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Pneumadin-Evoked Intracellular Free Ca^{2+} Responses in Rat Aortic Smooth Muscle Cells: Effect of Dexamethasone

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ABSTRACT. The direct vascular effect of pneumadin (PN) was determined by studying the changes in intracellular free calcium ($[Ca^{2+}]_i$) levels in cultured rat aortic smooth muscle cells maintained between the second and fifth passages. PN evoked a rapid, concentration-dependent, biphasic increase in $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ level rose from a basal value of 108 nM to a maximum increase in peak value of 170 nM. Although the level of maximal $[Ca^{2+}]_i$ response evoked by PN was less than with other vasoactive agonists, it was more potent (EC_{50} 0.5 nM) than even endothelin-1 (EC_{50} 3.1 nM). At concentrations > 100 nM, $[Ca^{2+}]_i$ elevations induced by PN above basal levels were no longer observed. Pretreatment with dexamethasone (100 nM for 24 hr) resulted in a significant increase ($P < 0.01$) in the peak $[Ca^{2+}]_i$ response (310 nM) to PN. However, the biphasic pattern in the peak $[Ca^{2+}]_i$ responses encountered with increasing concentrations of PN remained unaffected. The exaggerated $[Ca^{2+}]_i$ response to PN was abolished by preincubation of cells with either the glucocorticoid antagonist mifepristone (RU 486) or the protein synthesis inhibitor cycloheximide. Inclusion of either an AT_1 antagonist (losartan), a V_1 selective vasopressin antagonist ($d(Ch_2)_5$ Tyr (Me) AVP), or an α-adrenoceptor antagonist (phentolamine) failed to affect the increases in $[Ca^{2+}]_i$ induced by PN. PN-evoked increases in inositol 1,4,5-trisphosphate levels paralleled the $[Ca^{2+}]_i$ changes. These data suggest that PN increases Ca^{2+} mobilization in rat aortic smooth muscle cells via activation of phospholipase C coupled receptors. This effect is up-regulated by dexamethasone. BIOCHEM PHARMACOL **58**;1:177–182, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. $[Ca^{2+}]_i$; IP_3; pneumadin; aortic smooth muscle cells; dexamethasone; endothelin

PN‡ is a decapeptide found in mammalian lungs. It confers antidiuretic actions mediated by the release of AVP from the pituitary [1]. Using immunocytochemical approaches, studies have shown that PN is present in the bronchial mucosa and is discharged into the lumen. The presence of PN has been found in the cerebral cortex, thyroid, adrenal gland, and pyloric antrum of the stomach [2]. Besides releasing AVP, PN also is capable of increasing plasma concentrations of atrial natriuretic peptide and aldosterone [3]. Finally, acute administration of very low concentrations (< 4 nmol) of PN stimulates the rat pituitary-adrenal axis, resulting in the release of corticosterone and aldosterone production [4]. Except for these indirect biological responses, there have been no systematic studies conducted thus far to establish a direct action of PN either at the tissue or the cellular level. The present study attempts to address this issue at the cellular level in VSM cells.

AVP is a potent vasoconstrictor agonist that enhances $[Ca^{2+}]_i$ levels subsequent to the generation of IP_3 in VSM cells via activation of V_1 subtype receptors [5, 6]. Because other peptides including AVP, Ang II, and ET-1 exert their effects through activation of PLC and the subsequent increase in $[Ca^{2+}]_i$, we examined whether PN is capable of elevating $[Ca^{2+}]_i$ levels in early passage cultured rat ASMC. The effect of PN was compared with the $[Ca^{2+}]_i$ responses to ET-1, Ang II, and AVP. Previous studies have shown that pretreatment with dexamethasone enhances the vascular response to various vasoactive agonists [7–12]. Therefore, the effect of PN in dexamethasone-pretreated rat ASMC was also investigated.

