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Pituitary adenylate cyclase activating polypeptide (PACAP) dilates cerebellar arteries through activation of large-conductance Ca^{2+} -activated (BK) and ATP-sensitive (K_{ATP}) K^{+} channels

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Abstract

Pituitary adenylate cyclase activating polypeptide (PACAP) is a potent vasodilator of numerous vascular beds, including cerebral arteries. Although PACAP-induced cerebral artery dilation is suggested to be cyclic AMP (cAMP)-dependent, the downstream intracellular signaling pathways are still not fully understood. In this study, we examined the role of smooth muscle K^{+} channels and hypothesized that PACAP-mediated increases in cAMP levels and protein kinase A (PKA) activity result in the coordinate activation of ATP-sensitive K^{+} (K_{ATP}) and large-conductance Ca^{2+} -activated K^{+} (BK) channels for cerebral artery dilation. Using patch-clamp electrophysiology, we observed that PACAP enhanced whole-cell K_{ATP} channel activity and transient BK channel currents in freshly isolated rat cerebellar artery myocytes. The increased frequency of transient BK currents following PACAP treatment is indicative of increased intracellular Ca^{2+} release events termed Ca^{2+} sparks. Consistent with the electrophysiology data, the PACAP-induced vasodilations of cannulated cerebellar artery preparations were attenuated by approximately 50% in the presence of glibenclamide (a K_{ATP} channel blocker) or paxilline (a BK channel blocker). Further, in the presence of both blockers, PACAP failed to cause vasodilation. In conclusion, our results indicate that PACAP causes cerebellar artery dilation through two mechanisms: 1) K_{ATP} channel activation and 2) enhanced BK channel activity, likely through increased Ca^{2+} spark frequency.

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Disclosures

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Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP) is a peptide that is highly conserved over species and widely distributed in the brain and peripheral organs. While acting as a neurotransmitter and neurotrophic peptide in the central and peripheral nervous systems (reviewed in Vaudry et al. 2009), PACAP is also a potent vasodilator (Warren et al. 1992; Vaudry et al. 2009; Syed et al. 2012). PACAP-induced vasodilation has been observed in various vascular beds including coronary arteries (Bruch et al. 1997; Dalsgaard et al. 2003), pulmonary arteries (Cheng et al. 1993), mesenteric arteries (Wilson et al. 1993) and cerebral arteries (Tong et al. 1993; Anzai et al. 1995). For example, *in vivo* treatment of PACAP via an open cranial window resulted in brain pial artery dilation in newborn pigs (Tong et al. 1993), and intracisternal administration of PACAP caused dose-dependent canine basilar artery dilation detected by angiography (Seki et al. 1995). Further, in *ex vivo* studies, PACAP caused concentration-dependent relaxation of isolated canine basilar arteries (Anzai et al. 1995; Seki et al. 1995), cat cerebral arteries (Uddman et al. 1993), rabbit posterior cerebral arteries (Dalsgaard et al. 2003), rat middle cerebral arteries (Erdling et al. 2013) and rat intracerebral arterioles (Anzai et al. 1995). This vasodilatory effect is, at least in part, a direct effect of PACAP on arterial smooth muscle cells. PACAP binds to three types of receptors; PAC1, VPAC1 and VPAC2 (Vaudry et al. 2009; Harmar et al. 2012), and all three receptors are expressed in cerebral artery smooth muscle cells (Syed et al. 2012; Erdling et al. 2013). Binding of PACAP to PACAP receptors increases production of adenosine 3', 5'-cyclic monophosphate (cAMP) (Tong et al. 1993; Vaudry et al. 2009) in arterial myocytes which likely accounts for the mechanism of PACAP-induced cerebral artery dilation.

