Available online at www.sciencedirect.com

SCIENCE DIRECT

Regulatory Peptides 125 (2005) 179–184

REGULATORY PEPTIDES

www.elsevier.com/locate/regpep

Inhibition of phospholipase C-independent exocytotic responses in rat peritoneal mast cells by U73122

Wiebke Gloyna[b], Frank Schmitz[a], Jörg Seebeck[b,*]

[a]St. Josef Hospital, Ruhr-University of Bochum, Gudrunstr. 56, D-44791 Bochum, Germany
[b]Schleswig-Holstein Medical Center, University of Kiel, Department of Pharmacology, Hospitalstr. 4, D-24105 Kiel, Germany

Received 13 January 2004; received in revised form 20 August 2004; accepted 26 August 2004
Available online 22 September 2004

Abstract

The aminosteroid U73122 has been established as potent, selective, and cell-permeable inhibitor C-type phosphatidylinositol-specific phospholipases (PI-PLCs), and has been used to define a contribution of PI-PLCs as part of exocytotic signalling pathways in rat peritoneal mast cells (RPMCs). However, doubts have been raised regarding its PI-PLC selectivity of action. Therefore, in the present study, U73122 was tested in RPMCs under experimental conditions allowing to elicit exocytosis PI-PLC independently (streptolysin O [SLO]-permeabilised cells; stimulated by GTPγS; in the presence of low concentrations of free Ca^{2+}). The release of [^{3}H]5-hydroxytryptamine ([^{3}H]5-HT) from [^{3}H]5-HT-loaded RPMCs served as measure of secretion. U73122 potently inhibited the exocytotic response induced by 10 μM GTPγS (Ca^{2+}: 10^{-6} M) in permeabilised cells (IC_{50}: 0.6 μM, n=5) in an insurmountable manner. In intact RPMCs, with a nearly equal potency (IC_{50}: 4 μM, n=4), U73122 also inhibited the PI-PLC-dependent exocytotic response induced by concomitant application of nerve growth factor and lyso-phosphatidylserine (NGF/lyso-PS).
Conclusion: U73122 exerts potent PI-PLC-independent secretostatic effects, limiting its use to define PI-PLC function within exocytotic processes.

Keywords: U73122; Mast cells; Exocytosis; Phospholipase C; Streptolysin O; GTPγS; Mastoparan; G-protein

1. Introduction

Rat peritoneal mast cells (RPMCs) express a number of mono- and heterotrimeric G-proteins involved in the regulation of exocytosis [1]. Some of these G-proteins have been shown to regulate of phosphatidylinositol-specific phospholipases (PI-PLCs), which catalyse the production of the intracellular messenger molecules inositoltrisphosphate (IP_3) and diacylglycerol (DAG) [2,3]. IP_3 facilitates the release of Ca^{2+} into the cytoplasm by interacting with endoplasmatic Ca^{2+}channels; DAG activates serine/threonine-kinases of the C-type (PKC). Among the G-proteins regulating exocytosis in RPMCs, a distinction has been made between those regulating PI-PLC activity (previously termed G_P), and those not regulating PI-PLC activity (previously termed G_E-proteins) [4]. G_E-proteins are assumed to act in parallel or downstream (within the exocytotic signalling pathway) to G_P-proteins, since a complete set of PI-PLC functions, as demonstrated by means of the PI-PLC inhibitor neomycin, does not impair the exocytotic response induced by the GTP-analogue GTPγS, which activates both G_P- and G_E-proteins [5,6].

A number of basic peptides (e.g., mastoparan, substance P) and some polycationic compounds (e.g., compound 48/80) have previously been shown to stimulate PI-PLC activity and exocytosis in RPMCs [7]. In addition, also PI-PLC-independent exocytotic effects of these compounds have been described [8].

The aminosteroid U73122 has been established as potent, cell-permeable and selective inhibitor of PI-PLC-type phospholipases. U73343 has been established as negative control to U73343, because it is a close structural analogue

* Corresponding author. Tel.: +49 228 2073796; fax: +49 228 2073315.
E-mail address: joerg.seebeck@web.de (J. Seebeck).

0167-0115/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.regpep.2004.08.023

but inhibits PI-PLC activity above a factor of 10 less potently [9,10].

In a recent publication, we reported that U73122 inhibits the exocytotic response induced by the basic peptide pituitary adenylate cyclase activating polypeptide (PACAP) in RPMCs more potently than U73343. This finding, together with other data, has led us to conclude that the signalling cascade underlying the PACAP response may involve a PI-PLC-dependent step [11]. However, the possibility has not been ruled out that U73122 might bring about secretostasis via PI-PLC-independent mechanisms. This suspicion is also supported by the fact that it has been shown to inhibit exocytosis in nearly all systems tested thereupon. Therefore, the aim of the present study was to investigate if U73122 might possess PI-PLC-independent secretostatic properties.

