and MC. $n$ = 48, 51, and 49 monolayers from seven isolations for control, GC, and MC, respectively. * $P < 0.05$ by ANOVA.

## Discussion

The results of these studies demonstrate some similarities and some differences between the mechanisms by which MC and GC agonists stimulate $Na^+$ transport by primary cultures of rat IMCD cells. These steroid effects are dependent upon protein synthesis and intact cytoskeletal function. Both GC and MC exposure cause only a small increase in citrate synthase activity; the increase is not attenuated by inhibition of apical $Na^+$ entry. Neither MC or GC stimulate $Na^+/K^+$ ATPase activity in permeabilized cells, but both steroids cause an increase in ouabain binding to whole cell monolayers. The increase in ouabain binding caused by MC is independent of $Na^+$ entry across the apical membrane. In contrast, the GC-induced increase in ouabain binding is prevented by the inhibition of apical $Na^+$ entry.

*Citrate synthase activity.* An increase in citrate synthase activity after exposure to aldosterone has been demonstrated in toad bladder (30), adrenalectomized rat kidney (31), and adrenalectomized rabbit cortical collecting duct (CCD) (32). The stimulation of citrate synthase activity in our cells is minimal in comparison to the severalfold stimulation that has been demonstrated in rabbit CCD in response to aldosterone (32). It has been suggested that this increase in enzyme activity provides the capacity for increased ATP synthesis required for steroid-induced augmentation of active $Na^+$ transport, and that stimulation of citrate synthase is in fact a prerequisite for the physiological action of aldosterone (32).

*Table IV. Steroid Effects on $I_{sc}$ and ATPase Activities*

| | n | | | *Total ATPase* activity | *Mg-ATPase* activity | *NaK-ATPase* activity |
|---|---|---|---|---|---|---|
| | Filters | Isolations | $I_{sc}$ | | | |
| | | | $\mu A/cm^2$ | $\mu mol$ Pi/mg protein per 10 min | $\mu mol$ Pi/mg protein per 10 min | $\mu mol$ Pi/mg protein per 10 min |
| Control | 48 | 7 | 6.0±0.7 | 1.47±0.04 | 1.15±0.03 | 0.32±0.04 |
| Glucocorticoid | 51 | 7 | 19.3±2.1* | 1.44±0.03 | 1.09±0.03 | 0.36±0.03 |
| Mineralocorticoid | 49 | 7 | 19.8±2.4* | 1.48±0.04 | 1.08±0.04 | 0.39±0.04 |

$I_{sc}$ and ATPase activities were measured in the same monolayers after 24-h incubations with steroids. * Values greater than controls, $P < 0.05$ by ANOVA.

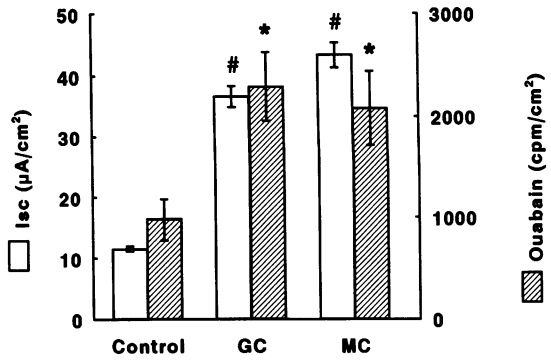


*Figure 4.* Effect of steroids on $I_{sc}$ and specific $^3$H-ouabain binding measured in the same monolayers. GC and MC stimulated both $I_{sc}$ and specific ouabain binding. $n$ = 36 monolayers from three isolations. *#$P < 0.05$ versus control by ANOVA.

