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VASOACTIVE INTESTINAL PEPTIDE (VIP) ACTIVATION OF NUCLEAR PROTEIN KINASE C IN PURIFIED NUCLEI OF RAT SPLENOCYTES

NANCY E. ZORN* and DIANE HADDOCK RUSSELL

Department of Pharmacology and Therapeutics, University of South Florida College of Medicine and H. Lee Moffitt Cancer Research Institute, Tampa, FL 33612, U.S.A.

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Abstract—We have examined the actions of vasoactive intestinal peptide (VIP) and certain other known immune modulators on a nuclear pool(s) of protein kinase C (PKC) in isolated rat splenocyte nuclei. Rat splenocyte nuclei pure by enzymatic and electron microscope criteria demonstrated a time- and concentration-dependent activation of nuclear PKC (nPKC) by VIP. A biphasic pattern of three bell-shaped curves was observed with peak phosphorylation at 10^{-15}, 10^{-9} and 10^{-6} M VIP. The phosphorylation of endogenous nuclear substrates was characterized as a PKC-mediated event by use of three known PKC inhibitors, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7), sphingosine, and staurosporine, which produced similar phosphate incorporation measurements. Also, this activity was blocked with the addition of a monoclonal antibody to PKC. Inhibitors of the ability of VIP to activate nPKC included somatostatin, 8-bromo-cAMP, peripheral benzodiazepine receptor modulators, and the PKC inhibitors, sphingosine and staurosporine. These data have direct relevance to our knowledge of cell-mediated immunity.

Vasoactive intestinal peptide (VIP) is a polypeptide comprised of 28 amino acids with a broad tissue distribution. Although its precise physiologic role has yet to be established, VIP may function as a neurotransmitter, hormone, and immunoregulator [1]. Research has indicated that VIP released from nerve endings in the intestine regulates the secretion of water and electrolytes [2]; in the pituitary system, it may serve as a prolactin releasing hormone [3]; and in the immune system, VIP appears to modulate lymphocyte migration, mast cell mediator release, and natural killer cell activity [4–6]. Ottaway and Greenberg [7] have further demonstrated the regulatory role of VIP in immune responses. In the presence of VIP, the *in vitro* response of lymphocytes to the T cell mitogens concanavalin A (Con A) and phytohemagglutinin (PHA) was inhibited.

Specific surface receptors for VIP have been identified on potential target tissues in a number of mammals [2, 8–11]. Recently, nuclear receptors for VIP were detected in a human colonic adenocarcinoma cell line [12]. While a biologic role for these nuclear receptors has not been defined, evidence exists for a direct action in the nucleus [13–15].

Protein phosphorylation may be one biochemical consequence of VIP action [16]. The phosphorylations may be mediated by different kinases. One possibility is the activation of protein kinase C (PKC) which has been shown to mediate a wide variety of cellular responses including lymphocyte activation [17]. PKC has been localized immunochemically and by its calcium and phospholipid activity requirements in liver nuclei [18] and in NIH 3T3 cell nuclei [19]. Recently, nuclear PKC from rat liver nuclei has been partially purified and characterized [20]. This nuclear pool demonstrated a higher calcium requirement for optimal activation.

Substantial literature now demonstrates both the presence of PKC and growth factor receptors in the nucleus in a variety of tissues [13–15, 21–23]. The activation of a nuclear pool(s) of PKC by trophic factors may provide a mechanism by which genetic transcription could be altered rapidly. We have demonstrated recently that prolactin, which is mitogenic and trophic for both liver and spleen cells, activates a nuclear pool of protein kinase C in isolated nuclei [24, 25]. Additionally, nerve growth factor has been shown to result in the phosphorylation of endogenous nuclear substrates in isolated cortical nuclei and this phosphorylation was mediated by PKC.† These observations suggested that VIP might also affect the activation of nuclear PKC in splenocytes. Therefore, the purpose of this investigation was to determine if VIP affects the phosphorylation state of endogenous nuclear substrates in isolated rat splenocytes and if this phosphorylation is mediated by protein kinase C.

