Neuroscience 134 (2005) 745–756

PITUITARY ADENYLATE CYCLASE ACTIVATING POLYPEPTIDE REDUCES A-TYPE K^+ CURRENTS AND CASPASE ACTIVITY IN CULTURED ADULT MOUSE OLFACTORY NEURONS

P. HAN[a] AND M. T. LUCERO[b]*

[a]*Interdepartmental Neuroscience Program, University of Utah, Salt Lake City, UT 84108, USA*

[b]*Department of Physiology, University of Utah, 410 Chipeta Way, Room 156, Salt Lake City, UT 84108-1297, USA*

Abstract—Pituitary adenylate cyclase activating polypeptide has been shown to reduce apoptosis in neonatal cerebellar and olfactory receptor neurons, however the underlying mechanisms have not been elucidated. In addition, the neuroprotective effects of pituitary adenylate cyclase activating polypeptide have not been examined in adult tissues. To study the effects of pituitary adenylate cyclase activating polypeptide on neurons in apoptosis, we measured caspase activation in adult olfactory receptor neurons *in vitro*. Interestingly, we found that the protective effects of pituitary adenylate cyclase activating polypeptide were related to the absence of a 4-aminopyridine (IC_{50}=144 μM) sensitive rapidly inactivating potassium current often referred to as A-type current. In the presence of 40 nM pituitary adenylate cyclase activating polypeptide 38, both A-type current and activated caspases were significantly reduced. A-type current reduction by pituitary adenylate cyclase activating polypeptide was blocked by inhibiting the phospholipase C pathway, but not the adenylyl cyclase pathway. Our observation that 5 mM 4-aminopyridine mimicked the caspase inhibiting effects of pituitary adenylate cyclase activating polypeptide indicates that A-type current is involved in apoptosis. This work contributes to our growing understanding that potassium currents are involved with the activation of caspases to affect the balance between cell life and death. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: K channels, apoptosis, neuroprotection, patch clamp, PLC, 4AP.

Pituitary adenylate cyclase activating polypeptide (PACAP) is a pleiotropic peptide belonging to the vascular intestinal peptide–corticotrophin-releasing-hormone–glucagon-secretin superfamily. The high affinity pituitary adenylate cyclase activating polypeptide receptor (PAC-1) belongs to the 7-transmembrane spanning G-protein-coupled receptor family (Pisegna, 1993). In the nervous system, PACAP serves as neurotransmitter and neuromodulator (Arimura, 1998). It also promotes the proliferation of neuronal precursors, neuronal differentiation and survival by facilitating neurogenesis and inhibiting apoptosis (Vaudry et al., 2000b; Vaudry et al., 2002b). By inhibiting apoptosis, PACAP is important in the neurodevelopmental process and in providing neuroprotection under pathological conditions (Gillardon et al., 1998).

Apoptosis usually involves activating a cascade of caspases (Nicotera et al., 2000). PACAP prevents apoptosis by inhibiting activation of caspase-3 and other upstream components including cytochrome C and caspase-9 (Vaudry et al., 2000a). However, the detailed mechanisms underlying PACAP's anti-apoptotic effects have not been identified. Neuronal apoptosis can be initiated by several mechanisms including axonal target removal, serum or trophic factor deprivation, potassium withdrawal and excitotoxicity. Different classes of ion channels can positively or negatively affect cell survival (Blondeau et al., 2000; Krick et al., 2001; Ekhterae et al., 2001; Xia et al., 2002). Potassium (K^+) channels in particular, play important roles in apoptosis (Villalba et al., 1997a), and perturbations of potassium concentration markedly affect caspase activation (Thompson et al., 2001). Like most ion channels, the intracellular segments of K^+ channels have modulatory sites through which second messengers, kinases and phosphatases, can change gating and conductance (Yi et al., 2001). Depending on receptor subtype, PACAP can activate either the adenylyl cyclase (AC) or phospholipase C (PLC) pathways (Spengler et al., 1993) and it is therefore possible that PACAP modulates ionic currents that are involved in apoptosis.

