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Glucocorticoid Amplifies Vasopressin-Induced Phosphoinositide Hydrolysis in Aortic Smooth Muscle Cells

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Abstract It has been reported that glucocorticoid modifies phosphoinositide (PI) hydrolysis stimulated by vasoactive agents in vascular smooth muscle cells. In the present study, we investigated the point at which glucocorticoid affects vasopressin-induced PI hydrolysis in primary cultured rat aortic smooth muscle cells. The pretreatment with dexamethasone significantly amplified the formation of inositol trisphosphate (IP_3) induced by vasopressin in a dose-dependent manner in a range of 1 pM to 10 nM. The effect of dexamethasone was dependent on the time of pretreatment up to 8 h. Dexamethasone had little effect on the number of vasopressin receptor and its affinity to vasopressin. The pretreatment with dexamethasone also amplified the formation of IP_3 induced by NaF, a GTP-binding protein activator, or angiotensin II. 12-O-Tetradecanoylphorbol-13-acetate, a protein kinase C (PKC)-activating phorbol ester, significantly reduced the dexamethasone-induced enhancement of IP_3 formation stimulated by vasopressin, angiotensin II or NaF. 4α-Phorbol-12,13-didecanoate, a PKC-nonactivating phorbol ester, had little effect on the enhancement by dexamethasone. These results strongly suggest that glucocorticoid amplifies vasopressin-induced PI hydrolysis at a point downstream from GTP-binding protein in primary cultured rat aortic smooth muscle cells, and that the activation of PKC has a negative feedback effect on the amplification by glucocorticoid of vasopressin-induced PI hydrolysis.

Key words: glucocorticoid, vasopressin, angiotensin II, phosphoinositide, protein kinase C, aortic smooth muscle cells

It is generally accepted that aortic smooth muscle cells play an important role in the development of atherosclerosis and hypertension [Schwartz, 1983; Ross, 1993]. It has been reported that vasopressin stimulates phosphoinositide (PI) hydrolysis through its binding to V_1 receptor in aortic smooth muscle cells [Nabika et al., 1985; Aiyar et al., 1986; Vallotton et al., 1986; Grillone et al., 1988] and leads them toward proliferation [Altura and Altura, 1977; Campbell-Boswell and Robertson, 1981]. It is well recognized that PI hydrolysis results in the formation of diacylglycerol and inositol phosphates. Among these products, diacylglycerol and inositol trisphosphate (IP_3) serve as messengers for the activation of protein kinase C and the mobilization of intracellular Ca^{2+}, respectively [Berridge and Irvine, 1989; Nishizuka, 1992]. It is generally accepted that as well as adenylate cyclase-cAMP system, a GTP-binding protein is involved in the signaling between the receptor and phospholipase C that hydrolyzes PIs [Gilman, 1987; Birnbaumer et al., 1990]. In a previous study, we have shown that pertussis toxin (PTX)-insensitive GTP-binding protein is involved in the coupling of vasopressin receptor to phospholipase C in primary cultured rat aortic smooth muscle cells [Kondo et al., 1989]. In addition, it has been reported that protein kinase C has a negative feedback effect on PI hydrolysis in rat vascular smooth muscle cells [Brock et al., 1985; Caramelo et al., 1988; Pfeilschifter et al., 1989].

Hypertension has been identified as one of the most common manifestations of glucocorticoid excess, and atherosclerosis is noted as well as hypertension in patients with glucocorticoid

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excess, so-called Cushing syndrome [Gomez-Sanchez, 1986]. In vitro, the receptor for glucocorticoid has been reported to exist in aortic smooth muscle cells [Duval et al., 1977; Nichols et al., 1985]. It has been reported that glucocorticoid shifts the dose–response curves of angiotensin II- and vasopressin-induced IP_3 formation to the left [Sato et al., 1991] and that glucocorticoid potentiates norepinephrine-induced inositol polyphosphate formation in primary cultured rat vascular smooth muscle cells [Liu et al., 1992]. However, the point exerted by glucocorticoid in PI hydrolysis has not yet been clarified. In the present study, we examined the effect of dexamethasone on PI hydrolysis induced by vasopressin and the interaction with protein kinase C in primary cultured rat aortic smooth muscle cells. Herein, we show that dexamethasone amplifies vasopressin-induced PI hydrolysis at a point downstream from GTP-binding protein, and that the activation of protein kinase C has a negative feedback effect on the amplification by dexamethasone of vasopressin-induced PI hydrolysis in these cells.