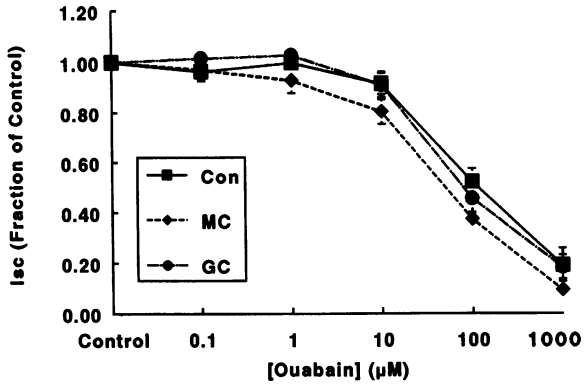


*Figure 5.* Ouabain inhibition of $I_{sc}$. The $IC_{50}$ values for $I_{sc}$ are 84, 60, and 48 μM for control, GC, and MC, respectively. There is no difference between groups by ANOVA. $n$ = 6 monolayers for control, 6 for GC, and 7 for MC.

In our IMCD cell monolayers, it appears that an increase in citrate synthase activity is not necessary for steroid stimulation of $Na^+$ transport. The small rise in enzyme activity probably contributes only minimally to the increase in energy requirement resulting from the increased rate of $Na^+$ transport. Approximately 25% of basal energy production by mitochondria in the IMCD is devoted to $Na^+$ reabsorption, according to measurements of ouabain-inhibitable $O_2$ consumption (11). A tripling of $Na^+$ transport would therefore require a 50% increase in total cell energy production. Citrate synthase activity rose by only 10%, an increment that falls far short of that required to match the increased demand assuming that energy production is near maximal. It therefore appears that the cultured IMCD cells have adequate energy reserve to support a substantial increase in $Na^+$ transport.

This deduction is consistent with the previous demonstration that freshly isolated rat IMCD cells possess considerable mitochondrial reserve (11). Others have also found minimal or no increase in citrate synthase activity in cultured cells that respond to aldosterone by increasing $Na^+$ transport. Enzyme activity is not stimulated in either aldosterone-treated A6 cells or cultured toad bladder cells (33, 34), and only a 30% rise has been demonstrated in primary cultures of rabbit CCD (35). These results in cultured cells stand in contrast to the marked stimulation seen in native tubules (32). Reasons for the reduced responsiveness of cultured cells are not known but may be related to the absence of factors present only in vivo that enable the induction of citrate synthase. An alternative explanation for the relative lack of enzyme stimulation in the present studies (aside from the fact that cultured cells were used) may be that IMCD cells have a fundamentally different response in this regard than do CCD cells.

*$Na^+/K^+$ pump effects.* The absence of stimulation of $Na^+/K^+$ ATPase activity in the present studies differs from previous results in the mammalian collecting duct. A three- to sixfold increase in enzyme activity has been demonstrated in isolated CCD segments of both the rat and the rabbit in response to aldosterone (36–40). Two- to threefold increases in $Na^+/K^+$ ATPase activity have also been demonstrated in primary cultures of rabbit CCD in response to both dexamethasone and aldosterone (41). Increases in $Na^+/K^+$ ATPase activity have also been found in isolated rat IMCD after in vivo treatment with deoxycorticosterone (42). We do not understand the reasons for the discrepancy between these results and ours. The most straightforward explanation is that the in vivo studies produce changes that cause the IMCD cells to respond differently than do freshly isolated or primary cultures of IMCD cells.

It is unlikely that the failure to detect an increase in $Na^+/K^+$ ATPase activity was due to technical limitations of the procedure we used. Based on the standard errors of the means, our method should have been sensitive enough to detect a 30% increase in activity. A result comparable to those of others (an increase of severalfold) should, therefore, have been readily apparent.

