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Vasoactive intestinal peptide increases intracellular calcium in astroglia: Synergism with α-adrenergic receptors

(astrocytes/vasoactive intestinal peptide receptors)

ALESSANDRO FATATIS†, LYNNE A. HOLTZCLAW*, RINA AVIDOR*, DOUGLAS E. BRENNEMAN*, AND JAMES T. RUSSELL*

*Laboratory of Developmental Neurobiology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892; and †Department of Pharmacology, II School of Medicine, University of Naples, Italy

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ABSTRACT **In type I astrocytes from rat cerebral cortex, vasoactive intestinal peptide (VIP) at concentrations below 1 nM evoked an increase in intracellular calcium ion concentration. This response, however, was observed in only 18% of the astrocytes examined. α-Adrenergic stimulation with phenylephrine or norepinephrine also resulted in an intracellular calcium response in these cells and the threshold sensitivity of astrocytes to phenylephrine was vastly different from cell to cell. Treatment of these astrocytes with VIP (0.1 nM) together with phenylephrine at subthreshold concentrations produced large increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and oscillations. The continued occupation of the α-adrenergic receptor was required for sustained synergism. Both α-receptor stimulation and stimulation with the mixture of agonists induced the cellular calcium response by triggering release of calcium from cellular stores, since the response persisted in the absence of extracellular calcium. Furthermore, thapsigargin pretreatment, which depletes intracellular stores, abolished the agonist-induced $[Ca^{2+}]_i$ response. VIP (0.1 nM) and phenylephrine were found to increase cellular levels of inositol phosphates; however, there was no apparent additivity in this response when the agonists were added together. These observations suggest a calcium-mediated second messenger system for the high-affinity VIP receptor in astrocytes and that α-adrenergic receptors act synergistically with the VIP receptor to augment an intracellular calcium signal. The synergism between diverse receptor types may constitute an important mode of cellular signaling in astroglia.**

Vasoactive intestinal peptide (VIP) is a widely distributed neuropeptide with a broad array of biological functions (1). Specific receptor sites for VIP have been demonstrated in various regions of rat brain (2, 3), and glial cells in particular have been shown to possess receptors, as shown by binding studies (4). More recently, two separate receptor systems for VIP in the central nervous system have been observed: one is a low-affinity receptor (K_d = 10 nM), and another is a high-affinity receptor (K_d = 30 pM) (5). The low-affinity receptor is coupled to the adenylate cyclase system as shown by the VIP-stimulated cAMP increase observed in various areas of the central nervous system (6, 7). Stimulation of VIP receptors on glial cells from rat brain and Schwann cells from squid giant axon was found to have significant effects on glycogenolysis, morphology, and physiology (8–10). The mitogenic and secretagogue effects of VIP, on the other hand, were elicited only at low concentrations (1–100 pM) of peptide, amounts that are 1000–10,000 times lower than concentrations required to stimulate adenylate cyclase and thereby increase cAMP in astrocytes. The coupling of the astroglial high-affinity VIP receptor, which has been shown to be important in the release of neurotrophic substances (11), is hitherto unidentified. In one study, low concentrations of VIP were shown to activate protein kinase C in nuclei isolated from the rat hippocampus (12). In the present work, we have examined the possibility that stimulation of the high-affinity VIP receptor on glial cells might result in calcium mobilization, with the idea that elevations of calcium might mediate the VIP-induced astroglial mitogenic effect and/or the release of neurotrophic substances from glial cells. In addition, we have examined the synergistic interaction between these VIP receptors and α-adrenergic receptors.