MATERIALS AND METHODS

Materials

Myo-[^{3}H]inositol (90 Ci/mmol) and [*tyrosyl*-^{3}H]vasopressin [arg^8] (17.6 Ci/mmol) were purchased from Amersham Japan (Tokyo, Japan). Dexamethasone, NaF, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and 4α-phorbol 12,13-didecanoate (4α-PDD) were purchased from Sigma Chemical Co. (St. Louis, MO). Vasopressin and angiotensin II were purchased from Peptide Institute Inc. (Minoh, Japan). The protein assay reagent kit was purchased from Pierce (Rockford, IL). Other materials and chemicals were obtained from commercial sources. Dexamethasone was dissolved in ethanol. TPA and 4α-PDD were dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of ethanol or DMSO in the culture medium was 0.1% and this did not affect the measurement of the formation of IP_3.

Cell Culture

Aortic smooth muscle cells were obtained from thoracic aorta of male Sprague-Dawley rats by the explantation method as described by Kariya et al. [1987]. The cells (1×10^5) were seeded into 35-mm-diameter dishes and maintained in 2 ml of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO_2–95% air. The cells were used for experiments between the third and sixth passages. After 6 days, the medium was exchanged for 2 ml of serum-free DMEM. The cells were used for experiments after 48 h. In the experiments for the formation of IP_3, the medium was exchanged for 2 ml of inositol-free DMEM. When indicated, the cells were pretreated with TPA or 4α-PDD for 20 min prior to the stimulation by vasopressin, NaF, or angiotensin II.

Measurement of the Formation of IP_3

The cultured cells were labeled with *myo*-[^{3}H]inositol (3 μCi/dish) for 48 h. The labeled cells were pretreated with various doses of dexamethasone for the indicated periods, and then preincubated with 10 mM LiCl at 37°C for 10 min in 1 ml of an assay buffer [5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 150 mM NaCl, 5 mM KCl, 5.5 mM glucose, 0.8 mM $MgSO_4$ and 1 mM $CaCl_2$] containing 0.01% bovine serum albumin (BSA). The cells were then stimulated by vasopressin, NaF, or angiotensin II at 37°C for the indicated periods. The reaction was terminated by adding 1 ml of 30% trichloroacetic acid (TCA). The acid supernatant was treated with diethyl ether to remove the acid and neutralized with 0.1 M NaOH. The supernatant was applied to an anion-exchange column containing 1 ml of Dowex AG1-X8 (100–200 mesh, formate form). To remove inositol monophosphate and inositol bisphosphate, 8 ml of 0.1 M formic acid containing 0.4 M ammonium formate was applied to the column. The radioactive IP_3 was then eluted from the column with 8 ml of 0.1 M formic acid containing 1 M ammonium formate [Berridge et al., 1983, 1984].

Determination of [^{3}H]Vasopressin Binding

The cultured cells were subjected to the binding assay, essentially as described by Brönnegård et al. [1986], with minor modifications. In brief, the cells were pretreated with 10 nM dexamethasone or vehicle at 37°C for 8 h, and then incubated in 1 ml of 5 mM HEPES-buffered DMEM (pH 7.4) containing various doses of [^{3}H]vasopressin with or without a 200-fold molar excess of nonradioactive vasopressin at 20°C for 2 h. At the end of the incubation, the cells were sufficiently washed with ice-cold phosphate-buffered saline (PBS) and solubilized with

1 ml of 0.1% sodium dodecyl sulfate (SDS). The radioactivity of the lysate was then determined. Binding capacity, expressed as number of binding sites per cell and apparent dissociation constant were calculated by the method of Scatchard [1949].

Determinations

The radioactivity of ^3H samples was determined with a Beckman LS 5000TD liquid scintillation spectrometer. Protein concentration was determined by using a protein assay reagent kit with BSA as a reference protein.

Statistical Analysis

The data were analyzed by Student's t-test, and $P < 0.05$ was considered significant. All data are presented as the mean ±SD of triplicate determinations.