Although our results regarding $Na^+/K^+$ ATPase activity differ from those obtained in mammalian CCD, they are more consistent with findings observed in amphibian models of the distal nephron. In toad bladder, stimulation of $Na^+/K^+$ ATPase activity or of total pump number by aldosterone has not been clearly demonstrated, even under conditions in which $Na^+$ transport was increased (43–45). On the other hand, $Na^+/K^+$ ATPase activity has been shown to increase in re-

*Table V. Steroid Effects on $I_{sc}$ and Ouabain Binding*

| | $I_{sc}$ | Total $^3$H-ouabain binding | Nonspecific $^3$H-ouabain binding | Specific $^3$H-ouabain binding |
|---|---|---|---|---|
| | *μA/cm²* | | *CPM/cm²* | |
| Control | 11.5±0.4 | 3,017±141 | 2,040±144 | 978±202 |
| Glucocorticoid | 36.6±1.8* | 4,768±305* | 2,476±139* | 2,292±335* |
| Mineralocorticoid | 43.3±2.0* | 4,691±325* | 2,611±168* | 2,080±366* |

Values are means±SE; $n$ = 36 monolayers from three isolations. * Values greater than controls, $P < 0.05$ by ANOVA.

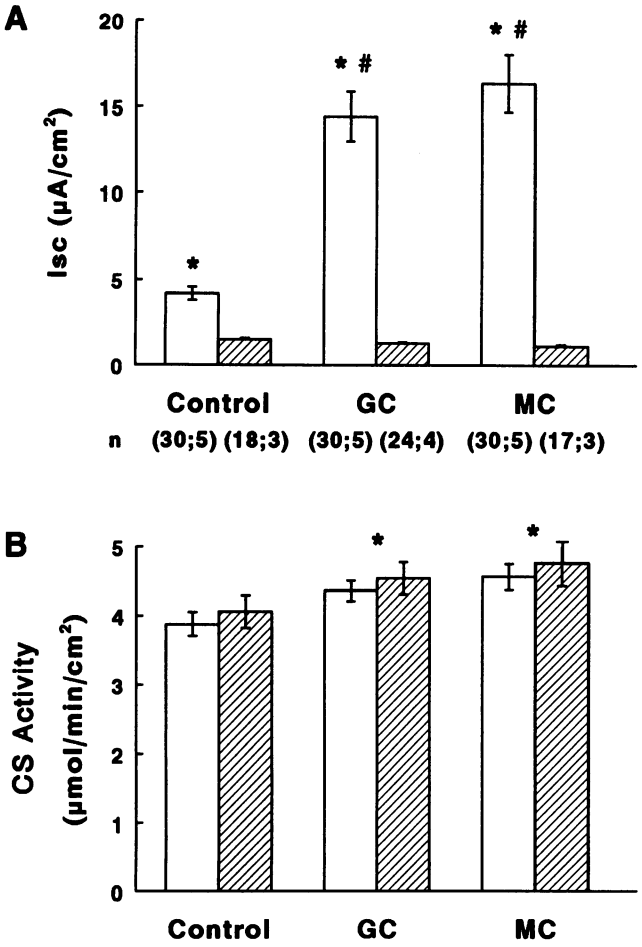


*Figure 6.* Effect of a 24-h incubation with 10 μM benzamil on $I_{sc}$ and citrate synthase activity. Both measurements were conducted in the same monolayers. (*A*) Benzamil (*hatched bars*) inhibited $I_{sc}$ to less than control levels (*open bars*) in all groups. $n$ = (monolayers; isolations). *Values different from benzamil-treated groups; #values different from control. $P < 0.05$ by two-way ANOVA. (*B*) Citrate synthase activity. Both steroids induced a small but significant stimulation of citrate synthase activity (*open bars*). This increase was not changed by benzamil treatment (*hatched bars*). *$P < 0.05$ by two-way ANOVA.

sponse to aldosterone in cultured cells derived from both toad kidney (A6) and toad bladder (TB6), but only when assays were performed on basolateral membrane-enriched fractions of cell homogenates (46).

Aldosterone causes increased synthetic rates of $Na^+/K^+$ ATPase $\alpha$ and $\beta$ subunits in the toad bladder and in monolayers of A6 cells (47, 48). This effect on subunit synthesis is mediated through stimulation of $\alpha$ and $\beta$ subunit gene transcription, as demonstrated by an increase in mRNA appearance in A6 cells after exposure to aldosterone (49, 50). Interestingly, despite the clear increase in subunit synthesis after aldosterone treatment, an increase in the total cellular pool of $Na^+/K^+$ ATPase has not been detected. The finding that ouabain binding to A6 monolayers is stimulated by aldosterone treatment (33), taken together with the lack of stimulation of total $Na^+/K^+$ ATPase activity or total cellular subunit number in amphibian models, does not conflict with our results. Rather the results suggest that the IMCD may share some similarities with amphibian systems while exhibiting some differences from the CCD.