Increased cAMP production and subsequent activation of cAMP-dependent protein kinase (protein kinase A, PKA) can produce vasodilation by several mechanisms including activation of smooth muscle K⁺ channels (Nelson et al. 1995b; Wellman et al. 1998). Potassium channels play an important role in the regulation of smooth muscle membrane potential, intracellular Ca²⁺ concentration and arterial diameter; activation of smooth muscle K⁺ channels causes membrane potential hyperpolarization, decreased open-state probability of voltage-dependent Ca²⁺ channels and reduced intracellular Ca²⁺ concentration resulting in vasodilation (Standen et al. 1989; Nelson et al. 1995b; Standen et al. 1998). Dilations induced by a number of endogenous vasoactive substances such as calcitonin gene-related peptide (CGRP) have been shown to result from increased ATP-sensitive K⁺ (K_{ATP}) channel currents via mechanisms involving adenylyl cyclase activation, increased cAMP production and PKA activation (Nelson et al. 1990; Quayle et al. 1994; Kleppisch et al. 1995a; Wellman et al. 1998). Similarly, K_{ATP} channel blockers were able to partially inhibit PACAP-induced vasodilation in coronary and pulmonary arteries, suggesting that PACAP may also activate K_{ATP} channels (Bruch et al. 1997; Bruch et al. 1998).

Stimulated cAMP/PKA activity however, can also augment the frequency of transient large-conductance Ca²⁺-activated K⁺ (BK) channel currents (previously referred to as spontaneous transient outward currents or STOCs) following intracellular Ca²⁺ spark events (Nelson et al. 1995a; Wellman et al. 2003). Transient outward BK currents occur as a result of increases in intracellular Ca²⁺, called Ca²⁺ sparks, and represent an important

physiological vasodilatory pathway in cerebral arteries (Nelson et al. 1995a; Wellman et al. 2003). Ca^{2+} sparks are transient and localized Ca^{2+} increases resulting from the opening of a small cluster of ryanodine-sensitive Ca^{2+} channels (ryanodine receptors, RyRs) located on the sarcoplasmic reticulum (SR). These localized increases in intracellular Ca^{2+} lead to activation of nearby BK channels on the plasma membrane (transient outward BK currents), resulting in smooth muscle membrane potential hyperpolarization and vasodilation (Nelson et al. 1995a; Wellman et al. 2003). Considering that the treatment of cerebral artery myocytes with cAMP analogues or adenylyl cyclase activators (to elevate intracellular cAMP levels) can increase the frequency and amplitude of transient outward BK currents (Porter et al. 1998; Wellman et al. 2001; Hayoz et al. 2007), we hypothesized that PACAP-induced increases in cAMP can also activate the Ca^{2+} spark/transient outward BK current pathway to promote cerebral vasodilation.

Because of its vasodilatory, neurotrophic and neuroprotective effects, PACAP has been examined in animal models as possible treatments for cerebral vasospasm after subarachnoid hemorrhage (Kaminuma et al. 1998) and ischemic stroke (Reglodi et al. 2000; Ohtaki et al. 2006). However, despite its therapeutic potential, the detailed intracellular signaling mechanisms involved in PACAP-induced cerebral artery dilation are not fully understood. In this study, we have examined the vasodilatory mechanisms of PACAP, focusing specifically on the role of smooth muscle K^{+} channels in freshly isolated rat superior cerebellar arteries. Our results indicate that activation of both K_{ATP} and BK channels contribute to PACAP-induced cerebral artery dilation.

Methods

Measurements of K_{ATP} currents and transient outward BK currents

All experiments and protocols were performed in accordance with the Guide for the care and Use of Laboratory animals (eighth edition, 2011) and were approved by the Institutional Animal Care Committee of the University of Vermont. Male Sprague-Dawley rats (300–350g; Charles River Laboratories, Saint Constant, QC, Canada) were euthanized by decapitation under deep pentobarbital anesthesia. The brain was carefully removed and superior cerebellar arteries were isolated in ice cold artificial cerebrospinal fluid (aCSF: 125 mM NaCl, 3 mM KCl, 18 mM NaHCO_3 , 1.25 mM NaH_2PO_4 , 1 mM MgCl_2 , 2 mM CaCl_2 , and 5 mM glucose, aerated with 5 % CO_2 , 20 % O_2 , 75 % N_2 , pH 7.35–7.40).