MATERIALS AND METHODS

Materials. Male Sprague–Dawley rats (120–150 g) were obtained from Harlan Sprague Dawley, Indianapolis, IN. The polypeptide hormones vasoactive intestinal peptide (VIP), zinc-free insulin, somatostatin, and nerve growth factor (NGF) were purchased from Sigma, St. Louis, MO. In addition, 8-bromo-cAMP, sphingosine sulfate, and 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7)

* Send correspondence to: Dr. Nancy E. Zorn, Department of Pharmacology and Therapeutics, University of South Florida College of Medicine, MDC Box 9, 12901 Bruce B. Downs Blvd., Tampa, FL 33612.

† Weill CL, unpublished data, cited with permission.

were obtained from Sigma. Staurosporine was obtained from Kamiya, Thousand Oaks, CA. Insulin-like growth factor 1 (IGF-1) was acquired from the Calbiochem Corp., San Diego, CA. The anti-PKC monoclonal antibody was purchased from Amersham, Arlington Heights, IL. The benzodiazepene R05-4864 was supplied by Dr. Peter Sorter of Hoffmann-LaRoche, Nutley, NJ, and PK 11195 by Dr. R. Michaud of Pharmuka Laboratory, Genevilliers, France.

Preparation of splenocyte nuclei. Rats were killed by cervical dislocation. The spleens were excised and cells obtained by teasing apart the splenic tissue with ground-glass microscope slides and forceps in Hanks' balanced salt solution plus 0.3% bovine serum albumin. The cellular suspension was rocked gently for 10 min at 4°, filtered through a nylon cloth, and centrifuged at 300 *g* for 10 min at 4°. The cell pellet was resuspended at a concentration of 3×10^7 cells/mL and incubated in a hypotonic buffer containing 0.4 mM KH_2PO_4, pH 6.7, 2.5 mM $MgCl_2$, 0.5 mM ethyleneglycolbis(aminoethylether)tetraacetate (EGTA), 0.1 mM spermine and 0.1% Triton X-100 (buffer A). After 1 min, the preparation was filtered and the sucrose concentration was adjusted to 0.32 M with buffer A containing 1.6 M sucrose and centrifuged at 300 *g* for 10 min at 4°. The pellet was resuspended at a concentration of 1×10^7 cells/mL in a buffer containing 0.4 mM KH_2PO_4, pH 6.7, 2.5 mM $MgCl_2$, 0.32 M sucrose, 0.5 mM EDTA, 0.1 mM spermine, 0.1% Triton X-100, 1.0 mM phenylmethylsulfonyl fluoride (PMSF), and 25 μg/mL of leupeptin and homogenized with 15 strokes of the A-pestle using a glass Dounce homogenizer. The homogenate was centrifuged at 450 *g* for 15 min at 4°. The nuclear pellet was washed with 25–50 mL of incubation buffer (40 mM Tris–HCl, pH 8.0, 0.25 M sucrose, 1.0 mM dithiothreitol, 4 mM $MgCl_2$, and 20 μM (NaF) and centrifuged at 450 *g* for 15 min at 4°. The nuclear pellet was subsequently resuspended at a concentration of 2×10^7 nuclei/mL in incubation buffer. Purity of the nuclear preparation was verified by the absence of 5′-nucleotidase activity and by electron microscopy [25].

PKC assay using endogenous nuclear protein as substrates for phosphorylation. This assay was developed in our laboratory with calcium and ATP concentrations as well as incubation temperatures and times adjusted for optimal stimulation of nuclear PKC in isolated splenocyte nuclei. Splenic nuclei (3×10^6 nuclei/250 μL) in incubation buffer were preincubated for 2 min at 37° in the presence and absence of 6 μM H-7. The reaction was initiated by the addition of the hormone or drug to be tested in the presence of 0.5 mM $CaCl_2$ and 10 μM ATP (containing 2.5 to 3×10^6 cpm [γ-^{32}P]ATP). The reaction was terminated after 10 sec by the addition of 3.0 mL of 25% trichloroacetic acid (TCA). Endogenous nuclear proteins served as the substrates for phosphorylation. To examine the effect of modifiers and/or inhibitors on VIP-stimulated PKC activity, the modifier or inhibitor was added to the nuclei preparation either simultaneously with VIP or 1 min prior to VIP initiation.

