VIP and PACAP regulate localized Ca\(^{2+}\) transients via cAMP-dependent mechanism

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Hagen, Brian M., Orline Bayguinov, and Kenton M. Sanders. VIP and PACAP regulate localized Ca\(^{2+}\) transients via cAMP-dependent mechanism. Am J Physiol Cell Physiol 291: C375–C385, 2006. First published March 29, 2006; doi:10.1152/ajpcell.00495.2005.—Vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) have been suggested as participants in enteric inhibitory neural regulation of gastrointestinal motility. These peptides cause a variety of postjunctional responses including membrane hyperpolarization and inhibition of contraction. Neuropeptides released from enteric motor neurons can elicit responses by direct stimulation of smooth muscle cells as opposed to other transmitters that rely on synapses between motor nerve terminals and interstitial cells of Cajal (ICC). Therefore, we studied the responses of murine colonic smooth muscle cells to VIP and PACAP (1–38) with confocal microscopy and patch-clamp technique. Localized Ca\(^{2+}\) transients (Ca\(^{2+}\) puffs) were observed in colonic myocytes, and these events coupled to spontaneous transient outward currents (STOCs). VIP and PACAP increased Ca\(^{2+}\) transients and STOC frequency and amplitude. Application of dibutyl cAMP had similar effects. The adenyl cyclase blocker MDL-12,330A alone did not affect spontaneous Ca\(^{2+}\) puffs and STOCs but prevented responses to VIP. Disruption of A-kinase-anchoring protein (AKAP) associations by application of AKAP St-Ht31 inhibitory peptide had effects similar to those of MDL-12,330A. Inhibition of ryanodine receptor channels did not block spontaneous Ca\(^{2+}\) puffs and STOCs but prevented the effects of dibutyl cAMP. These findings suggest that regulation of Ca\(^{2+}\) transients (which couple to activation of STOCs) may contribute to the inhibitory effects of VIP and PACAP. Regulation of Ca\(^{2+}\) transients by VIP and PACAP occurs via adenyl cyclase, increased synthesis of cAMP, and PKA-dependent regulation of ryanodine receptor channels.

Calcium puffs; ryanodine receptor channels; enteric nervous system; gastrointestinal motility

**METHODS**

Several neurotransmitters, including NO, purines, and neuropeptides, mediate enteric inhibitory neural responses in the gastrointestinal (GI) tract (12). The study of postjunctional responses to inhibitory neurotransmission is complicated because both smooth muscle cells and interstitial cells of Cajal (ICC) express receptors and second messenger systems capable of transducing inhibitory neurotransmitter signals. Studies on animals that lack intramuscular ICC (ICC-IM) have shown that the inhibitory electrical responses to exogenous sodium nitroprusside are greatly reduced compared with control animals. Thus the inhibitory effects of NO released from neurons are mediated by ICC-IM (5, 7). The short half-life of NO necessitates close physical association between nitricergic nerve terminals and postjunctional cells. In contrast, inhibitory responses to purines and peptides persist in the absence of ICC-IM (5), suggesting that these substance may escape the neuro-ICC-IM junctions and directly stimulate smooth muscle cells.

We previously characterized the effects of purines (ATP and 2-methylthio-ATP) on smooth muscle cells from murine large intestine (1). Voltage-clamped single colonic myocytes display spontaneous transient outward currents (STOCs) that are due to localized spontaneous Ca\(^{2+}\) transients. Spontaneous Ca\(^{2+}\) transients and STOCs have been recorded from a variety of smooth muscle cells (17, 31, 34, 47, 48), and the source of Ca\(^{2+}\) appears to vary in different cell types. For example, in vascular myocytes block of Ca\(^{2+}\) released from the sarcoplasmic reticulum (SR) via ryanodine receptor (RyR) channels abolishes localized Ca\(^{2+}\) transients and STOCs (30). In this case the Ca\(^{2+}\) transients are referred to as sparks. In murine colonic muscles the localized Ca\(^{2+}\) transients are due to inositol 1,4,5-trisphosphate (IP\(_3\)) receptor-operated Ca\(^{2+}\) release, and ryanodine is without effect on these events. In this case exposure of cells to purines increases Ca\(^{2+}\) release from IP\(_3\) receptors (referred to as Ca\(^{2+}\) puffs) and increases the occurrence of STOCs. STOCs are due to activation of large-conductance Ca\(^{2+}\)-activated K\(^+\) (BK) and small-conductance Ca\(^{2+}\)-activated K\(^+\) (SK) channels; however, at the negative potentials of colonic cells the major response appears to be due to SK channels. Purines bind to P2X receptors, activate phospholipase C\(_\beta\), and increase IP\(_3\) production. Enhanced IP\(_3\) levels in colonic muscle cells stimulate Ca\(^{2+}\) transients and STOCs, causing hyperpolarization responses to ATP (1, 22).