Arterial myocytes were enzymatically isolated using a previously described protocol (Ishiguro et al. 2005; Koide et al. 2007; Koide et al. 2011). Briefly, arteries were incubated in a solution containing papain (0.3 mg/mL papain, 0.7 mg/mL 1,4-dithioerythriol) for 17 min at 37 °C, and then in collagenase solution (0.7 mg/mL collagenase type F, 0.3 mg/mL collagenase type H, 0.1 mM CaCl_2) for 20 min at 37 °C. Enzyme solutions were made using a glutamate-containing isolation solution (GIS: 55 mM NaCl, 5.6 mM KCl, 80 mM L-glutamic acid-Na, 2 mM MgCl_2 , 2 mM CaCl_2 , 10 mM HEPES, and 10 mM glucose; pH 7.3). Finally, arteries were washed three times (10 minutes each wash) in GIS containing 2 mM CaCl_2 on ice then gently triturated into individual myocytes using a fire-polished Pasteur pipette.

Using the conventional whole cell patch clamp configuration, K_{ATP} currents were recorded in freshly isolated cerebellar arterial myocyte at room temperature (Wellman et al. 1999). The bath solution contained 134 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES (pH 7.4). The “140 mM K^+ ” bath solution was made by iso-osmotic replacement of NaCl with KCl. The pipette solution contained 107 mM KCl, 1.0 mM $MgCl_2$, 1.9 mM $CaCl_2$, 10 mM HEPES, 5 mM EGTA, 25 mM KOH (pH 7.2 adjusted with KOH, free Ca^{2+} ~100 nM). On the day of the experiment, 0.1 mM Na_2ATP , 0.1 mM NaADP and 0.1 mM LiGTP were freshly added into an aliquot of pipette solution. K_{ATP} currents were recorded at -60 mV. After a stable baseline was obtained, the extracellular solution was changed from “6 mM K^+ ” bath solution to “140 mM K^+ ” solution. PACAP was treated by superfusion into the bath in the presence of 140 mM K^+ .

Transient outward BK currents were measured using the perforated whole-cell patch-clamp technique at room temperature (Wellman et al. 2002; Koide et al. 2011). The bath solution contained 134 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES (pH 7.4). Patch pipettes (8–10 M Ω) were filled with a pipette solution that contained 110 mM K^+ -aspartate, 30 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES, 0.05 mM EGTA and 0.2 mM amphotericin B (pH 7.2). Transient outward currents were recorded at -40 mV. PACAP was treated by bath superfusion. Recordings were analyzed using Mini Analysis 6.0.3 software (Synaptosoft, Inc., Decatur, GA, USA) to determine transient BK current amplitude, frequency, rise time (time from 10 % to 90 % of the peak), decay time (time from 90% to 37 % of the peak) and half-width (time at 50% of the peak). The threshold for current peak detection was set at two and half times the single channel amplitude of the BK channel; 5.0 pA at -40 mV (Perez et al. 2001).

Diameter measurements of isolated cerebellar arteries

Superior cerebellar arteries were cannulated onto glass micropipettes mounted in 5 ml myograph chamber and the intravascular pressure was increased using a servo controller and a peristaltic pump (Living Systems Instruments, St. Albans, VT) as previously described (Ishiguro et al. 2002; Nystoriak et al. 2011; Koide et al. 2012). The arteries were continuously superfused with aCSF warmed at 37°C. Luminal diameter was measured and recorded using video edge detection (Living Systems Instrumentation, St Albans, VT) and WinDaq data acquisition software (Dataq Instruments; Akron, OH). Following a 30 minute equilibration period at 20 mmHg, arteries were briefly exposed to 60 mM KCl-aCSF (<2 min; iso-osmotic replacement of NaCl with KCl) to test their viability. Only arteries that exhibited constriction of 50 % or more to 60 mM KCl-aCSF were included for study. Following an additional 30 minutes, intraluminal pressure was increased to 60 mmHg. Over the course of approximately 1 hour, arteries constricted to the increase in intravascular pressure, i.e. developed pressure-induced myogenic tone. Once arterial diameter was stabilized at 60 mmHg, PACAP (3 nM) was superfused into the chamber in the absence and presence of K^+ channel blocker(s). Percent constriction in response to elevation of intravascular pressure was determined using the following equation: $[(DP-DA)/DP] \times 100$; where DP = the (passive) diameter of the artery in Ca^{2+} -free aCSF containing the vasodilators diltiazem (100 μ M) and forskolin (1 μ M), and DA = the (active) diameter of the artery in Ca^{2+} -containing aCSF. Dilation responses to PACAP are expressed as a reduction