Nuclear precipitate was collected on glass fiber filters with a cell harvester (Brandel Research and Development Laboratories, Gaithersburg, MD). The filters were washed three times with 10% TCA and dried, and radioactivity was counted by liquid scintillation spectroscopy. Enzyme activity was determined by subtracting the amount of ^{32}P incorporated into endogenous protein substrates in the presence of H-7 from that incorporated in the absence of H-7.

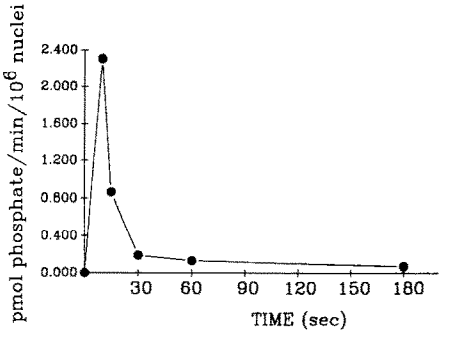

Fig. 1. Rate of VIP activation of nuclear protein kinase C activity. Rat splenic nuclei were incubated in the presence of 10^{-10} M VIP at 37° for the times indicated, and the protein kinase C activity was assayed in triplicate as described in Materials and Methods. A representative experiment is shown. Data were normalized to per minute per 10^6 nuclei. This experiment was repeated three times with equivalent results. The basal (non-VIP stimulated) amount of ^{32}P incorporated into endogenous proteins was 0.08 pmol and the phosphorylation (without H-7) that could be attributed to PKC was 3.5%. VIP induced phosphorylation by 22% (without H-7), and this increase was totally attributed to PKC.

Statistical analysis. Statistical differences between treatments were determined by an ANOVA followed by Fisher's LSD test for multiple comparisons or by Student's *t*-test for unpaired data. Differences were considered significant at the 95% confidence level.

RESULTS

Time course for VIP activation of PKC in rat splenic nuclei. Activation of PKC in isolated splenocyte nuclei was measured as a function of time from 0.12 to 3 min. The maximum incorporation of ^{32}P into endogenous substrates occurred within 10 sec after the addition of VIP compared to vehicle controls (Fig. 1). VIP increased phosphorylation by 22% over vehicle controls and this increase was totally attributed to PKC. A decline in the rate of phosphate incorporation was subsequently observed at 15 sec with a return to basal level within 1 min. Thus, assay conditions of 10 sec, 37°, and 3×10^6 nuclei were used throughout these studies.

Concentration dependency of VIP-stimulated rat splenic nuclear PKC activity. PKC activity was assayed in intact purified splenic nuclei by the addition of VIP plus [γ-^{32}P]ATP and calcium in the presence and absence of a known PKC inhibitor, H-7. The concentration response exhibited was complex, displaying three biphasic peaks at 10^{-15}, 10^{-9} and 10^{-6} M VIP (Fig. 2). Maximal phosphate incor-