MATERIALS AND METHODS

Astrocyte Cultures. Astrocytes were isolated from 1- to 3-day-old rat cerebral cortices as described by McCarthy and de Vellis (13). Briefly, tissue was dissociated with trypsin in the presence of DNase and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mM glutamine, and 2.5 μg of Fungizone per ml. Cells were plated in 250-ml plastic flasks and upon confluency (10 days) were vigorously shaken to remove nonadherent cells. The tightly adherent type I astrocytes were then collected by trypsin treatment and plated on 25-mm number 1 glass coverslips and were used in experiments between 4 and 6 days in culture. The cultures were maintained at 37°C in 5% CO_2/95% air. Immunocytochemical labeling in parallel experiments with anti-GFAP (glial fibrillary acidic protein) antibody showed that >95% of the cells in the cultures consisted of type I astrocytes, and very little contamination with neuronal and oligodendroglial cells was observed. Contamination of cultures with type II astrocytes was <2% as described (13).

Intracellular Calcium Measurements. Astrocytes grown on coverslips were placed in a balanced salt solution of the following composition: 159 mM NaCl, 4.5 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM Hepes (pH 7.32), 10.0 mM dextrose, 2 mg of bovine serum albumin per ml (osmolality, 320 mosmol/kg). Cells were loaded with fura-2 as described (14). The average fura-2 concentration in cells was determined spectrofluorometrically after Triton X-100 lysis to be between 50 and 100 μM. Fura-2 fluorescence was imaged with an inverted microscope on a vertical optical bench using a Nikon ×40/1.3 numerical aperture, CF fluor DL objective lens. A complete description of the imaging instrumentation and calibration procedure has been published (14–16). The ratioing procedure corrected satisfactorily for intracellular heterogeneity in fura-2 distribution and cell shape.

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Abbreviations: VIP, vasoactive intestinal peptide; TCA, trichloroacetic acid; IP_3, inositol 1,4,5-trisphosphate; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; G protein, GTP-binding protein.

Measurement of Inositol Phospholipids. Astrocytes (0.2×10^6 cells) were plated on 35-mm dishes in minimal essential medium (MEM) with 10% fetal calf serum. Twenty-four hours after plating, fresh medium was added plus 1 μCi of *myo*-[^{3}H]inositol (1 Ci = 37 GBq). After 3 days of incubation, the cultures were washed twice in MEM plus 20 mM Hepes (pH 7.4; without fetal calf serum). After 1 hr of additional incubation at 37°C, fresh medium was added along with 10 mM LiCl. Cultures were treated for 30 min with VIP (0.1 nM) and/or phenylephrine (1 μM). The medium was aspirated and 0.7 ml of ice-cold 10% trichloroacetic acid (TCA) was added to each dish and incubated for 30 min at 4°C. TCA was extracted with a 3:1 solution for 1,1,2-trichloro-1,2,2,2-trifluoroethane and trioctylamine (17). Inositol 1-phosphate was separated on an ion-exchange column (AG1-XS, formate form; Bio-Rad; ref. 18). For measurement of inositol 1,4,5-trisphosphate (IP_3), cultures were maintained as described above. Agonists were added for 1–3 min and then terminated by aspiration and addition of ice-cold 10% TCA. IP_3 was assayed with a radioreceptor assay kit (DuPont/NEN) as described (19).

RESULTS

Calcium Transients Evoked by VIP and Adrenergic Agonists. Very low concentrations (0.1 nM) of VIP evoked an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in type I astrocytes grown on coverslips. The amplitude of these increases, however, was small but significant and was observed in 18% of the 218 cells examined in 28 separate trials (Fig. 1*A*). In all responding cells, the $[Ca^{2+}]_i$ increase was elicited by VIP at concentrations as low as 1 pM and up to 0.2 nM and the responses began after a latent period of ≈100 s.

α-Adrenergic stimulation with norepinephrine or phenylephrine also resulted in an increase in $[Ca^{2+}]_i$ in astrocytes and the threshold sensitivities were significantly different from cell to cell (Fig. 1 *B* and *C*) as reported (20). These responses began promptly upon addition of the α-agonist, and the temporal characteristics of responses elicited by phenylephrine were consistently different from norepinephrine-induced responses. In the case of VIP alone and with phenylephrine, frequently the increase in cytoplasmic calcium spread within cells like waves, and these waves propagated between cells.