in arterial constriction using the following equation: % dilation = $[(DV-DA)/(DP-DA)] \times 100$, where DV = arterial diameter in the presence of vasodilator, DA = the active diameter of the artery prior to addition of vasodilator, and DP = the passive diameter of the artery. All aliquots of compounds used in the study were diluted to their final concentrations immediately before use and were administered to arteries by bath superfusion (5 mL/min flow rate) for a minimum of 10 min.

Chemicals

PACAP was obtained from American Peptide Company (Sunnyvale, CA). All other compounds were purchased from Sigma-Aldrich (St. Louis, MO).

Statistics

Data are expressed as mean $\pm$ SEM. Comparisons between multiple groups were analyzed by one-way ANOVA followed by post hoc comparison of means using Tukey test. Student's paired *t*-test was used to compare data between two groups. Statistical significance was considered at $p < 0.05$.

Results

PACAP activates K_{ATP} channels in freshly isolated rat cerebellar artery myocytes

Several endogenous vasodilators (e.g. CGRP, adenosine) linked to increased adenylyl cyclase activity have been shown to activate K_{ATP} channels in arterial smooth muscle cells (Nelson et al. 1990; Quayle et al. 1994; Kleppisch et al. 1995a; Kleppisch et al. 1995b; Wellman et al. 1998). To examine whether PACAP can increase K_{ATP} channel activity in freshly isolated cerebellar artery myocytes, membrane K^+ currents were measured by patch clamp electrophysiology. Here, recording conditions using the conventional whole-cell configuration were designed to optimize K_{ATP} currents and minimize BK and voltage-dependent K^+ (K_V) channel activity (e.g. 0.1 mM ATP, 0.1 mM ADP and 5 mM EGTA in intracellular pipette solution; holding potential of -60 mV) (Wellman et al. 1998). In symmetrical (intracellular and extracellular) 140 mM K^+ , the K^+ equilibrium potential (E_K) predicted by the Nerst equation is 0 mV. Thus, at a holding potential negative to E_K , e.g. -60 mV, K_{ATP} channel activation will cause K^+ ions to move into the cell creating an inward electrical current (Quayle et al. 1994; Kleppisch et al. 1995b; Wellman et al. 1998). As shown in figure 1A, treatment of a cerebral artery myocyte with the synthetic K_{ATP} channel opener, pinacidil (10 μ M), caused a significant increase in inward K^+ current that was inhibited by the K_{ATP} channel blocker, glibenclamide (10 μ M). Using similar recording conditions, PACAP (3 nM) treatment also caused an increase in inward K^+ current (figure 1B). This data suggests that PACAP can activate K_{ATP} channels in rat cerebellar artery myocytes.

