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REGULATION OF NEURONAL PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE EXPRESSION DURING CULTURE OF GUINEA-PIG CARDIAC GANGLIA

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Abstract—The trophic neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) increases in many different neuron types following injury; a response postulated to support cell survival and regeneration. In acutely isolated cardiac ganglia, approximately 1% of the cardiac neurons exhibited PACAP immunoreactivity whereas after 72 h in culture, ~25% of the neurons were PACAP immunoreactive. In contrast, there was no increase in vasoactive intestinal polypeptide (VIP)-immunoreactive (IR) cells. Using a combination of immunocytochemical and molecular techniques, we have quantified PACAP expression, during explant culture of guinea-pig cardiac ganglia. Using real time polymerase chain reaction, PACAP transcript levels increased progressively up to 48 h in culture with no further increase after 72 h. PACAP transcript levels were reduced by neurturin at 48 h in culture but not after 24 or 72 h in culture. In addition, neurturin partially suppressed the percentage of PACAP-IR neurons after 72 h in culture, an effect mediated by activation of the phosphatidylinositol 3-kinase and mitogen-activated protein kinase signaling pathways. The addition of different known regulatory molecules, including ciliary neurotrophic factor (CNTF), interleukin-1 beta (Il-1β), tumor necrosis factor-alpha (TNFα), fibroblast growth factor basic (bFGF), transforming growth factor-beta (TGF-β) and nerve growth factor (NGF) did not increase the percentage of PACAP-IR neurons after 24 h in culture; a result indicating that the generation and secretion of these factors did not stimulate PACAP expression. The presence of 20 nM PACAP or 10 μM forskolin increased the percentage of PACAP-IR cardiac neurons in 24 h cultures, but not in 72 h cultures. Neither treatment enhanced the number of VIP-IR neurons. The addition of the PACAP selective receptor (PAC_1) receptor antagonist, M65 (100 nM) suppressed the 20 nM PACAP-induced increase in percentage of PACAP-IR cells in 24 h cultures indicating the effect of PACAP was mediated through the PAC_1 receptor. However, 100 nM M65 had no effect on the percentage of PACAP-IR cells in either 24 or 48 h cultures not treated with exogenous PACAP, suggesting that endogenous release of PACAP likely did not contribute to the enhanced peptide expression. We postulate that the enhanced PACAP expression, which occurs in response to injury is facilitated in the explant cultured cardiac ganglia by the loss of a target-derived inhibitory factor, very likely neurturin. In intact tissues the presence of neurturin would normally suppress PACAP expression. Lastly, our results indicate that many common trophic factors do not enhance PACAP expression in the cultured cardiac neurons. However, the stimulatory role of an, as yet, unidentified factor cannot be excluded.

Key words: parasympathetic neurons, trophic factors, choline acetyltransferase, phosphatidylinositol 3-kinase, mitogen-activated protein kinase, explant culture.

Pituitary adenylate cyclase activating polypeptide (PACAP) is a potent trophic and intercellular signaling molecule, distributed widely throughout both the CNS and peripheral nervous system (Arimura, 1998; Vaudry et al., 2000). In control peripheral nervous system structures, such as parasympathetic and sympathetic ganglia, dorsal root ganglia, vagal sensory ganglia and spinal and cranial motor nuclei, limited numbers of neurons exhibit PACAP immunoreactivity. However, PACAP expression increases markedly in these neurons following axotomy or explant culture (Zhang et al., 1995, 1996; Moller et al., 1997a,b; Zhou et al., 1999; Pettersson et al., 2004). It is hypothesized that the increased expression of PACAP may be a response to injury that supports cell survival and regeneration (Moller et al., 1997a,b; Armstrong et al., 2003; Boeshore et al., 2004; Pettersson et al., 2004; Suarez et al., 2006).

In acutely isolated guinea-pig cardiac ganglia, all the intrinsic neurons are innervated by extrinsic PACAP-immunoreactive (IR) fibers, but very few of the cardiac neurons exhibit PACAP immunoreactivity (Braas et al., 1998; Calupca et al., 2000; Parsons et al., 2006). Following explant culture, the extrinsic PACAP-IR fibers degenerate and the percentage of cholinergic neurons expressing PACAP increases significantly (Calupca et al., 2000; Girard et al., 2006b).

