0021-972X/93/7706-1452$03.00/0
Journal of Clinical Endocrinology and Metabolism

Vol. 77, No. 6
Printed in U.S.A.

The Inositol-1,4,5-Trisphosphate System Is Involved in Rapid Effects of Aldosterone in Human Mononuclear Leukocytes*

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ABSTRACT

There is increasing evidence for rapid steroid action on electrolyte transport in human mononuclear leukocytes (HML). In HML, aldosterone stimulates the Na^+/H^+ antiporter within a few minutes. Because a variety of hormones and growth factors activate the Na^+/H^+ antiporter via protein kinase C and inositol phospholipids, a possible involvement of inositol-1,4,5-trisphosphate (IP_3) in the rapid effects of aldosterone in HML was investigated.

The stimulation of IP_3 generation was started by the addition of aldosterone, concanavalin A, or other steroids. A significant increase in IP_3 levels by aldosterone (1 nmol/L, $P < 0.05$) was found after 1 min, similar to that found after concanavalin A (25 μg/mL). Aldosterone caused a concentration-dependent elevation of IP_3 levels, with an apparent EC_{50} of approximately 0.1 nmol/L. Fludrocortisone stimulated IP_3 generation at similar concentrations, whereas a weaker IP_3 stimulation by glucocorticoids (hydrocortisone, dexamethasone) occurred at micromolar concentrations only. Canrenone, a potent inhibitor of classical aldosterone action, was not effective up to a concentration of 100 nmol/L.

These findings show kinetic and pharmacological similarities with both the functional data on Na^+/H^+ antiport stimulation by aldosterone and the studies of ^{125}I-aldosterone binding to plasma membranes of HML. Thus, these data are the first to indicate an involvement of the phosphoinositide pathway in the rapid membrane effects of aldosterone. (*J Clin Endocrinol Metab* **77:** 1452–1457, 1993)

IN THE CLASSICAL model of genomic steroid action, steroids bind to intracellular receptors which act as ligand-dependent transcription factors. The pathway of genomic steroid action involving transcription, translation, and protein synthesis is characterized by late onset effects preceded by a latency of 1–4 h. In contrast to these well known mechanisms, there is increasing evidence for rapid, probably nongenomic, steroid actions. These include neural effects by local application of steroids, fast effects of steroids on the γ-aminobutyric $acid_A$ receptor, on LH-releasing peptide secretion, on dopamine release, and rapid effects of progesterone on the oocyte maturation and spermatozoan acrosome reaction (1). *In vitro* effects of aldosterone on intracellular electrolyte concentrations and cell volume have been demonstrated in human mononuclear leukocytes (HML) (2, 3) initiated by a rapid stimulation of the sodium-proton antiport of the cell membrane (4). This stimulation has been shown to share close pharmacological and kinetic similarities with the radioactive binding of ^{125}I-labeled aldosterone to plasma membranes from HML (5).

Although rapid increases of intracellular calcium in oocytes and spermatozoa after progesterone (6, 7) have been demonstrated, little is known about the involvement of other second messenger systems in fast steroid action. Changes of intracellular inositol-1,4,5-trisphosphate (IP_3) levels occurring in vascular smooth muscle cells (VSMC) 15 min after application of hydrocortisone (8), and the modulation of angiotensin II- and vasopressin-induced IP_3 production in VSMC (9) after glucocorticoid treatment, indicate a possible involvement of the IP_3 intracellular signaling system in rapid steroid action. In addition, in response to agents such as GH or angiotensin II, the Na^+/H^+ antiport is stimulated via calcium and phospholipid-dependent pathways (10); therefore, we investigated the potential role of IP_3 generation on short-term effects of aldosterone.

Received October 23, 1992. Accepted August 19, 1993.

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* The study was supported by the Wilhelm-Sander-Stiftung (88.015.2) and the Deutsche Forschungsgemeinschaft (We 1184/4-2).

