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PACAP induces plasticity at autonomic synapses by nAChR-dependent NOS1 activation and AKAP-mediated PKA targeting

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ABSTRACT

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a pleiotropic neuropeptide found at synapses throughout the central and autonomic nervous system. We previously found that PACAP engages a selective G-protein coupled receptor (PAC_1R) on ciliary ganglion neurons to rapidly enhance quantal acetylcholine (ACh) release from presynaptic terminals via neuronal nitric oxide synthase (NOS1) and cyclic AMP/protein kinase A (PKA) dependent processes. Here, we examined how PACAP stimulates NO production and targets resultant outcomes to synapses. Scavenging extracellular NO blocked PACAP-induced plasticity supporting a retrograde (post- to presynaptic) NO action on ACh release. Live-cell imaging revealed that PACAP stimulates NO production by mechanisms requiring NOS1, PKA and Ca^{2+} influx. Ca^{2+}-permeable nicotinic ACh receptors composed of α7 subunits (α7-nAChRs) are potentiated by PKA-dependent PACAP/PAC_1R signaling and were required for PACAP-induced NO production and synaptic plasticity since both outcomes were drastically reduced following their selective inhibition. Co-precipitation experiments showed that NOS1 associates with α7-nAChRs, many of which are perisynaptic, as well as with heteromeric α3*-nAChRs that generate the bulk of synaptic activity. NOS1–nAChR physical association could facilitate NO production at perisynaptic and adjacent postsynaptic sites to enhance focal ACh release from juxtaposed presynaptic terminals. The synaptic outcomes of PACAP/PAC_1R signaling are localized by PKA anchoring proteins (AKAPs). PKA regulatory-subunit overlay assays identified five AKAPs in ganglion lysates, including a prominent neuronal subtype. Moreover, PACAP-induced synaptic plasticity was selectively blocked when PKA regulatory-subunit binding to AKAPs was inhibited. Taken together, our findings indicate that PACAP/PAC_1R signaling coordinates nAChR, NOS1 and AKAP activities to induce targeted, retrograde plasticity at autonomic synapses. Such coordination has broad relevance for understanding the control of autonomic synapses and consequent visceral functions.

1. Introduction

PACAP is a neurotrophic, neuromodulatory, and anxiogenic neuropeptide found throughout the nervous system (Arimura, 1998; Hannibal, 2002; Sherwood et al., 2000). PACAP and a PAC_1R gene mutation have been proposed as biomarkers for post-traumatic stress disorder because they correlate with human and animal models of trauma, stress and fear (Hammack et al., 2009; Ressler et al., 2011; Vaudry et al., 2009). Consistent with its neuromodulatory and anxiogenic actions, PACAP potently influences synaptic function and plasticity. In the amygdala, where PACAP is present in presynaptic terminals, fear conditioning increases PAC_1R mRNA levels (Ressler et al., 2011) while exogenous PACAP enhances anxiogenic responses (Legradi et al., 2007) and intrinsic synaptic transmission (Cho et al., 2012). The autonomic nervous system (ANS) regulates cardiovascular, respiratory, and sexual functions that can become imbalanced in stress and anxiety disorders (Blechert et al., 2007; Chang et al., 2013; Cohen et al., 2000; Kotler et al., 2000; Shah et al., 2013; Williamson et al., 2013) and PACAP/PAC_1R signaling potently impacts autonomic synapses, regulating output to visceral targets (Pugh et al., 2010; Stroth et al., 2012; Tompkins et al., 2007). PACAP/PAC_1R signaling may therefore contribute to stress disorders by regulating central and autonomic synapses.