Peptide neurotransmitters, such as vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase-activating peptide (PACAP), also participate in postjunctional enteric inhibitory responses (14, 26). VIP and PACAP are abundant in enteric inhibitory neurons and colocalize with nitric oxide synthase (13, 16). Multiple distinct receptors exist for VIP and PACAP, including VPAC1, VPAC2 and various isoforms of the PAC1 receptor. All of these receptors are thought to couple via G\(_i\) to activation of adenyl cyclase (AC), increased synthesis of cAMP, and activation of cAMP-dependent protein kinase (PKA), leading to relaxation of GI smooth muscles.

In the present study we tested the effects of VIP and PACAP on Ca\(^{2+}\) transients and STOCs in murine colonic myocytes with laser scanning confocal microscopy and patch-clamp technique. We also investigated the second messenger pathway that links receptor activation to Ca\(^{2+}\) transients.
Fig. 1. Localized Ca\(^{2+}\) transients are amplified by vasoactive intestinal polypeptide (VIP). A: spontaneous Ca\(^{2+}\) transients in a colonic myocyte. B: increased Ca\(^{2+}\) transients and development of Ca\(^{2+}\) waves after VIP (10\(^{-6}\) M). Images were created by dividing frames (F) of interest by inactive averaged frames (F\(_0\)) to create fluorescent ratio images. Red circles (denoted 1–4 in A, left) outline regions of interest (ROIs). C and D: fluorescence ratios (F/F\(_0\)) for each ROI over 20-s scan periods. ROIs 1 and 2 displayed spontaneous Ca\(^{2+}\) transients during the control scan (traces 1 and 2 in C). The amplitudes of the Ca\(^{2+}\) transients in ROIs 1 and 2 increased after the application of VIP. ROIs 3 and 4 were essentially inactive during the control scan (traces 3 and 4 in C), but these regions displayed Ca\(^{2+}\) fluctuations after the application of VIP as a result of the development of Ca\(^{2+}\) waves into these regions.

Fig. 2. VIP increased spontaneous transient outward currents (STOCs). A: STOCs from a colonic myocyte recorded in the perforated-patch, whole cell configuration. The cell was held at -35 mV. B: dramatic increase in STOCs in the same cell after VIP (10\(^{-6}\) M). VIP increased the frequency and amplitude of STOCs. The effects of VIP on STOCs are summarized in a survival curve analysis (2) in C (P < 0.001, n = 5). y-Axis shows the fraction of STOCs of amplitude greater than values on x-axis. D: summary of increase in STOC frequency (*P < 0.05, n = 5) in response to VIP.
mice (60–90 days old) of either sex were anesthetized with isoflurane inhalation (AErrane, Baxter Healthcare, Deerfield, IL) and killed by cervical dislocation in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All protocols were approved by the Institutional Animal Use and Care Committee at the University of Nevada, Reno. Colonies were excised and opened along the mesenteric border. The luminal contents were removed with Krebs-Ringer bicarbonate buffer (KRB; see Solutions and drugs). Tissues were pinned to the base of a Sylgard-coated dish, and the mucosa and submucosa were dissected away.

Colonic muscles (both longitudinal and circular layers) were equilibrated in Ca\(^{2+}\)-free solution for 60 min at 4°C. Tissues were then digested at 37°C for 16 min without agitation in an enzyme solution containing collagenase F (Sigma, St. Louis, MO) (1). After digestion, tissues were washed with Ca\(^{2+}\)-free Hank's solution to remove enzymes and triturated with blunt-tipped pipettes to free single smooth muscle cells.

**Confocal microscopy.** Suspensions of cells were placed in 0.5-ml chambers with glass bottoms. The cells were incubated for 35 min at room temperature in Ca\(^{2+}\)-free buffer containing fluo-4 acetoxy-methyl ester (AM) (10 \(\mu\)g/ml; Molecular Probes, Eugene, OR) and pluronic acid (2.5 \(\mu\)g/ml; Telfabs, Austin, TX). Cell loading was followed by incubation in a solution containing 2 mM Ca\(^{2+}\) for 25 min to restore the normal concentration of extracellular Ca\(^{2+}\) and to allow the cells to adhere tightly to the bottom of the chambers during deesterification of fluo-4. All measurements were made at room temperature (22–25°C) and within 45 min after extracellular Ca\(^{2+}\) was restored.

