Vascular Dopamine-I Receptors

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The modulation of dopamine DA₁ receptors of cultured rat renal arterial smooth muscle cells by phorbol ester, glucocorticoid and sodium chloride was studied. The extent of [³H]Sch-23390 binding to phorbol ester-treated cell was increased without any change in the dissociation constant (Kₐ). At a concentration of 10 nmol/l, the synthetic glucocorticoid dexamethasone increased maximum receptor binding (Bₘₐₓ) but had no effect on the Kₐ. 100 mmol/l sodium chloride did not change Bₘₐₓ but increased the Kₐ for DA₁ receptor. The production of cAMP in response to DA₁ receptor stimulation was enhanced without any change of the adenylate cyclase activity. The glucocorticoid effect on DA₁ of arterial smooth muscle cells became apparent after hours of incubation in the presence of the steroid and was significantly inhibited by cycloheximide (10 µg/ml) and by the glucocorticoid receptor antagonist RU-38486, indicating that the effect required protein synthesis through glucocorticoid receptors. Treatment of cells with 1µmol/l dexamethasone for 24 h increased basal and DA₁-stimulated adenylate cyclase activity. Basal adenylate cyclase was decreased by sodium chloride in a dose-dependent manner. These results suggest differential control of DA₁ receptors on vascular smooth muscle cells by protein kinase C, glucocorticoid or sodium chloride. (Hypertens Res 1995; 18 Suppl. I: S29-S33)

Key Words: dopamine, vascular smooth muscle cell, adenylate cyclase, protein kinase C, glucocorticoid, sodium chloride

Two subtypes of dopamine receptor are thought to exist in peripheral tissue and are designated as DA₁ and DA₂ receptors. The DA₁ receptor is situated postjunctionally on vascular smooth muscle. Although several dopamine receptors have been cloned from the brain, DA₁ and DA₂ classification in peripheral tissue has been used to distinguish between the peripheral effect of dopamine as determined by physiological methods (1). Recently, biochemical evaluation of DA₁ receptors has been reported (2).

The interaction between phorbol ester, glucocorticoid and sodium chloride and the cardiovascular system has been shown to play an important role in cardiovascular function. Phorbol ester, glucocorticoid, sodium chloride, and catecholamines are involved in the regulation and maintenance of normal blood pressure (3-5). There is greater potentiation by phorbol esters of the contractile response of the candal arteries to norepinephrine, vasopressin and potassium in spontaneously hypertensive rats than that in normotensive Wistar Kyoto rats (5). Synthetic glucocorticoids have been shown to augment the presser response to angiotensin II and norepinephrine, and this effect is blocked by glucocorticoid antagonist. We have already reported that the DA₁ receptor response on vascular smooth muscle cells (2) as well as in the central nervous system (6) is regulated by glucocorticoids. The interaction between dopamine and sodium chloride has been extensively studied. Urinary excretion of dopamine increases during dietary salt loading, and it has been suggested that renal dopamine production participates in the hemostatic regulation of sodium balance, and sodium chloride increases dopamine release into circulation (7). However, few studies have focused on the effect of sodium chloride on the DA₁ receptor itself.

In the present study, the effects and mechanisms of action of phorbol ester, glucocorticoid or sodium chloride on DA₁ receptor responses were studied.

Materials and Methods

Materials
All tissue culture supplies were obtained from GIBCO Laboratories (Grand Island, NY). Sch-23390 was a gift from Schering Plough (Bloomfield, NJ); forskolin was from Nihon Kayaku (Tokyo, Japan). All other chemicals were analytic grade and were obtained from Sigma (St. Louis, MO). RIA kits for adenosine 3',5'-cyclic monophosphate (cAMP) and [N-methyl-³H]Sch-23390 (75 Ci/mm) were purchased from Amersham Japan (Tokyo, Japan). Multi well plates, pipettes, and flasks were purchased from Becton Dickinson (Oxnard, CA).

Tissue Culture
Vascular smooth muscle cells (VSMC) were grown

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from the explants of Wistar rat renal arteries as previously described (8, 9). VSMC were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Cells from passages 3-6 were used. No significant detectable differences in VSMC characteristics were observed in this range of passages. Cells were subcultured on a weekly basis after trypsinization. Each plate was replenished twice a week with fresh medium. Before the experiments, VSMC were placed in DMEM without FCS for at least 24 h.


