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RAPID ALDOSTERONE SIGNALING IN VASCULAR SMOOTH MUSCLE CELLS: INVOLVEMENT OF PHOSPHOLIPASE C, DIACYLGLYCEROL AND PROTEIN KINASE C α

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Rapid in vitro effects of aldosterone (ALDO) on intracellular sodium, potassium and calcium, cell volume and the sodium-proton-antiport have been described in human mononuclear leukocytes and rat vascular smooth muscle cells (VSMC). These nongenomic effects are signaled through membrane receptors with a high affinity for aldosterone, but not for hydrocortisone. Effects of ALDO on the production of diacylglycerol (DAG) and protein kinase C α (PKC) were measured in VSCM by enzymatic assay and immunoblotting. DAG production was stimulated twofold by ALDO (≥ 1 nM) within 30 sec while hydrocortisone was inactive at concentrations of up to 1 μM. The inhibitors of phospholipase C, neomycin and U-73122 completely blocked this effect. PKC translocation from cytosol to membranes by ALDO occured within 5 min, the extent of this effect was comparable to that of angiotensin II. These data demonstrate rapid intracellular signaling for ALDO in VSMC through phospholipase C, DAG and PKC in addition to calcium and inositol-1,4,5-trisphosphate as determined earlier.

Recently, rapid in vitro effects of aldosterone on sodium, potassium and calcium concentrations and cell volume of human mononuclear leukocytes (HML; 8, 9), and on the activity of the sodium-proton-exchanger of the cell membrane in HML and vascular smooth muscle cells (VSMC; 3, 10) have been demonstrated. Not being compatible with the involvement of the classical Type-I-mineralocorticoid receptors, these nongenomic effects rather suggested the existence of distinct receptors which subsequently have been detected in plasma membranes from HML by radiotracer studies (11). Inositol-1,4,5-trisphosphate appears to be involved in intracellular signaling in HML and VSMC (2, 3), same as free intracellular calcium $[Ca^{2+}]_i$ (13, 14). Here, rapid effects of aldosterone on diacylglycerol (DAG), the byproduct of phosphoinositide hydrolysis,

Abbreviations:
Free intracellular calcium: $[Ca^{2+}]_i$, ALDO: aldosterone, BCIP: 5-bromo-4-chloro-3-indolyl phosphate, DAG: diacylglycerol, DTT: dithiothreitol, EDTA: ethylenediaminetetraacetic acid, EGTA: ethyleneglycol-bis(b-aminoethyl ether)-N,N,N´,N´-tetraacetic acid, NBT: nitroblue tetrazolium, PMSF: phenylmethylsulfonyl fluoride, PKC: protein kinase C, PLC: phospholipase C, SDS-PAGE: sodium dodecylsulfate - polyacrylamide gel electrophoresis, VSMC: rat vascular smooth muscle cells.

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and on PKC translocation which is commonly thought to result from DAG stimulation (1) are investigated in VSMC.

