Molecular and Cellular Neuroscience 68 (2015) 177–185

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

Profiles of secreted neuropeptides and catecholamines illustrate similarities and differences in response to stimulation by distinct secretagogues

Sonia Podvin [a], Richard Bundey [b], Thomas Toneff [a], Michael Ziegler [b], Vivian Hook [a,c,*]

[a] Skaggs School of Pharmacy and Pharmaceutical Sciences, Univ. of Calif.—San Diego, La Jolla, CA 92093, United States
[b] Dept. of Medicine, Univ. of Calif.—San Diego, La Jolla, CA 92093, United States
[c] Dept. of Neuroscience and Dept. of Pharmacology, School of Medicine, Univ. of Calif.—San Diego, La Jolla, CA 92093, United States

ARTICLE INFO

Article history:
Received 16 January 2015
Revised 12 May 2015
Accepted 1 June 2015
Available online 16 June 2015

Keywords:
Neurotransmitter profiles
Secretion
Catecholamines
Neuropeptides
Secretagogues
Chromaffin cells
Stress response

ABSTRACT

The goal of this study was to define profiles of secreted neuropeptide and catecholamine neurotransmitters that undergo co-release from sympathoadrenal chromaffin cells upon stimulation by distinct secretagogues. Chromaffin cells of the adrenal medulla participate in the dynamic responses to stress, especially that of 'fight and flight', and, thus, analyses of the co-release of multiple neurotransmitters is necessary to gain knowledge of how the stress response regulates cell–cell communication among physiological systems. Results of this study demonstrated that six different secretagogues stimulated the co-release of the neuropeptides Met-enkephalin, galanin, NPY, and VIP with the catecholamines dopamine, norepinephrine, and epinephrine. Importantly, the quantitative profiles of the secreted neurotransmitters showed similarities and differences upon stimulation by the different secretagogues evaluated, composed of KCl depolarization, nicotine, carbachol, PACAP, bradykinin, and histamine. The rank-orders of the secreted profiles of the neurotransmitters were generally similar among these secretagogues, but differences in the secreted amounts of each neurotransmitter occurred with different secretagogues. Epinephrine among the catecholamines showed the highest level of secretion. (Met)enkephalin showed the largest levels of secretion compared to the other neuropeptides examined. Levels of secreted catecholamines were greater than that of the neuropeptides. These data support the hypothesis that profiles of secreted neuropeptide and catecholamine neurotransmitters show similarities and differences upon stimulation by distinct secretagogues. These results illustrate the co-release of concerted neurotransmitter profiles that participate in the stress response of the sympathoadrenal nervous system.

1. Introduction

Neurotransmitters are released from neurons to mediate cell–cell communication in the nervous system for the regulation of brain and peripheral physiological functions. Neurons have been classically viewed to secrete a predominant neurotransmitter that defined the transmitter phenotype of the indicated neuron (e.g., adrenergic, cholinergic, neuropeptidergic, and others). Subsequently, the dual release of two classical small molecule neurotransmitters has been demonstrated in numerous neuronal systems (Hnasko and Edwards, 2012; Gutierrez, 2009). Importantly, findings of multiple neurotransmitters within secretory vesicles, such as that of the large dense core secretory vesicles (LDCSVs) of neuronal-like chromaffin cells (Njus et al., 1985; Laslop and Mahata, 2002; Gupta et al., 2010), predict the co-release of multiple neurotransmitters in an activity-dependent, regulated manner. While many of these neurotransmitters have been studied individually with respect to their secretory properties, analyses of the 'profile' of multiple neurotransmitters released simultaneously have not been extensively studied. Therefore, this study evaluated the co-release of quantitative profiles of neuropeptides and catecholamines from adrenal medullary chromaffin cells to assess similarities and/or differences in secreted profiles of these neurotransmitters when stimulated by different secretagogue agents.