Confluent VSMC grown in six-well plates were washed twice with 2 ml of DMEM. The changes with time in total $[^3]$H]Sch-23390 binding were monitored by exposure of cell monolayers to DMEM containing 1 nmol/l $[^3]$H]Sch-23390 at 37°C at specified times. The cells were washed three times with fresh, ice-cold DMEM and solubilized in 0.2 mol/l acetic acid (pH 2.5) containing 0.5 mmol/l NaCl for 6 min at 4°C to extract only surface-bound ligands (10). Radioactivity was measured by scintillation counting.

Competitive displacement experiments were performed by incubation of VSMC with 1 nmol/l $[^3]$H]Sch-23390 for 15 min at 37°C.

A saturation binding study was conducted by incubating various concentrations of $[^3]$H]Sch-23390 (0.2-50 nmol/l) in the presence or absence of phorbol ester, dexamethasone or sodium chloride for 15 min at 37°C to achieve equilibrium. Because DMEM contains 110 mmol/l sodium chloride, control (110 mmol/l) and high (generally 210 mmol/l) sodium chloride-treated cells were compared. Cells were washed and treated with acetic acid in the above-mentioned manner. The specific binding is defined as the total binding minus nonspecific binding assayed in the presence of 10 μmol/l unlabeled Sch-23390.

Measurement of cAMP

After preincubation, cells grown in six-well plates were washed three times with 2 ml of DMEM containing 0.05% bovine serum albumin at 37°C and then stimulated with various concentrations of dopamine or other agonists dissolved in DMEM with 0.5 mmol/l 3-isobutyl-1-methylxanthine (IBMX) for 10 min. Dopamine (10 μmol/l)-mediated cAMP formation was not inhibited by propranolol (1 μmol/l) (10). However, this propranolol concentration was sufficient to inhibit completely 10 μmol/l isoproterenol-mediated cAMP formation. At each dopamine stimulation, 1 μmol/l propranolol was added to block the β-adrenergic effect of dopamine. Rapid aspiration and the addition of 1.5 ml of ice-cold 65% ethanol stopped the reaction. After evaporation by a centrifugal evaporator, the dry residue was dissolved in an assay buffer. The cAMP concentration was then determined by radioimmunoassay using the Amersham cAMP radioimmunoassay kit (11).

Adenylate Cyclase Assay

All reagents and materials were kept at 0°C, unless stated otherwise. Cells were homogenized in 50 mmol/l acetic buffer at pH 7.5 (2 × 10^6 cells/ml) containing 1 mmol/l EGTA, 4 mmol/l MgSO4, and 5 mmol/l IBMX. Aliquots (140 μl) of homogenate were placed in Eppendorf tubes, 0.1 mmol/l GTP (20 μl) was added, and each tube was transferred to a water bath for 6 min at 37°C. Thereafter, the reaction was initiated by additions of 40 μl of 50 mmol/l acetic buffer (pH 7.5) containing 2.5 mmol/l ATP and various agonists and antagonists as specified in the tables. Incubation was terminated by transferring the tubes to a boiling water bath for 3 min. The tubes were cooled, the precipitate was removed by centrifugation, and the cAMP content in the aliquots (100 μl) of the supernatant was determined in triplicate using the Amersham cAMP radioimmunoassay kit as described above. The coefficient of variation of each triplicate estimation of cAMP content was < 5%. The protein content of the cell homogenate (50 μl) was estimated by a dye-binding technique.

Statistical Methods

Statistical analysis was performed by analysis of variance (ANOVA) and Scheffe's modified t test. Values of p<0.05 were considered to be significant.

Results

$[^3]$H]Sch-23390 Binding to Control, Phorbol Ester-steroid-or Sodium Chloride-Treated Cells

Scatchard analysis of the data showed that there was a single class of binding sites for Sch-23390 with a dissociation constant ($K_d$) of 0.73 ± 0.10 nmol/l and a maximal binding capacity ($B_{max}$) of 2.6 ± 0.5 × 10^4 sites/cell. Preincubation with 1 μmol/l PMA for 4 h affected the binding of $[^3]$H]Sch-23390. No significant difference was observed in $K_d$, but there was a significant increase in the $B_{max}$ for Sch-23390 binding to VSMC after preincubation with PMA: 5.1 ± 0.8 × 10^4 sites/cell (1 μmol/l DMA) and 3.7 ± 0.4 × 10^4 sites/cell (10 nmol/l PMA) compared with the control value of 2.6 ± 0.5 × 10^4 sites/cell. Pretreatment of the cells with 1 μmol/l H-7 or 10 mmol/l staurosporine prevented the protein kinase C-induced increase in $B_{max}$.