Synergism Between VIP and α-Adrenergic Receptors. To determine whether a synergism between VIP receptors and α-adrenergic receptors existed (8, 21, 22) in cortical astrocytes, the effect of adding VIP (0.1 nM) together with phenylephrine (0.2 μM) was studied. Unlike with VIP alone, most cells (85%) responded to this mixture of agonists (Fig. 1*D*). Identical results were obtained when subthreshold concentrations of norepinephrine were used as the agonist (data not shown). Because of the marked difference in the threshold sensitivity of astrocytes to α-adrenergic stimulation (ref. 20; unpublished data), it was essential that the effect of VIP and phenylephrine together be tested in cells whose response to phenylephrine had been determined. Two different experimental paradigms were used. In both the experiments, the cells were initially tested for their sensitivity to phenylephrine and then were challenged with either a mixture of VIP and phenylephrine (Fig. 2*A*) or VIP alone and later a mixture of two agonists (Fig. 2*B*). The results showed that phenylephrine, at subthreshold concentrations, when applied together with VIP (0.1 nM) caused a maximal response in $[Ca^{2+}]_i$. The threshold concentration of phenylephrine was different from cell to cell. In control experiments, two successive applications of phenylephrine or norepinephrine by themselves were tested. In these experiments, the second application was similar to or lower than the first, suggesting that the responsiveness of cells was not increased by prior application of the same agonist. Similarly, previous stimulation with α-adrenergic agonist did not influence a subsequent application of VIP. The results outlined above demonstrated that while either agonist present alone may produce little change in $[Ca^{2+}]_i$, when present together they elicit a near-maximal cellular response.

Three major differences were observed when a mixture of the two agonists was present compared with VIP alone: (*i*) most cells (85%) now responded with increases in $[Ca^{2+}]_i$; (*ii*) the increases were larger in magnitude; and (*iii*) the responses