MATERIALS AND METHODS

Culture of ASMC

Male Sprague–Dawley rats purchased from Charles River Inc. were maintained in our animal quarters until 16 weeks of age (200–250 g). The detailed methodology adopted for the isolation and maintenance of cultures of rat ASMC has been described earlier [5, 13]. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing

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‡ *Abbreviations*: Ang II, angiotensin II; ASMC, aortic smooth muscle cells; AVP, arginine vasopressin; $[Ca^{2+}]_i$, intracellular free calcium; ET-1, endothelin-1; IP_3, inositol 1,4,5-trisphosphate; PLC, phospholipase C; PN, pneumadin; and VSM, vascular smooth muscle.

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10% heat-inactivated fetal bovine serum, 10% horse serum, penicillin (100 U/mL), streptomycin (100 μg/mL), glutamine (2 mM), and glucose (5 mM). The culture medium was replaced with a serum-free medium 24 hr prior to measurements in cells between the second and fifth passages. Earlier, we demonstrated that there are no significant differences in the $[Ca^{2+}]_i$ responses to agonists in primary cultures of rat ASMC and cells maintained between the second and fifth passages [5]. A group of cells were pretreated with dexamethasone (100 nM for 24 hr), while the other group served as a control. In a few experiments, the cells were pretreated with either cycloheximide (10 μg/mL) or mifepristone (RU 486, 1 μM) for 24 hr. These interacting agents were removed and the cells were washed with serum-free medium prior to fura-2 loading or IP_3 determination as described below.

Measurement of $[Ca^{2+}]_i$

Basal and agonist-evoked increases in peak $[Ca^{2+}]_i$ responses were determined by measuring fura-2 fluorescence in cell suspensions of ASMC maintained at 37° using a spectrofluorometer, CAF-100 Ca^{2+} analyzer (Japan Spectroscopic). The detailed methodology of dye loading and of calibration for $[Ca^{2+}]_i$ has been described previously [5, 14]. Cultured ASMC, following trypsinization, were suspended in Krebs-HEPES buffer of the following composition (in mM): NaCl, 145.0; KCl, 4.8; $CaCl_2 \cdot 2H_2O$, 1.8; $MgCl_2 \cdot 6H_2O$, 1.2; glucose, 10.0; HEPES, 10.0; and 0.2% bovine serum albumin (pH 7.4). Fura-2/AM prepared in DMSO at a stock concentration of 5 mM along with pluronic acid F 127 (0.02%) was used such that the final concentration of fura-2/AM was 5 μM in the incubation medium. For each determination, a fura-2 loaded suspension of $\sim 0.15 \times 10^6$ cells was added to a cuvette in a final volume of 0.5 mL and stirred at a rate of 800 rpm. Then each sample was exposed to a single concentration of either PN or another agonist. The excitation ratio (340/380 nm) of fura-2 fluorescence was monitored for a total period of 4 min after the addition of another agonist. Repetitive estimations were made using different cell suspensions to avoid possible receptor desensitization. Select experiments were also performed in which extracellular Ca^{2+} (1.8 mM) was replaced with 1.0 mM EGTA. In addition, the $[Ca^{2+}]_i$ responses to PN were determined in the presence and absence of a 1 μM concentration of either nifedipine, BQ-123 [cyclo(-D-Trp-D-Asp-Pro-D-Val-Leu)], losartan, d$(Ch_2)_5$ Tyr (Me) AVP (a V_1 selective antagonist), or phentolamine.