Members of the glial-derived family of trophic factors, particularly glial-derived neurotrophic factor (GDNF) and neurturin, are key regulatory molecules determining the migration and development of many parasympathetic gan-

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Abbreviations: ANOVA, analysis of variance; bFGF, fibroblast growth factor basic; CARTp, cocaine- and amphetamine-regulated transcript peptide; ChAT, choline acetyl transferase; CNTF, ciliary neurotrophic factor; C_T, threshold cycle; Cy3, indocarbocyanine; FITC, fluorescein isothiocyanate; GDNF, glial-derived neurotrophic factor; GFRα2, receptor for neurturin; Il-1β, interleukin-1 beta; IR, immunoreactive; LIF, leukemia inhibitory factor; MAPK, mitogen-activated protein kinase; M65, pituitary adenylate cyclase-activating polypeptide selective receptor antagonist; NGF, nerve growth factor; PACAP, pituitary adenylate cyclase-activating polypeptide; PAC_1, pituitary adenylate cyclase-activating polypeptide selective receptor; PCR, polymerase chain reaction; PI3-kinase, phosphatidylinositol 3-kinase; SCG, superior cervical ganglion; TGF-β, transforming growth factor-beta; TNFα, tumor necrosis factor-alpha; VIP, vasoactive intestinal polypeptide.

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glia, including the intrinsic cardiac ganglia (Enomoto et al., 2000; Hiltunen et al., 2000; Hashino et al., 2001; Airaksinen et al., 2002). In initial studies, we reported that the increase in PACAP-IR cardiac neurons is partially suppressed by two members of the glial-derived neurotrophic family, neurturin and GDNF (Girard et al., 2006b) although the intracellular signaling cascades mediating the effect of these two trophic factors were not identified.

In the present study, we have quantified using a combination of immunocytochemical and polymerase chain reaction (PCR) techniques the time-dependent increase in PACAP expression that occurs when cardiac ganglia are maintained in explant culture. Since we previously demonstrated that adult guinea-pig cardiac neurons preferentially expressed the neurturin specific receptor GFRα2 (Girard et al., 2006a), we also have analyzed potential signaling cascades underlying the neurturin modulation of PACAP expression in cultured cardiac neurons. Our results show that the neurturin-induced suppression of PACAP expression was mediated through the activation of phosphatidylinositol 3-kinase (PI3-kinase) and mitogen-activated protein kinase (MAPK) pathways. Furthermore, although a number of common regulatory or target-derived factors had no effect, the addition of PACAP or forskolin in 24 h cultures could promote PACAP expression.

A preliminary account of some aspects of this study was presented at the VIIth International Symposium on VIP, PACAP and Related Peptides (Girard et al., 2006b).

EXPERIMENTAL PROCEDURES

Experiments were performed *in vitro* on atrial whole mount preparations containing the cardiac ganglia from Hartley guinea pigs (either sex; 250–400 g) that were killed and exsanguinated. Protocols for use of guinea pigs were approved by the University of Vermont Institutional Animal Care and Use Committee and conformed to methods described in the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering. The heart was quickly removed and placed in cold standard Krebs' solution (in mM: 121 NaCl, 5.9 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 1.2 NaH_2CO_4, 8 glucose). The pH was maintained at 7.4 by aeration with 95% O_2–5% CO_2.

Cardiac ganglia preparations were prepared as described previously (Mawe et al., 1996; Braas et al., 1998; Calupca et al., 2000). The preparations were maintained in Sylgard-lined dishes with oxygenated Krebs solution at 35–37 °C. When prepared for immunocytochemistry, control and cultured preparations were fixed in 2% paraformaldehyde containing 0.2% picric acid for 2 h at room temperature (Mawe et al., 1996; Braas et al., 1998; Lynch et al., 1999; Calupca et al., 2000; Parsons et al., 2006).

For culture, the cardiac ganglia explants were dissected under sterile conditions and then maintained at 37 °C in culture media consisting of DMEM-F12 (1:1) containing 10% horse serum, gentamicin (10 μg/ml), amphotericin B (3.75 μg/ml), penicillin (100 units/ml) and streptomycin (100 μg/ml) (Sigma Chemical Company, St. Louis, MO, USA) (Lynch et al., 1999; Calupca et al., 2000; Braas et al., 2004; Girard et al., 2006a). The preparations were placed on a slowly rocking shaker table in a 37 °C, 5% CO_2 and 95% air incubator and kept for 24, 48, 72 or 96 h with the culture media replaced every 24 h.