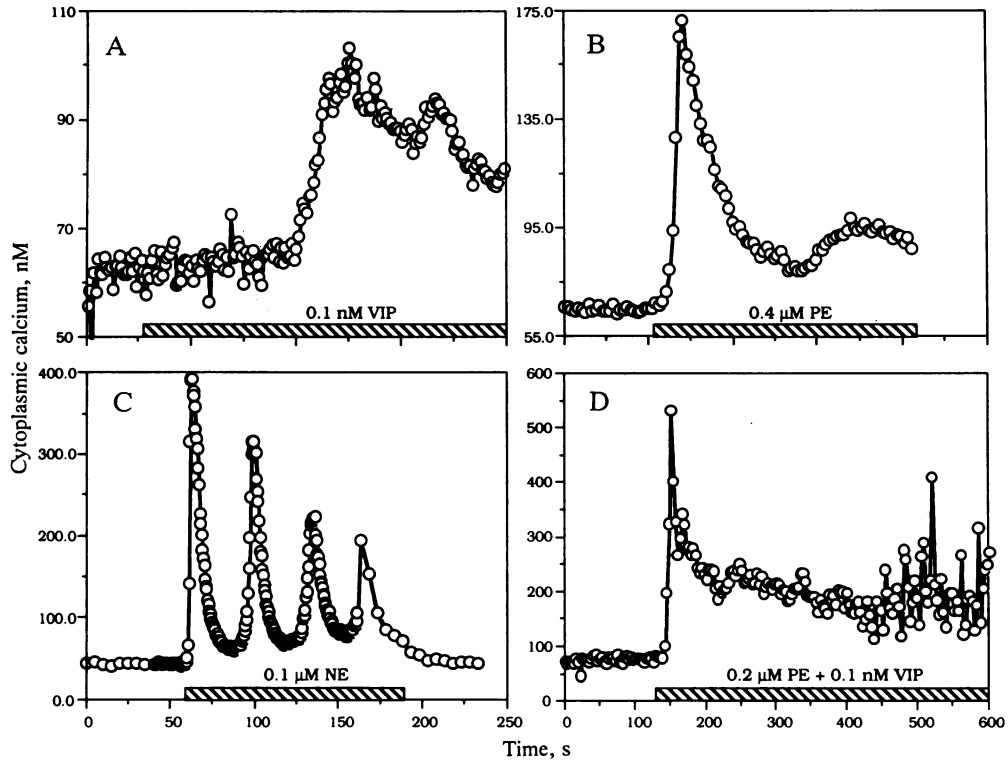

FIG. 1. Agonist-induced changes in $[Ca^{2+}]_i$ in cultured cortical astrocytes. Astrocytes on glass coverslips were perfused with a balanced salt solution containing VIP (*A*), phenylephrine (PE) (*B*), norepinephrine (NE) (*C*), or a mixture of PE and VIP (*D*) as shown by hatched bars. Fluorescence imaging was carried out as described (14). All four treatments resulted in a significant increase in $[Ca^{2+}]_i$. Note that the amplitude of the response was larger when VIP and PE were present together (*D*) compared with either agonist alone (*A* or *B*).

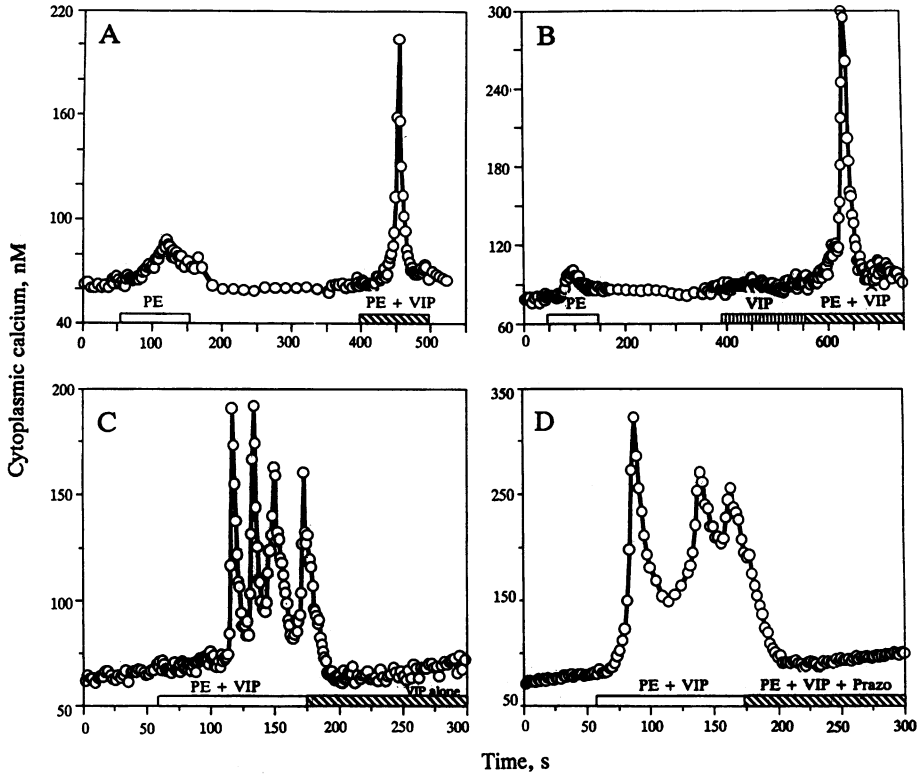

FIG. 2. Synergism between VIP and phenylephrine (PE) induced increases in $[Ca^{2+}]_i$. Cells were initially tested for threshold sensitivity to PE (open bars) and subsequently challenged with a mixture of VIP and PE (hatched bars) (*A*) or VIP alone (vertical hatched bar) followed by the mixture (*B*). PE concentration was 0.75 μM in *A* and 0.8 μM in *B*. VIP concentration was 0.1 nM when used alone or when mixed together with the threshold concentration of PE, which differed from experiment to experiment. Note that in each case, while PE or VIP alone produced minimal responses, the agonist mixture elicited maximal changes in $[Ca^{2+}]_i$. (*C* and *D*) Effects of removal or antagonism of α_1-receptor stimulation during dual receptor stimulation. In these experiments, astrocytes were stimulated with VIP (0.1 nM) and PE (0.2 μM) in a mixture, and after 200 s either PE was removed (*C*) or prazosin (Prazo) (0.5 μM) was added (*D*). Note that under both conditions, the $[Ca^{2+}]_i$ signal elicited by the mixture was inhibited. PE alone at 0.2 μM did not elicit a response in these cells.