Materials and Methods

Materials

Aldosterone was purchased from Serva Feinbiochemica (Heidelberg, Germany), other steroids and sodium propionate from Sigma (St. Louis, MO). Canrenone was a gift of Dr. D. Armanini (University of Padua, Padua, Italy), and ethylisopropylamiloride (EIPA) was kindly provided by Dr. H. J. Lang (Hoechst AG, Frankfurt, Germany). Percoll was obtained from Pharmacia (Uppsala, Sweden), Fura-2-AM from Boehringer Mannheim (Mannheim, Germany), thapsigargin and RPMI 1640 medium from GIBCO Life Technologies (Berlin, Germany). All other reagents (analytical grade) were from Merck (Darmstadt, Germany). The IP_3 assay kit (TRK 1000) was purchased from Amersham Buchler (Braunschweig, Germany).

Separation of HML

The preparation of HML has already been described in detail elsewhere (3). Briefly, 150 mL peripheral blood from fasting healthy male volunteers (mean age ± SD, 27.5 ± 3.8 yr, range 21–34 yr) was drawn into heparinized syringes (Heparin Novo, Novo Industries, Germany) between 0800 and 0900 h. Informed consent was obtained in compliance

with the Declaration of Helsinki. The blood was centrifuged at 120 × *g* for 20 min. Platelet-rich plasma was removed, and the remaining pellet was mixed with 10% platelet-poor plasma. Mononuclear cells were separated by a Percoll gradient according to Hjorth *et al.* (11) and washed in RPMI 1640 medium. After another centrifugation at 120 × *g*, the preparation of human mononuclear cells contained 4–8% monocytes and 92–96% lymphocytes but no granulocytes and few platelets. Cell viability amounted to at least 95% before stimulation, as judged by trypan blue exclusion.

Measurement of IP_3 in HML

Levels of IP_3 in HML were measured by a radioreceptor assay kit according to Sato *et al.* (9) and Palmer and Wakelam (12). HML were suspended in RPMI 1640 at a concentration of 6.8–7.5 × 10^7 cells/mL. Addition of steroids and concanavalin A started the stimulation of IP_3 generation. Steroids were dissolved in ethanol in a 10 mmol/L stock solution. The reaction was stopped by 60 μL ice-cold 20% perchloric acid, and the mixture was kept on ice for 20 min. Centrifugation at 2000 × *g* for 15 min at 4 C resulted in protein sedimentation. Supernatants were kept on ice and titrated to pH 7.5 with 1.5 N KOH and 60 mmol/L HEPES in siliconized tubes to minimize losses of phosphorylated inositol species, according to the method of Palmer *et al.* (13). After sedimentation of precipitated $KClO_4$, 100 μL supernatant were mixed with equal amounts of assay buffer containing 0.1 mmol/L Tris/HCl, 4 mmol/L ethylenediaminetetraacetic acid, and 4 mg/mL BSA (pH 9.0). D-Myo-[^{3}H]IP_3 (100 μL) and bovine adrenal binding protein were added and vortexed. After incubation for 15 min on ice, the protein-bound fraction was separated by centrifugation at 2000 × *g* for 15 minutes at 4 C. The supernatant was carefully decanted, and absorbent tissue was used to remove adhering liquid droplets in the tubes. The pellets were resuspended in 200 μL distilled water, and radioactivity was measured by β-scintillation counting. The amount of IP_3 was calculated from a standard curve by plotting the percent B/B_0 as a function of the $\log_{10}$ of IP_3 standards.

In this assay, the cross-reactivities of other inositolphosphates are less than 0.006% for inositol-1-phosphate, 0.02% for inositol-4-phosphate, 0.015% for inositol-1,4-bisphosphate, 0.22% for inositol-1,3,4-trisphosphate, 1% for inositol-2,4,5-trisphosphate, and less than 1% for cyclic inositol-trisphosphate and inositol-1,3,4,5-tetrakisphosphate.

Measurement of the activity of the Na^+/H^+ antiporter in HML

The activity of the Na^+/H^+ antiporter in HML was determined by the time-dependent cell swelling in isotonic sodium propionate as described recently (4). HML were incubated in a buffer containing 140 mmol/L Na^+ propionate, 1 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 10 mmol/L glucose, and 20 mmol/L HEPES, adjusted to a pH of 6.7. Aldosterone was added at concentrations indicated; maximum ethanol concentration in these experiments was 0.0001%. The cell diameters were determined by a Coulter Channelyzer 256 (Coulter Electronics, Luton, Great Britain). Plateau values of diameter after 30 min are considered as a measure of the initial activity of the sodium proton exchanger (14) and are used in the dose-response curve for this effect (see Fig. 2).