IP_3 Measurement

Either control or dexamethasone-pretreated adherent ASMC maintained in 24-well culture plates at 37° were utilized for these studies. Various concentrations of either PN (100 pM–100 nM) or maximal concentrations of other agonists (1 μM) were added in a final volume of 10 μL. The reaction was stopped after 30 sec by the addition of 0.2 vol. of ice-cold 20% perchloric acid. The mixture was incubated on ice for an additional 20 min. Protein was removed by centrifugation at 2000 *g* for 15 min at 4°. The collected supernatant was adjusted to pH 7.5 with 10 N KOH and 60 mM HEPES, and kept on ice for an additional 60 min. After removal of insoluble potassium perchlorate by centrifugation, 100 μL of supernatant was mixed with the assay buffer containing 0.1 M Tris buffer, 4.0 mM EGTA, and 4 mg/mL of BSA (pH 9.0). One hundred microliters of tracer (D-*myo*[^{3}H]inositol 1,4,5-trisphosphate) and 100 μL of bovine adrenal binding protein were added. The tubes were kept on ice for 30 min. Unbound tracer was removed by centrifugation and decantation. The pellet was dissolved in water, and the bound radioactivity was measured by scintillation counting. Based on the competition between unlabeled IP_3 and fixed amount of [^{3}H]IP_3 for the limited number of binding sites on bovine adrenal binding protein, the amount of IP_3 generated was calculated by interpolation from the standard curve (0.19 to 25 pmol/tube). The bovine adrenal glands were collected fresh from a local abattoir, and the microsomal fractions from the isolated cortex portions were prepared as described [15]. The final pellet was resuspended in the homogenization buffer and stored as the binding protein in small aliquots at −70°. Each time, sufficient aliquots of binding protein were taken out, thawed, and used in competition experiments.

Chemicals and Reagents

Rat PN was purchased from Bachem Bioscience Inc. Ang II octapeptide, ET-1, and AVP were from Peninsula Laboratories. D-*myo*[^{3}H]Inositol 1,4,5-trisphosphate was from DuPont Canada. The following competitive antagonists were obtained from sources shown in the parentheses: ET_A selective antagonist BQ-123 (American Peptide Co.); AT_1 selective antagonist losartan (gift from DuPont Merck Pharmaceutical Co.); V_1 selective antagonist d$(CH_2)_5$ Tyr (Me) AVP (Peninsula Laboratories); and glucocorticoid receptor antagonist RU 486 (gift from Roussel-UCLAF). Dexamethasone 21 acetate, cycloheximide, Triton X-100, EGTA, DMSO, and the α-adrenoceptor blocker phentolamine methanesulfonate were purchased from the Sigma Chemical Co. All other chemicals for culture studies were purchased from Gibco. Fura-2/AM ester and pluronic acid F-127 were from Molecular Probes.

Data Analyses

Each concentration–response (C–R) curve was analyzed individually for the estimation of the concentration required to produce 50% of the maximal response (EC_{50}) and the maximal increases in $[Ca^{2+}]_i$ and IP_3 levels after the addition of either PN or other agonists. All results are expressed as means ±

TABLE 1. Effect of dexamethasone pretreatment (100 nM for 24 hr) on peak $[Ca^{2+}]_i$ responses evoked by pneumadin (PN), endothelin-1 (ET-1), arginine vasopressin (AVP), and angiotensin II (Ang II) in cultured rat ASMC

	Control group		Dexamethasone group	
	EC_{50} (nM)	Peak $[Ca^{2+}]_i$ (nM)	EC_{50} (nM)	Peak $[Ca^{2+}]_i$ (nM)
PN	0.5	170 ± 20	0.5	310 ± 50*
ET-1	4.0	407 ± 50	5.0	212 ± 30†
AVP	10.0	260 ± 30	12.5	360 ± 20*
Ang II	20.0	700 ± 80	25.0	1000 ± 99*

Values are means ± SEM of 6–8 observations using different batches of ASMC. There were no significant differences in basal $[Ca^{2+}]_i$ values between the control group (108 ± 12 nM) and the dexamethasone-treated (105 ± 9 nM) group.
*$P < 0.05$, compared with respective control value.
†$P < 0.01$, compared with control value.

SEM. The comparison of data obtained for each group was subjected to analyses using ANOVA (SUPERANOVA software, Macintosh). Simultaneous multiple comparisons were examined using Scheffe's F-test.