Neurturin, interleukin-1 beta (Il-1β) and tumor necrosis factor-alpha (TNFα) were obtained from R&D Systems Inc. (Minneapolis, MN, USA). Recombinant rat ciliary neurotrophic factor (CNTF) (expressed as $[his]_6$CNTFyyy in a bacterial expression vector) (Heller et al., 1993), fibroblast growth factor basic (bFGF), transforming growth factor-beta (TGF-β) and nerve growth factor (NGF) were generously supplied by Dr. Rae Nishi (Department of Anatomy and Neurobiology, University of Vermont, Burlington, VT, USA). Wortmannin, PD980059, forskolin and veratridine (all from Calbiochem, La Jolla, CA, USA) were prepared as stock solutions in DMSO and diluted in culture media just prior to use. PACAP27, referred to simply as PACAP throughout the text, was obtained from American Peptide Co, Sunnyvale, CA, USA. Pituitary adenylate cyclase-activating polypeptide selective receptor antagonist (M65) was the generous gift of Dr. Ethan Lerner (Harvard Medical School, Cambridge, MA, USA).

Immunohistochemistry

The cardiac ganglia preparations were immunolabeled using procedures described previously (Mawe et al., 1996; Braas et al., 1998; Calupca et al., 2000; Parsons et al., 2006; Girard et al., 2006a,b). The fixed atrial tissue was washed in phosphate buffered saline, permeabilized with 0.5% Triton X-100 and incubated at 4 °C overnight with primary antiserum (mouse monoclonal anti-PACAP 1:10, from Dr. Jan Fahrenkrug, Copenhagen, Denmark; rabbit anti-vasoactive intestinal polypeptide (VIP) 1:100 from CURE Research Center, UCLA, Los Angeles, CA, USA; goat anti-choline acetyl transferase (ChAT) 1:100, obtained from Chemicon International, Temecula, CA, USA; and rabbit anti-PGP 9.5 1:1000 from Ultraclomne Ltd., Isle of Wright, UK). All primary antisera have been used extensively in our prior studies where specificity of staining was determined using the appropriate control procedures (Mawe et al., 1996; Calupca et al., 2000; Parsons et al., 2006). The preparations were washed and incubated for 2 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated or indocarbocyanine (Cy3)-conjugated secondary antiserum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Each cardiac ganglia preparation was washed, mounted with Citifluor (UKA Chemical Laboratory, Canterbury, UK) and viewed with a Nikon fluorescence photomicroscope with HBO 100 W UV light source and filters for FITC and Cy3.

Quantification of peptide-IR cardiac neurons

Quantification of PACAP-IR parasympathetic neurons present in the cardiac ganglia followed procedures similar to those published previously (Lynch et al., 1999; Braas et al., 2004; Parsons et al., 2006). The number of cardiac neurons in each preparation was determined by counting ChAT-IR or PGP-9.5-IR cells (Mawe et al., 1996; Braas et al., 1998, 2004; Kennedy et al., 1998; Lynch et al., 1999; Calupca et al., 2000; Parsons et al., 2006). The percentage of cardiac ganglia neurons that expressed PACAP was then determined as a fraction of the total number of neurons that were ChAT or PGP-95 IR: [(PACAP-IR neurons/ChAT-IR or PGP-95-IR neurons)$\times$100=%]. This allowed comparison between different whole mount preparations that contained varying numbers of neurons (Lynch et al., 1999).

Real time quantitative reverse transcription–PCR (Q-PCR)

Cardiac ganglia preparations were dissected under RNase-free conditions, and total RNA was extracted from individual preparations using TRI Reagent (Sigma). The total RNA quantity for each whole mount preparation was determined using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). One microgram of RNA per sample was used to synthesize complementary DNA with the Omniscript reverse transcription (Qiagen Inc., Valencia, CA, USA) and oligo-dT primers.

Real time PCR to quantify PACAP transcripts was performed as described previously (Girard et al., 2002a, 2006a; Braas et al.,