An Odyssey XL confocal laser scanning head (Noran Instruments, Middleton, WI) connected to a Nikon Diaphot 300 microscope with \(\times60\) water immersion lens (numerical aperture = 1.2) was used to image the cells. The cells were scanned with INTERVISION software (Noran Instruments) running on an Indy workstation (Silicon Graphics, Mountain View, CA). Changes in the fluo-4 fluorescence (indicating fluctuations in cytosolic Ca\(^{2+}\)) were recorded for 20-s test periods with T series acquisition and a laser wavelength of 488 nm (excitation for FITC). Six hundred frames were acquired per test period (1 frame every 33 ms), creating 20-s movie files.

**Ionic currents of single cells.** Ionic currents were measured in isolated muscle cells with the whole cell perforated-patch (amphotericin B) configuration of the patch-clamp technique. An Axopatch 200B amplifier with a CV 203BU head stage (Axon Instruments, Foster City, CA) was used to measure ionic currents. Membrane

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**Fig. 3.** Pituitary adenylate cyclase-activating peptide [PACAP(1–38)] increased Ca\(^{2+}\) transients and STOCs. Amplitude and frequency of the Ca\(^{2+}\) transients, represented by the Fr/F\(_0\) oscillations in a single ROI, increased in response to PACAP(1–38) (A and B). Similarly, STOC amplitude and frequency also increased in response to PACAP(1–38) (C and D). Effects of PACAP(1–38) on STOC amplitude (*\(P < 0.005, n = 5\)) and frequency (\(*P < 0.05, n = 5\)) are summarized in E and F, respectively.
currents were recorded with pCLAMP software (version 9.0, Axon Instruments) while cells were held between −30 and −40 mV (after correction of a −9 mV junction potential). Currents were digitized at 1 kHz. All experiments were performed at room temperature (22–25°C).

**Solutions and drugs.** The standard KRB used to dissect intact organs contained (mM) 120 NaCl, 5.9 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 15.5 NaHCO₃, 1.2 NaH₂PO₄, and 11.5 dextrose. This solution had a final pH of 7.3–7.4 after equilibration with 97% O₂-3% CO₂. The enzyme solution used to disperse smooth muscle cells contained 1.3 mg/ml collagenase F, 2 mg/ml papain, 1 mg/ml BSA, and 0.154 mg/ml l-DTT in a Ca²⁺-free Hanks’ solution (pH 7.4). The bathing solution used in confocal microscopy and whole cell patch-clamp studies contained (mM) 134 NaCl, 6 KCl, 1 MgCl₂, 2 CaCl₂, 10 glucose, and 10 HEPES (pH 7.4). The pipette solution used in whole cell patch-clamp experiments contained (mM) 110 K-aspartate, 30 KCl, 10 NaCl, 1 MgCl₂, 10 HEPES, and 0.05 EGTA (pH 7.2) with 250 μg/ml amphotericin B. VIP, PACAP(1–38), N⁶,2⁰-O-dibutyryl-adenosine 3⁰,5⁰'-cyclic monophosphate sodium salt (dbcAMP), nicardipine, ryanodine [ryanodol 3-(1H-pyrole-2-carboxylate) from *Rynia speciosa*] and cis-N-(2-phenylcyclopentyl)-azacyclotridec-1-en-2-amine hydrochloride (MDL-12,330A) were purchased from Sigma-Aldrich. InCELLect AKAP St-Ht31 inhibitor peptide (AKAP-IP) was obtained from Promega (Madison, WI). The concentrations of drugs used in experiments were ascertained from the literature or by empirical determinations of effective concentrations on murine colonic myocytes.