began promptly on addition of the agonists without a latent period, unlike when VIP was added alone (see Fig. 1*A*). Thus, phenylephrine, when present with VIP, lowered the threshold and altered the character of the responses, suggesting synergism between the two receptor types. The synergism between VIP receptor activation and adrenergic receptors was specific to α-adrenergic receptors, since addition of isoproterenol (1 μM) by itself and in combination with VIP (0.1 nM) was ineffective in causing any change in $[Ca^{2+}]_i$ in astrocytes (data not shown).

The $[Ca^{2+}]_i$ response caused by the mixture of VIP and phenylephrine depended on the continued occupation of the α-adrenergic receptor. In two sets of experiments, the effect of the removal of phenylephrine or addition of an α-receptor antagonist on the $[Ca^{2+}]_i$ response elicited by a mixture of VIP and phenylephrine was tested. Cells were first challenged with phenylephrine alone to determine the threshold sensitivity (data not shown) and then exposed to the same concentration of the agonist together with 0.1 nM VIP. The increase in $[Ca^{2+}]_i$ and the oscillations caused by VIP and phenylephrine were almost completely abolished upon removal of phenylephrine and continued perfusion with VIP alone (Fig. 2*C*). Similarly, addition of an α_1-receptor antagonist, prazosin (0.26 μM) in the presence of VIP and phenylephrine also abolished the cellular calcium increase caused by the agonists (Fig. 2*D*).

Mechanism of Receptor Effector Coupling. The $[Ca^{2+}]_i$ response in astrocytes to a mixture of VIP and phenylephrine or phenylephrine alone persisted in the complete absence of extracellular calcium. In one set of experiments, calcium was removed from the perfusion medium without addition of EGTA. The response to stimulation with a mixture of VIP and phenylephrine or α-adrenergic stimulation alone was still observed and the amplitudes of the responses were smaller (data not shown). In another set of experiments, the medium was changed to one without calcium and containing 10 μM EGTA (pCa 8.5). These cells were challenged with the agonist mixture or phenylephrine alone after 3 min (Fig. 3*A*). Under these conditions, both treatments resulted in a spike-like increase in $[Ca^{2+}]_i$ without the sustained elevation seen in normal solution. In calcium-free solutions, VIP alone produced transient responses of reduced amplitude in 16% of 48 cells (data not shown).

In another set of experiments, astrocytes were initially tested for agonist responses under control conditions and then exposed to 20 μM thapsigargin in the absence of extracellular calcium (1 mM EGTA). Thapsigargin, a sesquiterpene lactone tumor promoter, depletes intracellular calcium stores by specifically inhibiting the intracellular calcium transport ATPase (23). The addition of thapsigargin after a 7-min exposure to a calcium-free EGTA-containing medium caused a $[Ca^{2+}]_i$ increase, presumably due to release of calcium from the cellular stores (Fig. 3*B*). Subsequent challenge with phenylephrine did not elicit the characteristic changes in $[Ca^{2+}]_i$. Similar results were obtained in experiments in which the agonists were added together. These experiments clearly demonstrated that the agonist response originates from release of calcium from a thapsigargin-depletable intracellular calcium store.

