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Phorbol Ester-Mediated Inhibition of Vasopressin and β-Adrenergic Responses in a Vascular Smooth Muscle Cell line

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SUMMARY

We have reported previously that in the vascular smooth muscle cell line A-10 (ATCC CRL 1476), vasopressin stimulated phosphatidylinositol turnover Ca^{2+} efflux and inhibited isoproterenol-stimulated cAMP accumulation. Here we report that pretreatment of these cells with phorbol dibutyrate, an activator of protein kinase C, attenuated the responses to vasopressin and isoproterenol. This effect was concentration dependent and could be observed after pretreatment for 2 min. 4α Phorbol 12,13-dideconoate, which does not activate protein kinase C, did not attenuate the responses. These data suggest that activation of protein kinase C by phorbol dibutyrate attenuates the responses of vascular smooth muscle cells to isoproterenol and vasopressin. Although phorbol ester did not affect [^{3}H]-8-arginine vasopressin binding to intact cells, it appeared to uncouple vasopressin receptors from guanine nucleotide-binding protein.

The mechanisms of hormonal regulation of cellular function have elicited a great deal of investigative interest. In addition to the adenylate cyclase system, another major second messenger pathway has been described recently (1, 2). This pathway involves receptor-mediated stimulation of PI turnover that leads to the formation of DG and IP_3. IP_3 has been shown to induce the release of Ca^{2+} from intracellular storage sites and DG activates PKC (1, 2).

Phorbol esters such as 4β phorbol 12β-myristate 13α acetate and PDBu are structurally similar to DG and activate PKC (3, 4). Phorbol esters have been shown to affect hormonal stimulation of adenylate cyclase and PI turnover in different experimental systems. In duck and turkey erythrocytes, phorbol esters mimicked β-adrenergic-induced receptor desensitization (5, 6). Phorbol ester treatment of human platelets (7), PC12 cells (8), DDT_1 MF-2 cells (9), and isolated hepatocytes (10, 11) decreased agonist-induced PI turnover.

We have recently reported that rat aortic smooth muscle cells (A-10) express vascular V_1 vasopressin receptors (12) and β-adrenergic receptors (13). In these cells, vasopressin increased PI turnover (14) and decreased cAMP accumulation induced by β-adrenergic agonists (15). The cells were subcloned to ensure a monoclonal cell population and thus provided an excellent model to study vasopressin regulation of these responses in the same cells. In the present study we have employed PDBu and αPDD to assess the role of PKC in the vasopressin-induced responses.

Materials and Methods

myo-[2-^{3}H]Inositol (15.6 Ci/mmol) was obtained from Amersham Corp. (Chicago, IL), 8-arginine vasopressin (AVP) from Bachem (Torrance, CA); [^{3}H]AVP (53 Ci/mmol) and $^{45}CaCl_2$ (16.16 mCi/mg) from New England Nuclear (Boston, MA); AG-1 x8 resin (formate form), 100–200 mesh, from Bio-Rad (Richmond, CA); and (−)-isoproterenol, isobutylmethylxanthine, PDBu, and αPDD from Sigma Chemical Co. (St. Louis, MO). [^{3}H]SK&F 101926 (37.90 Ci/mmol) was prepared at Smith, Kline and French Laboratories (Philadelphia, PA).

Culture of smooth muscle cells. Rat aortic vascular smooth muscle cells (A-10; ATCC CRL 1476) were obtained from the American Type Culture Collection Rockville, MD and cultured as described (15). Experiments were performed 3 days after inoculation of the culture wells (35 mm diameter) with 75,000 cells.