To this purpose, the effect of U73122 on exocytosis was studied in RPMCs under experimental conditions which have previously been demonstrated to elicit exocytosis independent of PI-PLC function [5,6]. The aforementioned experimental conditions comprise the use of the stable GTP analogue GTPγS (as stimulus) in the presence of the pore-forming toxin streptolysin O (SLO), and the presence of an effective EGTA-based Ca^{2+} buffer in the incubation medium. The chosen Ca^{2+} buffer has been shown to be suitable to maintain low and stable concentrations of free Ca^{2+} ($[Ca^{2+}]$); unimpaired, for example, by variations in the concentration of IP_3 [12]. Thus also an interference between the G_P- and G_E-mediated exocytotic signalling pathway due to changes in $[Ca^{2+}]$ are precluded [13]. In addition, experiments with staurosporine were performed, in order to estimate the contribution of a putative DAG-mediated PKC activation to the GTPγS-induced exocytotic response. Staurosporine is a cell-permeable PKC inhibitor, which has previously been shown to inhibit all relevant PKC isoforms in the nanomolar range [14]. Complementary to experiments in GTPγS-stimulated, SLO-permeabilised RPMCs, the effect of staurosporine was also tested in intact RPMCs stimulated by mastoparan or nerve growth factor (NGF). The latter has previously been demonstrated to stimulate exocytosis in RPMCs by interfering with high-affinity TrkA-subtype NGF receptors in the presence of lyso-phosphatidylserine (lyso-PS) [15,16]. The NGF/lyso-PS-induced exocytotic response in RPMCs has previously been described to depend on PI-PLC activation (positive control) [17]. For the same reasons, also the effects of U73122 and U73343 were tested on the mastoparan- and NGF/lyso-PS-induced exocytotic response.

2. Materials and methods

2.1. Solutions and reagents

If not otherwise indicated, substances were obtained from Sigma (FRG). The following compounds were ordered from different manufacturers: GTPγS (Boehringer Mannheim, FRG), mastoparan H-I-N-W-K-G-I-A-A-M-A-K-K-L-L-NH_2 (Bachem, FRG), streptolysin-O (RBI, FRG), U73122, U73343 (Biomol, FRG), [^{3}H]5-hydroxytryptamine ([^{3}H]5-HT; NEN, FRG).

2.2. Preparation of cells

Peritoneal mast cells were obtained from Wistar rats of both sexes (weight > 300 g). They were isolated by means of a BSA-gradient centrifugation procedure, as previously described [3]; the mast cell purity was 80–95%, while the cell viability, as assessed by Trypan blue staining, was always above 95%. Unless otherwise stated, cells were suspended in a modified KRH buffer of the following composition: NaCl 137 mM, KCl 2.7 mM, $CaCl_2$ 0.3 mM, $MgCl_2$ 1.0 mM, NaH_2PO_4 0.4 mM, HEPES 10.0 mM, Glucose 5.6 mM, BSA 0.2% (pH 7.3).

2.3. Secretion studies

For secretion assays purified mast cells were loaded for 2 h in the presence of 1 μCi/ml [^{3}H]5-HT, washed and resuspended in the incubation medium. The above-described modified KRH buffer was used to study secretion in intact RPMCs. Secretion in permeabilised cells was studies by means of a potassium-rich, BSA-free permeabilisation buffer (PB) of the following composition: NaCl 30 mM, KCl 110 mM, NaH_2PO_4 0.4 mM, HEPES 15 mM (pH 7.3). Low concentrations of Ca^{2+} were adjusted by means of a Ca^{2+}/EGTA buffer system (final EGTA concentration: 2.5 mM), prepared as previously described [12]. For standard assays, a final concentration of 1.0 U/ml SLO and a free Ca^{2+} concentration of 10^{-6} M was used. Test compounds were applied at different time points, as indicated. Usually, GTPγS was added 2–3 min after application of SLO. Radioactivity was determined by means of scintillation counting. Secretion was expressed as the percentage of cellular radioactivity released into the supernatant. All conditions were determined in duplicate.

2.4. Statistical analysis, curve fittings

Results are given as means ± S.E.M. Whenever possible, agonist and antagonist concentration–response curves were fitted to a four-parameter logistic equation through computer-assisted curve fitting (Prism 2, GraphPad software, San Diego, USA).

3. Results

3.1. Characterisation of SLO-permeabilized RPMCs

In a potassium-rich permeabilisation buffer ($[Ca^{2+}]$ 10^{-7} M) SLO (0.0–2.0 U/ml) influenced the release of [^{3}H]5HT