PACAP increases the frequency of transient outward BK currents in rat cerebellar artery myocytes

An increase in the frequency of smooth muscle transient outward BK currents represents another potential mechanism involved in PACAP-induced cerebral artery dilation. Transient BK currents represent the coordinated opening of up to 100 individual BK channels in

regions of the cell membrane ($\approx 1\%$ of the total cell surface area) exposed to brief local increases in Ca^{2+} sparks (Wellman et al. 2003). Treatment of vascular smooth muscle cells with either a cAMP analogue, an adenylyl cyclase activator (e.g. forskolin) or endogenous vasodilators that elevate intracellular cAMP levels causes an increase in the frequency of Ca^{2+} spark-induced transient BK currents triggered by Ca^{2+} sparks (Porter et al. 1998; Wellman et al. 2001). Considering that PACAP can potentially increase intracellular cAMP levels, activation of the Ca^{2+} spark/transient BK current pathway may also contribute to PACAP-induced cerebral artery dilation. To examine this hypothesis, we directly measured transient BK currents in freshly isolated cerebellar artery myocytes using the whole-cell perforated patch configuration of the patch clamp technique. In this experimental series, cells were held at the physiological membrane potential of -40 mV in an extracellular (bath) solution containing 6 mM K^+ . Spontaneous transient outward BK currents were observed in these cells and PACAP (3 nM) significantly increased the frequency of these events by approximately 50% (from $1.38 \pm 0.47\text{ Hz}$ to $2.02 \pm 0.51\text{ Hz}$; $n = 6$ cells from 3 animals, figure 2A and 2B). Although PACAP markedly increased the transient BK current frequency, the amplitude of these events was not dramatically altered by PACAP treatment (before treatment: $14.71 \pm 2.11\text{ pA}$, after PACAP treatment: $17.49 \pm 2.98\text{ pA}$, figure 2C). Further, the temporal characteristics of transient BK currents were similar before and after PACAP treatment: rise-time_{10–90%} (msec): $7.94 \pm 0.85/7.28 \pm 0.30$; decay time_{90–37%} (msec): $25.03 \pm 3.84/23.57 \pm 4.16$; half-width (msec): $30.33 \pm 3.21/31.33 \pm 3.34$ ($n = 6$ cells from 3 animals, before/after PACAP treatment, respectively). In summary, these data demonstrate that PACAP increased the frequency of Ca^{2+} spark-mediated transient BK currents in isolated rat cerebellar artery myocytes.

PACAP-induced cerebellar artery dilation involves activation of both BK and K_{ATP} channels

The above studies indicate that PACAP can increase the activity of both BK and K_{ATP} channels in freshly isolated cerebellar artery myocytes. Next, the functional contribution of these K^+ channels in PACAP-induced dilation was examined in isolated and pressurized rat superior cerebellar arteries. At a physiological intravascular pressure of 60 mmHg , arteries superfused with aCSF developed pressure-induced constriction (myogenic tone) representing a $23.0 \pm 1.3\%$ decrease in diameter ($n = 19$ from 19 animals). At 60 mmHg , the average diameter of cerebellar arteries in aCSF with myogenic tone was $191.1 \pm 7.5\text{ }\mu\text{m}$ compared to a fully-dilated diameter of $247.2 \pm 7.5\text{ }\mu\text{m}$ obtained in Ca^{2+} -free aCSF containing diltiazem ($100\text{ }\mu\text{M}$, a voltage-dependent Ca^{2+} channel blocker) and forskolin ($1\text{ }\mu\text{M}$). PACAP (3 nM) caused significant dilation ($52.2 \pm 10.7\%$ of maximal dilation), increasing the average arterial diameter from $189.8 \pm 9.1\text{ }\mu\text{m}$ to $220.8 \pm 14.0\text{ }\mu\text{m}$ ($n = 4$ from 4 animals, figure 3A and 3E). To examine whether K_{ATP} channel activation (shown in figure 1B) contributes to PACAP-induced cerebellar artery dilation, arteries were treated with the K_{ATP} channel blocker, glibenclamide ($10\text{ }\mu\text{M}$). Glibenclamide treatment alone, did not change arterial diameter ($187.0 \pm 14.7\text{ }\mu\text{m}$ and $185.8 \pm 16.4\text{ }\mu\text{m}$, in the absence and presence of glibenclamide, respectively, $n = 4$ from 4 animals), suggesting minimal basal K_{ATP} channel activity in these arteries. However, in the presence of glibenclamide, the PACAP-induced dilations were significantly attenuated (approximately 50% of maximal dilation with PACAP alone vs $22.6 \pm 11.8\%$ of maximal dilation in PACAP +

glibenclamide; $n = 4$ from 4 animals, figure 3B). This data indicates that PACAP-induced vasodilation is partially caused by K_{ATP} channel activation.