The olfactory epithelium is a good model for studies in neuronal proliferation, differentiation and survival because olfactory receptor neurons (ORNs) are generated from underlying progenitor cells throughout life (Calof et al., 1996). Mammalian ORNs within the olfactory epithelium send their axons to the olfactory bulb. Surgical removal of the olfactory bulb (bulbectomy) or direct severance of axons (axotomy) will initiate apoptosis in the entire mature ORN population; becoming apparent at 12 h and peaking at 24–36 h post-bulbectomy (Cowan et al., 2001). An identified cascade of caspases is activated during olfactory apoptosis following olfactory bulb ablation, starting with caspase-9 and followed by caspase-3 (Cowan et al.,

*Corresponding author. Tel: +1-801-585-5601; fax: +1-801-581-3476.
E-mail address: Mary.lucero@m.cc.utah.edu (M. T. Lucero).

Abbreviations: AC, adenylyl cyclase; BDNF, brain-derived neurotrophic factor; cAMP, cyclic AMP; CaspACE, CaspACE–FITC–VAD–FMK; CNG, cyclic nucleotide gated; DMEM, Dulbecco's Modified Eagle Medium; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; I_A, A-type potassium current; I–V, current–voltage; NGF, nerve growth factor; OEC, olfactory ensheathing cell; ORN, olfactory receptor neuron; PAC-1, high affinity pituitary adenylate cyclase activating polypeptide receptor; PACAP, pituitary adenylate cyclase activating polypeptide; PACAP6-38, high affinity pituitary adenylate cyclase activating polypeptide receptor antagonist; PBS, phosphate-buffered saline; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; $V_{1/2}$, voltage for half activation; 4AP, 4-aminopyridine; 8-Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate.

0306-4522/05$30.00+0.00 © 2005 Published by Elsevier Ltd on behalf of IBRO.
doi:10.1016/j.neuroscience.2005.05.007

2001). PACAP is present in both embryonic and adult olfactory epithelium (Hansel et al., 2001; Hegg et al., 2003), and promotes functional maturation of an olfactory cell line (Illing et al., 2002). PACAP also maintains the survival of neonatal rat ORNs by inhibiting apoptosis and promoting proliferation (Hansel et al., 2001). Thus far, the molecular and cellular pathways underlying neuroprotection by PACAP appear to vary with cell type (Shioda et al., 1998; Vaudry et al., 2000a), and have not previously been described for olfactory neurons except in abstract form (Han et al., 2002).

Here, we used ORNs dissociated from adult mouse olfactory epithelium to investigate the anti-apoptotic effect of PACAP. This *in vitro* study mimics both bulbectomy and axotomy in that dissociated ORNs lose their target and are devoid of axons. In theory, apoptosis of cells *in vitro* should replicate the course of neurodegeneration following bulbectomy. Accordingly, we focused on the role of K^+ currents, especially a transient A-type potassium (I_A) current, in apoptosis within the initial 24 h after dissociation. Specifically, we monitored caspase activation and K^+ current density from the same cells. This study is intended to investigate the involvement of I_A within the anti-apoptotic effect of PACAP.

EXPERIMENTAL PROCEDURES

Materials

Dulbecco's Modified Eagle Medium (DMEM), DMEM-F-12, fetal bovine serum (FBS), insulin–transferrin–selenium, streptomycin-antimycotics, dispase and collagenase type I were purchased from GIBCO BRL (Grand Island, NY, USA). CaspACE-FITC-VAD-FMK was purchased from Promega (Madison, WI, USA). PACAP38 and PACAP6-38 were purchased from Peninsula Laboratories, Inc. (Torrance, CA, USA). U73122 and U73343 were obtained from BIOMOL (Plymouth Meeting, PA, USA). SQ22538 was from Calbiochem (La Jolla, CA, USA). The remaining chemicals were from Sigma (St. Louis, MO, USA). Stock solutions of U73122, U73343, SQ22538, phorbol 12-myristate 13-acetate (PMA) and 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP) were made in anhydrous dimethylsulfoxide (DMSO) and diluted in cell culture media so that the DMSO concentration did not exceed 0.01%. Aliquots of U73122 and U73343 were made according to the manufacturer's instructions before dissolving in DMSO and media.