RESULTS

Effect of Dexamethasone on Vasopressin-Induced Formation of IP_3 in Rat Aortic Smooth Muscle Cells

In a recent study [Ito et al., 1993], we showed that vasopressin stimulates the formation of IP_3 in a dose-dependent manner in a range of 0.1 nM to 1 μM in primary cultured rat aortic smooth muscle cells and that the maximum effect of vasopressin was observed at 1 μM. We first examined the effect of dexamethasone on the vasopressin-induced formation of IP_3 in these cells. The 8-h pretreatment with 10 nM dexamethasone did not affect basal levels, but amplified vasopressin-induced formation of IP_3 (Figs. 1, 2). Dexamethasone potentiated the IP_3 formation stimulated by 1 μM vasopressin, which causes maximum stimulation (Fig. 2). The 8-h pretreatment with dexamethasone amplified vasopressin (0.1 μM)-induced formation of IP_3 in a dose-dependent manner in a range of 1 pM to 10 nM (Fig. 3). The maximum effect of dexamethasone was observed at 10 nM, resulting in about 50% amplification in vasopressin-induced IP_3 formation. The amplification by dexamethasone of vasopressin-induced IP_3 formation was dependent on the time of pretreatment up to 8 h (Fig. 4). Dexamethasone had little effect on the protein contents of the cultured cells up to 8 h (0.346 ± 0.014 mg/dish for control; 0.345 ± 0.021 mg/dish for 10 nM dexamethasone for 8 h, there was no significant difference from control).

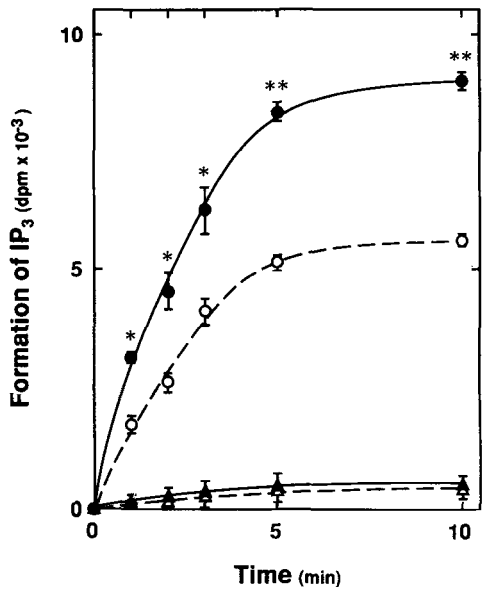

Fig. 1. Effect of dexamethasone on vasopressin-induced formation of IP_3 in rat aortic smooth muscle cells. The [^{3}H]inositol-labeled cells were pretreated with 10 nM dexamethasone (●,▲) or vehicle (○,△) for 8 h and then stimulated by 0.1 μM vasopressin (●,○) or vehicle (▲,△) for the indicated periods. Each value represents the mean ±SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, **$P < 0.01$ compared to the value stimulated by vasopressin without dexamethasone pretreatment.

Effect of Dexamethasone on Vasopressin Binding in Rat Aortic Smooth Muscle Cells

To clarify the point at which dexamethasone exerts the amplified effect on the vasopressin-induced PI hydrolysis, we next examined the effect of dexamethasone on vasopressin binding to the specific receptor in rat aortic smooth muscle cells. Dexamethasone had little effect on the affinity of vasopressin receptor and the binding capacity (Fig. 5). The apparent dissociation constant (Kd) and maximum binding (Bmax) were 4.7 ± 0.8 nM and 5,520 ± 480 sites/cell for control, and 4.6 ± 0.7 nM and 5,160 ± 470 sites/cell for dexamethasone-treated cells, respectively (Fig. 5).

Effect of Dexamethasone on NaF-Induced Formation of IP_3 in Rat Aortic Smooth Muscle Cells

GTP-binding protein is well known to be involved in the coupling of the receptor to phospholipase C in the signaling of vasopressin in vascular smooth muscle cells [Birnbaumer et al., 1990]. We previously showed that PTX-insensi-

tive GTP-binding protein is involved in the coupling of vasopressin receptor to phospholipase C in primary cultured rat aortic smooth muscle cells [Kondo et al., 1989]. We next examined the effect of dexamethasone on the formation of IP_3 induced by NaF, a GTP-binding protein activator [Gilman, 1987]. NaF stimulated the formation of IP_3 in a dose-dependent manner in a range of 5 to 20 mM in these cells; the maximum effect of NaF was observed at 20 mM (data not shown). Dexamethasone also amplified the formation of IP_3 induced by 20 mM NaF (Fig. 6). The effect of dexamethasone was dose dependent in the range of 1 pM to 10 nM. Patterns of the dose dependency of both dexamethasone effects on vasopressin- and NaF-induced IP_3 formations appear to be similar.