RESULTS

Basal $[Ca^{2+}]_i$ Values

The basal or resting $[Ca^{2+}]_i$ value (mean ± SEM) before agonist stimulation in dispersed rat ASMC suspensions was 108 ± 12 nM (Table 1). These data are similar to the $[Ca^{2+}]_i$ value reported previously in cultured ASMC of normotensive rat strains [13]. The basal $[Ca^{2+}]_i$ values in cells pretreated with dexamethasone, RU 486, or cycloheximide were 105 ± 9, 117 ± 12, and 113 ± 8 nM, respectively. These values were not significantly different from the basal $[Ca^{2+}]_i$ value seen in the untreated control group of cells.

PN-Evoked Increases in $[Ca^{2+}]_i$ in Rat ASMC: Regulation by Dexamethasone

PN evoked a rapid, concentration-dependent, biphasic peak $[Ca^{2+}]_i$ response; an initial increase in the peak response was followed by a decline to basal levels at higher concentrations. Data from a single experiment are shown in Fig. 1 (upper panel). The threshold concentration was 200 pM, and the maximal increase was evident at 5 nM. The PN-evoked peak $[Ca^{2+}]_i$ level was 170 ± 20 nM, and the EC_{50} value for PN was 0. 5 nM (Table 1). At concentrations > 5 nM, PN-evoked peak $[Ca^{2+}]_i$ responses decreased, and at concentrations above 100 nM there was no appreciable increase from the basal level. Inclusion of dexamethasone led to significant increases in the peak responses to PN at 2 nM ($P < 0.05$) and 5 nM ($P < 0.01$) compared to peak $[Ca^{2+}]_i$ responses for similar data points in the control group. This is evident from the tracing of a single experiment (lower panel, Fig. 1) as well as pooled data of several C–R curves (Fig. 2). Similar to the control group, the C–R curve to PN was biphasic; there were no significant differences in the EC_{50} values between control and treatment groups, but the peak $[Ca^{2+}]_i$ response to PN was higher in the dexamethasone treatment group (Table 1 and Fig. 2).

Pretreatment of ASMC with the glucocorticoid antagonist RU 486 *per se* did not affect the responses to PN in control ASMC, but attenuated the exaggerated $[Ca^{2+}]_i$ responses to PN in the presence of dexamethasone (Fig. 3). Similarly, inclusion of the protein synthesis inhibitor cycloheximide (10 μg/mL) in the medium *per se* failed to affect the $[Ca^{2+}]_i$ responses to PN in the control group but antagonized the elevated $[Ca^{2+}]_i$ response to PN observed in the presence of dexamethasone (Fig. 3).