MATERIAL AND METHODS

Aldosterone was obtained from Fluka (Buchs, Switzerland), hydrocortisone, phorbol myristate acetate and phenylmethylsulfonyl fluoride (PMSF) from Sigma (St. Louis, MA). Dithiothreitol (DTT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), nitroblue tetrazolium (NBT), neomycin, U-73122 and U-73343 were obtained from Biomol (Hamburg, Germany). Waymouth's 752/1 medium (WM-752), Ham's nutrient mixture F-12 (H-F12), antibiotics, and trypsin-EDTA were from Gibco BRL GmbH (Eggenstein, Germany), elastase, leupeptin and angiotensin II from Boehringer Mannheim GmbH (Mannheim, Germany), and fetal calf serum (FCS) from c.c.pro GmbH (Neustadt, Germany). Collagenase (CLS I) was from Worthington Biochemical (distributor: Pansystems, Aidenbach, Germany), bovine serum albumin (BSA) and soybean trypsin inhibitor from Serva Feinbiochemica GmbH (Heidelberg, Germany).
The monoclonal mouse antibody against the α - isoform of protein kinase C (PKC-III) was purchased from Upstate Biotechnology Inc. (distributor: Biomol, Hamburg, Germany), the alkaline-phosphatase coupled anti-mouse IgG, and other equipment for gel-electrophoresis and immunoblotting from BioRad (Munich, Germany). Other reagents were from Merck (Darmstadt, Germany; analytical grade). The *sn*-1,2-diacylglycerol-assay was purchased from Amersham Buchler GmbH & Co. KG (Braunschweig, Germany).
VSMC from Sprague Dawley rat thoracic aorta were obtained enzymatically (incubation with 0.25 mg/mL elastase, 1 mg/mL CLS-I, 0.375 mg/mL trypsin inhibitor for 90 min at 37 °C) as described recently (3) and cultured in H-F12/WM-752 medium (1:1) supplemented with 10% fetal calf serum in a 5% CO_2 atmosphere. Early passage cells (passages 2 - 5) grown either in 6-well-dishes (Nunc, Wiesbaden, Germany) for DAG-experiments or in 10 cm petridishes (Falcon, Becton Dickinson, Heidelberg, Germany) were used, since late passage cells alter their morphological and functional characteristics (5).
Intracellular DAG levels were determined enzymatically according to the modified method of Preiss et al. (6) using the *sn*-diacylglycerol-assay supplied by Amersham. In brief, VSMC were grown to subconfluency in 6-well-dishes and growth arrested in H-F12/WM-752 supplemented with 0.1 % BSA 24 hours before the experiments. After preincubation of the cells in HEPES-buffered medium for 2 hours, cells were stimulated by different concentrations of aldosterone or hydrocortisone, 100 nmol/L angiotensin II, or vehicle alone (maximum final ethanol concentration 0.01 %). Reactions were stopped by addition of ice-cold methanol and lipids were extracted by chloroform/methanol (1:2, v/v). Aliquots of the lipid extracts were evaporated under a stream of nitrogen, solubilized in 1 mmol/L diethylenetriaminepentaacetic acid with n-octyl-b-glucopyranoside (7.5 % w/v), and cardiolipin (5 mmol/L), and enzymatic conversion of DAG to [^{32}P] phosphatidic acid was started by addition of *E. coli* DAG-kinase and [^{32}P]-γ-ATP. After 30 min the reaction was stopped, lipids were extracted with 1 % perchloric acid (v/v) and chloroform/methanol (1:2, v/v), and phosphatidic acid was separated by thin layer chromatography (silica gel, chloroform:methanol:acetic acid - 65:15:5, v/v/v). The zone corresponding to [^{32}P] phosphatidic acid was visualized by submitting the dried plates to autoradiography, and the amount of DAG was calculated after liquid scintillation counting of samples and comparing the counts with DAG - standards (31.25 to 1000 pmol/tube) in each assay.
For immunoblotting of PKC III in VSMC, cells were washed three times in ice-cold 250 mmol/L sucrose and 50 mmol/L Tris-HCl (pH 7.5). Cells were harvested by a cell-scraper and homogenized in a lysis buffer (250 mmol/L sucrose, 2 mmol/L EDTA, 2 mmol/L EGTA, 200 µmol/L PMSF, 10 µg/mL leupeptin, 10 mmol/L DTT, and 50 mmol/L Tris-HCl (pH 7.5)) by a tight fitting pestle of a dounce homogenizer. Homogenates were subfractionated by ultracentrifugation (100 000 g, 15 min), cytosolic and membrane fractions were separated by SDS-PAGE on a 10 % slab gel and transferred to nitrocellulose membranes according to the method of Towbin et al. (7). Anti-PKC antibody binding was

carried out according to Church et al. (4), using a monoclonal antibody specifically directed against the α-isoform of PKC (PKC-III). Following incubation with alkaline phosphatase coupled anti-mouse IgG and color development in NBT/BCIP, color densities were determined by densitometry (Pharmacia, Freiburg, Germany).
Results of intracellular diacylglycerol levels and color densities were presented as mean ± standard error (SE). For statistical comparisons, Student's t-test was used.

RESULTS

Effects of aldosterone and hydrocortisone on intracellular diacylglycerol levels

The basal intracellular DAG content of VSMC was 293.4 ± 31.1 pmol per 10^6 cells (n = 4). During incubation of the cells in WM-752/H-F12 medium containing 0.01 % ethanol (maximal ethanol concentration), a stable baseline of intracellular DAG was obtained (Fig 1A). 30 sec after addition of aldosterone (10 nmol/L) intracellular DAG levels were increased to a maximum of 740.0 ± 102.9 pmol per 10^6 cells (254.9 ± 36.1 % of basal levels, $p < 0.05$), whereas 15 sec after addition of 100 nmol/L angiotensin II a rise of intracellular DAG to a maximum of 625.5 ± 143.1 pmol per 10^6 cells was seen (187.6 ± 14.0 %, $p < 0.05$). Fig. 1B shows the dose-response curves for aldosterone and hydrocortisone. Aldosterone produced near maximal effects at a concentration of 1 nmol/L. Hydrocortisone did not stimulate DAG generation at concentrations up to 1 µmol/L.
Preincubation of the cells with inhibitors of phospholipase C (PLC), neomycin (300 µmol/L) or U-73122 (10 µmol/L), for 30 min blocked the effects of aldosterone on intracellular DAG levels (Fig. 2). The inactive congener of the U-compound, U-73343 (10 µmol/L), did not antagonize the effect of aldosterone. After preincubation of the cells with these inhibitors alone, intracellular DAG levels were not different from the baseline values without inhibitors (not shown).