Scatchard analysis was performed on $[^3]$H]Sch-23390 binding at different concentrations before and after dexamethasone treatment (10 nmol/l to 1 μmol/l) to determine whether the observed increase in $[^3]$H]Sch-23390 binding measured at 1 nmol/l was due to a change in the affinity or number of binding sites in VSMC with dexamethasone resulted in an increase in the maximal binding capacity ($B_{max}$) for $[^3]$H]Sch-23390. The results of $[^3]$H]Sch-23390 binding experiments indicate that dexamethasone did not change the dissociation constant ($K_d$) (0.73 ± 0.10 nmol/l for control and 0.74 ± 0.15 nmol/l for dexamethasone-treated VSMC); therefore it did not change the affinity. But the glucocorticoid significantly increased $B_{max}$ (1.5 ± 0.1-fold, p<0.05).

Sodium chloride 100 mmol/l added for 4 h did not
change the $B_{max}$ (2.6 ± 0.4 × 10^4 sites/cell vs. 2.5 ± 0.3 × 10^4 sites/cell) but increased the $K_d$ value from 0.72 ± 0.10 to 1.06 ± 0.10 nmol/l, values obtained from the Scatchard plot. Thus high sodium chloride decreased the affinity for [3H]Sch-23390 binding.

To determine if the effect of glucocorticoids requires protein synthesis, VSMC were added to plain medium and medium containing cycloheximide (10 μg/ml) alone, dexamethasone (1 μmol/l) plus cycloheximide, or dexamethasone alone. To see if the effect was mediated through the glucocorticoid receptor, VSMC were added to plain medium alone (control) or plain medium containing dexamethasone (10 nmol/l) or dexamethasone (10 nmol/l) plus the glucocorticoid antagonist RU-38486 (1 μmol/l). While RU-38486 alone had no effect on [3H]Sch-23390 binding, it significantly inhibited the dexamethasone effect.

**Effect of Phorbol Ester, Steroid or Sodium Chloride Treatment on Dopamine-Induced cAMP Production**

The induction of cAMP formation by dopamine was increased 1.6 fold by preincubation of VSMC with 1 μmol/l PMA for 4 h, although PMA alone had no significant effect on cAMP formation.

With respect to cAMP measured before and after dexamethasone (1 μmol/l) treatment of VSMC, 10 μmol/l dopamine-stimulated cAMP levels, as well as the basal cAMP levels of dexamethasone-treated VSMC, were significantly higher than those of their respective controls.

The stimulatory effect of dopamine on cAMP formation was decreased by prior exposure of the VSMC to 100 mmol/l sodium chloride added to DMEM for 4 h. Sodium chloride at a dose of 100 mmol/l also decreased basal cAMP formation. The shortest preincubation period with high concentration of sodium chloride that resulted in decreased cAMP formation after the subsequent addition of dopamine was 30 min, and the degree of the decrease became greater as preincubation was prolonged until it leveled off at 4 h. Decrease in cAMP formation was observed after 1 min of stimulation by dopamine in VSMC treated with 100 mmol/l sodium chloride. This decrease reached its maximum after 5 min and persisted after 15 min. The amount of the decrease was dependent on the concentration of sodium chloride added to DMEM (25-100 mmol/l).

**Effect of Phorbol Ester, Steroid or Sodium Chloride Treatment on Adenylate Cyclase Activity**

To further investigate the possible mechanism of the observed glucocorticoid effect, adenylate cyclase was measured in cell homogenate. This approach allowed us to study not only the dopamine stimulation of adenylate cyclase but also the activation of adenylate cyclase by agents acting on the guanine nucleotide regulatory protein [Gpp(NH)p] and the catalytic unit (forskolin).

In homogenates prepared from cells treated with 1 μmol/l dopamine for 24 h, there was a significant increase in adenylate cyclase activity in response to dopamine stimulation. However there was no significant difference between PMA-treated and untreated cells with respect to activation by Gpp(NH)p and forskolin.

In homogenates prepared from cells treated with 1 μmol/l dexamethasone for 24 h, the alteration in adenylate cyclase stimulation by dopamine was very similar to the alteration in intact cells observed during cAMP formation. In addition there was a significant increase in dexamethasone-treated cells compared with untreated cells regarding activation by Gpp(NH)p and forskolin.