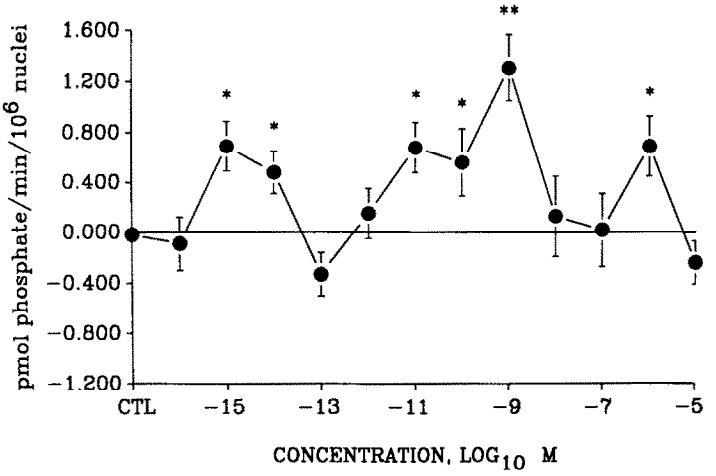

Fig. 2. Concentration response for VIP activation of nuclear PKC in rat splenic nuclei. Concentration-dependent activation of nuclear protein kinase C in rat splenocyte nuclei by VIP using endogenous substrate as described in Materials and Methods. The data are the means ± SE of three separate experiments and are normalized to per minute per 10^6 nuclei. Key: Significant stimulation over basal: * $P < 0.05$, and ** $P < 0.001$.

poration was observed at 10^{-9} M VIP ($P < 0.001$). However, levels of phosphorylation were statistically different from background at 10^{-15}, 10^{-14}, 10^{-11}, 10^{-10} and 10^{-6} M VIP. Furthermore, a phorbol ester known to directly activate PKC, phorbol 12-tetradecanoate-13-acetate (PTA), produced maximal stimulation at 10^{-11} M PTA, whereas the inactive form, phorbol 12-myristate 13-acetate 4-0 methyl ether did not stimulate nuclear PKC (unpublished observations).

Properties of protein kinase C in nuclear kinase activity. The VIP-stimulated incorporation of ^{32}P into endogenous nuclear substrates as mediated by protein kinase C was examined by using three different known PKC inhibitors, H-7, sphingosine, and staurosporine. PKC activity was assessed in intact purified splenic nuclei by the addition of VIP (10^{-9} M) in the presence and absence of the known PKC inhibitors. Enzyme activity was determined by subtracting the amount of ^{32}P incorporated into endogenous nuclear substrates in the presence of each inhibitor from the amount of ^{32}P incorporated in the absence of each inhibitor. There was no difference in the VIP-stimulated incorporation of ^{32}P using any of these three PKC inhibitors (Table 1). Thus, the assay conditions assessed nPKC activity by measuring the level of phosphorylation in the presence and absence of H-7.

Effects of known inhibitors of protein kinase C, various immune modulators, and anti-PKC monoclonal antibody on VIP-stimulated PKC activity in isolated rat splenic nuclei. A peripheral benzodiazepine agonist, R05-4864, and antagonist, PK 11195, although not active by themselves in stimulating nuclear PKC activity, blocked the ability of 10^{-9} M VIP to activate nuclear PKC (Table 2). In addition, the cAMP analog, 8-bromo-cAMP, did not stimulate nPKC phosphorylation by itself, and when added simultaneously with VIP, totally blocked VIP-stimulated incorporation of ^{32}P into endogenous substrates.

When either sphingosine or staurosporine was added to the assay concurrently with VIP (10^{-9} M), the VIP-stimulated phosphate incorporation into endogenous substrates was blocked (Table 2). In this case, either sphingosine or stauroporine was added to all tubes to assess their abilities to block the VIP-mediated incorporation of ^{32}P into endogenous nuclear substrates as measured in the presence and absence of H-7.

In addition, PKC activity was evaluated using a monoclonal antibody to PKC. The level of VIP-stimulated phosphorylation was greatly attenuated with the addition of the PKC antibody (Table 2). To verify that the antibody portion of this agent had blocked the action of VIP, a normal immunoglobin of the correct class was included. As shown, the addition of IgG_{2a} did not affect the VIP-stimulated phosphorylation events. This further substantiates a PKC-mediated event within the splenic nucleus as a result of VIP stimulation.

