Modulation of Cytosolic Protein Kinase C and Calcium Ion Activity by Steroid Hormones in Rat Distal Colon*

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Studies from our laboratory have demonstrated rapid (<1 min) non-genomic activation of Na⁺-H⁺ exchange and potassium recycling by mineralocorticoids in human and rat colonic epithelium. It has previously been demonstrated that Na⁺-H⁺ exchange may be stimulated by protein kinase C (PKC) activation; therefore, we examined the effect of mineralocorticoids on PKC activity in rat colonic epithelium. Activation (after 15 min of incubation) of basal PKC activity was observed in cytosolic fractions of rat colonic epithelium by aldosterone, fludrocortisone, and deoxycorticosterone acetate. In all instances, PKC activation was inhibited by the PKC inhibitor bisindolylmaleimide (GF109203X). Hydrocortisone failed to activate PKC activity. Stimulation of basal intracellular free calcium [Ca²⁺], was observed, in isolated rat colonic crypts, following aldosterone addition. This stimulatory effect was inhibited by the PKC inhibitor, chelerythrine chloride. Hydrocortisone failed to increase [Ca²⁺]. These results indicate that intracellular signaling for aldosterone involves changes in [Ca²⁺], via activation of PKC. Since the stimulation of PKC and increase in [Ca²⁺], are apparent at normal circulating levels of aldosterone, our findings have major implications for the reassessment of mineralocorticoid effects on electrolyte homeostasis.

Mineralocorticoid hormones increase sodium reabsorption and promote potassium and hydrogen secretion in high resistance epithelia. This effector mechanism involves binding of aldosterone to intracellular type 1 mineralocorticoid receptors initiating genomic events. The genomic effects of aldosterone are characterized by a latency of 2–8 h and a sensitivity to inhibitors of transcription or translation (cyclohexamide and actinomycin D).

The aims of this study were to investigate the effect of mineralocorticoids on PKC activity in rat distal colonic epithelium and the effects of aldosterone on [Ca²⁺], in rat colonic crypts.

**EXPERIMENTAL PROCEDURES**

Materials—[γ-32P]ATP (3000 Ci mmol⁻¹) and protein kinase C assay kit (RPN 77) were purchased from Amersham. Aldosterone, fludrocortisone, deoxycorticosterone acetate (DOCA), and hydrocortisone were obtained from Sigma. Bisindolylmaleimide GF 109203X (K, 14 μm) and chelerythrine chloride (K, 0.66 μm) were purchased from Calbiochem. Fura-2/AM (acetoxymethoxy ester) was obtained from Molecular Probes (Eugene, OR). All other chemicals were of the highest purity commercially available.

Female Sprague-Dawley rats were sacrificed by cervical dislocation, the distal colon was removed, the epithelium stripped by microdissection and homogenized in 3 ml of homogenization buffer (50 mM Tris-HCl, pH 7.5, containing: 5 mM EDTA, 10 mM EGTA, 0.3% mercaptoethanol, 10 mM benzamidine, 50 μM phenylmethylsulfonyl fluoride, and 50 μg/ml leupeptin) using a Camlab Omni homogenizer at 20,000 rpm for five 40-s bursts. The homogenate was spun at 86,000 x g for 30 min. The supernatant (cytosolic fraction) was retained, and the pellet (membrane fraction) was resuspended in homogenization buffer by six passages through a 21-gauge needle. Protein content of cytosolic and membrane fractions was determined using the method described (16).

**PKC Activity Assay—**PKC activity was measured using an assay based on the transfer of the terminal phosphate of [γ-32P]ATP to a synthetic peptide substrate. Assays were carried out at 25 °C in a final volume of 100 μl of incubation mixture containing 30 μg of protein (either cytosolic or membrane fraction), 225 μg of peptide substrate, 7.5 mM dithiothreitol, stimulators/inhibitors as appropriate, in 50 mM Tris-HCl, pH 7.5. Calcium acetate (3 mM) was present in all cases except where basal activity was measured. Following a 2-min preincubation period, the reaction was initiated by addition of 37.5 μM [γ-32P]ATP (1–2 x 10⁴ cpm/μmol) containing 11.3 mCi magnesium acetate. Classical

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1 The abbreviations used are: PKC, protein kinase C; DOCA, deoxycorticosterone acetate.
PKC (α, β, β1, and γ isozymes) has an absolute requirement for calcium (17) and magnesium ions, and these ions were present at saturating levels in the assay. Assays were stopped after 15 min and processed by a modification of the method of Witt and Roskoski (18); assay mixture was spotted onto P81 phosphocellulose ion exchange chromatography filter papers, which were allowed to dry for 30 s and placed in 75 mM phosphoric acid solution (10 ml/filter). Filters were then washed (on ice) for 2 × 10 min in phosphoric acid. Incorporated radioactivity was determined by scintillation counting, and activities were expressed as picomoles of phosphate transferred/mg of protein/min.