To determine whether a pertussis toxin-sensitive GTP-binding protein (G protein) is involved in this dual receptor-mediated effect, astrocytes were treated with pertussis toxin (1 μg/ml) for 16 hr and challenged with VIP (0.1 nM) and phenylephrine (0.2 μM). In these cells, the response to either phenylephrine alone or a mixture of VIP and phenylephrine remained unchanged from untreated controls (data not shown). Thus, the receptor systems were not coupled to intracellular calcium response by a pertussis toxin-sensitive G protein.

In three separate trials, addition of 1 mM 8-Br-cAMP did not show any measurable changes in $[Ca^{2+}]_i$. Furthermore, previous experiments from this laboratory have established that VIP at the concentration ranges used (<1.0 nM) here does not increase cAMP levels in type I astrocytes (5, 7). Therefore, unless small local changes in cAMP induced by this low concentration of VIP went undetected, the VIP-mediated changes in $[Ca^{2+}]_i$ are likely not due to an increase in cellular cAMP levels. The possible involvement of phosphatidylinositol turnover in the VIP- and phenylephrine-mediated increase in $[Ca^{2+}]_i$ was examined. The cellular

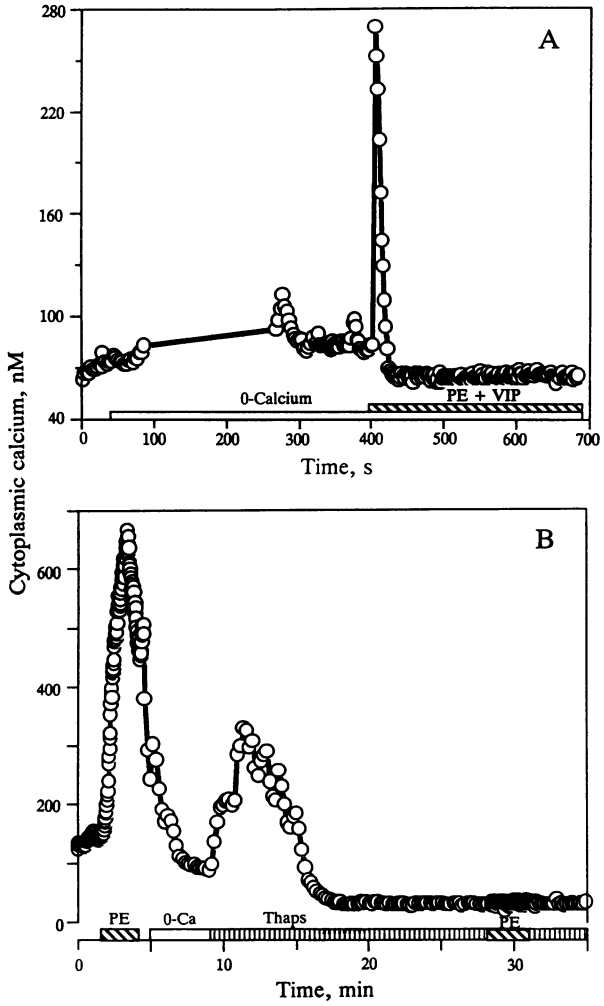

FIG. 3. Effect of removal of calcium and emptying intracellular calcium stores on responses elicited by phenylephrine (PE) alone or together with VIP. (*A*) At 30 s after the onset of the experiment, the perfusion medium was changed to one without calcium but with 10 μM EGTA (open bar). Agonist addition (0.1 nM VIP plus 0.2 μM PE) is depicted by the hatched bar. Note that the response persisted in the complete absence of extracellular calcium. (*B*) Effect of exposure to thapsigargin (Thaps) on agonist-induced changes in $[Ca^{2+}]_i$. In this experiment, a control exposure to PE (1.0 μM) produced a robust response. Then the cells were exposed to a medium containing no calcium but 1 mM EGTA (open bar) followed by the same medium containing 2 μM Thaps (vertical hatched bar). The cells were again challenged with the same concentration of PE in EGTA-containing medium with Thaps (hatched bar). Note that while the initial exposure to PE elicited the typical increase in $[Ca^{2+}]_i$, after Thaps treatment the cells were refractory to PE.