Measurement of the free intracellular calcium concentration in HML

Measurements of the free intracellular calcium concentration in HML were performed as described by Grynkiewicz *et al.* (15). Lymphocytes were incubated in 1 μmol/L Fura-2-acetoxymethylester at 37 C for 30 min, washed, and resuspended in physiological buffer (140 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L $MgCl_2$, 5 mmol/L glucose, 1 mmol/L Na_2HPO_4, and 10 mmol/L Tris/HCl, pH 7.4) in the presence or absence of external calcium (1 mmol/L). Changes in fluorescence of the Fura-2-loaded lymphocytes at the excitation wavelength of 340 and 380 nm and at the emission wavelength of 510 nm were measured. The ratios of maximum and minimum fluorescence were determined by the addition of 250 μmol/L digitonin in the presence of 1 mmol/L free calcium and in the presence of 10 mmol/L EGTA (pH ≥ 8.5), respectively.

Statistical analysis

The two-tailed Wilcoxon test for paired data was used, including multiple comparison corrections according to the method of Bonferoni. Values of $P < 0.05$ were considered significant. The results were expressed as mean ± SEM. Linear regression was calculated by least square analysis.

Results

Time course of the aldosterone-, hydrocortisone-, and concanavalin A-induced IP_3 production

The basal intracellular IP_3 content of HML was 0.27 ± 0.02 pmol/10^6 cells. During incubation of HML in RPMI 1640 with 0.01% ethanol (maximum vehicle concentration), a stable baseline of intracellular IP_3 levels was obtained for up to 15 min. When HML were incubated with aldosterone, intracellular IP_3 levels increased to a maximum of 0.58 ± 0.07 pmol/10^6 cells, or 239 ± 33% of initial values after 60 s (n = 7; $P < 0.05$ *vs.* initial values), and declined to 0.37 ± 0.03 pmol/10^6 cells (138 ± 15%) after 5 min ($P < 0.05$). Intracellular IP_3 levels were still significantly ($P < 0.05$) above baseline values after 15 min (0.39 ± 0.05 pmol/10^6 cells; 145 ± 15%) (Fig. 1). IP_3 levels after stimulation with concanavalin A were 0.53 ± 0.05 pmol/10^6 cells (206 ± 21%) after 60 s (n = 7; $P < 0.05$), being not significantly different from values 60 s after aldosterone stimulation; however, after 5 and 15 min of incubation with concanavalin A, IP_3 levels were not different from baseline values. Intracellular IP_3 levels were slightly increased by 1 μmol/L hydrocortisone up to 0.37 ± 0.03 pmol/10^6 cells after 60 s (n = 4; 137 ± 14%), which reached baseline values again after 5 and 15 min of incubation.

Effects of aldosterone on the Na^+/H^+ antiporter activity and IP_3 production in HML

A dose-related stimulation of the Na^+/H^+ antiporter (4) by aldosterone as measured by the time-dependent cell swelling

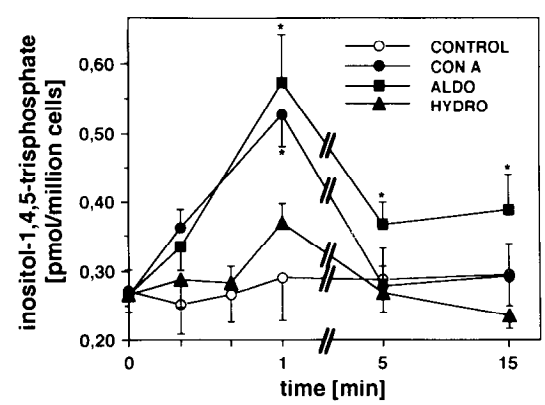

FIG. 1. Time course of IP_3 stimulation in HML by aldosterone, concanavalin A, and hydrocortisone is shown. HML were incubated in RPMI 1640 medium plus 0.01% ethanol (maximum vehicle concentration during steroid stimulation) alone (CONTROL), with 25 μg/mL concanavalin A (CON A), with 1 nmol/L aldosterone (ALDO), or with 1 μmol/L hydrocortisone (HYDRO) at room temperature. (*, $P < 0.05$ for comparison with time 0 value; mean ± SEM, n = 7; for hydrocortisone, n = 4).

in isotonic sodium propionate (Fig. 2) was found at half-maximal effects of approximately 0.1 nmol/L. Maximum increases of cell diameters after 30 min are shown as the differences of values obtained after incubation with and without 60 μmol/L EIPA.