Isolation of Rat Colonic Crypts—Rat distal colon was removed, everted on a glass rod, and placed in crypt isolation buffer, pH 7.4 (96 mM NaCl, 1.5 mM KCl, 10 mM HEPES-Tris, 27 mM EDTA, 55 mM sorbitol, 44 mM sucrose, 1 mM dithiothreitol) at room temperature for 40 min. The crypts were detached after the incubation by vigorous shaking, followed by centrifugation at 1000 × g for 10 min. The pellet of crypts obtained was resuspended in Krebs solution (140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES, 10 mM Tris-HCl, 10 mM glucose).

Spectrofluorescence—Colonic crypts were loaded with 5 μM FURA-2/AM for 30 min at 22 °C. The crypts were washed three times in Krebs solution and transferred to glass coverslips treated with poly-L-lysine. All experiments were performed at room temperature (20–22 °C) to minimize dye leakage and colonic crypt disintegration. The coverslips were mounted on an inverted epifluorescence microscope (Diaphot 200, Nikon). The light from a xenon lamp (Nikon) was filtered through alternating 340 and 380 nm interference filters (10-nm bandwidth, Nikon). The resultant fluorescence was passed through a 400 nm dichroic mirror, filtered at 510 nm, and then collected using an intensified CCD camera system (Darkstar, Photonic Science). Images were digitized and analyzed using the Starwise Fluo system (Imstar, Paris, France) as described in detail (19).

The Ca²⁺ concentration was calculated according to Equation 1,

\[ [Ca^{2+}] = K' \cdot \frac{R - R_{min}}{R_{max} - R} \]  

where \( K' \) is the product of the dissociation constant of the Ca²⁺/Fura-2 complex and a constant related to the optical characteristics of the particular system, \( R \) is the experimental ratio of \( F_{340}/F_{380} \) from which the background fluorescence has been subtracted, and \( R_{min} \) and \( R_{max} \) are the values of \( R \) in the presence of zero and saturating calcium, respectively (19). \( R_{min} \) and \( R_{max} \) values were obtained using the following solutions containing: 150 mM KCl, 10 mM EGTA, and 25 μM ionomycin with or without 10 mM CaCl₂. A micropipette perfusion system was used to apply test solutions to isolated colonic crypts plated on a glass coverslip. Where the effect of the PKC inhibitor was examined, the rat colonic crypts were preincubated with chelerythrine chloride for 5 min at room temperature prior to the addition of aldosterone. In all experiments the test solution was identical to the bathing solution except for the dose of steroid used.

Statistical Analysis—Measurements of PKC activities are presented as mean values ± S.E. of n experiments performed in duplicate. Statistically significant differences were determined by an unpaired Student’s t test, and differences were deemed significant if \( p \leq 0.05 \).

RESULTS

The presence of PKC activity in both cytosolic and membrane fractions isolated from rat colonic epithelium has been identified. Stimulation of PKC activity was observed in the presence of a mixture containing phorbol 12-myristate 13-acetate (6 μg/ml), phosphatidylserine (2 mol%), and protein kinase C inhibitor bisindolylmaleimide GF109203X (25 nm), and these results are shown in Fig. 1. Although the intracellular localization of PKC varies with cell type, in most tissues the enzyme is recovered mainly in the soluble fraction and would translocate to the membrane only in the presence of sustained activating signals (20, 21). In fractionated samples of rat colonic epithelium, approximately 75% of PKC activity is associated with the cytosolic fraction and the remainder localized to the membrane fraction.

Since it has been shown that Na⁺−H⁺ exchange may be stimulated by both mineralocorticoids (4, 22) and PKC activation (12), the effect of mineralocorticoids on PKC activity was investigated in rat colonic epithelium. As we had shown that PKC activity was recovered mainly in the cytosolic fraction (Fig. 1), this was the fraction chosen to assay for the effect of steroid hormones on PKC activity. Basal PKC activity was significantly stimulated, following a 15-min incubation, in the presence of aldosterone (0.01–100 nM; \( p < 0.005 \)), fludrocortisone (0.01–100 nM; \( p < 0.005 \)), and DOCA (0.01–100 nM; \( p < 0.01 \)) in cytosolic fractions isolated from rat distal colonic epithelium. Results are expressed as phosphate transferred (pmol min⁻¹ mg⁻¹ protein). Data represent the mean ± S.E. of six experiments performed in duplicate. Asterisks indicate significant differences between values linked by horizontal bars. * \( p < 0.0005 \), ** \( p < 0.005 \).

