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VIP induces PKA-mediated rapid and sustained phosphorylation of HSP20

Robert R. Gilmont, Sita Somara, and Khalil N. Bitar*

Department of Pediatrics-Gastroenterology, University of Michigan Medical Center, Ann Arbor, MI 48109

Abstract

The small molecular weight heat shock protein HSP20 has been proposed to regulate smooth muscle relaxation in a manner dependent on its phosphorylated state. We present the first evidence of HSP20 phosphorylation in response to a naturally occurring neurotransmitter. HSP20 was rapidly phosphorylated in colonic circular smooth muscle cells exposed to the physiologically relevant relaxant neuropeptide, Vasoactive Intestinal Peptide (VIP). HSP20 phosphorylation was significantly and substantially increased by 30 seconds following VIP treatment and remained elevated for 30 minutes. VIP-induced HSP20 phosphorylation was dose dependent. Both basal and VIP-induced HSP20 phosphorylations were solely mediated by Protein Kinase A. Maximal phosphorylation of HSP20 was induced by the same VIP concentration range which induces maximal relaxation. Increased phosphorylation of HSP20 occurred in both cytosolic and particulate cell fractions. Our findings represent evidence for neurogenic modulation of the cyclic molecular regulation of relaxation required for peristalsis via a VIP-PKA-HSP20 pathway.

Keywords

Phosphorylation; Thin filaments; Smooth muscle relaxation; Heat shock proteins; Peristalsis; PKA; H-89

Introduction

Colonic smooth muscle contraction/relaxation is regulated by the release of contractile agonists by cholinergic neurons and the release of relaxants by Non-Adrenergic Non-Cholinergic (NANC) neurons. The primary excitatory neurotransmitter responsible for contraction is acetylcholine (Ach) while relaxation is primarily mediated by Vasoactive Intestinal Peptide (VIP) its homolog pituitary adenylate cyclase activating peptide (PACAP) and nitric oxide (NO). While VIP is thought to work primarily through the activation of adenylate cyclase, it can also induce NO synthesis in colonic smooth muscle cells and regulate L-type channel dependent Ca^{+2} influx [1;2]

The cyclic nature of colonic contraction-relaxation is regulated at the level of the smooth muscle cell. In the gut, the contractile agonist, Ach binds to M2 and M3 muscarinic receptors

*Address Correspondence to: Khalil N. Bitar, Ph.D., AGAF, University of Michigan Medical School, 1150 W. Medical Center Dr., MSRB I, Room A520, Ann Arbor, MI 48109-5656, Phone: (734) 763-2005, FAX: (734) 647-9703, E-mail: bitar@umich.edu.

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resulting in the activation of myosin light chain kinase (MLCK), the phosphorylation of myosin light chain (MLC 20) and contraction [3]. Relaxant agonists work primarily through reducing MLC20 phosphorylation. NANC neurotransmitters such as VIP are co-released with Ach [4]. For peristalsis to occur there is a requirement for the inhibition of contraction in the presence of contractile agonist.

The low molecular weight heat shock protein, HSP20, has been well documented as a substrate for cyclic AMP-dependent protein kinase (PKA) or cyclic GMP-dependent protein kinase (PKG) and has been strongly implicated as a modulator of cyclic nucleotide mediated smooth muscle relaxation[5;6]. Both PKA and PKG phosphorylate HSP20 *in vitro*, with the phosphorylation site being serine residue 16 (S16) [7]. The phosphorylation of the S16 residue is associated with smooth muscle relaxation. Relaxation associated with HSP20 phosphorylation appears independent of Ca^{+2} concentration or MLC20 phosphorylation [8] and has been termed force suppression [9]. Force suppression has been observed in vascular smooth muscle cells (where HSP20 is abundant and phosphorylated) but does not occur in bladder smooth muscle cells which contain substantially lower levels of HSP20 [10] or in umbilical artery where phospho-HSP20 is not observed in response to cyclic nucleotide induction [11]. Force suppression through HSP20 phosphorylation could be a component of peristaltic regulation; however, the kinetics of HSP20 phosphorylation in experimental systems described to date is slower than that required in the dynamic process of peristalsis. Substantial phosphorylation of HSP20 is typically not observed until after several minute's treatment [6; 9].