Effect of Dexamethasone on Angiotensin II-Induced Formation of IP_3 in Rat Aortic Smooth Muscle Cells

Angiotensin II is known to be a potent vasoactive agent [Altura and Altura, 1977] and has been reported to induce PI hydrolysis via GTP-binding protein in vascular smooth muscle cells [Birnbaumer et al., 1990]. We next examined the effect of dexamethasone on angiotensin II-induced formation of IP_3 in primary cultured rat aortic smooth muscle cells. The pretreatment with dexamethasone also amplified angiotensin II-induced formation of IP_3. This effect of dexamethasone on angiotensin II-induced IP_3 formation was dependent on the time of pretreatment up to 8 h (Fig. 7).

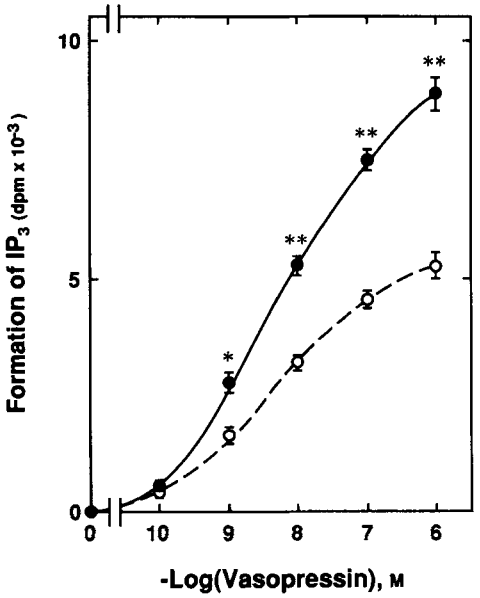

Fig. 2. Effect of dexamethasone on vasopressin-induced formation of IP_3 in rat aortic smooth muscle cells. The [^{3}H]inositol-labeled cells were pretreated with 10 nM dexamethasone (●) or vehicle (○) for 8 h and then stimulated by various doses of vasopressin for 5 min. Values for unstimulated cells have been subtracted from each data point. Each value represents the mean ±SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, **$P < 0.01$ compared to the value stimulated by vasopressin without dexamethasone pretreatment.

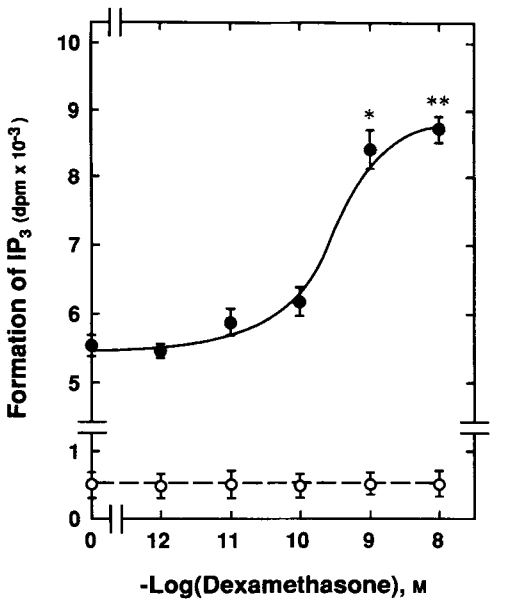

Fig. 3. Dose-dependent effect of dexamethasone on vasopressin-induced formation of IP_3 in rat aortic smooth muscle cells. The [^{3}H]inositol-labeled cells were pretreated with various doses of dexamethasone for 8 h and then stimulated by 0.1 μM vasopressin (●) or vehicle (○) for 5 min. Each value represents the mean ±SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, **$P < 0.01$ compared to the value stimulated by vasopressin without dexamethasone pretreatment.