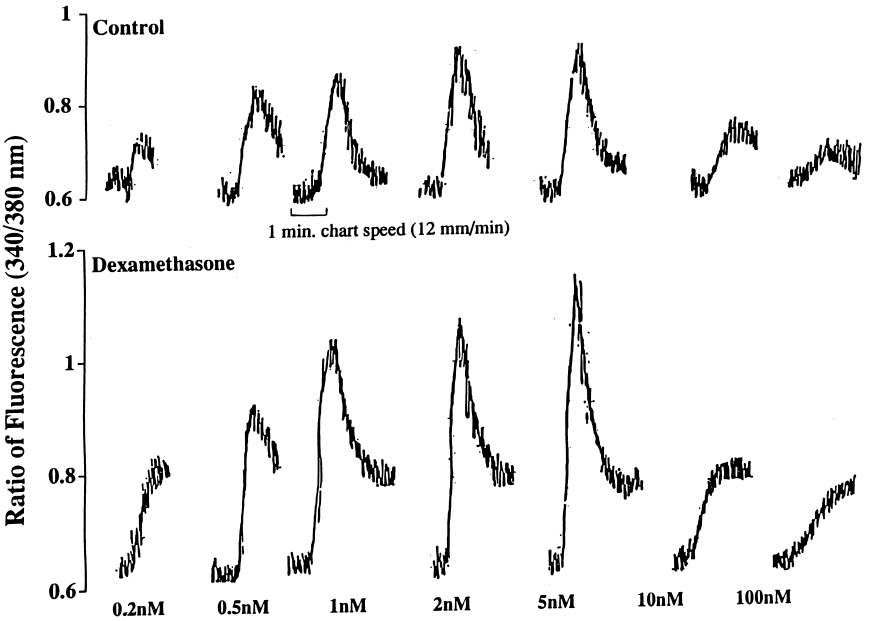

FIG. 1. Representative experiment that shows the pattern of PN-evoked increases in the ratio of fura-2 fluorescence. Values shown under each response on the x-axis represent PN concentration. Each response was obtained with a fresh cell suspension (0.15×10^6 cells in 500 μL buffer). The upper panel shows the responses in control ASMC. The lower panel shows the responses to similar concentrations of PN in dexamethasone-pretreated (100 nM; 24 hr) ASMC. *Note*: Dexamethasone was not present during fura-2 fluorescence measurement. Similar results were obtained in 5 separate determinations.

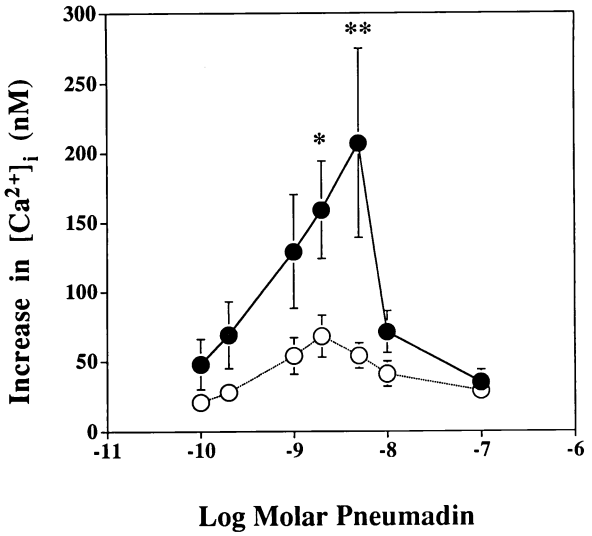

FIG. 2. Relationship between PN concentration and peak $[Ca^{2+}]_i$ response attained above basal levels in ASMC of control (○–○) and dexamethasone-pretreated (●–●) groups. Each data point is the mean ± SEM of 5 separate determinations performed with different batches of cells. Key: (*) $P < 0.05$ and (**) $P < 0.01$ compared with values at similar data points for the control group.

Comparison of Peak $[Ca^{2+}]_i$ and IP_3 Responses to PN with Other Agonists

A comparative evaluation of $[Ca^{2+}]_i$ responses to other Ca^{2+} mobilizing vasoactive peptides revealed that PN was the most potent amongst them. Based on the data of EC_{50} values for these agonists, in both control and dexamethasone pretreatment groups, the rank order of potency was: PN > ET-1 > AVP > Ang II (Table 1). The rank order of maximal $[Ca^{2+}]_i$ increase for the control group of cells was: Ang II > ET-1 > AVP > PN (Fig. 4, upper panel). Thus, PN-evoked peak $[Ca^{2+}]_i$ responses were found to be highly sensitive and were exaggerated by dexamethasone pretreatment. Dexamethasone pretreatment significantly increased the peak $[Ca^{2+}]_i$ responses to PN, AVP, and Ang II, whereas it decreased ($P < 0.01$) the responses to ET-1 (Fig. 4, upper panel, and Table 1). The maximal increases in IP_3 levels attained with each of these agonists followed a pattern similar to that of changes in $[Ca^{2+}]_i$ (Fig. 4, lower panel). The addition of PN led to a concentration-dependent biphasic increase in IP_3 levels in both control and dexamethasone treatment groups. Increases in IP_3 generation induced by PN were higher in dexamethasone-pretreated cells. Moreover, the biphasic pattern of IP_3 increase (Fig. 5) followed the same characteristics observed with $[Ca^{2+}]_i$ changes to PN.