We next examined if activation of the Ca^{2+} spark/transient BK current pathway also plays a role in PACAP-induced cerebellar artery dilation. To inhibit this pathway, arteries were treated with the BK channel blocker, paxilline (1 μM), which alone constricted arteries in aCSF from $204.0 \pm 13.4 \mu m$ to $174.4 \pm 14.7 \mu m$, an approximate 15% decrease in diameter on top of myogenic tone (fig. 3C, note paxilline-induced vasoconstriction before PACAP application; fully-dilated diameter: $262.6 \pm 11.1 \mu m$, $n = 7$ from 7 animals). This paxilline-induced vasoconstriction is consistent with previous studies demonstrating that the Ca^{2+} spark/transient BK current pathway plays an important negative-feedback role in the regulation of cerebral artery myogenic tone (Nelson et al. 1995a; Koide et al. 2011; Nystoriak et al. 2014). The PACAP-induced vasodilation was significantly attenuated in the presence of paxilline (approximately 50% maximal dilation with PACAP alone vs $14.8 \pm 2.6\%$ of maximal dilation in PACAP + paxilline; $n = 7$ from 7 animals), suggesting that the Ca^{2+} spark/transient BK current pathway is also involved in PACAP-induced dilation (figure 3C and 3E). Thus, when applied separately, blockers of K_{ATP} channels (glibenclamide) and BK channels (paxilline) both decreased PACAP-induced dilations by approximately 50%. Interestingly, the combined treatment of arteries with both glibenclamide and paxilline abolished PACAP-induced cerebellar artery dilation. In the presence of glibenclamide and paxilline, arterial diameter before and after PACAP treatment was $174.0 \pm 21.9 \mu m$ and $163.5 \pm 23.7 \mu m$, respectively ($n = 4$ from 4 animals, figure 3D and 3E). In summary, these data indicate that PACAP causes cerebellar artery dilation through activation of both K_{ATP} and BK channels.

Discussion

Here we demonstrate that two distinct mechanisms contribute to PACAP-induced cerebellar artery dilation; activation of K_{ATP} channels and BK channels. The involvement of smooth muscle K_{ATP} and BK channels in cerebral artery dilations caused by PACAP (depicted in figure 4) is supported by our following observations: 1) PACAP increased K_{ATP} channel activity in isolated rat cerebellar artery myocytes, 2) PACAP also increased the frequency of cerebral artery smooth muscle transient BK currents, 3) Individual application of either the K_{ATP} channel blocker (glibenclamide) or the BK channel blocker (paxilline) significantly attenuated PACAP-induced cerebral artery dilation (~ 50 % attenuation in the presence of either blocker) and 4) the combined treatment of arteries with glibenclamide and paxilline abolished PACAP-induced dilation of pressurized rat cerebellar arteries.

In vascular smooth muscle, functional K_{ATP} channels are thought to be composed of four Kir 6.1 pore-forming subunits encoded by the gene KCNJ8 and four SUR2B accessory subunits encoded by the gene ABCC9 (Flagg et al. 2010). K_{ATP} channels of arterial myocytes are activated by a variety of endogenous vasodilators such as CGRP (Nelson et al. 1990; Quayle et al. 1994; Kleppisch et al. 1995a; Kleppisch et al. 1995b; Wellman et al. 1998) as well as cAMP analogs and cell dialysis with the catalytic subunit of PKA (Quayle et al. 1994; Wellman et al. 1998). PKA-mediated phosphorylation of serine and threonine residues within the nucleotide binding folds of SUR2B subunits is thought to promote ADP

