Regulatory Peptides 165 (2010) 111–116

Contents lists available at ScienceDirect

Regulatory Peptides

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

Melittin promotes exocytosis in neuroendocrine cells through the activation of phospholipase A_2

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ARTICLE INFO

Article history:
Received 17 July 2009
Received in revised form 17 September 2009
Accepted 24 September 2009
Available online 2 October 2009

Keywords:
Arachidonic acid
Chromaffin
Exocytosis
Melittin
PLA2

ABSTRACT

Regulated exocytosis requires the formation of trans-SNARE complexes that assemble at the interface between vesicles and the plasma membrane. Recent evidence has also highlighted the importance of lipid dynamic in this process. For instance, small cone-shaped lipids generating membrane curvature of the plasma membrane are synthesized at the exocytotic sites. Among those lipids, phosphatidic acid (PA) synthesized through the activity of phospholipase D (PLD) has been recently shown to be necessary to hormonal release in various cell types as well as in neurotransmitter release. In this paper we examined the possible role of arachidonic acid (AA), a fatty acid that is generated by the activity of phospholipase A_2 (PLA_2). Melittin a well-known activator of PLA_2 was found to concomitantly promote catecholamine and chromogranin A (CGA) release in a calcium-dependent manner and also to increase AA synthesis in chromaffin cells. The effects of melittin on exocytosis and AA synthesis did not involve heterotrimeric G protein activation, but were suppressed by PLA_2 inhibitors. Accordingly addition of exogenous PLA_2 stimulated AA synthesis and catecholamine release in permeabilized chromaffin cells, whereas provision of exogenous AA directly increased exocytosis. These results suggest that AA produced by PLA_2 activation during exocytosis may play an important regulatory role in hormonal and neurotransmitter release. The possibility that CGA-derived peptides released during exocytosis mimic the activity of melittin is discussed.

1. Introduction

Hormones and neurotransmitters are released from cells by exocytosis, following a rise in cytosolic calcium, which triggers fusion of the secretory vesicle membrane with the plasma membrane. Exocytosis of neurotransmitters involves the assembly of complexes composed of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, the synaptic vesicle VAMP (synaptobrevin) and the plasma membrane proteins syntaxin and SNAP-25 [1]. SNAREs are targets for botulinum and tetanus toxins. These toxins selectively inhibit synaptic vesicle fusion by site-specific proteolysis of SNAREs [2], suggesting a central function for SNARE in exocytosis. SNARE proteins have been implicated in the fusion machinery of all cellular systems investigated to date, including neuroendocrine cells. However, other cellular elements regulating the kinetics, the extent of fusion, and the preparation of vesicle for release have received scant attention.

Among those factors, lipids appear to play important functions both at the level of secretory vesicle recruitment and at the late membrane fusion steps [3]. For instance phosphoinositides such as PIP_2 regulate the number of active exocytotic sites [4,5], whereas a number of other phospholipids and fatty acids have been proposed to directly promote membrane fusion based on their cone-shaped structure [6]. We have previously shown that phosphatidic acid (PA) produced by PLD1 is required for exocytosis in a number different cell types [7]. Indeed depletion of endogenous PLD1 by RNA interference, strongly reduced exocytosis from chromaffin cells [8]. Furthermore using a PA-binding probe to visualize PA at the ultrastructural level, we were able to show that PA is specifically enriched at the exocytotic sites after cell stimulation [8]. Supporting the notion that the accumulation of PA on the inner leaflet of the plasma membrane favors membrane fusion during exocytosis by directly affecting the plasma membrane topology, PLD1 depletion could be rescued by the addition of lysophosphatidylcholine (an inverted cone-shaped lipid) in the outer leaflet of the membrane [8].

