NeuroToxicology 30 (2009) 174–181

Contents lists available at ScienceDirect

NeuroToxicology

Neurotoxicity of inverted-cone shaped lipids

Paola Caccin, Ornella Rossetto, Cesare Montecucco *

Dipartimento di Scienze Biomediche and Istituto C.N.R. Neuroscienze, Università di Padova, Viale G. Colombo n. 3, 35121 Padova, Italy

ARTICLE INFO

Article history:
Received 5 September 2008
Accepted 24 November 2008
Available online 9 December 2008

Keywords:
Neuromuscular junction
Neurotoxicity
Lysophospholipids
Alkylphosphocholine
LysoPAF
Inverted-cone shaped lipid

ABSTRACT

Many amphipatic molecules are characterized by an inverted-cone shape capable of altering the curvature and other properties of the plasma membrane of cells. We have recently shown that several lysophospholipids which have this shape impair nerve terminals by promoting neuroexocytosis and inhibiting endocytosis. This results in a bulging of neurites and nerve terminals and block of neurotransmission with paralysis of the neuromuscular junction. Here, we have determined the neurotoxicity of four inverted-cone shaped molecules of great interest because of their biological and pharmacological activities: miltefosine, perifosine, lysoPAF and lysophosphatidylcholine. These compounds were found to cause a complete, but reversible, paralysis of the nerve-hemidiaphragm preparation and to induce bulging of neurons in culture with entry of calcium from the external medium.
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1. Introduction

Biological membranes are composed of variable proportions of proteins, phospholipids, glycolipids and sterols. The lipid component includes many different classes of molecules which assemble together to form lipid bilayers that are physical barriers to the passage of ions, sugars, amino acids and many other molecules. Ion permeability is strictly controlled by channels and pumps and this allow cells to perform a variety of physiological processes. The transmission of the nerve impulse to muscles at the neuromuscular junction takes place via the controlled fusion of synaptic vesicles (SV) with the presynaptic membrane of the nerve terminal with release of acethylcholine (Kandel et al., 2001). This neurotransmitter crosses the intersynaptic space and binds to receptors located on the post-synaptic muscle membrane causing an allosteric transition to open ion channels (Taly et al., 2006). Similar events take place at synapses of the central nervous system with the release of this or other neurotransmitters. Different pools of SV are present within a nerve terminal (Rizzoli and Betz, 2005). A ready to release pool of SV is docked to the cytosolic face of the presynaptic membrane and release its content within hundreds of microseconds from the membrane depolarization which opens voltage-gated calcium channels. There is evidence that such a rapid release can take place because these SV are already half-fused with the membrane, i.e. the cis monolayers are fused, whilst the trans monolayers are not (Zimmerberg and Chernomordik, 2005; Wong et al., 2007; Chernomordik and Kozlov, 2008). A recycling pool and a reserve pool of vesicles can be released only upon a large elevation of the cytosolic [Ca^{2+}] (Ceccarelli et al., 1972; Rizzoli and Betz, 2005).

We recently found that some snake presynaptic phospholipase A2 neurotoxins (SPANs) cause nerve terminal paralysis by inducing SV exocytosis and blocking the ensuing SV endocytosis via the hydrolysis of phospholipids with release of lysophospholipids (LysoPL) and fatty acids (FA) (Bonanomi et al., 2005; Rigoni et al., 2005). Indeed a mixture of lysophosphatidylcholine (LysoPC, the major form of released phospholipid) and FA was as effective as the toxin itself. The more active component of the mixture was LysoPC which is an inverted-cone shaped lipid which remains confined to the outer plasma membrane monolayer and, as such, it induce a positive curvature of the bilayer (Chernomordik et al., 1997; Chernomordik and Kozlov, 2008). This would promote the fusion of a SV with the membrane because it favours the transition from the half-fused intermediate to pore formation; for the same biophysical reason it inhibits the inverse process of membrane fission which is essential for SV recycling and reuse (Sudhof, 2004). As such, the toxin action was taken as an indirect indication of the existence of the hemifusion intermediate in the process of neuroexocytosis (Zimmerberg and Chernomordik, 2005; Chernomordik and Kozlov, 2008). Later on we found that the accumulation of LysoPC and FA within the plasma membrane of neurons caused by the action of SPANs increases the membrane permeability to Ca^{2+} with ensuing fusion of all the SV contained within the nerve terminals (Rigoni et al., 2007). In addition, lysoPC was shown to be the dominant effector and other inverted-cone shaped

* Corresponding author at: Dipartimento di Scienze Biomediche Sperimentali, Viale G. Colombo 3, 35121 Padova, Italy. Tel.: +39 0498276058; fax: +39 0498276049.
E-mail address: cesare.montecucco@unipd.it (C. Montecucco).
Abbreviations: LysoPL, lysophospholipids; LysoPC, lysophosphatidylcholine; LysoPAF, lyso derivative of the platelet-activating factor; SPANs, snake presynaptic phospholipase A2 neurotoxins; SV, synaptic vesicles.