In parasympathetic ciliary ganglion (CG) neurons, PAC_1Rs couple via Gαs to adenylate cyclase (AC) signaling, potentiating the function of α3*- and α7-nAChRs (Margiotta and Pardi, 1995; Pardi and Margiotta, 1999; Pugh and Margiotta, 2006) that underlie synaptic transmission (Chen et al., 2001; Sargent, 2009; Ullian et al., 1997). PACAP is present in cholinergic presynaptic terminals on CG neurons and released by depolarization, supporting a functional role during ganglionic

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transmission (Pugh et al., 2010). Functional synapses established between CG neurons in cell culture are mediated exclusively by nAChRs clustered in the postsynaptic membrane adjacent to juxtaposed presynaptic terminals, and thereby provide a useful model for synapses formed on CG neurons in vivo (Chen et al., 2001; Jayakar and Margiotta, 2011; Margiotta and Berg, 1982; Pugh et al., 2010; Zhou et al., 2004). Consistent with its actions in the amygdala, exogenous PACAP enhances synaptic transmission in CG cultures within minutes, increasing the frequency and amplitude of impulse-driven, spontaneous nAChR-mediated excitatory postsynaptic currents (sEPSCs) by enhancing vesicular ACh release from presynaptic terminals (quantal content) without affecting the unitary postsynaptic response (i.e. quantal size; miniature EPSC amplitude) (Pugh et al., 2010). The rapid PACAP-induced plasticity requires PAC_1Rs, AC, and subsequent activation of PKA to drive protein phosphorylation, as well as NOS1 to synthesize NO (Pugh et al., 2010).

NOS1 is activated by calmodulin binding in response to Ca^{2+} elevation (Abu-Soud et al., 1994; Bredt and Snyder, 1990). In central nervous system (CNS) neurons, Ca^{2+} influx via N-methyl-D-aspartate (NMDA)-type glutamate receptors (NMDARs) likely triggers NOS1 activation (Garthwaite, 2008; Garthwaite et al., 1988) and NO produced in this manner acts as a retrograde messenger to regulate presynaptic function (Regehr et al., 2009). In the ANS, elevated NO production has been similarly linked to long-term presynaptic potentiation resulting from increased quantal content (Lin and Bennett, 1994). Interestingly PACAP stimulates NOS1 in pituitary gonadotrophs (Garrel et al., 2002), and in sensory neurons NOS1 is activated in both a Ca^{2+}/calmodulin- and PKA-dependent manner (Bredt and Snyder, 1990; Bredt et al., 1992; Hurt et al., 2012). Given these precedents we examined the interplay between NOS1- and PKA-dependent mechanisms in transducing the actions of PACAP at synapses on CG neurons where transmission is mediated by Ca^{2+}-permeable nAChRs. A NO scavenger (C-PTIO) was first used to show that NO likely acts in a retrograde manner to induce synaptic plasticity. We then examined mechanisms whereby PACAP/PAC_1R signaling activates NOS1 to increase NO levels in CG neurons, and localizes these effects to synaptic components via PKA-anchoring proteins (AKAPs) to enhance synaptic function. The results reveal that PACAP/PAC_1R signaling tightly coordinates nAChR with NOS1 and AKAP activities to induce targeted, retrograde synaptic plasticity. Such coordination has broad relevance for understanding the control of autonomic synapses and consequent visceral functions, possibly including those impacted by stress disorders.

2. Results

2.1. PACAP Stimulates NOS1 to Increase NO Levels

We previously reported that PACAP signaling via PAC_1Rs rapidly and potently increases the frequency and amplitude of sEPSCs on CG neurons in culture by increasing presynaptic ACh release (quantal content) (Pugh et al., 2010). We also found that this plasticity required NOS1 activity and could be mimicked by an NO donor (Pugh et al., 2010). The latter finding predicts that PACAP/PAC_1R signaling leads to NOS1 activation and NO production responsible for the subsequent presynaptic plasticity. We therefore used an NO imaging approach based on the fluorescent indicator dye DAF-FM to assess the ability of PACAP to increase NO production and reveal underlying mechanisms. Throughout this study, PACAP was applied at 100 nM, a concentration we previously found that maximally enhances PAC_1R signaling and subsequent synaptic function (Margiotta and Pardi, 1995; Pardi and Margiotta, 1999; Pugh et al., 2010). PACAP increased the fluorescence intensity of DAF-FM-loaded CG neuron somas compared to sham-treated control neurons from the same cultures (Fig. 1A, B). The higher fluorescence intensity associated with PACAP application was indicative of increased cellular NO since the NO donor MAHMA-NANOate similarly increased DAF-FM soma fluorescence. Subsequent quantification revealed that PACAP and MAHMA-NANOate significantly increased specific DAF-FM