Chromaffin cells of the sympathoadrenal medullary system are key regulators of the body's responses to emergency 'fight or flight' reactions and mediate stress responses by releasing neurotransmitter and neurohumoral chemical molecules targeted at regulating multiple physiological systems (Burgoyne, 1995; Aunis, 1998; Arun, 2004; Eisenhofer et al., 2004; Kvetnansky et al., 2009; Purves et al., 2001). Epinephrine is a key stress neurotransmitter released from the adrenal medulla for the regulation of metabolic, cardiac, lung, and related physiological functions, as well as emotions, memory, and related behaviors (Joëls et al., 2011; Shansky and Lipps, 2013). Stress responses involve sympathetic nerve

* Corresponding author at: Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, 9500 Gilman Drive #0744, La Jolla, CA 92093-0744, United States.
E-mail address: vhook@ucsd.edu (V. Hook).

http://dx.doi.org/10.1016/j.mcn.2015.06.008
1044-7431/

activation of the adrenal medulla via multiple secretagogues (Carmichael and Winkler, 1985; Fulop et al., 2005; Smith and Eiden, 2012; Stroth et al., 2013) that stimulate the secretion of epinephrine with peptide neurotransmitters (neuropeptides) and other catecholamines in an activity-dependent manner (Holman et al., 1994).

Epinephrine is present in the adrenal medulla together with the catecholamines dopamine and norepinephrine, as well as with numerous neuropeptides (Eisenhofer et al., 2004; Kvetnansky et al., 2009; Purves et al., 2001). These neurotransmitters are secreted from chromaffin cells in a regulated manner from secretory vesicles upon stimulation by sympathetic activation. Secretion of these catecholamine and neuropeptide transmitters (i.e., enkephalin, NPY, galanin, and others) has been studied individually from chromaffin cells (Zhang et al., 2006; Hook et al., 2008; Smith and Eiden, 2012; Ges et al., 2013) and other neuronal cell types (Eiden, 2013). But characterization of the secreted profiles of co-released neurotransmitters in response to stimulation by different secretagogues has not yet been fully defined. Therefore, the goal of this study was to assess the quantitative profiles of neuropeptides and catecholamines co-secreted from chromaffin cells upon stimulation by different secretagogues that were composed of KCl depolarization, nicotine, carbachol, PACAP, bradykinin, and histamine (Toneff et al., 2013; Taylor et al., 2000; Stroth et al., 2013; Kuwashima et al., 2000; Wallace et al., 2002).

Results showed that quantitative profiles of secreted neuropeptide and catecholamine neurotransmitters show similarities and differences upon stimulation by distinct secretagogues. These findings demonstrate that specific profiles of secreted neurotransmitters, with similarities and differences, are released from chromaffin cells by distinct secretagogues. These data illustrate the co-release of multiple neurotransmitter profiles from adrenal medullary chromaffin cells related to stress responses.

2. Materials and methods

2.1. Chromaffin cells of the adrenal medulla in primary culture

Primary cultures of bovine adrenal chromaffin cells were prepared using a modification of a method described previously (O'Connor et al., 2007; Hook et al., 2008). Ten fresh bovine adrenal glands received from a local slaughterhouse were placed onto ice and trimmed of excess fat. The glands were sterilized by brief immersion in 70% ethanol prior to washing of the glands' lumen with standard release medium (SRM was composed of 188 mM NaCl, 4.6 mM KCl, 10 mM D-Glucose, 25 mM HEPES pH 7.3, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 100 units/ml penicillin, 100 μg/ml streptomycin). The chromaffin cells were dissociated by washing the gland lumens with collagenase (1.5 mg/ml) at 37 °C for 20 min, repeated three times. Adrenal glands were dissected to obtain the adrenal medulla tissue, placed into SRM, and dissociated cells were collected as the supernatant after centrifugation (500 ×*g*, 30 s). The pellet of undissociated tissue was subjected to another incubation with collagenase solution at 37 °C for 30 min, and dissociated cells were collected as the supernatant after centrifugation (500 ×*g*, 30 s). The resultant pellet was washed with SRM another two times and the supernatants (obtained by centrifugation) pooled with the previous supernatant fractions. The collagenase activity of the supernatant was neutralized by the addition of SRM containing 1% BSA. The cell suspension was passed through filters with progressively smaller pore sizes (surgical gauze of 1 sheet, 2 sheets, and 4 sheets followed by cell strainer (Falcon) of 100 μm and then 70 μm). The filtered cell suspension was subjected to centrifugation (550 ×*g*, 5 min) and the pellet was washed with SRM/1% BSA three times. The final pellet was resuspended in cell culture medium (Dulbecco's Modified Eagle's Medium containing 10% FCS, cytosine arabinofuranoside (10 μM) and antibiotics) and seeded onto fibronectin-coated (2.5 μg/cm^2) 6 well plates at a density of 1.5 million cells/well. Cells were kept at 37 °C in 95% humidified air/5% CO_2. Chromaffin cells in primary culture have been shown to be stable in neurotransmitter content up to 2–3 weeks in culture (Kilpatrick et al., 1980).