Effects of TPA on the Amplification by Dexamethasone of the Formation of IP_3 Induced by Vasopressin, Angiotensin II and NaF in Rat Aortic Smooth Muscle Cells

Since it has been reported that protein kinase C has a negative feedback effect on PI hydrolysis in rat vascular smooth muscle cells [Brock et al., 1985; Caramelo et al., 1988; Pfeilschifter et al., 1989], we examined the effects of protein kinase C activation on the enhancement by dexamethasone of the formation of IP_3 induced by vasopressin, angiotensin II and NaF in primary cultured rat aortic smooth muscle cells. We confirmed that TPA, a protein kinase C-activating phorbol

ester [Nishizuka, 1986], significantly inhibited the formation of IP_3 induced by vasopressin (Table I). In addition, TPA also inhibited the NaF-induced IP_3 formation (Table I). Moreover, TPA markedly reduced the amplified effect of dexamethasone on the formation of IP_3 induced by vasopressin or NaF (Table I). However, 4α-PDD, a protein kinase C-nonactivating phorbol ester [Nishizuka, 1986], had little effects (data not shown). TPA also reduced the amplification by dexamethasone of the angiotensin II-induced formation of IP_3 (data not shown).

DISCUSSION

In the present study, we showed that glucocorticoid potentiates vasopressin-induced IP_3 formation in primary cultured rat aortic smooth muscle cells. It is well established that vasopressin stimulates PI hydrolysis by phospholipase C through its binding to V_1 receptor in aortic smooth muscle cells [Nabika et al., 1985; Aiyar et al., 1986; Vallotton et al., 1986; Grillone et al., 1988]. We focused our attention to the point where dexamethasone affects the signaling of vasopressin between the receptor and phospholipase C. We demonstrated that dexamethasone had little effect on the affinity and the binding capacity of vasopressin receptor in these cells. Therefore, it is unlikely that the effect of dexamethasone on the vasopressin-induced PI hydrolysis is exerted at the point of vasopressin receptor in these cells. In other words, dexamethasone affects vasopressin-induced PI hydrolysis at a point downstream from vasopressin receptor. It is generally recognized that GTP-binding protein is involved in the coupling of vasopressin receptor to phospholipase C [Birnbaumer et al., 1990], and we previously showed that PTX-insensitive GTP-binding protein is involved in the vasopressin-induced PI hydrolysis in primary cultured rat aortic smooth muscle cells [Kondo et al., 1989]. We next examined the effect of dexamethasone on PI hydrolysis induced by NaF, a GTP-binding protein activator [Gilman, 1987], and showed that dexamethasone also significantly amplified the NaF-induced PI hydrolysis. The patterns of the dose dependency of both dexamethasone effects on vasopressin- and NaF-induced IP_3 formations seem to be similar. These results suggest that the effect of dexamethasone on vasopressin-induced PI hydrolysis is exerted at a point downstream from GTP-binding protein. Thus, our findings lead us to speculate that glucocorticoid

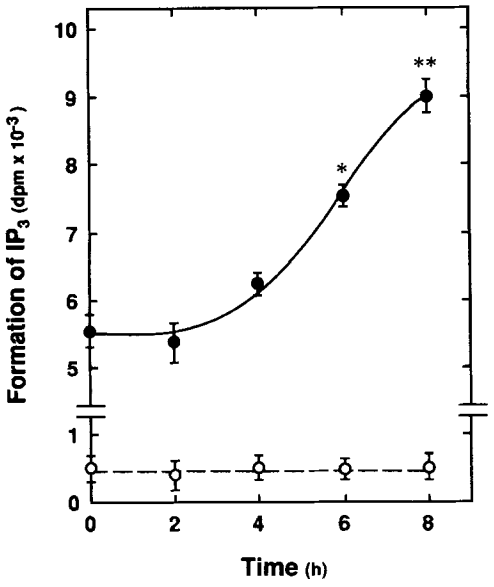

Fig. 4. Effect of dexamethasone on vasopressin-induced formation of IP_3 in rat aortic smooth muscle cells: time course of pretreatment. The [^{3}H]inositol-labeled cells were pretreated with 10 nM dexamethasone for the indicated periods and then stimulated by 0.1 μM vasopressin (●) or vehicle (○) for 5 min. Each value represents the mean ±SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$, **$P < 0.01$ compared to the value stimulated by vasopressin without dexamethasone pretreatment.