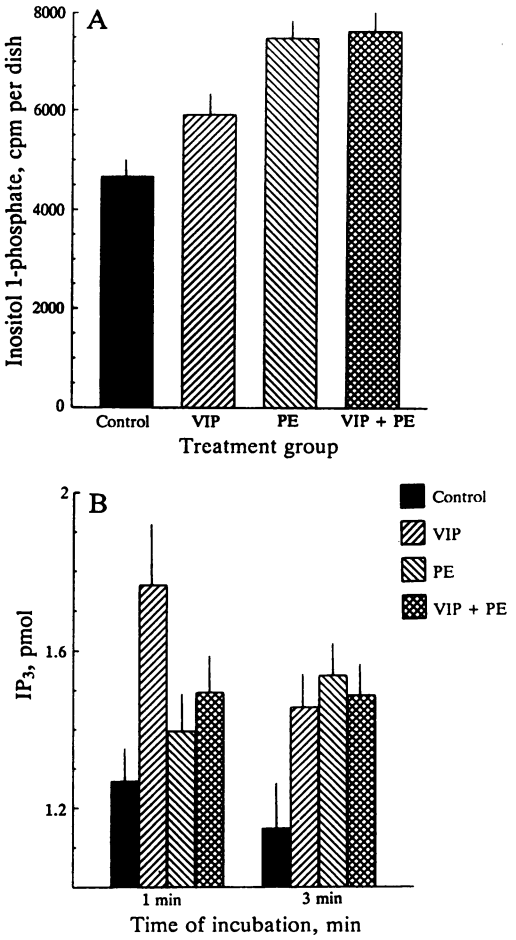

FIG. 4. Agonist-induced increase in inositol phospholipid turnover in astrocytes. (*A*) Effect of VIP and phenylephrine (PE) on inositol 1-phosphate levels in astrocytes labeled with *myo*-[^{3}H]inositol. Cultures were incubated with the agonist for 30 min and the inositol phospholipids were extracted as described. After separation on ion-exchange gels, the amount of radioactivity recovered in the fraction eluting with inositol 1-phosphate was compared in cultures treated with VIP, PE, and VIP plus PE (VIP + PE) versus that of controls. Each value is the mean of three or four determinations ± SEM. Significant increases from control were observed for all treatments ($P < 0.05$). Values from the VIP treatment group were significantly less than observed for cultures treated with PE or PE plus VIP. (*B*) IP_3 levels in astroglial cultures treated with VIP, PE, or VIP plus PE is compared with control. Significant increases from control were observed for all treatment groups at 1 and 3 min ($P < 0.02$). At 1 min, VIP treatment produced increases significantly greater than observed for PE alone or for PE plus VIP ($P < 0.05$); the same comparisons at 3 min revealed no significant differences. Each value is the mean of 5–7 determinations ± SEM.

levels of IP_3 and inositol 1-phosphate were measured in a series of experiments after treatment with VIP, phenylephrine, or a mixture of the two agonists. The inositol 1-phosphate levels were elevated over control values in cells treated with VIP or phenylephrine alone or with the agonists together (Fig. 4*A*). Similarly, the time course of appearance of IP_3 was compared during treatment with VIP or phenylephrine alone or with the agonists together (Fig. 4*B*). A significant increase in cellular levels of IP_3 was observed 1 min after agonist exposure under all three conditions. The increase in inositol phosphates induced by the combination of agonists (VIP and phenylephrine), however, did not differ from either agonist applied alone. These experiments suggested that both the α-adrenergic receptor and the high-affinity VIP receptor stimulation result in an increase of cellular inositol phospholipid turnover.