The response of intracellular IP_3 levels to aldosterone is shown in Fig. 2. There was a small effect on IP_3 at an aldosterone concentration of 0.01 nmol/L, and near-maximum IP_3 levels were reached at a concentration of 10 nmol/L. By increasing the concentration to 100 nmol/L, no further rise in IP_3 values was obtained (not shown). Half-maximal effects (EC_{50}) of aldosterone are seen at a concentration of approximately 0.1 nmol/L. Aldosterone increases intracellular IP_3 levels despite inhibition of the Na^+/H^+ exchanger by 60 μmol/L EIPA (Fig. 3). After incubation with aldosterone in the presence of 60 μmol/L EIPA, intracellular IP_3 levels increased to 0.37 ± 0.01 pmol/10^6 cells after 15 s and to 0.67 ± 0.03 pmol/10^6 cells after 60 s incubation; IP_3 levels obtained with aldosterone alone were 0.36 ± 0.02 and 0.51 ± 0.06 pmol/10^6 cells, respectively (n = 4).

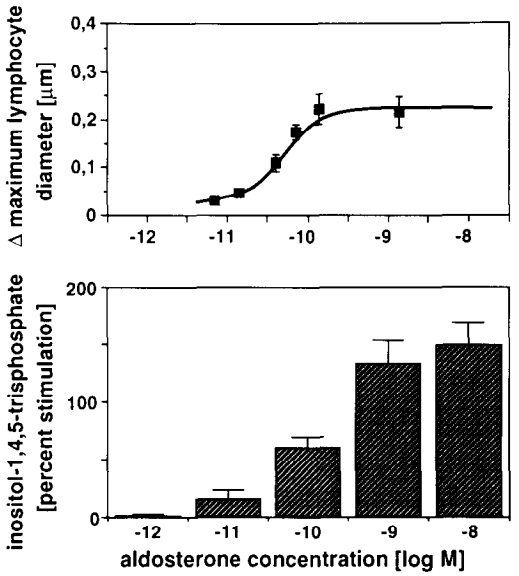

FIG. 2. *Top*, Dose-response curve for maximum increases of HML diameter by aldosterone after an incubation for 30 min in isotonic Na^+ propionate in excess of values obtained in the presence of 60 μmol/L EIPA (mean ± SEM, n = 7–8). *Bottom*, Dose-response curve for aldosterone effects on IP_3 in HML after an incubation for 1 min (mean ± SEM, n = 6).

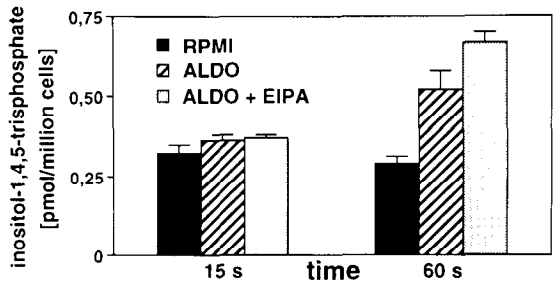

FIG. 3. IP_3 levels in HML were measured after incubation in RPMI 1640 medium alone (RPMI), with 1 nmol/L aldosterone (ALDO), and with 1 nmol/L aldosterone plus 60 μmol/L EIPA (ALDO + EIPA) for 15 and 60 s (mean ± SEM, n = 4).

