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Short communication

Cholesterol inhibits the lytic activity of melittin in erythrocytes

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Abstract

Although cell lysis by the hemolytic peptide, melittin, has been extensively studied, the role of specific lipids of the erythrocyte membrane on melittin-induced hemolysis remains unexplored. In this report, we have explored the modulatory role of cholesterol on the hemolytic activity of melittin by specifically depleting cholesterol from rat erythrocytes using methyl-β-cyclodextrin (MβCD). Our results show that the hemolytic activity of melittin is increased by ∼3-fold upon depletion of erythrocyte membrane cholesterol by ∼55% without any appreciable loss of phospholipids. This result constitutes the first report demonstrating that the presence of cholesterol inhibits the lytic activity of melittin in its natural target membrane, i.e., the erythrocyte membrane. These results are relevant in understanding the role of cholesterol in the mechanism of action of melittin in the erythrocyte membrane.

Keywords: Cholesterol depletion; Erythrocyte membrane; Hemolysis; Melittin; Methyl-β-cyclodextrin

1. Introduction

Melittin is the principal toxic component in the venom of the European honey bee, *Apis mellifera*. It is a small linear peptide composed of 26 amino acid residues (NH_2-GIGAVLKVLTTGLPALISWIKRKRQQ-$CONH_2$) and is known to have powerful hemolytic activity (Habermann, 1972; Tosteson et al., 1985; Dempsey, 1990). It is a cationic peptide in which the amino-terminal is composed predominantly of hydrophobic amino acids (residues 1–20), whereas the carboxy-terminal end has a stretch of predominantly hydrophilic amino acids (residues 21–26), which give rise to its amphiphilic character. This amphiphilic property of melittin makes it water-soluble, and yet it spontaneously associates with natural and artificial membranes (Dempsey, 1990). Such a sequence of amino acids, coupled with its amphiphilic nature, is characteristic of many membrane-bound peptides and putative transmembrane helices of membrane proteins (Dempsey, 1990). This has resulted in melittin being used as a convenient model to monitor lipid–protein interactions in membranes and membrane-mimetic systems (Hristova et al., 2001; Raghuraman and Chattopadhyay, 2003; Raghuraman and Chattopadhyay, 2004).

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Despite the availability of a high-resolution (2 Å) crystal structure of tetrameric melittin in aqueous solution (Terwilliger and Eisenberg, 1982), the structure of the membrane-bound form is not yet resolved by X-ray crystallography. Since the association of the peptide in the membrane is linked to its physiological effects (Dempsey, 1990), understanding the interaction of melittin with membrane components assumes significance. The importance of the membrane-bound form also stems from the observation that the amphiphilic α-helical conformation of this hemolytic toxin in membranes resembles those of signal peptides (Golding and O'Shea, 1995), and the envelope glycoprotein gp41 from the human immunodeficiency virus (HIV) (Rabenstein and Shin, 1995). Furthermore, understanding melittin–membrane interaction assumes greater significance due to the recent finding that melittin mimics the N-terminal of HIV-1 virulence factor Nef1-25 (Barnham et al., 1997).

Cholesterol is an essential component of eukaryotic membranes and plays a crucial role in membrane organization, dynamics, function and sorting (Yeagle, 1985; Simons and Ikonen, 2000). It is often found distributed non-randomly in domains or pools in biological and model membranes (Simons and Ikonen, 2000; Simons and Ikonen, 1997; Rukmini et al., 2001). Many of these domains are believed to be important for the maintenance of membrane structure and function. Recent observations suggest that cholesterol exerts many of its actions by maintaining a specialized type of membrane domain termed "lipid raft" in a functional state (Simons and Ikonen, 1997), although the existence of lipid rafts in membranes has not been unequivocally shown (Munro, 2003). In view of the importance of cholesterol in relation to membrane domains, the interaction of cholesterol with membrane proteins (Yeagle, 1985; Burger et al., 2000) represents an important determinant in functional studies of such proteins.