Labeling of smooth muscle cells with [^{3}H]inositol, treatment with phorbol esters, and extraction of inositol phosphates. The cells were labeled with 2 μCi of [^{3}H]inositol for 24 hr starting on day 3 as described (14). Cells were treated with phorbol esters in HEPES buffer (140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.6 mM $MgSO_4$, 1.0 mM Na_2HPO_4, 5.5 mM D-glucose, 25.0 mM HEPES, and 0.06% bovine serum albumin) for the times indicated. Subsequently, the cells were washed three times with 1 ml of HEPES buffer and incubated with 750 μl of HEPES buffer containing 10 mM LiCl for 5 min at 37°. The reaction was started by adding 250 μl of buffer containing 10 mM LiCl with AVP and the incubation was continued for 1 min at 37°. Incubations were terminated with 100 μl of 10% perchloric acid at 0° and neutralized with 125 μl of 1.53 M KOH, and buffered to pH 7.4 by the addition of 250 μl of 75 mM HEPES. The neutralized extract (1 ml)

ABBREVIATIONS: PI, phosphatidylinositol; DG, diacylglycerol; IP_3, inositol trisphosphate; PKC, protein kinase C; PDBu, phorbol 12,13-dibutyrate; αPDD, 4α phorbol 12β,13α acetate; AVP, 8-arginine vasopressin; HEPES, 4-(2-hydroxyethyl)-1-piperazinesthanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DPBS, Dulbecco's phosphate-buffered saline containing 10 mM $MgCl_2$; 0.1% glucose, 0.2% bovine serum albumin; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate.

after removal of $KClO_4$ was diluted to 5 ml with 5 mM sodium tetraborate/0.5 mM EDTA and applied to a column (5 cm × 0.4 cm) of Bio-Rad AG 1-x8 resin (formate form) and inositol phosphates were separated as described (14).

$^{45}Ca^{2+}$ efflux. Cells were labeled with $^{45}CaCl_2$ (5 μCi/ml) for 16 hr and then washed with HEPES buffer and preincubated with or without 100 nM phorbol esters for 10 min at 37°. Cell-associated $^{45}Ca^{2+}$ was determined as described (14) after incubating the cells in the presence or absence of 1 μM AVP for 5 min.

Phorbol ester pretreatment and cAMP determination. The cells were incubated in 1 ml of fresh medium with the phorbol esters for the times indicated. Subsequently, the cells were washed three times with DPBS. The cells were incubated with 1 μM (−)-isoproterenol with or without 10 nM AVP for 10 min at 37° in DPBS containing 0.5 mM isobutylmethylxanthine. cAMP was extracted as previously described (15) and determined by radioimmunoassay (New England Nuclear).

[^{3}H]AVP binding to A-10 cells. [^{3}H]AVP binding was performed on intact cell monolayers. Cells were washed with DPBS and incubated in 1 ml of DPBS with or without 1 μM PDBu for 60 min at 37°. At the end of incubation, the buffer was removed and the cells were washed two times with 1 ml of DPBS. The binding was initiated with 1 ml of DPBS containing 1–40 nM [^{3}H]AVP with or without 10 μM unlabeled AVP. Assays were carried out for 20 min at 37°. Assays were terminated by rapidly washing the monolayers two times with 1 ml of ice-cold DPBS. The cells were scraped in ice-cold DPBS and washed rapidly in Amicon filters; then, the cell-associated radioactivity was measured. The K_D and B_{max} values were determined by Scatchard analysis.

Membrane preparation from A-10 cells. A-10 cell membranes were prepared as described (13). Briefly, the cells were washed with DPBS, scraped, frozen in liquid N_2, and thawed. To this was added 10 volumes of hypotonic buffer (5 mM Tris, 2 mM Mg^{2+}, 1 mM EDTA, pH 7.5) and the cells were homogenized using a Dounce homogenizer (25 strokes). The homogenate was centrifuged at 300 ×*g* for 10 min at 4°.The supernatant was centrifuged at 40,000 ×*g* for 10 min and the pellet was resuspended in hypotonic buffer.

[^{3}H]SK&F 101926 binding to membranes. The cell membranes in hypotonic buffer were incubated for 60 min at 37° with [^{3}H]SK&F 101926 (2 nM), a newly developed AVP antagonist, (16), in the absence (total) and presence of increasing concentrations (competition) of AVP in a total volume of 500 μl. At the end of incubation the bound and free ligand were separated by centrifugation. The membrane pellet was solubilized with NCS solubilizer and radioactivity was determined by liquid scintillation counting.