Effects of other steroids and canrenone on IP_3 production in HML

Fludrocortisone was active at concentrations between 0.01 and 10 nmol/L (EC_{50} ~0.1 nmol/L, Fig. 4). Near-maximum stimulation of IP_3 was seen at 1 nmol/L, reaching absolute values similar to those after aldosterone stimulation. Hydrocortisone did not stimulate IP_3 generation up to a concentration of 10 nmol/L. At 100 and 1000 nmol/L, a minor stimulation of IP_3 was found; thus, the EC_{50} is estimated to be about 1 μmol/L. Dexamethasone stimulates IP_3 generation starting at about 100 nmol/L. Maximal IP_3 levels at 1000 nmol/L dexamethasone were not different from levels after stimulation with 1 nmol/L aldosterone. Incubation of the cells with 1 nmol/L aldosterone plus 100 nmol/L canrenone showed no significant inhibition of the aldosterone-induced IP_3 response (Fig. 5), and canrenone (100 nmol/L) alone was inactive.

Effects of aldosterone and thapsigargin on free intracellular calcium concentration in HML

Within 5 min, aldosterone (1–100 nmol/L) and thapsigargin (0.5 μmol/L), a specific inhibitor of the Ca^{2+} ATPase in IP_3-sensitive, intracellular calcium stores, do not significantly affect intracellular calcium concentrations in HML as determined by Fura-2. Experiments were done with and without 1 mmol/L Ca^{2+} in the incubation medium (not shown).

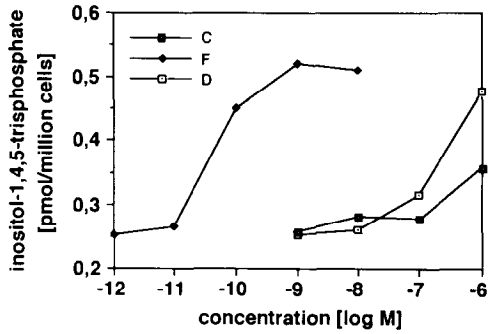

FIG. 4. Dose-response curve for IP_3 stimulation by fludrocortisone (F), hydrocortisone (C), and dexamethasone (D) in HML after an incubation for 1 min. Experiments were done in triplicate.

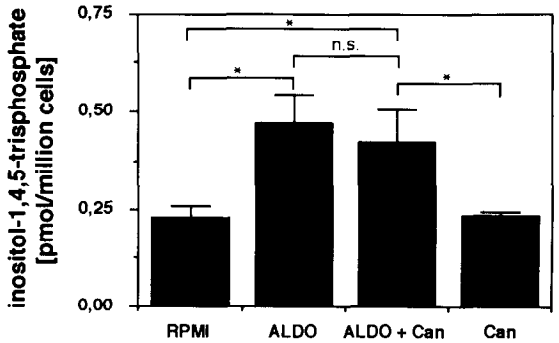

FIG. 5. IP_3 levels in HML were measured after incubation in RPMI 1640 medium alone (RPMI), with 1 nmol/L aldosterone (ALDO), 1 nmol/L aldosterone plus 100 nmol/L canrenone (Can), and 100 nmol/L canrenone alone for 1 min. (*, $P < 0.05$; mean ± SEM, n = 6).

Discussion

An activation of the sodium-proton exchanger by aldosterone could be demonstrated in HML within 1–2 min (4), initiating the electrolyte and water shifts mentioned above. The time interval between aldosterone application and stimulation of the Na^+/H^+ antiport in HML is too short to reflect a genomic response. Actinomycin D, an inhibitor of transcriptional processes, was not able to block this effect. Thus, the rapid action of aldosterone was assumed to result from direct interaction with specific membrane receptors for aldosterone that subsequently were demonstrated by binding studies (16) and characterized on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (17). In the present study, the involvement of phosphoinositide hydrolysis as a possible second messenger system in these rapid aldosterone effects was investigated.

The main findings of the present study may be summarized as follows: 1) In HML, aldosterone significantly stimulates the generation of IP_3 within 60 s; 2) IP_3 stimulation was concentration-dependent with an EC_{50} of approximately 0.1 nmol/L, and inhibition of the Na^+/H^+ antiporter by the specific inhibitor EIPA was not able to antagonize these aldosterone effects; 3) fludrocortisone had similar effects, whereas hydrocortisone and dexamethasone were partially active with an EC_{50} of approximately 1 μmol/L; and 4) the classical mineralocorticoid inhibitor, canrenone, did not block aldosterone effects at 100-fold higher concentrations.