In this investigation, we have undertaken studies to determine if HSP20 could be a modulator of colonic contraction/relaxation. We demonstrate that the physiologically relevant relaxant neuropeptide VIP could induce phosphorylation of HSP20 at physiological doses and determined the kinetics of phosphorylation. We determined if VIP-induced HSP20 phosphorylation was dependent on PKA activation, calcium influx or NO production. Additionally, we studied the relationship of VIP-induced HSP20 phosphorylation and sub-cellular localization.

Material and Methods

Reagents

Type II collagenase was purchased from Worthington Biochemical Company (Freehold NJ), ampholytes, H-89 hydrochloride and N_{ω} -Nitro-L-arginine methyl ester hydrochloride (L-NAME) were purchased from Sigma-Aldrich (St. Louis, MO.), rabbit polyclonal anti-HSP20 antibody was purchased from Abcam (Cambridge, MA.). Tissue culture reagents were purchased from Invitrogen (Carlsbad, CA.).

Determination of HSP20 phosphorylation status

Rabbit sigmoid colon was stripped of serosa and mucosa before being washed with HBSS containing antibiotics. The tissue was finely minced and digested twice with type II collagenase (0.1%) at 32°C for 1 hour. The cells were plated in DMEM with 10% FBS, antibiotics and 0.5% L-glutamine. Cells were grown to confluence and serum starved for 48 hours, treated with 1 μ M VIP, harvested in a lysis buffer containing 9 M urea and subjected to electrophoresis on slab glycerol/urea IEF gels (pH gradient 1% 3–10: 4% 4–7). Gels were transferred to PVDF membranes immunoblotted with 0.05 μ g/ml anti-HSP20 antibody. Following treatment with 1:5000 HRP-conjugated secondary antibody, HSP20 was detected using ECL. Phosphorylated forms of HSP20 appeared as more acidic bands in this analysis. Band densities were quantified by densitometric scanning (Quantity One, BioRad).

Sub-cellular fractionation of CSMC

Soluble and particulate fractions from cultured CSMC were isolated essentially as described for freshly isolated smooth muscle cells. [12]. Briefly, CSMC were incubated with or without 1 μ M VIP for 1 min, washed and suspended in a Tris buffered saline solution supplemented with protease and phosphatase inhibitors. The cell suspensions were then sonicated for 2 min, intact cells removed by low speed centrifugation, and centrifuged at $100,000 \times g$ for 1 hr. The supernatant fraction was collected and the pellet was suspended in RIPA buffer and sonicated for 1 min and the solubilized lysate collected as the particulate fraction. Twenty μ g protein samples from cytosol and particulate fractions were concentrated by ethanol precipitation and analyzed by IEF and immunoblotting.

Determination of inhibitor effect on VIP-induced HSP20 phosphorylation

Cultures were pre-treated for 30 minutes in HBSS containing 10 or 100 μ M H-89, 300 μ M L-NAME or in HBSS minus calcium containing 3 mM EGTA prior to the addition of 1 μ M VIP. Following treatment, cultures were harvested and subjected to IEF analysis as described above.

Data analysis

Results from all experiments are expressed as means $\pm$ SEM. An un-paired two tailed Students *t*-test was used to compare differences among groups.

Results

Basal HSP20 phosphorylation levels

Protein analysis by IEF and immunoblotting was used to determine changes in phosphorylation status as more phosphorylated forms of HSP20 become more acidic (Fig. 1–Fig. 4). Under control conditions with no VIP stimulation a small amount of the most acidic form of HSP20 was observed. The basal phosphorylation status of HSP20 was determined using 15 separate CSMC culture whole cell lysates. In these samples the mean percentage basal phosphorylation level (Mean % $\pm$ SEM) was 4.48 ± 0.59 . This phosphorylated form of HSP20 was not detectable in samples from cultures treated with 100 μ M H-89 (Fig 3B). These data demonstrate that the basal phosphorylation resulting in this form of HSP20 is PKA dependent.

Kinetics of VIP-induced HSP20 phosphorylation

CSMC were treated with 1 μ M VIP for times ranging from 30 seconds to 30 minutes. These samples were compared to untreated control (0 time-point) by IEF and immunoblotting as described in Material and Methods. Mean % $\pm$ SEM basal phosphorylation levels in untreated control cells were 4.54 ± 0.84 . By 30 seconds HSP20 phosphorylation was significantly increased to 31.81 ± 3.23 ($P \leq 0.001$; $n = 8$) and HSP20 phosphorylation continued to increase to 46.91 ± 5.15 by 1 minute. No significant further increase in phosphorylation was observed, but HSP20 phosphorylation levels remained high throughout the entire experiment. By 5 minutes HSP20 phosphorylation was at 41.48 ± 5.15 . High levels of HSP20 phosphorylation were observed at 15 minutes (47.67 ± 2.61) and after 30 minutes of treatment (45.43 ± 1.67). Increased HSP20 phosphorylation levels were significantly higher than un-treated controls at all time points measured post-VIP treatment ($P \leq 0.001$; $n = 8$) (Fig. 1A and B).