The importance of polyunsaturated fatty acids for neuronal function has been postulated, firstly on the basis that mutations in enzymes involved in their metabolism cause mental retardation in humans [9], and secondly because diets deficient in polyunsaturated fatty acids are associated with deficits in infant brain function [10]. Although there was some uncertainty regarding the effect of AA on

Abbreviations: AA, arachidonic acid; Ca^{2+}, calcium; CGA, chromogranin A; PLA_2, phospholipase A_2; PLD, phospholipase D; PLD1, phospholipase D1; PTX, pertussis toxin; SLO, Streptolysine-O.

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doi:10.1016/j.regpep.2009.09.008

catecholamine secretion [11,12], a recent study illustrated that among various fatty acid tested, provision of exogenous AA specifically potentiated catecholamine release evoked by various secretagogues [13]. In agreement with these observations the release of AA by PLA_2 has been postulated to be involved in vesicle fusion in many biological systems, including neurotransmitter release, insulin secretion, sperm acrosome exocytosis and neurite outgrowth (reviewed in [14]).

In this study we used the PLA_2 activator melittin to test the implication of AA in the late calcium-dependent exocytosis of large dense-core secretory granule in chromaffin cells. Melittin is the principal toxic component in the venom of the European honey bee *Apis mellifera* and is a cationic, hemolytic peptide. It is a small linear peptide composed of 26 amino acid residues in which the amino-terminal region is predominantly hydrophobic, whereas the carboxy-terminal region is hydrophilic due to the presence of a stretch of positively charged amino acids. Melittin has been described to promote AA synthesis through direct and indirect stimulation of PLA_2 activity in different cellular contexts [15]. AA is found at the *sn-2* position of membrane phospholipids, where it can be released by the deacylation of a variety of lipases, and is converted into eicosanoid by cyclooxygenases and lipoxygenases. Direct cleavage of AA is catalyzed by PLA_2 enzymes that can be classified into three distinct groups: secretory PLA_2, cytosolic PLA_2, and calcium-independent PLA_2.

Permeabilization of the plasma membrane of chromaffin cells allows the run-off of essential components and metabolite required for the exocytotic process. Whereas Ca^{2+} is the main trigger of exocytosis, studies in several cell types have demonstrated that ATP acts before Ca^{2+} in the sequence of events leading to exocytosis. Given the possibility that secretion can be dissected into distinct ATP-dependent and ATP-independent phases in SLO-permeabilized cell models [16–18], we explored the possibility that AA may promote the late calcium-dependent membrane fusion step. We previously reported that the amphipatic peptide mastoparan concomitantly increased AA release and secretion in a MgATP-independent way [16]. It was suggested that mastoparan acted through the stimulation of a PTX-sensitive $G\alpha i_3$ trimeric GTPase [17,18]. We show herein that melittin potentiates, in a PTX-insensitive manner, AA release and catecholamine secretion in permeabilized bovine chromaffin cells. Because intracellular proteins, as well as CGA-derived peptides released from large dense-core secretory granule, may activate PLA_2 like melittin, our finding may have broader implications for understanding of pathway regulating AA metabolism during exocytosis.

2. Materials and methods

2.1. Reagents

Arachidonyl trifluoromethyl ketone ($AACOCF_3$) was from Calbiochem. Melittin and other reagents were purchased from Sigma. The purity of melittin was greater than 97% as estimated by HPLC analysis. Using an anti-PLA_2 antibody, we were unable to detect the presence of contaminating bee PLA_2 in 50 µg of melittin. The anti-chromogranin A (CGA) antibody has been described previously [19].

2.2. Culture of chromaffin cells

Bovine adrenal chromaffin cell were isolated and maintained in primary culture essentially as previously described [20]. Briefly cells were isolated from bovine adrenal glands by retrograde perfusion with collagenase and purified on self-generating Percoll gradients. They were suspended in Dulbecco's modified Eagle medium supplemented with 10% (v/v) fetal calf serum containing streptomycin (50 µg/ml) and penicillin (50 units/ml). Growth of non-chromaffin cells was inhibited by the addition of cytosine arabinoside (10 µM) and 5-fluorodeoxyuridine (10 µM) to the culture medium. Cells were grown on 24 multiple 16-mm Costar plates (Cambridge, MA) at a density of 2.5×10^5 cells/well. Experiments were performed 3–6 days after the preparation of the cultures.