Recent work on clone 9 cells (a rat liver cell line) reveals that they increase $Na^+/K^+$ ATPase isozyme mRNA in response to dexamethasone, the increment in $\beta_1$ mRNA being much greater (~ 40-fold) than that of $\alpha_1$ (~ 1.8-fold) (51). $Na^+/K^+$ ATPase activity, however, was only slightly increased; the results were not quantitatively different from ours. This failure to demonstrate an increase in $Na^+/K^+$ ATPase activity in the face of a huge increase in $\beta_1$ mRNA and a small rise in $\alpha_1$ mRNA surprised the authors; interestingly, we find these results to be consistent with ours. The authors postulated that perhaps the $\beta_1$ subunit does not play an important role in the regulation of $Na^+/K^+$ ATPase activity in all tissues. They suggest that the $\beta_1$ peptide may have other functions that are important in the mediation of dexamethasone-induced responses. One such function might be related to the translocation of pumps from an intracellular pool to the plasma membrane.

The measurement of the $Na^+$ transport and $Na^+/K^+$ ATPase activity in the same monolayers allows us to address the question of whether a threefold increase in $Na^+$ transport could occur in the absence of an increase in $Na^+/K^+$ ATPase activity. Assuming that the hydrolysis of one molecule of ATP results in the transport of three $Na^+$ ions, the maximum number of $Na^+$ ions that could be transported given a certain level of ATPase activity can be calculated as follows: $Na^+$ transport ($\mu$mol/h per cm$^2$) = 3 × $Na^+/K^+$ ATPase activity ($\mu$mol $P_i$/mg protein per h) × 0.203 mg protein/cm$^2$, where 0.203 mg protein/cm$^2$ is the mean value for a single monolayer. We have previously demonstrated that the $I_{sc}$ is an accurate measure of $Na^+$ transport in these monolayers (5). Expressing the results as $I_{sc}$ ($\mu$A/cm$^2$), the measured $Na^+/K^+$ ATPase activity of the control, MC-, and GC-treated monolayers could have supported maximal transport rates of 30.8, 37.0, and 37.4 $\mu$A/cm$^2$, respectively. The measured $I_{sc}$ of control, MC-, and GC-treated monolayers (6.0, 19.8, and 19.3 $\mu$A/cm$^2$, respectively; Fig. 3) were well below the maximum possible transport rates. These calculations demonstrate that the IMCD cells had more than adequate $Na^+/K^+$ ATPase activity to support the threefold increase in $Na^+$ transport induced by steroid treatment.

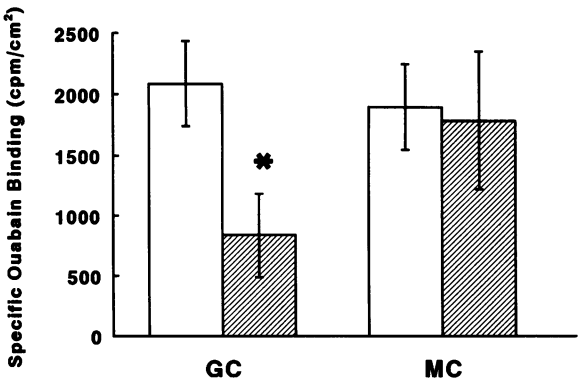


*Figure 7.* Effect of benzamil on specific $^3$H-ouabain binding. A 24-h incubation with 1 μM benzamil (*hatched bars*) prevented the GC-induced stimulation of ouabain binding. Benzamil did not alter the MC-induced stimulation. $n$ = 36 monolayers from three isolations for each group. *$P < 0.05$ versus GC control by $t$ test.