HSP20 phosphorylation in response to VIP was dose-dependent

CSMC were treated for 1 min with various concentrations of VIP ranging from 10^{-9} M to 10^{-6} M and HSP20 phosphorylation status analyzed. The basal phosphorylation levels in two independent experiments from untreated control cells (Mean % $\pm$ SEM) was 3.8 ± 1.8 . Treatment with 10^{-9} M VIP caused a significant increase in HSP20 phosphorylation to 29.3 ± 1.3 ($P \leq 0.05$; $n = 2$). Treatment with increasing concentrations of VIP caused increases in HSP20

phosphorylation. The dose response curve demonstrated saturation (Fig. 2 B). Phosphorylation levels peaked between 10^{-7} M and 5×10^{-6} M VIP with mean percentage phosphorylation values of 50.56 ± 1.85 ($P \leq 0.05$; $n = 2$) at 10^{-7} M VIP, and 49.55 ± 2.75 at 5×10^{-6} M VIP ($P \leq 0.05$; $n = 2$). The peak mean percentage phosphorylation increase (54 ± 12.3) occurred when using 5×10^{-7} M VIP, however this value was not significant by students *t* test ($P > 0.05$). The VIP concentration chosen for future studies was 10^{-6} M. At this concentration, very high phosphorylation levels (48.6 ± 5.6) were observed and were found to be significant ($P \leq 0.05$; $n = 2$) (Fig. 2A and B).

Inhibition of HSP20 phosphorylation

VIP is known to modulate calcium influx into the cell via L-type Ca^{+2} channels, cGMP production via NO production, and cAMP production via activation of adenylate cyclase. These three potential mediators of HSP20 phosphorylation were tested. CSMC were treated for 20 minutes in HBSS lacking Ca^{+2} and containing 3 mM EGTA, HBSS containing 300 μM L-NAME, 10 μM H-89 or 100 μM H-89 before being treated for 1 min with 10^{-6} M VIP. Treatment in medium lacking Ca^{+2} and containing 3 mM EGTA, treatment with L-NAME or treatment with 10 μM H-89 had no inhibitory effect on HSP20 phosphorylation, while treatment with 100 μM H-89 completely inhibited HSP20 phosphorylation (Fig. 3 A). These data demonstrate that VIP-induced HSP20 phosphorylation was completely mediated by PKA. Inhibition of VIP-induced HSP20 phosphorylation by 100 μM H-89 was also demonstrated using CSMC cultures treated in DMEM medium (Fig. 3 B).

Effects of cellular distribution on VIP-induced HSP20 phosphorylation

We determined the phosphorylation status of HSP20 in the cytosolic and particulate fractions of CSMC. The phosphorylation status of HSP20 in the cytosolic fraction of control cells was low ($<10\%$). VIP treatment caused an increase in cytosolic HSP20 phosphorylation to a mean value of 45.6%. The particulate fraction contained no detectable phosphorylated HSP20 as compared to that seen in the cytosolic fraction under basal conditions. However, after VIP treatment phosphorylated HSP20 was observed in the particulate fraction. HSP20 was more abundant in the cytosolic fraction both before and after VIP treatment. VIP treatment did not cause a dramatic shift in the distribution of HSP20 between these fractions.

Discussion

HSP20 is a member of the superfamily of mammalian small heat shock proteins (sHSPs) which contains 10 members [13]. Seven members of the superfamily are expressed in muscle. Among these seven members are HSP20, HSP27, and αB -crystallin. Sugiyama et al. [14] showed that HSP20 associated with HSP27 and αB -crystalline in multimer complexes that were approximately 500 kDa in molecular mass, co-immunoprecipitated HSP20, HSP27 and αB -crystalline, and demonstrated the presence of all three in human colon. *In vivo*, phosphorylation of HSP27 causes a reduction in the size of the large molecular weight form it is associated with [15]. Phosphorylation of HSP20 also causes a reduction in the size of the large macromolecular form it is associated with [16]. If these molecules form mixed oligomers in colonic smooth muscle, then phosphorylation of either would produce an oligomer of changed composition as well as free monomers and dimers of the phosphorylated protein. It is important to note that in smooth muscle, phosphorylation of HSP27, is associated with cell contraction [17;18] while phosphorylation of HSP20 is associated with relaxation [5;7;8;19;20].