TABLE I

| [Steroid]    | Aldosterone | Fludrocortisone | DOCA | Hydrocortisone |
|--------------|-------------|----------------|------|---------------|
| 100          | 12 ± 3      | 10 ± 2.5       | 5.2 ± 1.8 | 1.6 ± 0.1   |
| 10           | 11.2 ± 2.3  | 11.6 ± 2.9     | 4.6 ± 1.5 | 1.6 ± 0.27   |
| 1            | 17.6 ± 4.7  | 12.2 ± 3       | 5.1 ± 1.4 | 1.7 ± 0.4    |
| 0.1          | 15 ± 3      | 11.6 ± 3       | 5.2 ± 1.4 | 1.7 ± 0.2    |
| 0.01         | 10.5 ± 2.6  | 11.3 ± 2.9     | 4.9 ± 1.6 | 2 ± 0.2      |

![FIG. 1. Modulation of PKC activity by phorbol 12-myristate 13-acetate (6 μg/ml), phosphatidylserine (2 mol%), and protein kinase C inhibitor bisindolylmaleimide GF109203X (25 nm) in cytosol and membrane fractions isolated from rat distal colonic epithelium. Results are expressed as phosphate transferred (pmol min⁻¹ mg⁻¹ protein). Data represent the mean ± S.E. of six experiments performed in duplicate. Asterisks indicate significant differences between values linked by horizontal bars. * \( p < 0.0005 \), ** \( p < 0.005 \).]
The stimulatory effect of DOCA (0.01–100 nM) on PKC activity was approximately 50% of that observed in the presence of aldosterone and fludrocortisone (0.01–100 nM).

To examine whether stimulation of PKC activity could be detected following a shorter incubation time, the effect of aldosterone (0.1 nM) on basal PKC activity was examined following a 5-min aldosterone (0.1 nM) incubation. In these experiments, significant stimulation of basal PKC activity was observed after a 5-min incubation. This stimulatory effect of aldosterone was inhibited by the PKC inhibitor bisindolylmaleimide (25 nM), and these results are shown in Table II. Aldosterone (0.1 nM) stimulation of basal PKC activity was also observed in membrane fractions isolated from rat distal colonic epithelium. This stimulatory effect of aldosterone was significantly inhibited by the PKC inhibitor bisindolylmaleimide (25 nM), and these data are shown in Table III. The stimulation of PKC activity in membrane fractions, however, was approximately 4-fold less than that observed in cytosolic fractions. In a further series of experiments, the existence of PKC activity was also established in cytosolic and membrane fractions isolated from rat distal colonic crypts. Significant stimulation of basal PKC activity was observed, following aldosterone (0.1 nM), in both rat crypt cytosolic and membrane fractions, and this stimulation was significantly inhibited by the specific PKC inhibitor bisindolylmaleimide (25 nM). These results are shown in Table IV.

Sex steroid hormones, particularly β-estradiol, have salt-retaining properties, but their possible non-genomic effects on ion transport or cell signaling have not previously been examined. Fig. 2 shows the stimulation of basal PKC activity in rat colonic cytosolic fractions in the presence of β-estradiol (0.01–100 nM).

The stimulatory effect of all of the above steroid hormones on PKC activity from rat distal colonic cytosolic fractions was inhibited by the PKC inhibitor bisindolylmaleimide (25 nM). The effects of aldosterone (0.01–100 nM) on PKC activity, in the absence and presence of the PKC inhibitor bisindolylmaleimide (25 nM) are shown in Table V. Bisindolylmaleimide (25 nM) also inhibited the PKC stimulatory effect obtained in the presence of fludrocortisone, DOCA, and β-estradiol (0.01–100 nM) (data not shown).

As rapid effects of aldosterone on free [Ca\(^{2+}\)] have recently been demonstrated in endothelial cells (6), we determined the effect of both aldosterone and hydrocortisone on free [Ca\(^{2+}\)] in single isolated crypts from rat colonic epithelium. We also examined whether the effect of aldosterone to increase [Ca\(^{2+}\)], was due to protein kinase C activation, by use of the PKC inhibitor chelerythrine chloride. Fig. 3 shows a typical rat colonic crypt with surface cells attached. The regions of analysis corresponding to Fig. 4 and Tables VI and VII are indicated. (2 mM Ca\(^{2+}\) was present in the external bathing solution in all cases unless otherwise stated.) Basal free [Ca\(^{2+}\)] was significantly increased in all regions of the rat colonic crypt, 16 min following aldosterone (1 μM) ad-