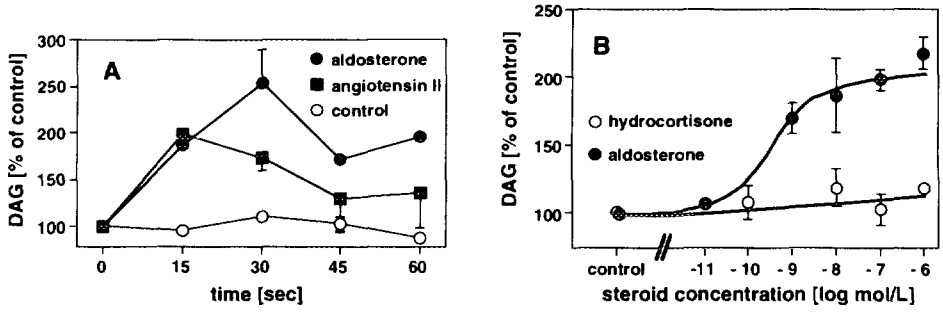

Figure 1
Time course of diacylglycerol (DAG) stimulation in growth arrested vascular smooth muscle cells by aldosterone and angiotensin II (A): cells were incubated in HEPES-buffered WM-752/H-F12 medium plus 0.01 % ethanol (maximum vehicle concentration during steroid stimulation) alone (control), with 10 nmol/L aldosterone or with 100 nmol/L angiotensin II.
Dose - response curves for DAG stimulation by aldosterone and hydrocortisone in vascular smooth muscle cells after an incubation for 30 seconds (B). DAG levels were measured enzymatically (see methods).
Means ± SE, 4 experiments were run in duplicates.

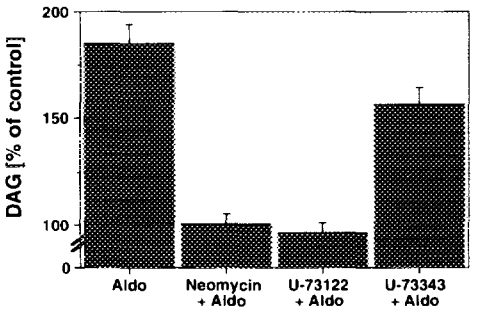

Figure 2
Effects of inhibitors of phospholipase C on aldosterone-induced changes of diacylglycerol (DAG). Growth arrested vascular smooth muscle cells were preincubated with 300 µM neomycin, 10 µM U-73122 or U-73343 for 30 min in HEPES-buffered medium (37 °C) and stimulated with aldosterone (10 nM) or vehicle alone (control); reactions were stopped after 30 sec.
Means ± SE of 4 experiments are shown.

Effects of aldosterone and other stimuli on PKC - α distribution

Aldosterone (100 nmol/L) stimulates the translocation of PKC - α from the cytosol to the plasma membrane (Fig. 3, left) as a measure of PKC activation. The optical density in the membrane fraction increased by 68.8 ± 16.3 %, 70.5 ± 11.2 % and 58.3 ± 23.8 % ($p < 0.05$) after 5, 10 and 15 min of stimulation by aldosterone (100 nmol/L, Fig. 4), while the optical density in the cytosol fraction decreased by 26.6 ± 7.8 %, 30.1 ± 10.3 % and 32.5 ± 10.1 % ($p < 0.05$) respectively ($n = 4 - 7$). The extent of membrane increase and cytosolic decrease of PKC - α was 84.1 ± 33.2 % and 35.6 ± 10.2 % 15 min after stimulation with angiotensin II (100 nmol/L; not shown), while the changes after 15 min incubation with phorbol myristate acetate (1 µmol/L) amounted to 106.0 ± 10.1 % and 87.6 ± 4.6 % (Fig. 3, right).

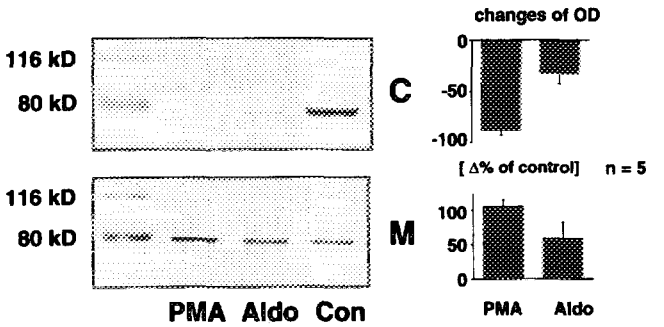

Figure 3
PKC - α immunoblotting (left) in cytosolic (C) and membrane fractions (M) from vascular smooth muscle cells is shown for a typical experiment (see methods). Cells were incubated in HEPES-buffered WM-752/H-F12 medium plus 0.01 % ethanol (maximum vehicle concentration during steroid stimulation) alone (Con), with 100 nmol/L aldosterone (Aldo) or with 1 µM phorbol myristate acetate (PMA) for 15 min. Optical densities (OD) are shown on the right, means ± SE of 5 experiments are given.