Researchers studying HSP27 have long made use of such naturally occurring ligands including bombesin, endothelin-1, platelet-derived growth factor and lisophosphatidic acid to study the relationship between HSP27 phosphorylation and contraction [17;18;21]. Researchers have used NO donor molecules, high extracellular Mg^{+2} , direct adenylate cyclase activation, cyclic

nucleotide analogs, phosphodiesterase inhibitors or direct phosphorylations with kinase catalytic subunits to study HSP20 phosphorylation [8;16;20;22]. Here we present the first evidence of the phosphorylation of HSP20 in response to a naturally occurring relaxant peptide.

VIP is a physiologically relevant modulator of smooth muscle relaxation. VIP knockout mice demonstrate significant defects in peristalsis and reduced motility [23]. At the molecular level VIP has been shown to modulate L-type Ca^{+2} channels, activate NOS and adenylate cyclase. To determine which of the signal transduction pathways was responsible for HSP20 phosphorylation, we performed studies where we inhibited calcium influx, NO production or PKA activity. We found no inhibitory effect on HSP20 phosphorylation by removing extracellular Ca^{+2} from the incubation medium or by inhibiting NO production by pre-incubation with L-NAME. We found that only inhibition of PKA activity was effective in reducing VIP-dependent phosphorylation of HSP20. Treatment with 100 μM H-89 completely inhibited HSP20 phosphorylation indicating that PKA activation is the only mechanism used by CSMC to regulate VIP-dependent phosphorylation of HSP20. The loss of the most acidic form of HSP20 under basal conditions also occurred with H-89 treatment. This loss of phosphorylation in control cells indicates that in the absence of exogenous stimulation there is a basal activation of PKA in CSMC and that the phosphatase required to dephosphorylate HSP20 is also active under basal conditions. This equilibrium between PKA and phosphatase activities would appear to be tightly controlled as there was little variation in the quantity of this form of HSP20 in the studies performed (4.48% $\pm$ 0.59%).

HSP20 phosphorylation has been proposed to be a modulator of muscle tone in systems where tonic regulation is required. In vasculature smooth muscle, low levels of HSP20 are associated with chronic hypertensive state [24] while treatments which cause vaso-relaxation induce HSP20 phosphorylation [5;8]. To date, all studies of HSP20 phosphorylation have demonstrated slow onset (several minutes) and incremental increases in phosphorylation in response to pharmacologically-induced activation of adenylate cyclase or guanylate cyclase. We found the kinetics of HSP20 phosphorylation in VIP-treated colonic smooth muscle is much more rapid and more substantial than previously described in any other system. VIP induced significant and substantial increases in HSP20 phosphorylation by 30 sec post treatment. The rapidity of VIP-induced HSP20 phosphorylation appears very similar to that described using purified HSP20 and PKA catalytic subunit *in vitro* [22]. Most importantly, we found that significant increases in HSP20 phosphorylation could be induced by VIP concentration as low as 10^{-9} M. Maximal phosphorylation of HSP20 was achieved using VIP concentrations between 10^{-7} and 5×10^{-6} M. This means that maximum phosphorylation of HSP20 occurs at the same physiological dose range in which VIP induces maximal relaxation [25]. Other researchers have noted a more basic (unphosphorylated) form of HSP 20 as well as the dominant monophosphorylated (PKC form) and the most acidic, diphosphorylated forms of the molecule [6;19]. We sometimes see a minor band which is more basic (less phosphorylated) than the major form or the VIP-induced form of HSP20. This typically occurs in gels which are overloaded and can sometimes be seen in overexposed films. These researchers used carotid artery strips for analysis while we used serum starved confluent cultures of pure populations of CSMC. Our cultures lack Interstitial Cells of Cajal (ICC) or neuronal cells as defined by negative staining using antibodies directed against c-Kit or NeuN respectively (data not shown), and are cultured under conditions designed to optimize myogenic response to contractile agonists (MLC20 phosphorylation). There is a fundamental difference between vascular and colonic smooth muscle in that vascular smooth muscle does not exhibit peristalsis. The regulation of HSP20 phosphorylation in the vasculature may not be as sensitive or tightly controlled as in the colon.