Results

Preincubation of [^{3}H]inositol-labeled A-10 cells with 100 nM PDBu had no significant effect on basal inositol phosphate levels. However, PDBu inhibited IP_3 formation induced by AVP. This inhibition by PDBu was dependent on the time of preincubation, being observed as early as 2 min (Fig. 1). αPDD, a non-tumor-promoting phorbol ester, had no effect on AVP-induced IP_3 formation (data not shown). The dose-dependent effect of phorbol ester pretreatment on AVP-induced IP_3 formation is shown in Fig. 2. With 20 min preincubation, PDBu significantly inhibited the AVP response at a concentration as low as 1 nM. At all concentrations tested, αPDD was inactive. To determine whether the PDBu-induced inhibition was due to an effect on the vasopressin receptor itself, the effect of PDBu on increasing concentrations of AVP was tested. As shown in Fig. 3, PDBu-induced inhibition was observed at all concentrations of AVP tested. Increasing the concentrations of AVP did not overcome the PDBu-mediated inhibitory effect. The attenuated formation of IP_3 after PDBu pretreatment does not seem to be a consequence of an accelerated degradation of IP_3 because levels of inositol bisphosphate and monophosphate (degradation products of IP_3) were also inhibited (data not shown). To test whether the inhibitory effect of PDBu on IP_3 formation is also reflected in the calcium efflux, $^{45}Ca^{2+}$-loaded cells were preincubated with 100 nM PDBu (10 min at 37°) and Ca^{2+} efflux in response to AVP was measured. As shown in Fig. 4, pretreatment with PDBu inhibited AVP-induced Ca^{2+} efflux by 60% without affecting the basal levels.

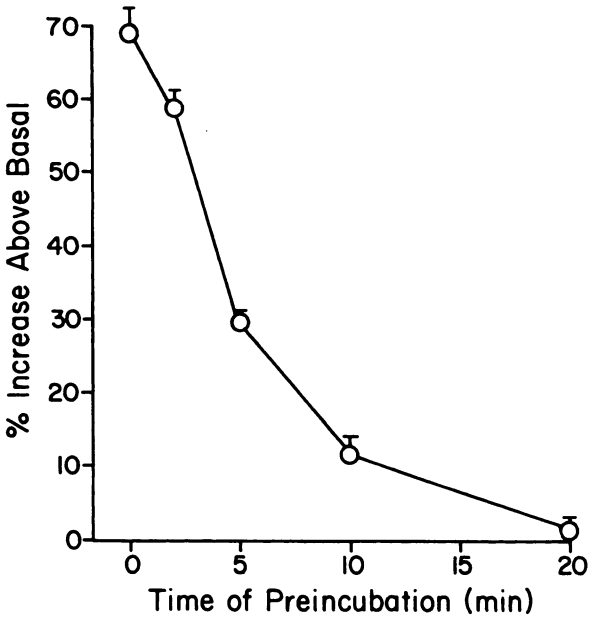

Fig. 1. Time course of phorbol ester pretreatment on vasopressin-induced PI turnover. Prelabeled A-10 cells were incubated with 100 nM PDBu for the indicated times and then exposed to 1 μM AVP for 1 min. Values are means of two experiments done in triplicate. The basal IP_3 value was 479 ± 9 dpm/10^6 cells.