In both control and dexamethasone-pretreated groups, addition of either the α-blocker phentolamine or selective blockers of ET-1 (BQ-123), AVP (d$(CH_2)_5$ Tyr (Me) AVP), or Ang II (losartan) failed to diminish the $[Ca^{2+}]_i$ increases in response to PN. However, it was ascertained that all of these agents completely blocked the $[Ca^{2+}]_i$ elevation in response to their respective agonists in both groups (data not shown). These data suggest that PN does

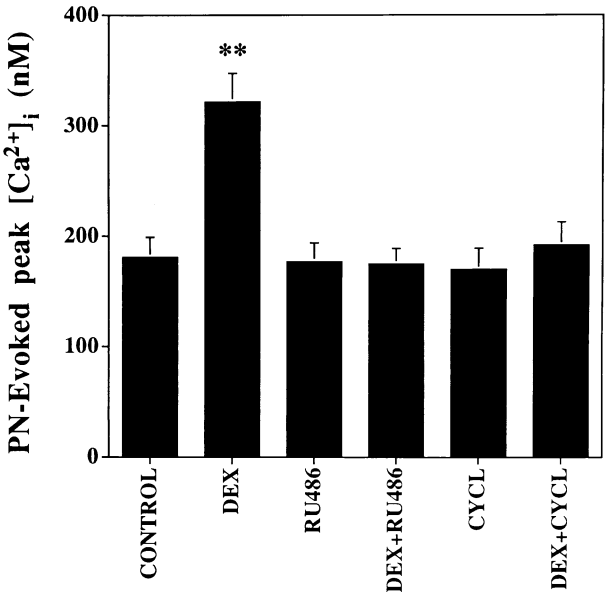

FIG. 3. PN-evoked peak $[Ca^{2+}]_i$ values after inclusion of either the glucocorticoid antagonist RU 486 (1 μM) or the protein synthesis inhibitor cycloheximide (CYCL; 10 μg/mL) in the incubation medium. Agents were included in the culture medium for 24 hr before PN challenge in both control (CONTROL) and dexamethasone (DEX) treated cells. Each data point is the mean ± SEM of 5 separate determinations. Key: (**) $P < 0.01$ compared with the control value.

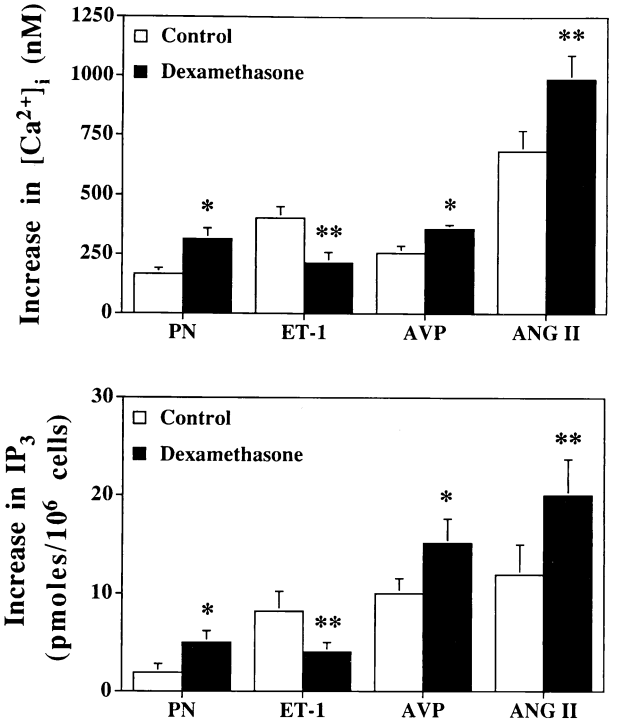

FIG. 4. Comparison of maximal increases in $[Ca^{2+}]_i$ (upper panel) and IP_3 (lower panel) levels to PN, ET-1, AVP, and Ang II in control and dexamethasone-pretreated ASMC. Each point is the mean ± SEM of 3–6 separate determinations. Key: (*) $P < 0.05$ and (**) $P < 0.01$ compared with the control group.