binding to increase channel activity (Quinn et al. 2004; Shi et al. 2007; Flagg et al. 2010). In this study, we demonstrate that PACAP, another endogenous vasodilator known to stimulate adenylyl cyclase activity (Tong et al. 1993; Vaudry et al. 2009), can activate K_{ATP} channels in rat cerebral artery smooth muscle. As shown Figure 1, activation of K_{ATP} currents by pinacidil and PACAP evoked inward membrane currents at -60 mV using intracellular and extracellular solutions that both contained 140 mM K^+ . However, with physiological K^+ concentrations (e.g. extracellular $K^+ \approx 5.4$ mM; intracellular $K^+ \approx 140$ mM), K_{ATP} channel activation causes outward membrane currents in isolated arterial smooth muscle cells and membrane potential hyperpolarization and vasodilation of intact arteries (Standen et al. 1989; Masuzawa et al. 1990; Quayle et al. 1994; Kleppisch et al. 1995b; Nelson et al. 1995b; Bruch et al. 1997; Bruch et al. 1998). Consistent with our electrophysiological recordings, PACAP-induced cerebellar artery dilation was significantly attenuated by the K_{ATP} channel blocker glibenclamide (Figure 3). Together, these data indicate that K_{ATP} channel activation contributes to PACAP-induced vasodilation in rat cerebellar arteries likely via activation of the cAMP/PKA pathway.

In addition to activation of K_{ATP} channels, increased intracellular cAMP and PKA activation can also enhance the Ca^{2+} spark/transient BK current signaling pathway to promote cerebral artery dilation (Porter et al. 1998; Wellman et al. 2001). Our data now shows that PACAP increases the frequency of transient BK currents in rat cerebellar artery myocytes (figure 2) and that BK channel activity is involved in PACAP-induced cerebral artery dilation (figure 3). Previous investigations have revealed three mechanisms that can contribute to PKA-mediated increased Ca^{2+} spark/BK channel activity. First, PKA can phosphorylate ryanodine receptors (RyRs), which are the sarcoplasmic reticulum (SR) Ca^{2+} release channels responsible for Ca^{2+} spark generation (Marx et al. 2000; Wehrens et al. 2006). Phosphorylation of RyRs by PKA increases their Ca^{2+} sensitivity resulting in elevated frequencies of Ca^{2+} sparks and Ca^{2+} -spark induced transient BK currents. Secondly, phosphorylation of phospholamban (PLB) can also contribute to PKA-mediated cerebral artery dilation (Simmerman et al. 1998; Wellman et al. 2001). Phospholamban is a regulatory protein that when bound to SR Ca^{2+} -ATPase (SERCA) causes a decrease in SERCA activity and reduced filling of the SR with Ca^{2+} . Phosphorylation by PKA causes PLB to dissociate from SERCA, leading to disinhibition of SERCA activity and elevation of SR Ca^{2+} load (Simmerman et al. 1998; Wellman et al. 2001) that can increase Ca^{2+} spark/BK current frequency and amplitude (ZhuGe et al. 1999). Lastly, PKA can increase BK channel activity via direct channel phosphorylation (Minami et al. 1993; Perez et al. 1994; Porter et al. 1998). Phosphorylation of BK channels by PKA causes a leftward-shift in the voltage-activation relationship (toward more negative membrane potential) without changing single channel conductance (Perez et al. 1994). Further, it has been suggested that cAMP can directly increase the open-state probability of BK channels based on the observation of increased single BK channel activity by application of cAMP to the intracellular side of excised membrane patches (Minami et al. 1993). A direct cAMP/PKA-mediated increase in BK channel activity would likely cause an increase in the amplitude of transient BK currents without altering Ca^{2+} spark frequency. In the present study, we demonstrate that PACAP increases the frequency, but not amplitude, of transient BK currents in isolated rat cerebellar artery myocytes. This observation is consistent with