**Analysis of data.** Image analysis was performed with custom analysis programs developed with Interactive Data Language software (Research Systems, Boulder, CO), as previously described (1). Baseline fluorescence (F₀) was determined by averaging 10 images (of 600) with no activity. Ratio images were then constructed and replayed for careful examination to detect active areas where sudden increases in ratio of fluorescence to baseline fluorescence (F/F₀) occurred. F/F₀ vs. time traces were further analyzed with Microcalf Origin (Microcal Software, Northampton, MA) and AcqKnowledge Software (Biopac Systems, Santa Barbara, CA). Fluorescence records from single colonic myocytes were composed of Ca²⁺ transients of multiple characteristics (i.e., single Ca²⁺ puffs, clusters of puffs, and Ca²⁺ waves). In many cells, especially after stimulation with VIP and PACAP, it was impossible to make measurements on single, discrete Ca²⁺ puffs. Therefore, as a measure of the Ca²⁺ released during the 20-s sampling periods, we integrated the area of signals above F₀. This measurement incorporates both the amplitude and the duration of Ca²⁺ transients. The amplitude and duration of the Ca²⁺ transients are both important parameters because an increase in either the amplitude or the duration of Ca²⁺ transients causes more openings of Ca²⁺-activated K⁺ channels. Therefore, it is likely that the fluorescence integrals are a better representation of the elevation in local Ca²⁺ for the purposes of this study.

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**Fig. 4.** N⁶,2⁰-O-dibutyryl-adenosine 3⁰,5⁰'-cyclic monophosphate (dbcAMP) mimics the effect of VIP and PACAP. Amplitude and frequency of the Ca²⁺ transients, represented by the F/F₀ oscillations in 2 ROIs (*traces 1 and 2*), increased in response to dbcAMP (10⁻³ M) (*A* and *B*). Similarly, STOC amplitude and frequency also increased in response to dbcAMP (*C* and *D*). Effects of dbcAMP on STOC amplitude (*P* < 0.005, *n* = 5) and frequency (*P* < 0.05, *n* = 5) are summarized in *E* and *F*. 
Statistical analysis. Results are expressed as means ± SE where applicable. Statistical analysis was made with SigmaStat 2.03 software (Jandel Scientific Software, San Rafael, CA). STOC amplitudes were measured with the Mini Analysis Program (Synaptosoft, Leonia, NJ), with a threshold for detection set at 15 pA. The distributions of STOC amplitudes were strongly skewed, resembling those of single-channel dwell times or survival curves. Accordingly, we have illustrated changes in STOC amplitudes in control and test conditions as cumulative distributions where the y-axis is the fraction of STOCs of amplitude greater than the picoampere value on the x-axis (2). In the text we have reported $P$ values from the log-rank tests, where $n$ represents the number of cells in each experiment.

RESULTS

Effect of VIP/PACAP on spontaneous Ca$^{2+}$ puffs and STOCs in murine colonic myocytes. Colonic myocytes loaded with fluo-4 AM generated spontaneous intracellular Ca$^{2+}$ transients that occurred either as highly localized events or as Ca$^{2+}$ waves. Imaging of cells under whole cell voltage clamp demonstrated that localized Ca$^{2+}$ events were associated with STOCs, as shown previously (1). Spontaneous Ca$^{2+}$ transients in colonic myocytes are due to Ca$^{2+}$ release from IP$_3$ receptors and therefore are termed Ca$^{2+}$ puffs.

VIP (10$^{-6}$ M) increased Ca$^{2+}$ transients in colonic myocytes by 40.6 ± 12.4% ($P < 0.005$, $n = 6$). The response to VIP was characterized by an increase in activity of sites that generated Ca$^{2+}$ puffs during control conditions and the development of localized Ca$^{2+}$ transients into Ca$^{2+}$ waves. These responses were apparent from analysis of changes in fluorescence within regions of interest (ROIs) at centers of spontaneous puffs and in ROIs outside the regions of spontaneous puffs. After VIP, ROIs that showed no spontaneous activity during the control period developed Ca$^{2+}$ transients (Fig. 1). This increase in Ca$^{2+}$ transients with VIP was associated with an increase in STOCs (Fig. 2, A and B). The amplitude of STOCs increased (Fig. 2C; $P < 0.001$, $n = 5$), and the frequency of STOCs increased by 68.7 ± 26.5% (Fig. 2D; $P < 0.05$, $n = 5$).

We showed previously (3) that substance P increases Ca$^{2+}$ transients and these effects are dependent on the stimulatory effects of the peptide on L-type Ca$^{2+}$ channels. We also found previously (21) that VIP, via activation of PKA, increased L-type Ca$^{2+}$ current in colonic myocytes by 16% at 10$^{-6}$ M. Therefore, we performed additional experiments testing the effects of VIP after pretreatment with nicardipine (10$^{-6}$ M). In the presence of nicardipine, VIP increased Ca$^{2+}$ transients by 67.1 ± 29.2% ($P < 0.05$, $n = 12$), which was not significantly different from responses in the absence of nicardipine.