In homogenates prepared from cells treated with 100 mmol/l sodium chloride for 24 h, the alteration in adenylate cyclase stimulation by dopamine was similar to the alteration in intact cells during cAMP formation. There was also a dose-dependent decrease in adenylate cyclase in sodium chloride-treated cells with respect to activation by Gpp(NH)p and forskolin. The rate of Gpp(NH)p activation was not reduced by sodium chloride. The effects of NaCl, KCl, choline chloride, and sodium sulfate on basal adenylate activity were also studied. The inhibitory effects already noted for NaCl were also observed with the other monovalent cations, although, in general, Na⁺ was the most potent. The potency order for inhibition was found to be Na⁺ > K⁺ > choline⁺. Sodium in the form of Na₂SO₄ was as potent as sodium chloride.

**Discussion**

Stimulation of DA₁ receptor caused vasodilation (1). The DA₁ response may be mediated through the production of cAMP, which in turn activates protein kinase. Subsequently, protein kinase activation caused a cascade of events that leads to the inactivation of myosine kinase by phosphorylation, thus preventing vascular muscle contraction and resulting in vessel relaxation or vasodilation.

Much more cAMP was produced in response to stimulation by dopamine receptor agonists after the VSMC had been exposed to PMA (a protein kinase C activator), and the enhancing effect of PMA was completely abolished by H-7 and staurosporine (protein kinase C inhibitors). It would therefore seem reasonable that the observed potentiation of cAMP formation in VSMC by PMA reflects the activation of endogenous protein kinase C. The increase in receptor expression on the cell surface is the most likely mechanism for the potentiation of DA₁ receptor-mediated cAMP formation triggered by protein kinase C, although it remains possible that receptor coupling to GS protein is also enhanced. This conclusion was further substantiated, since pretreatment of VSMC with H-7 or staurosporine prevented the PMA-induced increase in $B_{max}$.

In this study, we found an increase in the DA₁ receptor number induced by synthetic glucocorticoid in VSMC after 6-72 h of incubation. The glucocorticoid treatment of VSMC also resulted in enhanced adenylate cyclase activity, which is consistent with our previous findings (2, 12). Therefore, the enhanced cAMP response induced by dopamine is due...
to both increased receptor numbers and increased adenylate cyclase activity.

Our study showed that sodium chloride decreases DA$_1$ receptor-mediated responses. This decrease was not due to DA$_1$ receptor numbers but to a decrease in the receptor affinity and a decrease in adenylate cyclase activity. Sodium inhibits basal-, prostaglandin E$_2$- and DA$_1$-stimulated adenylate cyclase activity. It is tempting to speculate on the mechanism that underlies the inhibitory effects of sodium in VSMC in terms of our current understanding of the adenylate cyclase complex. Thus the ability of Na$^+$ to inhibit basal and DA$_1$ stimulated activity and not to reduce the rate of activation induced by Gpp(NH)$_p$ may indicate that the cation affects the adenylase catalytic unit.

It is difficult to explain the effects of phorbol ester, glucocorticoids or sodium chloride on blood pressure in animals or of sodium chloride in humans based on the results of our present study. The present study demonstrated the upregulation of the DA$_1$ receptors by protein kinase C. Protein kinase C appears to be involved in blood pressure regulation. Thus, although the physiological significance of this upregulation of the DA$_1$ receptors remains to be elucidated, protein kinase C may play some role in reducing as well as elevating blood pressure. While our results have demonstrated that the potential for vasodilation through DA$_1$ receptor binding and dopamine-induced cAMP response is increased after glucocorticoid treatment of VSMC, we can only speculate on the effect of endogenous glucocorticoids in vivo. However, if endogenous glucocorticoids act through the glucocorticoid receptor, as do synthetic glucocorticoids, it is very likely that they have similar effects on the DA$_1$ receptors of VSMC.

It seems that only the inhibitory effect of the sodium ion described in this paper is of functional significance, as the sodium ion concentration required to elicit these effects are well within the physiological range of extra- or intracellular fluid. Because sodium chloride increases dopamine release into circulation (7), this suppressed dopamine DA$_1$ response may be a feedback mechanism of the increased dopamine production by sodium chloride. The fact that sodium in the form of Na$_2$SO$_4$ was as potent as NaCl suggests that chloride is not necessarily required for the inhibition.

In summary, as shown in Fig. 1, the present study has shown that phorbol ester, pure synthetic glucocorticoid and sodium chloride have a different direct action on peripheral vascular tissue mediated through increased DA$_1$ receptor number, increased DA$_1$ receptor number and increased adenylate cyclase activity or decreased DA$_1$ receptor affinity and decreased adenylate cyclase activity.

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**References**