### Table II

| Steroid           | Amount of phosphate transferred at: |
|-------------------|-------------------------------------|
|                   | 0.01 nM    | 0.1 nM    | 1 nM      | 10 nM      | 100 nM     |
| Aldosterone       | 33 ± 8     | 32 ± 7.6  | 29 ± 7    | 29 ± 4     | 35 ± 4     |
| (p < 0.025)       |           | (p < 0.05)| (p < 0.05)| (p < 0.05)| (p < 0.05)|
| Aldosterone + PKC inhibitor | 8 ± 2 | 10 ± 4.5 | 9 ± 4 | 8.8 ± 2 | 9.4 ± 3 |

### Table III

| Steroid                  | Amount of phosphate transferred at: |
|--------------------------|-------------------------------------|
|                          | 0.01 nM | 0.1 nM | 1 nM | 10 nM | 100 nM |
| Aldosterone              | 33 ± 8  | 32 ± 7.6 | 29 ± 7 | 29 ± 4 | 35 ± 4 |
| ([Steroid])              |         | (p < 0.025) | (p < 0.05) | (p < 0.05) | (p < 0.05) |
| Aldosterone + PKC inhibitor | 8 ± 2 | 10 ± 4.5 | 9 ± 4 | 8.8 ± 2 | 9.4 ± 3 |

### Table IV

| Steroid                  | Amount of phosphate transferred at: |
|--------------------------|-------------------------------------|
|                          | 0.01 nM | 0.1 nM | 1 nM | 10 nM | 100 nM |
| Basal PKC activity       |        |       |     |      |       |
| Aldosterone (0.1 nM)     | 15 ± 1 | 18 ± 0.58 |       |      |       |
| (p < 0.025)              |        |       |     |      |       |
| Aldosterone (0.1 nM) + PKC inhibitor | 11 ± 2 | 14 ± 1.7 |       |      |       |
| (p < 0.01)               |        |       |     |      |       |

Fig. 2. Modulation of PKC activity, within 15 min by β-estradiol (100-0.01 nM) in cytosolic fractions isolated from rat distal colonic epithelium. Results are expressed as phosphate transferred (pmol min\(^{-1}\) mg\(^{-1}\) protein). Data represent the mean ± S.E. of three experiments performed in duplicate.
In the external bathing solution (data not shown).

In contrast, no increase in basal free \([\text{Ca}^{2+}]\) was observed in any region of the isolated rat colonic crypt following the addition of low concentrations of aldosterone (10 nM, 1 nM). Table VI (parts a and b, respectively) summarizes these results.

Basal free \([\text{Ca}^{2+}]i\) was significantly increased in all regions of the rat colonic crypt, 16 min following aldosterone (0.1 nM) addition. When the rat colonic crypts were preincubated (5 min) with the PKC inhibitor chelerythrine chloride (1 \(\mu M\)), this stimulatory effect of aldosterone on \([\text{Ca}^{2+}]i\) was abolished. These results are shown in Table VII.

**DISCUSSION**

In the present study, the involvement of PKC and \([\text{Ca}^{2+}]i\) as possible second messengers in rapid aldosterone effects was investigated.

This study demonstrates for the first time, rapid stimulation (after 5 and 15 min of incubation) of PKC activity and an increase in \([\text{Ca}^{2+}]i\) by mineralocorticoid hormones in rat distal colonic epithelium. Both of these steroid hormone effects were inhibited in the presence of a PKC inhibitor. These results indicate that intracellular signaling for aldosterone involves changes in \([\text{Ca}^{2+}]i\), via activation of PKC activity.

In cytosolic fractions isolated from rat distal colonic epithelium, basal PKC activity was significantly stimulated by aldosterone (0.01–100 nM). A dose-response relationship was not observed, as all doses of steroid stimulated PKC activity to the same extent. These results imply that under these conditions PKC activity is maximally stimulated at physiological concentrations of aldosterone. Sub-physiological (pM) concentrations of aldosterone failed to stimulate PKC activity in a significant manner. Fludrocortisone (0.01–100 nM) produced similar significant stimulation of PKC activity, whereas the stimulatory effect of DOCa (0.01–100 nM) was 50% of that observed in the presence of aldosterone or fludrocortisone. In membrane fractions, isolated from rat distal colonic epithelium, PKC could also be stimulated by aldosterone (0.1 nM). The stimulation observed was approximately 25% of that seen in cytosolic fractions. These results were expected, inasmuch as, in most tissues, PKC resides mainly in the cytosolic fraction and is translocated to the membrane post-activation. From these results it appears, therefore, in the rat colonic epithelium, that aldosterone stimulates the enzyme in both fractions. Protein kinase C activity in isolated rat colonic crypts was also significantly stimulated by aldosterone (0.1 nM). The sex steroid \(\beta\)-estradiol (0.01–100 nM) also produced a significant stimulation of PKC activity, but only to 20% of that seen with either aldosterone or fludrocortisone. In contrast to the stimulatory effects observed in the presence of the above steroids, no stimulation of PKC activity, in cytosolic fractions isolated from rat colonic epithelium, was observed in the presence of the glucocorticoid hydrocortisone (0.01–100 nM).