Receptor-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate and generation of IP_3 and diacylglycerol appear to play the key role for the activation of the Na^+/H^+ antiport by other hormones (10). Thus, a similar mechanism involving IP_3 generation may be assumed for the rapid activation of the Na^+/H^+ exchanger by aldosterone in HML. The finding of an aldosterone selectivity of IP_3 stimulation is in agreement with both the functional and membrane-binding data for aldosterone (4, 16) but is not compatible with the binding characteristics of the classical cloned type I mineralocorticoid receptor (18) that does not distinguish aldosterone from hydrocortisone. In addition, canrenone does not antagonize the aldosterone effect on IP_3 stimulation, again consistent with data for aldosterone on Na^+/H^+ stimulation and membrane binding. To our knowledge, there is no agonist that could serve as a positive control for aldosterone effects selectively stimulating phospholipase C without a stimulation of other signalling systems in HML. This includes angiotensin II, for which receptors are not found in HML (19).

The pharmacological properties of the cloned type I mineralocorticoid receptor (18), of aldosterone-related Na^+/H^+ and IP_3 stimulation, and of aldosterone membrane binding are compared in Table 1. It is obvious that membrane receptors for aldosterone, but not the classical type I mineralocorticoid receptors, may be ideal candidates for the transmission of rapid aldosterone effects including the stimulation of IP_3 generation. Specific inhibition of the Na^+/H^+ exchanger by EIPA did not antagonize the aldosterone-induced changes of intracellular IP_3 levels, thus ruling out a secondary increase of IP_3 in response to the primary activation of the antiporter. Thus, it is concluded that IP_3 may be a relevant second messenger in the intracellular signaling for the activation of the Na^+/H^+ exchanger by aldosterone.

The linkage between the membrane receptor/aldosterone complex and stimulation of phospholipase C as a prerequisite of increased IP_3 generation is still unclear. Orchinik *et al.* (20) suggested a possible involvement of guanine nucleotide-binding proteins in mediating agonist-receptor processes in steroid receptors of neuronal membranes. The binding of [^{3}H]-corticosterone to amphibian neuronal membranes was inhibited by nonhydrolyzable guanyl nucleotides both in equilibrium saturation and in titration studies. The enhancement of inhibition by Mg^{2+} was consistent with the formation of a ternary complex of steroid, receptor, and guanine nucleotide regulatory protein. In the literature, steroid-induced breakdown of phosphoinositides was first reported in rat VSMC by Steiner *et al.* (8, 21). The maximum rate of IP_3 formation in VSMC treated by $LiCl_2$ was up to 10-fold higher than basal levels at a concentration of about 6–9 μmol/L hydrocortisone after 15 min. RU 486, a potent antiglucocorticoid, inhibited hydrocortisone-induced response up to 50% (8). Glucocorticoid actions different from those depending on transcriptional and translational processes were postulated in that paper. For comparison with our data, it should be mentioned that a nonspecific inhibition of intracellular IP_3 breakdown was obtained by $LiCl_2$ in the paper of Steiner *et al.* (8), thus augmenting increases of IP_3 levels; in addition, membrane receptors for mineralocorticoids may be occupied by glucocorticoids in concentrations higher than micromolar, and the effect of cortisol on IP_3 may reflect an activation of the rapid aldosterone effector mechanism.

With regard to the involvement of second messengers in rapid steroid action, it should be mentioned that steroid membrane receptors, rapid steroid effects, and related second messengers have been described earlier in other cells and for other steroids. The successful identification of high affinity steroid-binding sites in preparations of pituitary membranes (22), liver membranes (23, 24), and, recently, neuronal membranes (25) supports the hypothesis of nongenomic steroid action involving plasma membrane receptors different from

TABLE 1. Comparison of properties of the cloned mineralocorticoid type I receptor (CMC),[a] of the membrane mineralocorticoid receptor (MMC), and of the functional results on rapid aldosterone (aldo) action on the Na^+/H^+ antiport in HML and IP_3 generation