Brophy et al.[19] used co-immunoprecipitation of actin with HSP20 and co-sedimentation analysis of recombinant HSP20 and actin reaction mixes to show that the phosphorylated form

of HSP20 associates with globular actin while the non-phosphorylated form associates with filamentous actin. Interestingly, Rembold et al. [8] showed that PKG-mediated phospho-HSP20 is preferentially distributed in pellets from cell lysates following centrifugation at $6000 \times g$, indicating increased association with thin filaments. Our studies demonstrate no dramatic shift of HSP20 to or from the particulate fraction following VIP treatment. HSP20 appears more abundant in the cytosol before and after VIP-induced phosphorylation and increased phosphorylation was observed in both fractions. It is important to note that we chose to analyze the effect of VIP on HSP20 and phospho-HSP20 subcellular distributions at 1 min post-treatment. This is a substantially earlier time post-treatment than other researchers have chosen to analyze. We chose this time because of the rapidity and substantial nature of VIP-induced HSP20 phosphorylation in CSMC. If HSP20 phosphorylation-induced changes in sub-cellular distribution were to play a major role in regulation of peristalsis, we would expect to see a dramatic change by 1 min. Our data allow us to neither clearly confirm nor refute hypotheses which require specific subcellular localization of phospho-HSP20 for functional regulation of relaxation.

These studies support a role for HSP20 as a modulator of peristalsis in colonic smooth muscle. We propose that phosphorylated HSP20 is a functional switch that determines the contraction/relaxation state in colonic smooth muscle. This is essential to peristalsis as VIP can be co-released with Ach [4]. We have shown that VIP induces a rapid and substantial phosphorylation of HSP20 and that maximal phosphorylation occurs within a concentration range of VIP known to induce maximal relaxation. These data are consistent with HSP20 modulating the inhibition of contraction in the presence of a contractile agonist.

Acknowledgments

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Fig. 1.

VIP-dependent HSP20 phosphorylation time-course. Confluent cultures of CSMC were treated with 1 μ M VIP for various times ranging from 30 sec to 30 min before being harvested and subjected to IEF and immunoblot analysis as described in Materials and Methods. A. A representative immunoblot of triplicate cultures treated with VIP demonstrating changed HSP20 phosphorylation status. B. Graphic representation of the combined results of 8 or greater samples. Data points represent Mean $\pm$ SEM at each time point. All treatments resulted in significantly increased HSP20 phosphorylation when compared to control ($P < 0.001$ by students t -test).

Fig. 2. HSP20 phosphorylation in response to 1 min VIP treatment. Confluent cultures of CSMC were treated for 1 min with varying concentrations of VIP ranging from 10^{-9} M – 5×10^{-6} M before being analyzed by IEF and immunoblotting as described in Materials and Methods. A. A representative immunoblot of changed HSP20 phosphorylation status. The upper HSP20 bands represent the more acidic (phosphorylated) form of the molecule. B. Graphic representation of the combined results of two dose-response experiments. Data points represent Mean $\pm$ SEM at each concentration of VIP. Significant differences ($P < 0.05$ by students *t*-test) are indicated by *.

Figure 3.

A) VIP-induced HSP20 phosphorylation is mediated by PKA. A. Duplicate confluent cultures of CSMS were incubated in HBSS, HBSS $-Ca^{++}$ containing 3 mM EGTA, HBSS containing 300 μ M L-NAME, HBSS containing 10 mM H-89 or HBSS containing 100 mM H-89 for 20 min before a 1 min treatment with 1mM VIP. Cultures were harvested and subjected to IEF and immunoblot analysis as described in Materials and Methods. B) Triplicate cultures of CSMC were incubated in DMEM medium or DMEM medium containing 100 mM H-89 for 20 min and the treated for 1 min with 1 mM VIP. Treatment with 100 mM H-89 completely inhibited HSP20 phosphorylation, while no inhibitory effect was seen using the other treatments.

Fig. 4.

VIP-induced HSP20 phosphorylation occurs primarily in the cytosol. Duplicate confluent cultures of CSMS were left untreated (control) or treated with 1 mM VIP for 1 min before the cells were harvested, fractionated into cytosol and particulate, and 20 mg protein of each subjected to IEF and immunoblot analysis as described in Materials and Methods. HSP20 was most abundant in the cytosol fraction in both control and VIP treated cultures. The most phosphorylated isoform of HSP20 was not detected in the particulate prior to VIP treatment, but was observed after stimulation with VIP. HSP20 phosphorylation also increased in the cytosol.