PACAP(1–38) (10$^{-6}$ M) also increased Ca$^{2+}$ transients by 69.5 ± 17.5% ($P < 0.05$, $n = 6$). STOC amplitude was...
increased by 56 ± 24.7% ($P < 0.005, n = 5$) by PACAP(1–38), and frequency increased by 102 ± 27.1% ($P < 0.05, n = 5$) (Fig. 3). Responses to VIP and PACAP were clearly noted within 3 min of addition of the drugs to the bath solution and reached maximum effects 5–7 min after application.

cAMP-dependent pathway modifiers on Ca$^{2+}$ transients and STOCs. VIP and PACAP are typically coupled via G$_s$ protein-coupled receptors to activation of AC. Therefore, we tested the effect of a membrane-permeant analog of cAMP on Ca$^{2+}$ puffs and STOCs. DBcAMP ($10^{-3}$ M) increased Ca$^{2+}$ puffs and

VIP increased STOCs in colonic myocytes from phospholamban-knockout (PLB$^{-/-}$) mouse. Some reports have suggested that cAMP-dependent protein kinase (PKA)-dependent activation of Ca$^{2+}$ transients and STOCs may be mediated by phosphorylation of PLB. A: STOCs in a cell from a PLB$^{-/-}$ myocyte held at −30 mV. B: VIP ($10^{-6}$ M) increased the frequency and amplitude of STOCs. Effects of VIP on STOC amplitude ($P < 0.0001, n = 5$) and frequency ($*P < 0.05, n = 5$) in PLB$^{-/-}$ myocytes are summarized in C and D.
STOCs in a manner similar to VIP and PACAP. Increasing the level of cytosolic cAMP increased the Ca\(^{2+}\) puffs by 37.3 ± 14.7% (P < 0.05, n = 6), increased STOC amplitude (P < 0.005), and increased frequency (to 176.3 ± 47.2%; P < 0.05, n = 5) (Fig. 4).

We also tested the effects of pretreating cells with the cell-permeant and irreversible inhibitor of AC MDL-12,330A on VIP responses. MDL-12,330A (5 × 10^{-4} M) did not significantly affect spontaneous Ca\(^{2+}\) transients (−4.9 ± 5.3%; P > 0.1, n = 6), but preexposure to this drug for 10 min before addition of VIP prevented responses such as those shown in Figs. 1 and 2. For example, in the presence of VIP responses. MDL-12,330A caused an average 14.7% (P < 0.05, n = 6), increased frequency (to 106.7 ± 29.0%; P < 0.05, n = 4) in colonic myocytes of PLB−/− mice was elevated compared with cells of BALB/c mice. VIP (10^{-6} M) increased STOC amplitude (P < 0.0001) and frequency (106.7 ± 29.0%; P < 0.05, n = 4) in colonic myocytes from the PLB−/− mice (Fig. 7).

Previous studies on cerebral arteries have shown that PLB mediates the increase in Ca\(^{2+}\) sparks caused by activation of AC (44). Activation of PKA, by forskolin or cAMP, increased Ca\(^{2+}\) sparks and STOCs in myocytes of control animals, but this effect was absent in cells of PLB−/− mice (44). In the present study we tested whether PLB is required for the activation of STOCs by VIP. STOC frequency in colonic myocytes of PLB−/− mice was elevated compared with cells of BALB/c mice. VIP (10^{-6} M) increased STOC amplitude (P < 0.0001) and frequency (106.7 ± 29.0%; P < 0.05, n = 4) in colonic myocytes of PLB−/− mice (Fig. 7).

Role of RyR channels in responses to DBcAMP. We investigated the effects of ryanodine on cAMP-mediated changes in Ca\(^{2+}\) transients and STOCs. As previously reported, ryanodine (10^{-5} M) did not significantly affect spontaneous Ca\(^{2+}\) transients in murine colonic myocytes. However, pretreatment of cells with ryanodine for 10 min blocked the increase in Ca\(^{2+}\) transients in response to DBcAMP (i.e., DBcAMP changed Ca\(^{2+}\) transients by −7.2 ± 13.9%; P > 0.1, n = 6; Fig. 8, A and B). Similar results were obtained in experiments in which STOCs were recorded. Ryanodine did not affect the amplitude (P > 0.1) or frequency (i.e., STOC frequency changed by 20.4 ± 10.4%; P > 0.1 in response to ryanodine) of STOCs.