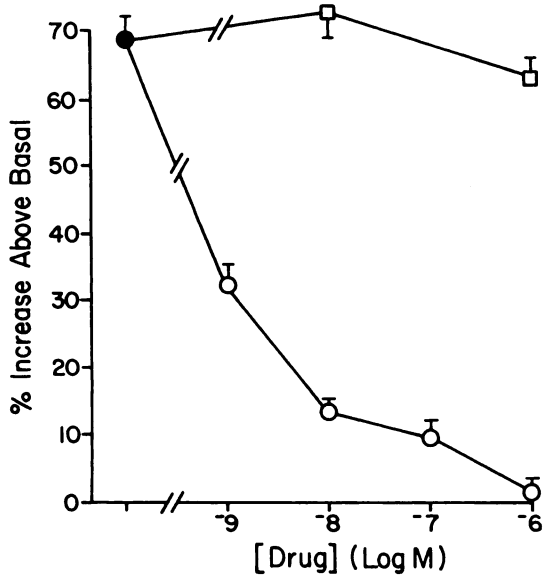

Fig. 2. Dose response of phorbol ester pretreatment. Prelabeled A-10 cells were incubated with various concentrations of phorbol esters for 20 min and then exposed to 1 μM AVP for 1 min in the presence of 10 mM LiCl: ●, untreated; ○, PDBu; or □, αPDD. The experiment was done in triplicate and repeated with similar results. The basal IP_3 value was 479 ± 9 dpm/10^6 cells.

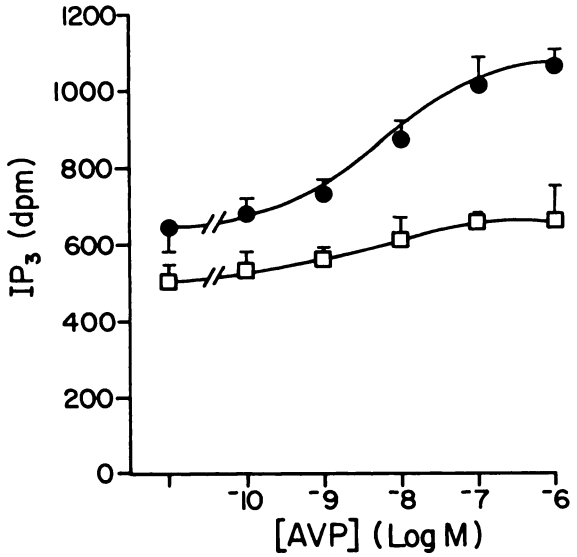

Fig. 3. Effect of phorbol ester pretreatment on IP_3 formation induced by increasing concentrations of AVP. Prelabeled A-10 cells were treated with (□) or without (●) 100 nM PDBu for 10 min and then exposed to increasing concentration of AVP for 1 min in the presence of 10 mM LiCl.

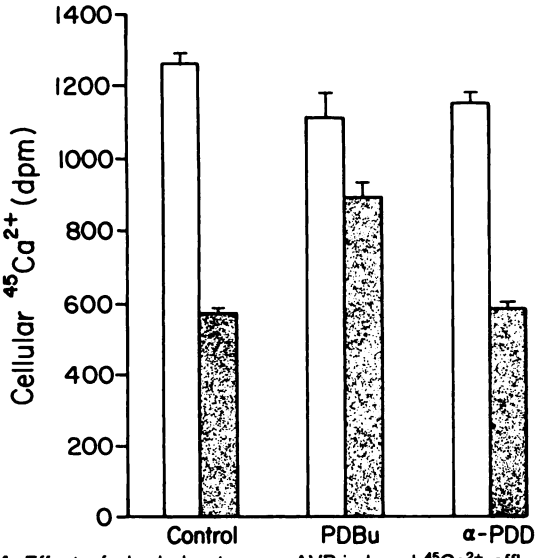

Fig. 4. Effect of phorbol esters on AVP-induced $^{45}Ca^{2+}$ efflux. $^{45}Ca^{2+}$-loaded A-10 cells were incubated with phorbol esters for 10 min and then exposed to 1 μM AVP for 5 min: □, no AVP; ▨, 1 μM AVP.

Another response induced by AVP in these cells is the inhibition of β-adrenergic agonist-stimulated cAMP formation (13). Pretreatment of the cells with PDBu resulted also in the inhibition of (a) isoproterenol-stimulated cAMP accumulation (45% with 5 min preincubation) and (b) vasopressin-induced inhibition of isoproterenol-stimulated cAMP accumulation (51% in control versus 25% in PDBu-treated cells) (Table 1). The dose-dependent effects of PDBu pretreatment on cAMP formation are shown in Table 2. Whereas αPDD was without effect, PDBu inhibited isoproterenol-stimulated cAMP accumulation in a dose-dependent fashion. The maximum inhibition was 48%. AVP induced inhibition in control and 1 μM PDBu-treated cells to 47% and 29%, respectively. The inhibitory effect of PDBu on AVP-induced inhibition was further investigated using increasing concentrations of AVP. As shown in Table 3, 1 nM PDBu diminished the inhibition at all AVP concentrations, e.g., 100 nM AVP inhibited isoproterenol-stimulated cAMP accumulation by 30% and 56% in PDBu-treated and control cells, respectively.