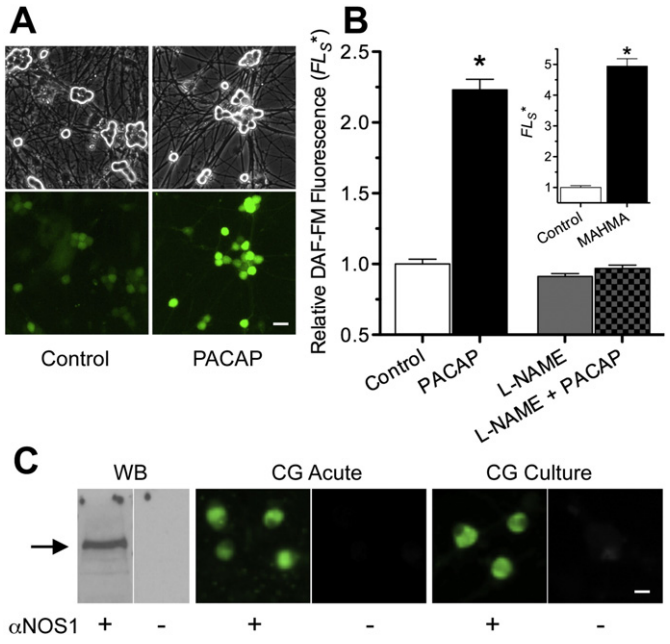

Fig. 1. PACAP increases NO production in CG neurons by activating endogenous NOS1. A. NO production assessed by DAF-FM fluorescence. Left and right pairs show bright field (*Top*) and DAF-FM fluorescence (*Bottom*) images from live CG neuron cultures treated with RS^{arg} incubation medium alone (Control, *Left*) or with RS^{arg} containing PACAP (*Right*, 30 min). Calibration: 25 μm. B. Specific neuronal DAF-FM fluorescence levels (FL_S*) associated with PACAP, L-NAME, or L-NAME + PACAP treatments are expressed here and subsequently relative to control levels from 204 to 739 neurons as described in Experimental Methods. *Inset* shows FL_S* quantification for neurons treated with MAHMA-NANOate (MAHMA) relative to untreated controls from 90 and 112 neurons, respectively. Here and subsequently, an asterisk above the treatment group value indicates a significant difference compared to the control group ($p < 0.05$). Concentrations of PACAP, MAHMA-NANOate, and L-NAME were 100 nM, 100 μM, and 1 mM, respectively. C. Endogenous NOS1 expression in CG and neurons. *Left:* E14 CG homogenates probed with NOS1 pAb (WB, +) revealed a major immunoreactive protein having the size predicted for chicken NOS1 (160 kDa, arrow) that was specific since it was absent when the primary antibody was omitted (WB, −). *Right:* Somas of acutely dissociated E14 CG neurons (*Left*) and E8 CG neurons in culture (*Right*) displayed NOS1-specific immunofluorescence. Calibration: 10 μm.

fluorescence (FL_S*) in neurons from CG cultures to levels that were on average 2.3 and 5.0 times higher, respectively, than those of untreated controls tested in parallel. No detectable increase in FL_S* was observed when the neurons were co-treated with PACAP and NOS inhibitor (L-NAME) indicating that the PACAP-stimulated increase required endogenous NOS1 activity. Functional NOS expression has been reported previously in CG neurons (Nichol et al., 1995) and consequent NO production linked to increased acetylcholine (ACh) release from preganglionic terminals (Lin and Bennett, 1994). Further evidence for the presence of endogenous NOS1 was obtained using an anti-NOS1 antibody that detected an ≈160 kDa protein in E14 CG extracts consistent with the predicted size of chicken NOS1, and revealed specific immunofluorescence localized to CG neurons (Fig. 1C). In accord with our previous findings indicating that NOS1 signaling is required for PACAP to induce synaptic plasticity, these results indicate that PACAP triggered signals converge on endogenous NOS1 to increase NO production in CG neurons.

2.2. NO Is a Retrograde Messenger

To determine whether NO generated by PACAP signaling acts as a retrograde messenger to impact presynaptic function, we examined the effect of the NO scavenger *2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide* (cPTIO). In accord with our previous findings, the frequency (F_s) and amplitude (A_s) of spontaneous