Fig. 8. Ryanodine prevents the effects of DBcAMP on Ca\(^{2+}\) transients and STOCs. Ryanodine did not prevent basal Ca\(^{2+}\) transients or STOCs in murine colonic myocytes (A, C, and D) but blocked the increase in Ca\(^{2+}\) transients in response to DBcAMP (A and B; P > 0.1, n = 6). STOC amplitude (P > 0.1, n = 5) and frequency (P > 0.1, n = 5) were also not significantly (NS) affected by DBcAMP in the presence of ryanodine (D and E). Effects of DBcAMP on STOC amplitude (P > 0.1, n = 5) and frequency (P > 0.1, n = 5) in the presence of ryanodine are summarized in F and G.
but prevented the increase in amplitude ($P > 0.1$) and frequency ($-4.2 \pm 16.2\% ; P > 0.1$, both $n = 5$) in response to DBcAMP (Fig. 8, C–G). Thus cAMP-mediated effects on Ca$^{2+}$ transients and STOCs appear to be due to recruitment of RyR channels.

In many cells regulation of PKA targeting is accomplished by AKAPs (45). RyR channels are among the cellular effectors that bind AKAP-anchored PKA (36, 37), and we reasoned that cAMP-dependent effects in colonic myocytes might be mediated through an AKAP. Peptides derived from anchoring proteins mimic the regulatory subunit of PKA, causing disruption of the interaction between PKA and AKAPs. We tested InCELLect AKAP-IP, which is stearated to make it cell permeant. AKAP-IP ($10^{-4} \text{M}$) did not significantly affect spontaneous Ca$^{2+}$ transients ($16.1 \pm 10.6\% ; P > 0.1$, $n = 6$), but pretreatment with this peptide prevented the increase in Ca$^{2+}$ transients in response to DBcAMP ($-0.6 \pm 11.8\% ; P > 0.1$, $n = 6$) (Fig. 9). STOC amplitude ($P > 0.05$) and frequency ($24.2 \pm 10.4\% ; P > 0.1$, both $n = 4$) were not affected by application of AKAP-IP, whereas application of DBcAMP in the presence of AKAP-IP caused no further increase in either amplitude ($P > 0.1$) or frequency ($12.5 \pm 11.62\% ; P > 0.5$, $n = 4$) of STOCs (Fig. 10). This suggests that regulation of the RyR channels by PKA requires binding of AKAP.

**DISCUSSION**

In the present study we investigated the role of VIP and PACAP, putative neurotransmitters released from enteric inhibitory neurons, on Ca$^{2+}$ transients and STOCs, which are due to openings of Ca$^{2+}$-activated K$^+$ channels in the plasma membranes of murine colonic myocytes (1). Previous contractile experiments have suggested the involvement of both small (SK) and large (BK) conductance K$^+$ channels in responses to enteric inhibitory neurotransmitters and the effects of VIP, which is transduced via the cAMP and PKA signaling pathway (19, 42). We found that VIP and PACAP increased Ca$^{2+}$ transients and STOCs in murine colonic myocytes by a cAMP-dependent increase in Ca$^{2+}$ release from RyR channels. The effects of VIP/PACAP on RyR channels were dependent on AKAPs that tether PKA to effector proteins. An increase in Ca$^{2+}$ transients and activation of Ca$^{2+}$-activated K$^+$ channels (BK and SK) (1) would lead to membrane hyperpolarization and relaxation.

Fig. 9. Disruption of the A-kinase anchoring proteins (AKAPs) prevented the increase in Ca$^{2+}$ transients by DBcAMP. AKAP St-Ht31 inhibitor peptide (AKAP-IP) has been shown to bind to the regulatory subunit RII of PKA and prevent AKAP-PKA interaction. AKAP-IP caused no significant effect on Ca$^{2+}$ transients ($P > 0.1$, $n = 6$), but it blocked the increase in Ca$^{2+}$ transients caused by DBcAMP (NS, $P > 0.1$, $n = 6$). D: summary of results from 6 experiments. NS, nonsignificant ($P > 0.1$).
as reported in other studies (1, 3). This is the first study on smooth muscle suggesting that the inhibitory effects of VIP/PACAP may be due, in part, to stimulation of Ca\textsuperscript{2+} release from RyR channels via PKA and AKAP.