There are several possible explanations for the steroid-induced increase in ouabain binding in the absence of an increase in total $Na^+/K^+$ ATPase activity. The most straightforward interpretations are that the steroids either stimulate the insertion of preexisting pumps into the basolateral membrane or that they activate pumps already present in the membrane. The results of Johnson et al. (46), demonstrating an increase in $Na^+/K^+$ ATPase activity in the basolateral membrane of A6 cells treated with aldosterone, are also consistent with either explanation. One must presume that if inactive pumps reside in the basolateral membrane of control tissues (those not exposed to steroids) that they can neither bind ouabain (33) nor hydrolyze ATP under assay conditions (46). If activation of pumps already present in the basolateral membrane were the sole explanation for our present results, we should have been able to detect a significant increase in $Na^+/K^+$ ATPase activity. The activation of enough pumps to increase $Na^+$ transport from 6 to 19 $\mu A/cm^2$ (13 $\mu A/cm^2$) should have produced a > 30% increase (i.e., detectable) in $Na^+/K^+$ ATPase activity. We therefore suspect that activation of pumps resident in the basolateral membrane cannot completely account for our results. It seems more likely that translocation of functional pumps from an intracellular compartment to the basolateral membrane (detectable by an increase in cell surface ouabain binding) is an important component of steroid-induced stimulation of $Na^+$ transport by IMCD cells.

*Inhibition of $Na^+$ entry.* Inhibition of $Na^+$ entry across the apical membrane with benzamil inhibits the GC-induced increase in ouabain binding but does not affect the MC-induced increase. These results provide strong evidence that MC and GC action produce different molecular events leading to increased $Na^+$ transport.

The role of $Na^+$ entry in MC-induced changes in $Na^+/K^+$ ATPase has been the subject of much investigation but nonetheless remains controversial. The effects of amiloride on $Na^+/K^+$ ATPase activity are quite variable and difficult to interpret for several reasons. Studies have been carried out in vivo and in vitro, in both native and cultured cells, in different nephron segments, and in several different species. Some studies have used adrenalectomized animals whereas others have not (37–40, 52, 53). In addition to these variables, two other factors may be important in dissecting primary versus secondary effects of aldosterone: (*a*) the specificity of aldosterone for the MC receptor and (*b*) the specificity of amiloride for inhibiting $Na^+$ transport only through (apical membrane) $Na^+$ channels. In the present studies, we have attempted to control these variables. The use of relatively specific antagonists of both GC and MC receptors permits specific agonist–receptor interactions with minimal crossover effects. The use of benzamil rather than amiloride greatly increases the likelihood that the benzamil-produced effects on ouabain binding result predominantly, if not exclusively, from inhibition of $Na^+$ entry across the apical membrane and are not secondary to unrecognized inhibition of (basolateral) Na/H exchange or Na/Ca exchange (29).

In the present studies, the use of specific agonists of GC and MC receptors together with inhibition of $Na^+$ entry across the apical membrane uncovered clear differences in ouabain binding. Inhibition of apical $Na^+$ entry prevented stimulation of ouabain binding by GC but had no effect on the MC-induced stimulation (Fig. 7). These results suggest that the GC-induced insertion of pumps into the basolateral membrane (or activation of membrane-associated pumps) is a consequence of increased cell $Na^+$ entry, whereas the stimulation of pump insertion (or activation) by MC is a primary steroid effect, independent of $Na^+$ entry. An alternative but perhaps less likely explanation is that $Na^+$ entry also plays an important role in the MC effect on the pump but that MC agonists increase $Na^+$ entry not only via the apical $Na^+$ channel but across the basolateral membrane as well, possibly via the $Na^+$-H exchanger (54). If this were the case, the difference between GC and MC would result from their differential effects on a second (not a channel) $Na^+$ entry mechanism. Regardless of the ultimate cause, the significance of these findings is that they represent basic and previously unrecognized differences between the mechanisms of MC- and GC-stimulated $Na^+$ transport.

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