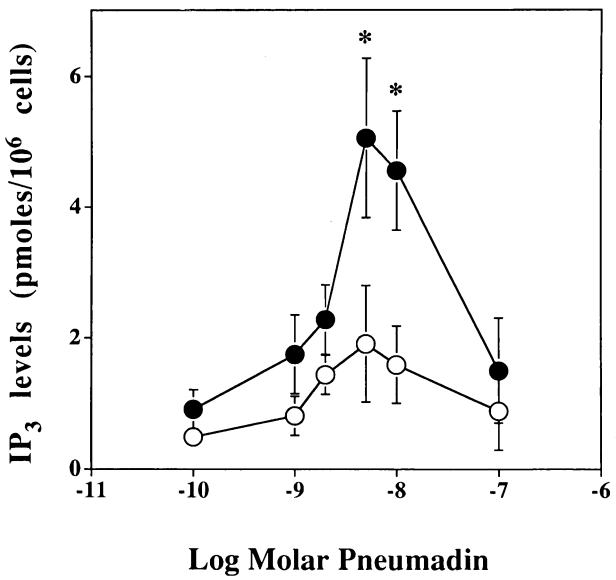

FIG. 5. Relationship between PN concentration and increase in IP_3 attained above basal levels in control (○–○) and dexamethasone-pretreated (●–●) ASMC. Each data point is the mean ± SEM of 5 separate determinations. Key: (*) $P < 0.05$ compared with values at similar data points for the control group.

not interact at cell surface receptors of other agonists in evoking its $[Ca^{2+}]_i$ response. In addition, nifedipine (1 μM) failed to diminish the $[Ca^{2+}]_i$ responses to PN in both control and dexamethasone groups, whereas removal of extracellular Ca^{2+} led to only a partial diminution (to the extent of 20%) of the maximal $[Ca^{2+}]_i$ response to PN in both groups, suggesting that PN predominantly mobilizes Ca^{2+} from the intracellular stores (data not shown).

DISCUSSION

The data reported here indicate that PN evokes an increase in $[Ca^{2+}]_i$ through activation of PLC and the generation of IP_3, and that these events are triggered by activation of specific receptors distinct from those stimulated by Ang II, AVP, ET-1, or α-adrenoceptor agonists. Arguments in favor of this hypothesis are listed below.

PN Actions on Other G-protein Coupled Receptors

The observation of a lack of inhibition of the $[Ca^{2+}]_i$ responses to PN (5 nM) in both control and dexamethasone-pretreated cells in the presence of BQ-123 (ET_A antagonist), losartan (AT_1 antagonist), $d(CH_2)_5$ Tyr (Me) AVP (V_1 selective antagonist), or phentolamine confirmed that PN did not activate ET_A, AT_1, V_1, or α-adrenoceptors present on rat ASMC. These receptor-selective antagonists completely abolished the responses to their respective agonists. Activation of other ET subtype receptors by PN is unlikely since dexamethasone treatment decreased $[Ca^{2+}]_i$ responses to ET-1, whereas the responses to PN were augmented.

Evidence That PN Could Activate Distinct Receptor(s)

Several observations suggest that PN could activate distinct cell surface receptors that are coupled to activation of PLC and generation of IP_3. (a) PN evoked a rapid increase in $[Ca^{2+}]_i$ in a concentration-dependent manner similar to other Gq receptor agonists. (b) The increases in IP_3 levels paralleled the $[Ca^{2+}]_i$ changes to PN. (c) At concentrations above 5 nM, both IP_3 and $[Ca^{2+}]_i$ elevations decreased in a concentration-dependent manner. (d) While nifedipine failed to block the rise in $[Ca^{2+}]_i$ to PN, depletion of extracellular Ca^{2+} led to only partial diminution of the maximal response. These data indicate that PN recruits the release of Ca^{2+} mainly from the intracellular stores. The partial extracellular Ca^{2+} dependence could be secondary to Ca^{2+} influx through nifedipine-insensitive channels in order to replenish the intracellular stores.