The current study suggests that VIP and PACAP actions are due to increased levels of cAMP due to activation of AC (Figs. 4–6). Activation of the AC/cAMP/PKA pathway occurs in numerous smooth muscles in response to a variety of agonists, e.g., VIP, calcitonin gene-related peptide, adenosine, β-adrenergic agonists (18, 20, 27, 32, 43, 46). Evidence supporting the conclusion that the AC/cAMP/PKA pathway is utilized in relaxation responses to VIP and PACAP in GI muscles includes 1) relaxation of canine stomach in response to VIP was reduced by AC inhibitors (4); 2) VIP and PACAP (1–27) caused a concentration-dependent increase in cAMP levels (18, 26); and 3) VIP and PACAP failed to induce relaxation of colonic muscles when PKA was inhibited (18, 38). Other authors have suggested that part of the response to VIP is mediated via the NO/cGMP/PKG pathway (28, 29), but this idea does not appear to be correct because responses to VIP and PACAP were not affected by blockade of NO synthase (11, 16) and VIP responses were not reduced in mice deficient in all isoforms of NO synthase (10). Although activation of PKA appears to be the major signaling pathway for the inhibitory responses to VIP and PACAP, most cellular effectors have not been elucidated.

The inhibitory actions of VIP and PACAP on contractions of some smooth muscles have been attributed, in part, to increased open probability (\(N_{p_o}\)) of Ca\textsuperscript{2+}-activated K\textsuperscript{+} (K\textsubscript{Ca}) channels (6, 19, 39, 42). In these studies, relaxation or hyperpolarization responses to VIP and PACAP were diminished by blocking K\textsubscript{Ca} channels with apamin and/or charybdotoxin. Previous studies have shown that PKA enhances the open probability of BK channels (8, 23). A previous study showed that cAMP-dependent mechanisms can increase localized Ca\textsuperscript{2+} transients in vascular muscle cells (35). In the present study we have linked responses of VIP and PACAP in colonic muscles to similar mechanisms. Stimulation of VIP receptors resulted in increased Ca\textsuperscript{2+} transients that are enhanced via cAMP-dependent mechanisms. Others have calculated that Ca\textsuperscript{2+} can reach concentrations of at least 10\textsuperscript{−6} M in the microdomain between the SR and the plasma membrane during localized Ca\textsuperscript{2+} transients (33, 48). Changing Ca\textsuperscript{2+} concentration from 100 nM to 10\textsuperscript{−6} M during a Ca\textsuperscript{2+} spark would increase the \(N_{p_o}\) of BK channels by a factor of \(10^4\). In contrast, direct modulation of BK channels by PKA has been shown to increase \(N_{p_o}\) by approximately threefold (8, 23). Thus the increased release of Ca\textsuperscript{2+} from RyR receptors in response to VIP and PACAP stimulation that we have observed may be the major drive to increase STOCs in colonic myocytes. This idea is also supported by our experiments in which the enhancement in STOCs in response to DBcAMP was blocked by ryanodine. STOCs remaining after ryanodine, due to Ca\textsuperscript{2+} release from IP\textsubscript{3} receptors (1), were not increased by activating the cAMP/PKA pathway. SK channels also contribute to STOCs in murine colonic myocytes (1), and there is no known regulation of SK channels by cAMP-dependent mechanisms. Thus in-

![Fig. 10. AKAP-IP prevented the increase in STOCs caused by DBcAMP. AKAP-IP caused no significant change in frequency (NS, \(P > 0.1\); \(n = 4\)) and amplitude (\(P > 0.05\); \(n = 4\)) of STOCs. DBcAMP, in the presence of AKAP-IP, caused no significant increase in amplitude (\(P > 0.1\); \(n = 4\)) or frequency (NS, \(P > 0.5\); \(n = 4\)). D and E: summary of data from 4 experiments.](http://ajpcell.physiology.org/)
creases in SK openings in response to VIP and PACAP are most likely due to the increase in Ca\(^{2+}\) transients.