0161-813X/$ – see front matter © 2009 Published by Elsevier Inc.
doi:10.1016/j.neuro.2008.11.013

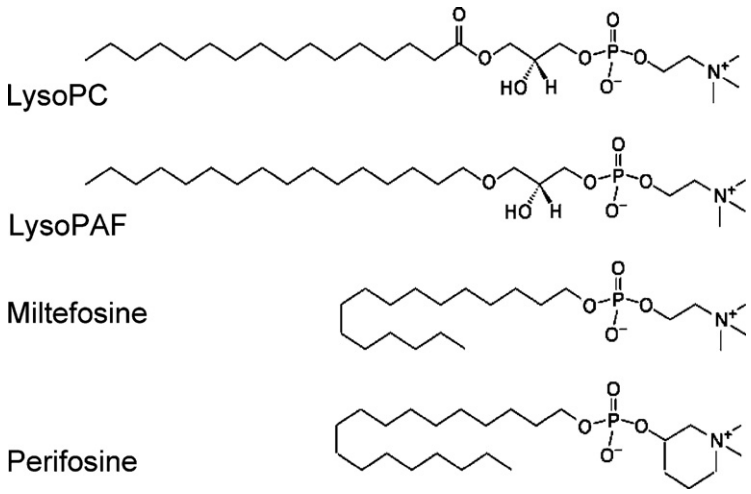

Fig. 1. Chemical structures of the inverted-cone shaped lipids used in this work. The polar head includes in any case the tetra-alkyl ammonium moiety. LysoPAF has an ether bond between glycerol and FA; in miltefosine and perifosine the glycerol moiety is lacking.

phospholipids were found to inhibit the neuromuscular junction (Caccin et al., 2006).

These findings prompted us to investigate the effects on neurons of other molecules of similar shape, but of entirely different chemical nature and biological properties, in order to determine if the effect is a general one and if it can be attributed mainly to the shape of the molecule rather than to the presence of a particular phospholipid head group. In addition, it was of great interest to evaluate potential side-effects of inverted-cone shaped drugs. Here, we report the effects on different neurons and on the neuromuscular junction of miltefosine, perifosine, and of the lyso derivative of the platelet-activating factor (lysoPAF) as compared to lysophosphatidylcholine (lysoPC).

Miltefosine and perifosine are alkylphosphocholines developed from alkylphospholipids, in which the glycerol backbone is lacking (Fig. 1). They have a strong anti-proliferative effect on many cellular models, but, unlike the majority of the other anticancer drugs, they are membrane-targeted and do not interact with DNA. Miltefosine is used in therapy for topical treatment of cutaneous lymphoma and cutaneous breast cancer metastasis (Vink et al., 2007). In addition, miltefosine is effective for the treatment of human visceral leishmaniasis (Chappuis et al., 2007; Palumbo, 2008). Perifosine differs from miltefosine for its cyclic polar head group (Fig. 1), and it is part of an ongoing phase II clinical trial in combination with other anticancer agents. These compounds interfere with multiple cellular processes including phospholipid turnover, signal transduction pathways and apoptosis. They are protein kinase B inhibitors and prolonged treatment can significantly change the gene expression pattern of treated cells (Kondapaka et al., 2003; Floryk and Thompson, 2008).

LysoPAF is a natural lipid produced in cells by a regulated $cPLA_2$ that acts on alkylphospholipids; it is an intermediate in one of the synthesis pathways of platelet activating factor, but the lack of the acetyl group makes it unable to bind the PAF receptor (Prescott et al., 2000). The main difference from LysoPC is the ether bond that links the hydrocarbon chain with the polar head (Fig. 1).

Notwithstanding their different structures, we found that these four molecules act on neurons very similarly and at the same range of concentrations. In particular: (a) they are neuroparalytic; (b) they cause bulging of neurons in culture and (c) they trigger calcium entry from the external medium not mediated by voltage-gated calcium channels. These effects are reversible, but, at higher doses, they are cytotoxic to neurons.

2. Materials and methods

2.1. Chemicals

Lysophosphatidylcholine (1-myristoyl-2-hidroxy-sn-glycero-3-phosphocholine) and LysoPAF (1-O-palmitoyl-sn-glycero-3-phosphocholine) were obtained from Sigma (Saint Louis, MS, USA). Miltefosine (1-hexadecylphosphorylcholine) and perifosine were from Cayman Chemical Company (Ann Arbor, MI, USA). Fura2-AM and Pluronic acid were from Molecular Probes (Eugene, OR, USA); all other chemicals were of analytical grade (Sigma).

2.2. Lipids preparation

Suitable amounts of lysoPC and lysoPAF were dissolved in small volumes of $CHCl_3$:CH_3OH (3:1, v/v), dried to a thin film under a gentle nitrogen flow and vacuum pumped for at least 2 h to remove residual traces of organic solvents. The dried lipid film was suspended in buffer L (Hepes-Na 10 mM, pH 7.4, NaCl 150 mM) at a final concentration of 5 mM, extensively vortexed and then ultrasonically dispersed in a bath sonicator at 37–40 °C, until optical clarity was achieved. Miltefosine and perifosine were suspended directly in buffer L (final concentration 5 mM), vortexed and ultrasonically dispersed with the same procedure, at room temperature.

2.3. Mouse phrenic nerve-hemidiaphragm preparation

All experimental procedures were carried out in accordance to the European Communities Council Directive no. 86/609/EEC. Mouse phrenic nerve hemidiaphragms were isolated from CD-1 mice weighing about 20–30 g following established procedures (Bulbring, 1946) and mounted in 5 ml oxygenated (95% O_2, 5% CO_2) solution (139 mM NaCl, 12 mM $NaHCO_3$, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM KH_2PO_4 and 11 mM glucose, pH 7.4). Two innervated hemidiaphragm preparations were isolated from each animal.

The phrenic nerve was stimulated via two ring platinum electrodes with supramaximal stimuli of 10 V amplitude and 0.1 ms pulse duration, with a frequency of 0.1 Hz (Stimulator 6002,

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