It is possible that PN, at its higher concentrations, could activate another effector system such as cyclic GMP (cGMP) either via nitric oxide generation or directly via activation of guanylate cyclase, which could attenuate PLC-mediated IP_3 generation and $[Ca^{2+}]_i$ elevations in ASMC. It has been shown that cGMP could uncouple receptor-PLC mediated events in addition to reducing $[Ca^{2+}]_i$ by promoting Ca^{2+} extrusion [16]. While the present observations provide indirect evidence of a distinct receptor for PN that could activate PLC, characterization of PN specific binding sites by radioligand binding studies and a demonstration of changes in other effectors would be required to adequately characterize the receptor and signaling activated by PN on ASMC.

Dexamethasone Effects on $[Ca^{2+}]_i$ Responses to PN

Glucocorticoid therapy, in addition to immunosuppressive and anti-inflammatory effects, is known to increase blood pressure [7, 8]. Although the exact mechanism(s) underlying glucocorticoid-induced hypertension is poorly understood, enhancement of vascular responsiveness to Ca^{2+} mobilizing agonists has been considered as one of the major contributing factors [7, 8]. Dexamethasone treatment has been shown to up-regulate Ang II and AVP receptors [11, 12]. However, conflicting evidence has been reported with regard to ET-1 receptors and action [10, 17]. The data from the present study reveal that ET-1-evoked $[Ca^{2+}]_i$ and IP_3 responses were down-regulated, whereas the responses to AVP, Ang II, and PN were up-regulated. However, PN-evoked responses differed from these agonists since it showed a biphasic response pattern in both IP_3 and $[Ca^{2+}]_i$ elevation, which was not observed with other agonists. The observation that cycloheximide and RU 486 pretreatment attenuated PN-evoked exaggerated peak $[Ca^{2+}]_i$ responses confirms that glucocorticoid-mediated protein synthesis is involved in the up-regulation of the PN response. These data and the fact that dexamethasone only up-regulates responses to PN without altering the qualitative, concentration-dependent, biphasic nature of the responses suggest

that dexamethasone acts at the level of the PN receptor. We have also ascertained that incubation with high concentrations of PN (100 nM) for several hours failed to alter the adherent ASMC morphology (data not shown). In the present study, PN was added just prior to either $[Ca^{2+}]_i$ or IP_3 measurement. Moreover, the selective blockade of the exaggerated responses to PN seen in dexamethasone-pretreated cells by the inclusion of either RU 486 or cycloheximide confirms that non-specific cell injury induced by PN could not account for the diminution of the peak $[Ca^{2+}]_i$ response.

In summary, the present study provides the first evidence that PN may have pronounced direct vascular actions in conditions characterized by elevated glucocorticoid levels, such as in Cushing's syndrome or during glucocorticoid therapy. At a low concentration of 5 nM, PN enhances $[Ca^{2+}]_i$ levels over 200 nM from the basal level in dexamethasone-treated cells. Although a direct vasoconstrictor response to PN has not been demonstrated yet, our *in vitro* observations with ASMC suggest that this peptide could contribute to an increase in VSM tone and/or vascular hypertrophy. It is not known whether PN-evoked $[Ca^{2+}]_i$ increases observed in the present study occur in the nuclear or cytosolic $[Ca^{2+}]$ compartments. Recent studies have shown that various G-protein activating agonists can produce remarkable increases in nuclear $[Ca^{2+}]$ levels [18–20]. Thus, the significant elevation in $[Ca^{2+}]_i$ and IP_3 levels reported in the present study provides the framework for further investigations to characterize PN specific receptor sites on VSM cells, to examine the effector systems coupled to these receptors, as well as to assess the spatio-temporal characteristics of $[Ca^{2+}]$ changes to PN in various subcellular compartments at the single cell level.

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