Previous studies have shown that \(\text{Na}^+\text{H}^+\) exchange may be stimulated by PKC activation (12) and by mineralocorticoids (4, 22). The results obtained in the first part of this study demonstrate a significant stimulation of basal PKC activity by aldosterone over a concentration range similar to that found to be effective to stimulate \(\text{Na}^+\text{H}^+\) exchange and inositol 1,4,5-

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**TABLE V**

| [Aldosterone] | 0.01 nM | 0.1 nM | 1 nM | 10 nM | 100 nM |
|---------------|---------|--------|------|-------|-------|
| Aldosterone   | 66 ± 15 | 72 ± 11| 80 ± 5 | 55 ± 10 | 62 ± 17 |
| Aldosterone + PKC inhibitor | 13 ± 2.3 | 13 ± 2.5 | 15 ± 3 | 15 ± 2 | 13 ± 3 |
| (p < 0.025)   | (p < 0.005) | (p < 0.0005) | (p < 0.01) | (p < 0.05) |
trisphosphate generation in vascular smooth muscle cells and human mononuclear leukocytes (5, 22). Recent studies from our laboratory provide evidence for rapid non-genomic activation of ATP-regulated K⁺ (KₐTP) channels by aldosterone in human distal colon and frog skin (9–11). Rapid activation of basolateral KₐTP channels by the hormone occurs within 5 min with an EC₅₀ of ~0.8 nM. This effect is insensitive to spironolactone, cyclohexamide, or actinomycin D, but can be prevented by pretreatment of the epithelium with PKC inhibitors or by inhibition of Na⁺-H⁺ exchange (amiloride or Na⁺-free medium). The estrogen β-estradiol (100 nM) also produced an immediate (<5 min) activation of KₐTP channels in human colonic epithelium (23). This rapid effect of β-estradiol on potassium-dependent SCC was abolished by inhibition of basolateral Na⁺-H⁺ exchange (100 μM amiloride or Na⁺-free medium). It is possible therefore that stimulation of PKC activity, by steroid hormones, may in turn lead to activation of Na⁺-H⁺ exchange.

In the second part of this study the effect of aldosterone on free [Ca²⁺] was investigated in isolated rat colonic crypts. Rapid stimulation of basal free [Ca²⁺] was observed in all regions of the rat colonic crypt following aldosterone addition (1 μM, 10 nM, 1 nM, and 0.1 nM), and this increase in [Ca²⁺] appeared to plateau within 12–16 min after aldosterone addition. This stimulatory effect of aldosterone (0.1 nM) on [Ca²⁺], was abolished in the presence of the PKC inhibitor chelerythrine chloride (1 μM) or in a Ca²⁺-free medium. No stimulation of [Ca²⁺] was observed following hydrocortisone (1 μM). Thus, aldosterone appears to stimulate the influx of extracellular Ca²⁺ via a PKC-sensitive pathway.

Clearly these rapid effects of aldosterone to: 1) stimulate Na⁺-H⁺ exchange in leukocytes and vascular smooth muscle, 2) increase [Ca²⁺] in vascular smooth muscle, 3) stimulate inositol 1,4,5-trisphosphate generation, and 4) in this study, stimulate PKC activity and increase [Ca²⁺], in rat colonic epithelium, are incompatible with classical genomic mechanisms of steroid action but indicate a non-genomic pathway with high affinity for mineralocorticoids and very low affinity for hydrocortisone. More importantly, the results obtained in our study demonstrate that the non-genomic signal transduction mechanism is operative in a classical steroid hormone target epithelium. These results therefore give additional support to the hypothesis of a novel rapid pathway for aldosterone action.

The physiological significance of non-genomic aldosterone action is supported by the low apparent Kₐ for the rapid in vitro effects of aldosterone, which are consistent with the physiological concentration of free circulating aldosterone (~0.1 nM). The selectivity for aldosterone is important, since it may explain the different effects of mineralocorticoids and glucocorticoids on sodium homeostasis.

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