2.3. [^{3}H]Noradrenaline release from permeabilized chromaffin cells

Chromaffin cells were loaded with [^{3}H]noradrenaline (New England Nuclear, 13.3 Ci mmol $^{-1}$) for 60 min and then washed four times with Locke's solution (140 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 0.01 mM EDTA, 11 mM glucose, 0.56 mM ascorbic acid and 15 mM HEPES, pH 7.2) and twice with Ca^{2+}-free Locke's solution (containing 1 mM EGTA). Cells were subsequently permeabilized for 2 min with 15 units/ml streptolysin-O (SLO, Institut Pasteur, Paris, France) in 200 µl/well Ca^{2+}-free KG medium (150 mM K-glutamate, 10 mM PIPES, pH 7.0, 5 mM nitrilotriacetic acid, 0.5 mM EGTA, 5 mM MgATP, 4.5 mM magnesium acetate, 0.2% bovine serum albumin). Extracellular fluids were then removed and cells were preincubated 6 min in 200 µl/well Ca^{2+}-free, MgATP-free KG medium in the presence of melittin and/or the compound to be tested as indicated in the figure legends. Then cells were stimulated for 10 min with 200 µl/well KG medium containing $CaCl_2$ and magnesium acetate to yield final free concentrations of 20 µM Ca^{2+} and 1 mM Mg^{2+}. Release of [^{3}H]noradrenaline is expressed as the percentage of total radioactivity present in the cells before Ca^{2+}-induced stimulation. Basal release was established by incubating cells for 10 min with Ca^{2+}-free KG medium. Release experiments were performed in triplicate on at least three different cell preparations.

2.4. [^{14}C]Arachidonic acid release

Cultured chromaffin cells grown on 24-well 16 mm Costar plates at a density of 2.5×10^5 cells/well were incubated for 5 h with culture medium (500 µl/well) containing 0.2 µCi of [^{14}C]arachidonate/ml (Amersham; 53 mCi/mmol). Cells were then washed four times with Locke's solution and twice with Ca^{2+}-free Locke's solution and permeabilized for 2 min with SLO (15 units/ ml) in Ca^{2+}-free MgATP-free KG medium. Subsequently cells were preincubated for 6 min in Ca^{2+}-free MgATP-free KG medium in the presence or absence of 7,7-dimethyl-5,8-eicosadienoic acid (DEDA) or neomycin and then stimulated for 2 min in the presence or absence (basal release) of 20 µM free Ca^{2+} in MgATP- free KG medium with or without melittin. [^{14}C]AA release after stimulation was determined by measuring the radioactivity present in the incubation medium after centrifugation for 5 min at 12,000 g and in cells after solubilization in 1% Triton X-100. Basal [^{14}C]AA release estimated in the absence of Ca^{2+} ($91+2.7$ pmol/10^6 cells) represented $1.12+0.07$% of the total radioactivity present in cells before stimulation assessed from the amount released plus the amount remaining in the cells. Release experiments were performed in triplicate on at least three different cell preparations.

2.5. Statistics

Statistical analysis was performed with Student's *t* test.

3. Results

Fig. 1 shows the effect of various melittin concentrations on secretion from SLO-permeabilized chromaffin cells. Cells were permeabilized, incubated in the absence of MgATP and then stimulated with increasing concentrations of melittin in a calcium-free or in a 20 µM free Ca^{2+} solution. Melittin moderately increased the catecholamine release in the absence of Ca^{2+} (+73%), but strongly increased Ca^{2+}-dependent secretion (+331%) (Fig. 1A). The mean stimulatory effect on Ca^{2+}-dependent secretion was observed at 1.2 µM of melittin. We also tested the ability of melittin to promote the secretion of the polypeptide CGA contained in the large dense-core chromaffin secretory granules. In agreement with the preceding