The effect of PDBu pretreatment on AVP binding to intact A-10 cells was also examined. [^{3}H]AVP bound to these cells in a saturable manner with an apparent K_d of 4.1 nM and B_{max} of 48 fmol/10^6 cells. Pretreatment of the cells with 1 μM PDBu for upto 60 min at 37° had no effect [^{3}H]AVP binding (K_d 4.8 nM, B_{max} = 45 fmol/10^6 cells).

The effect of 100 μM Gpp(NH)p on the binding of [^{3}H]SK&F 101926 to A-10 cell membranes in the presence of increasing concentrations of AVP is shown in Fig. 5. In membranes from control A-10 cells, the AVP competition of [^{3}H]SK&F 101926 binding yielded a shallow binding curve. Addition of Gpp(NH)p

TABLE 1

Phorbol ester effects on cAMP levels in response to isoproterenol and isoproterenol plus AVP in A10 cells

Cells were pretreated without and with 1 μM PDBu for 5 min at 37° and cAMP levels in response to 1 μM isoproterenol and 1 μM isoproterenol plus 10 nM AVP were measured. Each value is the mean ± standard error of triplicate determinations from one experiment. The experiment was repeated with similar results.

Addition	pmol of cAMP formed	
	Control	1 μM PDBu pretreated
Basal	12.4 ± 2.1	12.0 ± 2.0
Isoproterenol	133.0 ± 5.8	73.0 ± 4.0 (45)[a]
Isoproterenol + AVP	66.0 ± 1.2 (51[a])	54.5 ± 5 (25[a])
AVP	11.5 ± 3.0	12.0 ± 2.0

[a] Values in parentheses indicate the per cent decrease in cAMP accumulation.

TABLE 2

Dose response of phorbol ester effects on cAMP levels in response to isoproterenol and isoproterenol plus AVP

A-10 cells were pretreated with various concentrations of αPDD or PDBu for 30 min at 37° and cAMP levels in response to 1 μM (−)-isoproterenol and 1 μM (−)-isoproterenol + 10 nM AVP were measured. Each value is the mean ± standard error of triplicate determinations. The experiment was repeated with similar results.

Pretreatment	Basal	pmol of cAMP with isoproterenol	Percentage of inhibition[a]	pmol of cAMP with isoproterenol + AVP	Percentage of inhibition[b]
None	18.2 ± 2.2	124.0 ± 3.4	0	65 ± 2.5	47
αPDD					
10 nM	17.9 ± 2.5	122 ± 3.0	0	65 ± 2.0	47
1000 nM	18.8 ± 3.4	124 ± 4.0	0	63 ± 1.5	49
PDBu					
1 nM	18.8 ± 3.8	95 ± 2.5	23	52 ± 4.8	45
10 nM	17.5 ± 2.8	88 ± 6.0	29	53 ± 7.0	40
100 nM	17.4 ± 4.5	65 ± 4.0	48	50 ± 1.5	23
1000 nM	16.6 ± 2.8	68 ± 2.0	45	48 ± 2.5	29

[a] Per cent inhibition of isoproterenol-induced cAMP production by phorbol esters.
[b] Per cent inhibition of isoproterenol-induced cAMP production by AVP.

TABLE 3

Effect of phorbol ester on AVP-induced inhibition of isoproterenol-stimulated cAMP formation as a function of the AVP concentration

A-10 cells were pretreated without or with 1 μM PDBu for 10 min at 37°. The cAMP levels without (basal) and in response to 1 μM (−)-isoproterenol and 1 μM (−)-isoproterenol and various concentrations of AVP were measured. Each value is the mean ± standard error of triplicate determinations. The experiment was repeated with similar results.