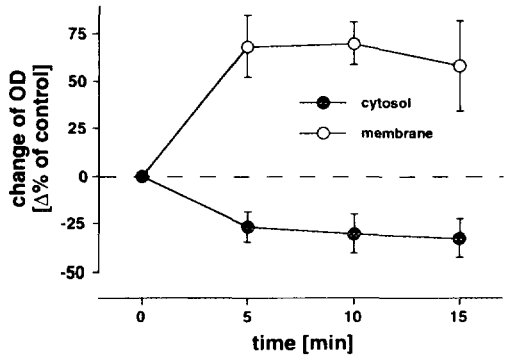

Figure 4
Time course of PKC - α translocation in cytosolic (C) and membrane fractions (M) from vascular smooth muscle cells by 100 nM aldosterone. Optical densities (OD) are given as means ± SE of 4 - 7 experiments.

DISCUSSION

The main finding of this paper is the demonstration of a rapid effect of aldosterone on DAG and PKC in VSMC, presumably mediated through activation of PLC. This enzyme induces hydrolytic breakdown of phosphoinositides into inositol-1,4,5-trisphosphate (IP_3) plus DAG. IP_3 is known to liberate calcium from IP_3-sensitive intracellular calcium stores which are abundant in the endoplasmic reticulum of VSMC, and DAG is thought to activate PKC (1). Aside from the rapid time course which is clearly incompatible with an involvement of the classical genomic pathway of steroid action, the specificity of aldosterone effects on DAG is in perfect agreement with that shown for those other nongenomic mineralocorticoid effects studied to date, and for aldosterone membrane binding (11, 12). This specificity is demonstrated by the high potency of aldosterone, being at least 1000fold more active than the glucocorticoid hydrocortisone. PKC translocation as a measure of PKC activation was demonstrated to be near maximal within 5 min, followed by a plateau up to 15 min. This mirrors the time course of aldosterone effects on $[Ca^{2+}]_i$ which reaches a plateau within 2-3 min in VSMC (13). These findings further support earlier, indirect evidences for PKC involvement in aldosterone signaling as suggested by effects of staurosporine and phorbolesters on the $[Ca^{2+}]_i$ response to aldosterone: inhibition of PKC by staurosporine resulted in a fourfold increase of the $[Ca^{2+}]_i$ signal, while stimulation of PKC by short term incubation with phorbol myristate acetate significantly suppressed the response. This is compatible with the concept of feed-back inhibition possibly through phosphorylation of the receptor and/or early signaling components by PKC as has been shown for other agonists (14).

The results reported here, thus, are fully compatible with and complementary to those of previous studies on rapid aldosterone effects both in HML and VSCM in which a significant stimulation of IP_3 production was found (2, 3) as early as 30 - 60 sec after application of aldosterone with an EC_{50} of ~ 0.1 nM; this effect depended upon activa-

tion of PLC as shown by specific inhibition of the enzyme by the same compounds as used in this paper. In addition, rises in $[Ca^{2+}]_i$ were demonstrated to occur both in VSMC and endothelial cells (13, 14). In VSMC, a predominant release of calcium from IP_3-sensitive calcium stores was shown both by cell imaging and by effects of thapsigargin which selectively empties those calcium stores and, thus, blocked the effects of aldosterone on $[Ca^{2+}]_i$. Therefore, combining those earlier findings with the findings presented here, rapid aldosterone signaling in VSMC seems to involve a common second messenger cascade with distal parts following two major tracks of sequential cellular activation: PLC $\Rightarrow$ IP_3 $\Rightarrow$ $[Ca^{2+}]_i$ and PLC $\Rightarrow$ DAG $\Rightarrow$ PKC. Whether or not activation of PKC is responsible for rapid aldosterone effects on the sodium-proton-exchanger as shown earlier in VSMC and HML, is still under debate.

The genomic theory of steroid action has been the unquestioned dogma for the explanation of steroid effects over the past four decades. Despite early observations on rapid steroid effects being clearly incompatible with this theory, only recently has nongenomic steroid action been more widely recognized and led to a critical re-upraisal of unsolved questions about this dogma. Evidences for nongenomic steroid effects come from all fields of steroid research now, and mechanisms of agonist action are studied with regard to membrane receptors and second messengers involved (12). Though the exact mechanisms remain to be elucidated, the findings reported here and those of related earlier studies could become the basis for the development of a membrane receptor antagonist for mineralocorticoids potentially initiating new strategies in the treatment of human cardiovascular disease.

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