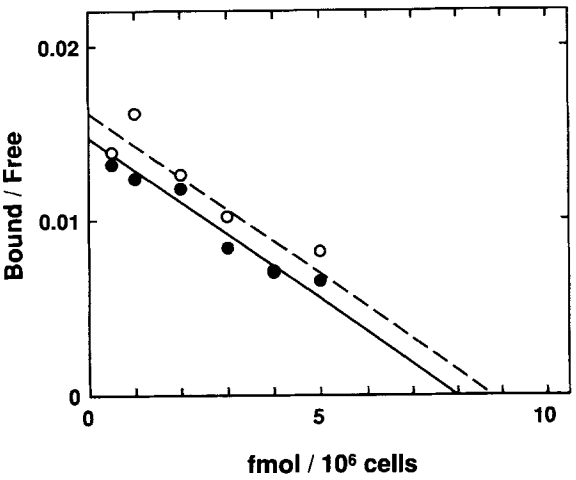

Fig. 5. Effect of dexamethasone on vasopressin binding in rat aortic smooth muscle cells. The cultured cells were pretreated with 10 nM dexamethasone (●) or vehicle (○) for 8 h, and then incubated in 1 ml of HEPES-buffered DMEM (pH 7.4) containing various doses of [^{3}H]vasopressin with or without a 200-fold molar excess of nonradioactive vasopressin at 20°C for 2 h. The number of binding sites is indicated at the intercept of the x-axis, while apparent dissociation constant is demonstrated by the slope of the line (slope = −1/kd). Data were representative of triplicate independent experiments.

also potentiates PI hydrolysis stimulated by other vasoactive agents which receptors are coupled to GTP-binding protein in rat aortic smooth muscle cells. We next examined the effect of dexamethasone on angiotensin II-induced IP_3 formation in these cells. It has been demonstrated that GTP-binding protein is involved in angiotensin II-induced PI hydrolysis in vascular smooth muscle cells [Birnbaumer et al., 1990]. Herein, we found that dexamethasone also amplified angiotensin II-induced formation of IP_3. It is most likely that dexamethasone commonly affects PI hydrolysis by vasoactive agents at a point downstream from GTP-binding protein and amplified them in primary cultured rat aortic smooth muscle cells.

It has been reported that protein kinase C has a negative feedback effect on PI hydrolysis in rat vascular smooth muscle cells [Brock et al., 1985; Caramelo et al., 1988; Pfeilschifter et al., 1989]. We showed here that TPA, a protein kinase C-activating phorbol ester [Nishizuka, 1986], inhibited the formation of IP_3 induced by NaF, a GTP-binding protein activator [Gilman, 1987] in primary cultured rat aortic smooth muscle

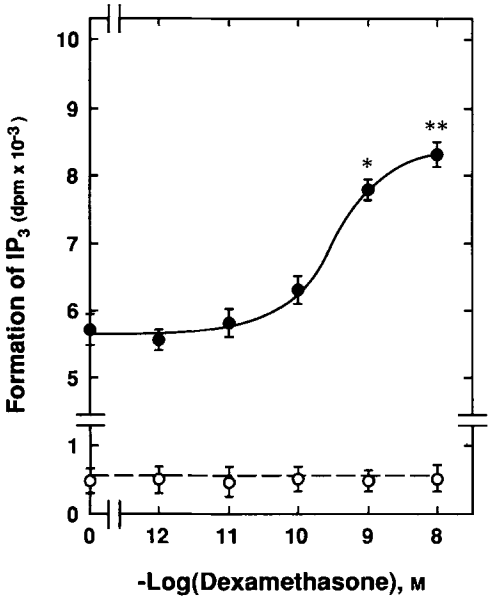

Fig. 6. Effect of dexamethasone on NaF-induced formation of IP_3 in rat aortic smooth muscle cells. The [^{3}H]inositol-labeled cells were pretreated with various doses of dexamethasone for 8 h and then stimulated by 20 mM NaF (●) or vehicle (○) for 10 min. Each value represents the mean ±SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $*P < 0.05$, $**P < 0.01$ compared to the value stimulated by NaF without dexamethasone pretreatment.