2.2. Secretagogue stimulation of neurotransmitter secretion from chromaffin cells

Chromaffin cells were maintained in culture for 7 days prior to stimulation with a variety of secretagogues. Culture media was aspirated from the cells and replaced with 1 ml SRM-B (SRM plus 0.5 μg/ml BSA). Following incubation for 15 min at 37 °C the SRM-B was aspirated and replaced with 1 ml incubation media (i.e. SRM-B containing secretagogues). Secretagogues evaluated were high KCl (50 mM) that produces depolarization, nicotine (up to 10 μM), carbachol (1 mM), PACAP (100 nM), bradykinin (300 nM), and histamine (10 μM). Cells were incubated with each secretagogue for 15 min at 37 °C, and the secretion media was collected and was centrifuged (500 ×*g*, 5 min) to remove residual cells. The collected media was stored at −70 °C for measurement of secreted neurotransmitters.

2.3. Isolation of chromaffin granules (large dense core secretory vesicles, LDCSVs)

Chromaffin granules (large dense core secretory vesicles, LDCSVs) were purified from fresh bovine adrenal medulla by differential sucrose density gradient centrifugation, as described previously (O'Connor et al., 2007; Wegrzyn et al., 2010), involving extensive wash steps (five washes) to obtain purified chromaffin granules. We have documented the high purity of this preparation of isolated secretory vesicles by electron microscopy (Wegrzyn et al., 2010) and biochemical markers (Wegrzyn et al., 2010). The sucrose gradient purification results in a preparation of purified, intact chromaffin secretory vesicles that lack biochemical markers for the subcellular organelles of lysosomes (acid phosphatase marker), cytoplasm (lactate dehydrogenase marker), mitochondria (fumarase and glutamate dehydrogenase markers), and endoplasmic reticulum (glucose-6-phosphatase marker) (Smith and Winkler, 1967; Gratzl et al., 1981; Wegrzyn et al., 2010). These results have established the high purity of the chromaffin granule preparation.

2.4. Neuropeptide and catecholamine assays

The secretion media was analyzed for levels of the neuropeptides (Met)enkephalin, galanin, NPY, and VIP were measured by radioimmunoassays (Peninsula Laboratories, CA). The catecholamines dopamine, norepinephrine, and epinephrine were each assayed as described previously (Kennedy and Ziegler, 1990). Measurements were conducted in triplicate for each sample, and each experiment utilized triplicate wells of cells treated under identical conditions. Further, experiments were repeated at least three times. Statistical significance of data was assessed by Student's *t*-test and one-way ANOVA analyses ($p < 0.05$) (using Prism GraphPad program).

Analyses of the purified chromaffin granules measured levels of the catecholamine and neuropeptide neurotransmitters. Protein content of the purified chromaffin granules was measured by the Bio-Rad DC protein assay kit (Biorad, Hercules, CA). The content of catecholamines and peptide neurotransmitters were expressed as fmol per μg protein.

3. Results

Regulated secretion of neuropeptides and catecholamines from chromaffin cells was evaluated for several different secretagogues. This study assessed six types of secretagogues (KCl, nicotine, carbachol, PACAP, bradykinin, and histamine) for their abilities to stimulate the release of several neuropeptides ((Met)enkephalin galanin, NPY, and VIP) and the catecholamines (dopamine, norepinephrine, and epinephrine). These experiments used secretagogues at concentrations known to stimulate secretion of catecholamines (dopamine, norepinephrine,