VIP and PACAP have been suggested to activate RyR channels through a PKA pathway in neuronal cells (9). Phosphorylation of RyR channel by PKA increases NP\(_{e}\) by causing more frequent openings and decreasing the mean closed time (40). The effector protein of PKA-mediated activation of sparks is controversial. Some studies have suggested that PKA-mediated phosphorylation of PLB increases Ca\(^{2+}\) sparks via enhanced filling of Ca\(^{2+}\) stores (25, 41, 44). When vascular tissues from the PLB\({\sim}\) mouse were stimulated with forskolin there was no significant increase in Ca\(^{2+}\) sparks but a reduction in caffeine-induced Ca\(^{2+}\) transients compared with responses in wild-type mice (44). These results suggest that activation of PKA in vascular myocytes leads to regulation of both PLB and RyR. In present study we found that VIP increased STOCs in cells from the PLB\({\sim}\) mouse. Thus it is likely that modulation of Ca\(^{2+}\) transients by activation of PKA is mainly mediated by effectors (such as RyR channel) downstream of PLB.

Recently, we reported that neurokinins regulate Ca\(^{2+}\) puffs and STOCs in murine colonic myocytes by PKC-dependent regulation of L-type Ca\(^{2+}\) current (3). Low concentrations of substance P (SP) enhanced Ca\(^{2+}\) transients via PKC-dependent activation of L-type Ca\(^{2+}\) channels and increased Ca\(^{2+}\) release from RyR channels. This increase in localized Ca\(^{2+}\) transients enhanced STOCs and hyperpolarized colonic muscles (3). We suspected that the increase in Ca\(^{2+}\) transients and enhanced STOCs in response to VIP/PACAP might be similar to the actions of SP in that VIP also increases L-type Ca\(^{2+}\) currents in GI smooth muscle myocytes (21, 24). We found, however, that application of VIP in the presence of nicardipine, to block L-type Ca\(^{2+}\) channels, resulted in responses that were equivalent to responses in the absence of the dihydropyridine. This suggests that increase in Ca\(^{2+}\) transients and enhanced STOCs in response to PACAP and VIP were not due to effects mediated by L-type Ca\(^{2+}\) current.

PKA is targeted to specific proteins, such as microtubule-associated protein-2, RyR channels, L-type Ca\(^{2+}\) channels, protein phosphatases, delayed rectifier K\(^{+}\) channels, troponin I, etc., via its association with AKAPs (see Refs. 15 and 45 for review). PKA is localized to specific proteins via binding of its dimerized regulatory subunits to a conserved anchoring motif in AKAPs. Compartmentalization of individual AKAP-PKA units is accomplished through specialized targeting domains present on each AKAP isoform. Selective compartmentalization of PKA by AKAPs ensures that particular PKA substrates can be rapidly and selectively phosphorylated in response to stimuli. On binding of cAMP to the regulatory subunits of PKA, the kinase is released from AKAPs and becomes active. In the present study we used an inhibitory protein, AKAP-IP, which attaches to the type 2 regulatory subunit of PKA, preventing PKA-AKAP binding and PKA localization. AKAP-IP blocked the cAMP-dependent regulation of Ca\(^{2+}\) transients and STOCs. Western blot analysis and immunocytochemistry have demonstrated that AKAPs are associated with RyR in skeletal muscles, and RyR is phosphorylated in response to enhanced cAMP levels (36, 37). Thus it is possible that the increase in Ca\(^{2+}\) transients and STOCs in response to cAMP in colonic myocytes was due to AKAP-mediated, PKA-dependent phosphorylation of RyR channels.

In summary, VIP and PACAP stimulate Ca\(^{2+}\) transients and STOCs in colonic muscle cells via cAMP-dependent protein kinase regulation of RyR channels. The effects of VIP and PACAP were indistinguishable, suggesting that these neuropeptides utilize the same receptors or separate receptors coupled to the same signaling pathway. VIP and PACAP appear to increase Ca\(^{2+}\) transients by binding to a Gs-coupled receptor, activation of AC, and increased production of cAMP. Activation of PKA causes an increase Ca\(^{2+}\) release from RyR channels. Increased Ca\(^{2+}\) release from RyR channels enhances activation of K\(_{Ca}\) channels in the plasma membrane. PKA is localized to RyR channels that mediate cAMP-dependent effects via binding to AKAP. These findings suggest a novel mechanism in which enteric inhibitory peptides are coupled to activation of K\(^{+}\) channels and yield hyperpolarization and relaxation of GI muscles.

GRANTS
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REFERENCES


47. ZhuGe R, Fogarty KE, Tuft RA, and Walsh JV Jr. Spontaneous transient outward currents arise from microdomains where BK channels are exposed to a mean Ca$_{2+}$ concentration on the order of 10 µM during a Ca$_{2+}$ spark. *J Gen Physiol* 120: 15–27, 2002.