Cell culture

All animal procedures were approved by the University of Utah Institutional Animal Care and Use Committee, and all applicable guidelines from the National Institutes of Health Guide for Care and Use of Laboratory Animals were followed. Concerted efforts were made to minimize both the number of animals used and their suffering. The 30 g Swiss-Webster mice (Charles River Laboratory, Wilmington, MA, USA) were deeply anesthetized with 150 μg/kg ketamine and 150 μg/ml xylazine and killed by decapitation. The olfactory epithelium from the nasal septum and turbinates was dissected in the presence of oxygen saturated divalent-free Ringer's solution. The divalent-free Ringer's solution contained NaCl (145 mM), KCl (5 mM), HEPES (10 mM), glucose (10 mM) and EGTA (4 mM), and was warmed to room temperature (22–25 °C) before use. The epithelium was incubated in divalent-free enzyme solution containing bovine serum albumin (1% W/V), dispase (44 U/ml), collagenase type I (1 g/l) and deoxyribonuclease type II (0.05 mg/l) for 1 h with gentle shaking at 37 °C. After rinsing in the divalent-free Ringer's solution, the tissue was gently triturated with a fire-polished Pasteur pipette until the solution became cloudy. The resulting cell suspension was filtered through a 105-μm nylon mesh (Small Parts, Miami Lakes, FL, USA). The filtered suspension was centrifuged, the pellet resuspended in the same divalent-free Ringer's solution, and the cells plated on Concanavalin A (ConA)-coated sterile coverslips, which prevents apoptotic and dead cell ghosts from detaching from the coverslip (unpublished observations). Dissociated cells were maintained in either Control Media or Test Media. Control Media consisted of DMEM supplemented by 5% FBS, 1% Insulin–Transferrin–Selenium-X, 1% streptomycin-antimycotics and freshly prepared 0.1 mM ascorbic acid, incubated at 37 °C with 5% CO_2 (pH 7.4). For Test Media, PACAP38, PACAP6-38, 4-aminopyridine (4AP), SQ22536, U73122, U73343, PMA, 8-Br-cAMP or vehicle controls were included or excluded from the Control Media depending on specific experimental conditions. All cultures were fed with their original media (i.e. Test Media with Test Media) at 20–24 h post-plating.

In some experiments, ORNs were co-cultured with olfactory ensheathing glial cells (OECs; generously provided by Dr. Jane Roskams, University of British Columbia, Canada) for 24 h. OECs were grown on poly-lysine-coated coverslips at 37 °C with 5% CO_2 in DMEM-F-12 media supplemented by 10% FBS until 95% confluent (Au and Roskams, 2003). The dissociated olfactory epithelial cells were seeded on top of the layer of OECs at 1×10^5 cell/ml, and the original DMEMF-12 media for OECs was replaced by Control Media for ORNs.

Whole cell voltage-clamp recording and analyses

Coverslips with adherent cells were placed into the recording chamber and perfused with external Ringer's bath solution at a rate of 80 ml/h. The bath was grounded with a 3 M KCl agar bridge. The Ringer's solution contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES and 10 mM glucose (pH 7.4, 320 mOsm). The internal solution contained 125 mM KF, 15 mM NaCl, 11 mM EGTA and 10 mM HEPES (pH 7.2, 320 mOsm). The 7.4 mV liquid junction potential between internal and external solutions was calculated using PClamp 8.2 software and was not corrected. Electrodes were pulled from borosilicate glass on a P87 Flaming/Brown puller (Sutter Instrument, CA, USA). The electrode resistances were 4–6 MΩ. Standard whole cell voltage patch-clamp techniques were used. Recordings were performed on an Olympus inverted microscope equipped with UV light source, using an Axon Instruments headstage (CV203 BU) mounted on a Burleigh micromanipulator. A Digidata 1320 interface and an Axopatch 200A amplifier (Molecular Devices, Union City, CA, USA) were controlled and monitored by pClamp 8.2 software. The recordings were low-pass filtered at 5 kHz. Cells were visualized under bright field and randomly selected for patch-clamp recording. In a subset of experiments, the cells were live labeled with a membrane permeable marker of activated caspases, CaspACE–FITC–VAD–FMK (CaspACE, 10 μM) for 20 min before recording. For these experiments, the cells were also randomly selected for recording under bright field before image capture under UV fluorescence. After gigaseal formation and seal rupture, the series resistance was measured (10–30 MΩ) and 60% maximal compensation applied. The membrane resistance (R_m) ranged from 1 to 12 GΩ. Whole-cell capacitances were measured using Pclamp 8.2 software. ORNs were identified and discerned from OECs in co-culture by morphology, size (whole-cell capacitance of ORNs=2.0–5.5 pF compared with 15–20 pF for OECs), and electrophysiological responses (both Na^+ and K^+ current were detectable in ORNs held at −70 mV and stepped up to +60 mV with an increment of 10 mV in each step). Cells not meeting all criteria were excluded from further analysis. All experiments were performed at room temperature (22–25 °C).