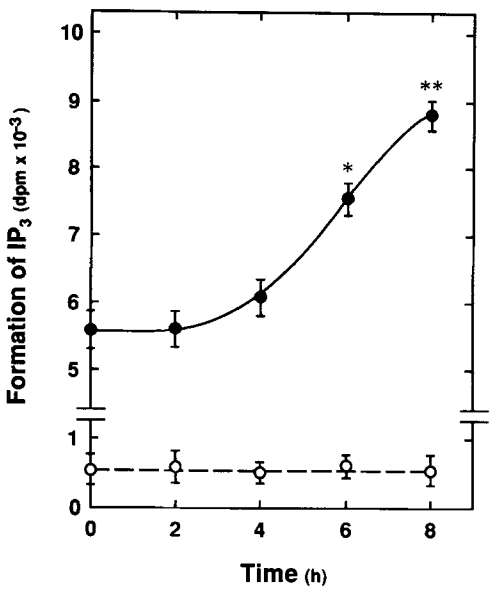

Fig. 7. Effect of dexamethasone on the angiotensin II-induced formation of IP_3 in rat aortic smooth muscle cells. The [^{3}H]inositol-labeled cells were pretreated with 10 nM dexamethasone for the indicated periods and then stimulated by 0.1 μM angiotensin II (●) or vehicle (○) for 5 min. Each value represents the mean ±SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $*P < 0.05$, $**P < 0.01$ compared to the value stimulated by angiotensin II without dexamethasone pretreatment.

cells, and that 4α-PDD, a protein kinase C non-activating phorbol ester [Nishizuka, 1986] had little effects. These results suggest that the activation of protein kinase C affects PI hydrolysis at the point downstream from GTP-binding protein in these cells. We next examined the effect of protein kinase C activation on the amplification by dexamethasone of PI hydrolysis. We showed that TPA significantly reduced the effect of dexamethasone on the formation of IP_3 induced by vasopressin, angiotensin II and NaF, and 4α-PDD was ineffective in these capacities. From our findings, it is most likely that the activation of protein kinase C also has a negative feedback effect on the glucocorticoid-induced amplification of PI hydrolysis at the point downstream from GTP-binding protein in these cells.

Vasoactive agents such as vasopressin and angiotensin II are well known to stimulate the proliferation of aortic smooth muscle cells through PI hydrolysis [Altura and Altura, 1977; Campbell-Boswell and Robertson, 1981; Nabika et al., 1985; Aiyar et al., 1986; Vallotton et al., 1986; Grillone et al., 1988] and that proliferation of these cells plays an important role in the development of atherosclerosis and hyperten-

TABLE I. Effects of TPA on Enhancement by Dexamethasone of Vasopressin (AVP)- and NaF-Induced Formation of IP_3 in Rat Aortic Smooth Muscle Cells*

Effector	IP_3 formation (dpm)
AVP	5,102 ± 198
TPA + AVP	2,143 ± 172[a]
Dexamethasone + AVP	8,520 ± 224[a]
Dexamethasone + TPA + AVP	4,643 ± 212[b]
NaF	5,361 ± 225
TPA + NaF	3,434 ± 176[c]
Dexamethasone + NaF	7,526 ± 189[c]
Dexamethasone + TPA + NaF	5,415 ± 231[d]

*The [^{3}H]inositol-labeled cells were pretreated with 10 nM dexamethasone or vehicle for 8 h. The cells were then stimulated by 0.1 μM AVP for 5 min or 20 mM NaF for 10 min. The pretreatment with TPA (0.1 μM) was performed for 20 min prior to stimulation. Values for unstimulated cells have been subtracted from each data point. Each value represents the mean ±SD of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

[a]$P < 0.01$, as compared to the value stimulated by AVP alone.

[b]$P < 0.01$, as compared to the value stimulated by AVP with dexamethasone pretreatment.

[c]$P < 0.01$, as compared to the value stimulated by NaF alone.

[d]$P < 0.01$, as compared to the value stimulated by NaF with dexamethasone pretreatment.

sion [Schwartz, 1983; Ross, 1993]. So, it is possible that glucocorticoid excess develops atherosclerosis and hypertension by amplifying the PI hydrolysis induced by vasoactive agents. The effect of glucocorticoid on aortic smooth muscle cells might contribute to the pathogenesis of aortic changes observed in patients with glucocorticoid excess, so-called Cushing syndrome.

In conclusion, our results suggest that glucocorticoid amplifies vasopressin-induced PI hydrolysis at a point downstream from GTP-binding protein in rat aortic smooth muscle cells, and that the activation of protein kinase c has a negative feedback effect on the amplification by glucocorticoid of vasopressin-induced PI hydrolysis.

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