DISCUSSION

Our results show that VIP in subnanomolar concentrations induces an increase in intracellular Ca^{2+} in type I astrocytes in culture. Furthermore, we show that a synergism exists between VIP receptors and α-adrenergic receptors in rat cortical astrocytes to increase $[Ca^{2+}]_i$. In many different cell types, including astrocytes, the actions of VIP are recognized to be mediated through the adenylate cyclase system (6, 7, 24, 25). This cAMP-elevating effect of VIP is produced at concentrations in the range of 0.1 to 1.0 μM and not at concentrations below 1.0 nM. Recent ligand binding data demonstrate the presence of a high-affinity and another low-affinity VIP binding site on astroglia (5). Neither the coupling mechanism nor the biological significance of the high-affinity receptor is yet clearly understood. One possibility is that the

high-affinity receptor mediates the mitogenic and secretagogue actions of VIP (see ref. 11). The VIP-mediated increase in $[Ca^{2+}]_i$ observed in this study demonstrates an alternative coupling mechanism for the intracellular effects produced by VIP in astroglia. We show here that this calcium-mobilizing effect of VIP occurs coincident with IP_3 generation. Furthermore, the $[Ca^{2+}]_i$ signal results from release of calcium from a thapsigargin-depletable intracellular pool. Similarly, α-adrenergic receptor-mediated calcium transients in astroglia also are derived from release of calcium from stores (20). It is not clear why VIP elicits this response in only a small percentage (18%) of the naive type I astrocytes under our experimental conditions and may reflect cellular heterogeneity in astrocytes isolated from cerebral cortex (26, 27).

Synergism between VIP receptors and α-adrenergic receptors has been observed previously in central nervous system cells (8, 21, 22). The cellular basis for the synergistic response between the two receptors is not clear and the α-adrenergic receptor needs to be occupied in the presence of VIP to elicit maximal cellular response. One possible mechanism may be that activation of one receptor reveals existing "hidden receptors" for the second agonist through changes in cytoskeleton or membrane. Isoproterenol and cAMP have been recently shown to cause marked changes in glial cell shape and cytoskeleton (28). Alternatively, synergism at the level of the G proteins coupled to the two receptors may be operative. In this scenario, although no measurable increase in cellular cAMP levels during activation of the high-affinity VIP receptor have been observed (5, 7), it is possible that subthreshold activation of the inhibitory G protein, G_i, which is coupled to adenylate cyclase, occurs during this stimulation. While the α_i subunit activates adenylate cyclase, the $\beta\gamma$ subunits of G_i protein are known to activate phospholipase β (29, 30). α-Adrenergic receptors are known to increase IP_3 by activation of the pertussis toxin-insensitive G_q protein and by interaction of the α subunit of G_q protein (α_q) with phospholipase β. Similarly, the high-affinity VIP receptors also cause IP_3 generation, measured in this study, possibly by acting through G_q protein. Thus, when both the α-adrenergic receptors and the high-affinity VIP receptors are activated simultaneously, maximal activation of phospholipase β might occur since both G_i and G_q proteins are activated. Although such a mechanism for synergism is likely, we did not observe a net increase in IP_3 levels during simultaneous stimulation.

This study directly demonstrates a VIP receptor-mediated intracellular calcium response in astroglia. Furthermore, a synergism between high-affinity VIP receptors and α-adrenergic receptors has been identified. Astrocytes are known to play a crucial role in development of the nervous system and are known to release neurotrophic substances that influence neuronal survival and/or neuronal sprouting (31, 32). Such glial cell-mediated signals are initiated by activation of membrane receptors (33, 34). VIP has been shown to be neurotrophic by acting as an astroglial mitogen and a secretagogue for glial-derived neurotrophic substances (1). The simultaneous multiple receptor activation of astrocytes might provide for dual receptor-mediated regulation of release of trophic factors and could form the basis for activity-dependent developmental plasticity. Since astrocytes are endowed with numerous types of neurotransmitter and neuropeptide receptors (35, 36), the synergism between two different receptor types may represent a model for many such interactions to orchestrate glial function via neural input both during development and in adulthood. The high-affinity VIP receptor coupled to an increase in $[Ca^{2+}]_i$ in astrocytes could function to promote secretion of neurotrophic factors by these glial cells.

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