Concentration of AVP (nM)	Control		PDBu pretreatment	
	pmol of cAMP	% Inhibition	pmol of cAMP	% Inhibition
Basal	14 ± 2		13 ± 2	
0	180 ± 8		114 ± 6	
0.1	155 ± 5	14	113 ± 3	1
1.0	118 ± 2	35	105 ± 2	8
10.0	95 ± 2	47	92 ± 4	20
100.0	78 ± 3	56	80 ± 2	30

shifted the competition curve to the right (Fig. 5A). However, with the membranes obtained from PDBu-pretreated cells, the competition curves in the absence and presence of Gpp(NH)p were not significantly different (Fig. 5B). The curves were similar to that observed in control cell membranes with the Gpp(NH)p. These data suggest that the vasopressin receptors are coupled to a guanine nucleotide-binding protein, and that pretreatment with PDBu resulted in the uncoupling of those receptors from the guanine nucleotide-binding protein.

Discussion

The key findings of the present study are that treatment of cultured vascular smooth muscle cells with PDBu, a potent activator of PKC, resulted in an attenuation of vasopressin-induced responses, i.e., stimulation of PI turnover, $^{45}Ca^{2+}$ efflux, and inhibition of isoproterenol-stimulated cAMP formation. The inhibition was dependent on the time of preincubation and the concentration of phorbol esters used. Higher concentrations of vasopressin (1 μM) could not overcome the PDBu-induced inhibition. This is in contrast to studies using hepatocytes in which phorbol ester treatment inhibited vasopressin-mediated effects only at concentrations below 1 nM AVP (10, 11). In A-10 cells, PDBu inhibition of the vasopressin-induced effects was likely mediated via activation of PKC, because αPDD, which does not activate PKC, was ineffective.

PDBu also inhibited isoproterenol-stimulated cAMP formation in A-10 cells. It is possible that attenuation of the isoproterenol-stimulated cAMP formation in A-10 cells was the result of phosphorylation of the β-adrenergic receptor by phorbol ester-activated PKC. This event has been reported for the β-adrenergic receptor in the duck and turkey erythrocyte (5, 6). In these studies, phosphorylation of the β-adrenergic receptor appeared to uncouple the receptors from adenylate cyclase. In the present study, due to high nonspecific binding and very low density of β-adrenergic receptors (3–5 fmol/mg of protein) in these cells, satisfactory binding studies could not be performed to identify the site of modification induced by PDBu.

Attenuation of vasopressin-induced responses by PDBu could be caused either at the receptor or at a postreceptor site. Phosphorylation of non-adenylate cyclase-coupled receptors induced by phorbol ester, and a concomitant decrease in stimulation of PI turnover, has recently been reported by Lundberg *et al.* (9). They showed that phorbol ester treatment of DDT$_1$ MF-2 smooth muscle cells caused phosphorylation of α_1-adrenergic receptor and decreased α_1-agonist-stimulated PI turnover. In hepatocytes PDBu increased phosphorylation of certain proteins in response to α_1-adrenergic agonists, vasopressin, and angiotensin II (17). It has also been shown that phorbol esters decreased binding of epidermal growth factors and α_1-adrenergic ligands in A431 cells (18) and hepatocytes (10), respectively. In contrast, phorbol esters did not affect binding of vasopressin in hepatocytes (10), angiotensin II in smooth muscle cells (19), chemotactic peptides in neutrophils (20), or insulin in cultured hepatoma cells (21). Our studies also show that PDBu had no effect on [^{3}H]AVP binding to intact cells, suggesting that the inhibitory effect may be at a postreceptor site.