	CMC	MMC	Na^+/H^+ antiport	IP_3 generation
Aldo required for dissociation constant (K_d)/EC_{50} (nmol/L)	1.4	~0.1 nmol/L	~0.1 nmol/L	~0.1 nmol/L
Aldo/hydrocortisone selectivity	~1:1	~10,000:1	~10,000:1	~1,000:1
Aldo/canrenone selectivity	~5:1	>1,000:1	~10,000:1	>100:1
Assumed location	Cytosol/nucleus	Plasma membrane	Plasma membrane	Plasma membrane

[a] See Ref. 18.

the classical intracellular steroid receptors. In addition, functional studies have shown rapid steroid effects on neurons (26), on neuronal LH-releasing peptide release (27), and on the acrosome reaction in spermatozoa (7). In terms of the second messengers involved in these rapid steroid effects, Sadler and Maller (28) have demonstrated that progesterone specifically inhibits adenylate cyclase in the *Xenopus laevis* oocyte plasma membrane. This effect appears to depend on a guanine nucleotide regulatory protein. These findings were consistent with experiments by Maller and Krebs (29) showing that the level of cAMP-dependent protein kinase activity is the critical factor for the regulation of oocyte meiotic cell division. Functional studies in oocytes, spermatozoa, and neurons identified early changes in the intracellular calcium concentration as a possible intracellular second messenger signal. Dufy *et al.* (30) observed an increased Ca^{2+}-dependent spiking activity in pituitary cells after estradiol application. Progesterone-induced Ca^{2+} mobilization appears to be involved in meiotic maturation of the oocyte (6). Similar effects on intracellular Ca^{2+} concentration were found during acrosome reaction in spermatozoa (7).

Concerning a possible involvement of calcium in rapid aldosterone effects on intracellular IP_3 levels, the following findings in lymphocytes should be mentioned. Activation of lymphocytes by lectins such as concanavalin A or phytohemagglutinin involves the stimulation of the phospholipase C turnover and concomitant increases of intracellular calcium due to the influx of extracellular calcium (31, 32). Grinstein *et al.* (33) demonstrated a concanavalin A-dependent stimulation of the Na^+/H^+ exchanger, which may be regulated by products of the phosphoinositide hydrolysis in thymic lymphocytes but is not accompanied by an increase of intracellular Ca^{2+} in Ca^{2+}-free solution. In contrast to the small aldosterone effects found in HML so far (2, 3, 34), lectins drastically (and in part irreversibly) change lymphocyte function on almost all cellular levels; this also involves the influx of extracellular calcium. Inasmuch as the increase of free intracellular calcium by IP_3 appears to depend upon intracellular calcium stores only (35), the effects of thapsigargin, a specific inhibitor of Ca^{2+} ATPase of IP_3-sensitive intracellular calcium stores (36), were investigated in HML. There was no significant increase of intracellular calcium in HML by thapsigargin in the presence and absence of extracellular calcium. This indicates that no significant intracellular calcium stores are present in HML; therefore, it is assumed that an intracellular liberation, but not an influx of extracellular calcium, may occur in response to aldosterone-stimulated increases of IP_3. However, this intracellular liberation of calcium is not easily detectable in HML because these cells contain only few intracellular organelles, and the buffering capacity of the fluorescent dyes may obscure such minute changes. In earlier experiments, a minor increase of free intracellular calcium was observed in HML after incubation with aldosterone for 1 h (34). This late response probably involves a genomic component and thus may not be related to the fast effects on the Na^+/H^+ antiport and IP_3 studied here.

In conclusion, aldosterone selectively increases intracellular IP_3 levels in HML with an apparent EC_{50} of about 0.1 nmol/L, and hydrocortisone and dexamethasone are active only at much higher concentrations. These findings give additional support to the hypothesis of a novel rapid pathway for aldosterone action. Further intracellular signaling mechanisms such as the involvement of diacylglycerol and protein kinase C clearly need to be examined in terms of their relevance for these rapid, nongenomic aldosterone effects. In addition, the physiological significance of HML plasma membrane receptors and the related rapid effects on electrolyte transport for immune responses or cell differentiation have not yet been determined.

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