Effects of other hormones and growth factors on VIP-stimulated PKC activity in isolated rat splenic nuclei. Although both VIP and somatostatin elevated nuclear PKC activity, somatostatin preincubated for 1 min with the nuclei totally blocked the ability of 10^{-9} M VIP to stimulate nPKC (Table 3). While both VIP and IGF-I (10^{-12} M) elevated nuclear PKC activity significantly, the simultaneous addition of these two hormones resulted in attenuation of the incorporation of ^{32}P into endogenous proteins. This decrease in nPKC activity was also observed when VIP was combined with insulin (10^{-8} M) and NGF (10^{-10} M).

DISCUSSION

It has been suggested that VIP plays an important

Table 1. Characterization of phosphate incorporation in isolated rat splenic nuclei as mediated by protein kinase C

Treatment	PKC activity (pmol phosphate/min/10^6 nuclei)
Vehicle control	-0.19 ± 0.08
VIP (10^{-9} M)	
−/+ H-7 (6 μM)	1.31 ± 0.26*
−/+ Staurosporine (3.5 nM)	1.41 ± 0.05*
−/+ Sphingosine (20 μM)	1.53 ± 0.27*

Rat splenic nuclei were incubated for 10 sec at 37° with 10^{-9} M VIP in the presence and absence of the PKC inhibitors, H-7, staurosporine, or sphingosine at the concentrations shown. PKC activity was determined in triplicate by subtracting the amount of ^{32}P incorporated into endogenous nuclear substrates in the presence of inhibitor from the amount of ^{32}P incorporated in the absence of inhibitor. Data are the means ± SE of four separate experiments.

* Significant stimulation over basal: $P < 0.025$.

Table 2. Effects of known inhibitors of protein kinase C (PKC), various immune modulators, and anti-PKC monoclonal antibody on VIP-stimulated nuclear phosphorylation in isolated rat splenic nuclei

Treatment	PKC activity (pmol phosphate/min/10^6 nuclei)
Vehicle control	-0.07 ± 0.03
VIP (10^{-9} M)	1.31 ± 0.26*
+R05-4864 (10^{-10} M)	-0.02 ± 0.03†
+PK 11195 (10^{-10} M)	0.05 ± 0.19
+R05-4864 (10^{-10} M) + PK 11195 (10^{-10} M)	-0.01 ± 0.12
+8-Bromo-cAMP (10^{-5} M)	-0.07 ± 0.03‡
+Sphingosine (20 μM)	-0.01 ± 0.23
+Staurosporine (7 nM)	-0.09 ± 0.16
+Anti-PKC monoclonal Ab	0.04 ± 0.09‡
+Normal immunoglobin (IgG_{2a})	1.19 ± 0.09

Rat splenic nuclei were incubated for 10 sec at 37° with 10^{-9} M VIP in the presence and absence of the inhibitory substance at the concentration indicated. The assay was run by adding the inhibitory substance simultaneously with VIP. The monoclonal antibody to PKC was preincubated with nuclei for 2 min prior to the addition of VIP at a concentration of 0.25 μg/mL. PKC activity was determined in triplicate as the incorporation of radiolabeled phosphate into nuclear substrates in the presence and absence of H-7. Data are the means ± SE of four separate experiments.

* Significant stimulation over basal; $P < 0.025$.

†‡ Significant inhibition of VIP-stimulated PKC activity: † $P < 0.05$, and ‡ $P < 0.025$.

role in immune function through modulation of lymphocyte function [26]. VIP has been shown to inhibit the *in vitro* response of lymphocytes to the mitogens Con A and phytohemagglutinin [7] and significantly inhibits the NK cell activity of normal human peripheral blood mononuclear cells on K562 targer cells [27]. Thus, these studies suggest VIP receptors are present on a subset of lymphocytes. While the biological effect of VIP varies according to the target organs, its actions are thought to be mediated by alterations in adenyl cyclase activity and cyclic AMP concentration [10].