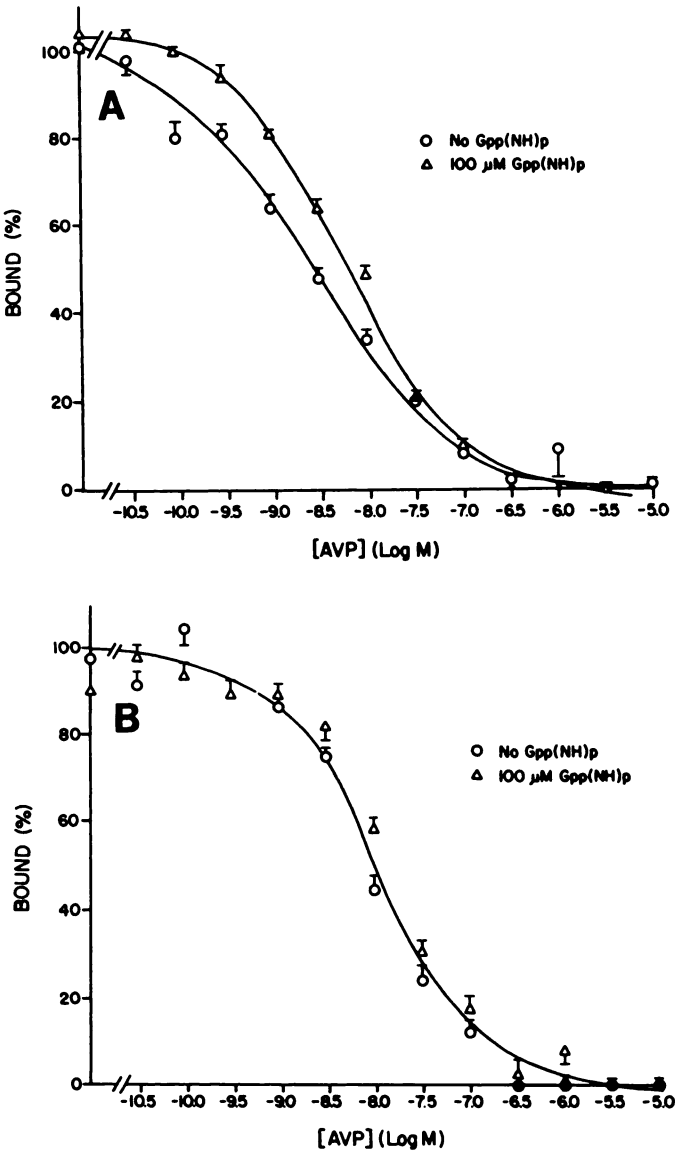

Fig. 5. Effect of Gpp(NH)p on AVP competition curves of [^{3}H]SK&F 101926 binding in control and PDBu-treated A-10 cell membranes. Shown are the AVP competition curves of [^{3}H]SK&F 101926 binding performed on membranes prepared from control A-10 cell membranes (A) and PDBu-treated A-10 cell membranes (B) in the absence (○) and presence (△) of 100 μM Gpp(NH)p. Membranes were incubated with 2 nM [^{3}H]SK&F 101926 in the absence and presence of increasing concentrations of AVP for 60 min at 37° and processed as explained in Materials and Methods. Each value is the mean ± standard error from triplicate determinations. The data are representative of the results of two separate experiments.

Recent studies have shown an involvement of guanine nucleotide-binding protein(s) in regulating the activity of phospholipase C in a number of systems (22, 23). Pertussis toxin pretreatment of A-10 cells caused ADP-ribosylation of a membrane protein (molecular weight 36,000–38,000) when incubated with [α-^{32}P]NAD (data not shown) and marginally inhibited (15–18%) AVP-induced PI turnover (14). It is conceivable that, in addition to this pertussis toxin substrate, other guanine nucleotide protein(s) might be involved in AVP-mediated responses. PDBu, through the stimulation of protein kinase C, could cause phosphorylation of the guanine nucleotide-binding protein(s) and thereby uncouple them from AVP receptors resulting in an inhibitory vasopressin response.

In conclusion, our studies show that treatment of cultured vascular smooth muscle cells with active phorbol ester (PDBu) causes inhibition of: (a) isoproterenol-stimulated cAMP formation, (b) vasopressin-induced stimulation of IP_3 formation, $^{45}Ca^{2+}$ efflux, and inhibition of isoproterenol-stimulated cAMP formation. It is likely that these effects of phorbol ester are mediated by PKC and the key interactions are at a postreceptor site. To our knowledge, this is the first report in which phorbol ester has been shown to inhibit several responses induced by one hormone, vasopressin.

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