PACAP causing an increase in Ca^{2+} spark frequency rather than directly altering the voltage-sensitivity of plasma membrane BK channels. Future studies are required to confirm the involvement of the cAMP/PKA pathway in PACAP-induced cerebral artery dilation, as well as the specific proteins involved in the observed increase in Ca^{2+} spark/transient BK current frequency. However, we clearly demonstrate the functional importance of the Ca^{2+} spark/BK channel pathway in PACAP-induced cerebellar artery dilation. As illustrated in figure 3, the BK channel blocker, paxilline, attenuated PACAP-induced vasodilation by approximately ~50%. Furthermore, the combined treatment of cerebral arteries with paxilline and the K_{ATP} channel blocker, glibenclamide, completely inhibited cerebral artery responses to PACAP. These data suggested that two pathways, activation of K_{ATP} and BK channels, are independent and both play an important role in PACAP-induced cerebellar artery dilation.

In summary, here we demonstrate that two distinct cell signaling mechanisms contribute to PACAP-induced dilation in rat cerebellar arteries; K_{ATP} channel activation and enhanced BK channel activity. Both mechanisms likely involve activation of the cAMP/PKA pathway, however further studies are needed to determine additional details of the intracellular signaling pathways linking PACAP receptor activation to these ion channels.

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Figure 1. K_{ATP} channel currents in rat cerebellar artery myocytes

A) K_{ATP} current measurement obtained from an isolated cerebellar artery myocyte. Whole-cell patch clamp recording conditions were used to optimize K_{ATP} currents and minimize other BK and K_V channel currents (e.g. 140 mM K^+ in extracellular bath solutions, 0.1 mM ATP and 0.1 mM ADP in intracellular pipette solution, -60 mV of holding potential). The synthetic K_{ATP} channel opener, pinacidil (10 μ M), caused an increase in inward current that was inhibited by the K_{ATP} channel blocker, glibenclamide (10 μ M). B) Original recording of PACAP treatment on inward K^+ current using the same recording conditions described panel A. PACAP (3 nM) increased inward K^+ current, suggesting increased K_{ATP} current.

Figure 2. PACAP enhanced transient BK current activity in rat cerebellar artery myocytes

A) Effect of PACAP (3 nM) on transient outward BK currents, showing increased frequency, but not amplitude, in rat cerebellar artery myocytes. Transient BK currents were measured at -40 mV. Expanded traces of transient outward BK currents are shown in lower panels. B and C) Summary data of transient outward BK current frequency (B) and amplitude (C). $n = 6$ cells from 3 animals. ** $P < 0.01$ vs before PACAP treatment ($-$ PACAP).

Figure 3. PACAP induced vasodilation is mediated via both K_{ATP} and BK channels in isolated rat cerebellar arteries

A) Representative trace of PACAP-induced dilation of a cerebellar artery. The artery was pressurized to 60 mmHg and treated with PACAP (3 nM) by bath superfusion. B and C) PACAP-induced vasodilation was significantly attenuated in the presence of the K_{ATP} channel blocker, glibenclamide (10 μM, panel B) or the BK channel blocker, paxilline (1 μM, panel C). D) The combined treatment of glibenclamide (Glib) and paxilline (Pax) abolished PACAP-induced cerebellar artery dilation. E) Summary data of PACAP-induced dilations in the absence and presence of glibenclamide and paxilline (n = 4–7). Data are expressed as mean ± SE. * P < 0.05, ** P < 0.01 vs PACAP-induced dilation in the absence of K⁺ channel blockers (Control).

Figure 4. Proposed mechanisms of PACAP-induced vasodilation in rat cerebellar artery
 AC: adenylyl cyclase, ATP: adenosine tri-phosphate, BK: large-conductance Ca²⁺-activated potassium (K⁺) channels, cAMP: cyclic adenosine mono-phosphate, K_{ATP}: ATP-sensitive potassium (K⁺) channels, PACAP: pituitary adenylate cyclase activating polypeptide, PKA: protein kinase A, PLB: phospholamban, RyR: ryanodine receptors, SERCA: sarcoplasmic reticulum calcium transport ATPase, SR: sarcoplasmic reticulum, VDCC: voltage-dependent Ca²⁺ channels.