Previously, VIP receptors have been identified in the nucleus [12] suggesting a role for VIP in altering genetic transcription. Recently, a protein with the characteristics of PKC has been isolated from rat liver nuclei [18, 20]. Activation of a nuclear pool(s) of protein kinase C may therefore be a common target for hormones and growth factors which affect the trophic state of cells. The present studies characterize VIP activation of a nuclear kinase activity in isolated rat splenocyte nuclei. This kinase activation by VIP was both time and concentration dependent, with a maximal response at 1 nM VIP which is a concentration in the range of the reported peripheral blood concentrations of VIP (less than 10^{-11} M) for mammals [7] and the K_D for VIP binding to nuclear receptors in adenocarcinoma cells [12]. Kinase activation by VIP was abolished by sphingosine, a precursor of sphingomyelin and

Table 3. Effects of various modifiers on VIP-stimulated protein kinase C (PKC) activity in isolated rat splenic nuclei

Treatment	PKC activity (pmol phosphate/min/10^6 nuclei)
Vehicle controls	-0.07 ± 0.03
VIP (10^{-9} M)	1.31 ± 0.26*
Somatostatin (10^{-10} M)	0.16 ± 0.01†
VIP (10^{-9} M) + somatostatin (10^{-10} M)	-0.20 ± 0.04§
IGF-I (10^{-12} M)	0.54 ± 0.12‡
VIP (10^{-9} M) + IGF-I (10^{-12} M)	-0.30 ± 0.18‖
Insulin (10^{-8} M)	0.49 ± 0.04†
VIP (10^{-9} M) + insulin (10^{-8} M)	-0.05 ± 0.12‖
NGF (10^{-10} M)	0.38 ± 0.05†
VIP (10^{-9} M) + NGF (10^{-10} M)	-0.01 ± 0.04¶

Rat splenic nuclei were incubated for 10 sec at 37° with the indicated hormones or growth factors at the concentrations shown. PKC activity was determined in triplicate as the incorporation of radiolabeled phosphate into nuclear substrates in the presence and absence of H-7, as indicated in Materials and Methods. Hormones were combined with VIP and added simultaneously except for somatostatin which was preincubated with the nuclei 1 min prior to VIP addition. Data are expressed as the means ± SE of four separate experiments.

*–‡ Significant stimulation over basal; * $P < 0.025$, † $P < 0.001$, and ‡ $P < 0.005$.

§–¶ Significant inhibition of VIP-stimulated PKC activity: § $P < 0.01$, ‖ $P < 0.05$, and ¶ $P < 0.025$.

gangliosides that inhibits protein kinase C [28, 29] and staurosporine, a bacterial protein kinase C inhibitor [30]. PKC activity was abolished also by the addition of a monoclonal antibody to PKC. Therefore, these data suggest that isolated rat splenic nuclei contain a kinase activity with pharmacological properties of PKC.

While insulin, NGF, and IGF-I caused stimulation of a nuclear pool(s) of PKC, co-addition of these hormones with VIP resulted in attenuation of the phosphate incorporation. This would suggest a possible mechanism for control within the nucleus. We have demonstrated recently that the ability of the known hepatic mitogen, prolactin, to activate nuclear PKC in rat splenic nuclei is blocked by the presence of VIP [25]. Thus, VIP may be an important adversary to the mitogens of immune function. These agents may counteract the phosphorylation patterns and thus serve to perform on/off functions. This modulation may occur by stimulation of different pools of guanine nucleotide-binding proteins which has been demonstrated in both pituitary lactotrophs and lymphoblasts in which somatostatin antagonizes the effect of VIP [1, 31, 32]. However, additional research is needed to elucidate the exact mechanism of action for these agents.

Thus, the evidence presented here supports a role for VIP in isolated rat splenic nuclei. After VIP binds to its cytoplasmic surface receptor, it may become internalized and subsequently interact with a nuclear VIP receptor. The activation of nuclear protein kinase C by VIP is a new concept since past evidence has supported a cAMP activation. A nuclear action is attractive since it provides a mechanism for new genetic transcription and, thus, rapidly alters immune modulation. Further study is necessary to determine the biochemical events which occur as a consequence of nuclear PKC activation.

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