The most characteristic effect of melittin on cell membranes is its lytic activity (Habermann, 1972; Tosteson et al., 1985; Dempsey, 1990). The natural target for melittin is the erythrocyte membrane, which contains high amounts (∼45 mol%) of cholesterol (Yeagle, 1985). It has been shown that melittin binds rapidly to erythrocytes and induces the release of hemoglobin (Tosteson et al., 1985; Dempsey, 1990). Melittin-induced hemolysis has been reported to occur by a colloid-osmotic mechanism (Tosteson et al., 1985). However, the role of membrane lipids of the erythrocyte membrane on the melittin-induced hemolysis is not clear. Studies using model membranes have shown that the presence of cholesterol in the membrane inhibits the lytic activity of melittin (Benachir et al., 1997; Raghuraman and Chattopadhyay, 2004). We have recently shown that melittin could interact preferentially with cholesterol in model membranes (Raghuraman and Chattopadhyay, 2004). However, the effect of cholesterol on the lytic activity of melittin in erythrocyte membranes is not yet known. In this paper, we have explored the modulatory role of cholesterol on the hemolytic activity of melittin by specifically depleting cholesterol from rat erythrocytes using methyl-β-cyclodextrin (MβCD), which selectively and efficiently extracts cholesterol from membranes by including it in a central nonpolar cavity (Christian et al., 1997; Steck et al., 2002). Our results show that depletion of cholesterol by ∼55% without any appreciable loss of phospholipids increases the hemolytic activity of melittin by ∼3-fold. This novel result constitutes the first report demonstrating that the presence of cholesterol inhibits the lytic activity of melittin in its natural target membrane, i.e., the erythrocyte membrane.

2. Experimental procedures

2.1. Materials

Melittin, DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine), and HEPES were obtained from Sigma Chemical Co. (St. Louis, MO, USA). To check for any residual phospholipase A_2 contamination in melittin, phospholipase activity was assayed using ^{14}C-labeled DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) (Amersham International, Buckinghamshire, UK) as described earlier (Ghosh et al., 1997). The concentration of melittin in aqueous solution was calculated from its molar extinction coefficient (ε) of 5570 M^{-1} cm^{-1} at 280 nm (Ghosh et al., 1997). Amplex Red cholesterol assay kit was from Molecular Probes (Eugene, OR, USA). All other chemicals used were of the highest purity available. Solvents used were of spectroscopic grade. Water was purified through a Millipore (Bedford, MA, USA) Milli-Q system and used throughout.

2.2. Methods

2.2.1. Assay for hemolytic activity

The hemolytic activity of melittin was evaluated using Wistar rat erythrocytes as described previously (Subbalakshmi et al., 1999). Erythrocytes were isolated from heparinized blood by centrifugation at 3000 rpm for 5 min and washed three times with HEPES buffer (5 mM HEPES, 150 mM NaCl, pH 7.4). An aliquot of washed erythrocytes was kept separately for carrying out cholesterol depletion. Increasing concentrations of melittin were added to aliquots of 0.5 ml suspension, which correspond to $\sim 10^7$ cells in Eppendorf tubes and were incubated at 37 °C in duplicates for 30 min with gentle mixing. The tubes were then centrifuged and the absorbance of the released hemoglobin in the supernatants was measured at 540 nm in a 96-well microtitre plate. The hemolysis obtained with water was taken as 100%. The cell suspension in buffer in the absence of melittin served as background control. The hemolytic activity of melittin in cholesterol-depleted erythrocyte membranes was evaluated the same way. All hemolytic assays were carried out on the same day of blood collection.

2.2.2. Cholesterol depletion of rat erythrocyte membranes

Rat erythrocyte membranes were depleted of cholesterol using MβCD as described earlier (Samuel et al., 2001). Briefly, 2×10^9 intact erythrocytes, washed free of serum, were resuspended in HEPES buffer containing 5 mM MβCD and incubated for 20 min at 37 °C with mild shaking, following which excess MβCD was washed off twice and resuspended in HEPES buffer.

2.2.3. Extraction of lipids from rat erythrocytes

Lipids were extracted from RBC using both Bligh–Dyer (Kates, 1986) and Rose–Oklander method (Rose and Oklander, 1965). All extractions were preceded by complete lysis of the packed erythrocytes with an equal volume of distilled water. Total phospholipid content of both control and cholesterol-depleted erythrocyte membranes was determined after lipid extraction as described below and cholesterol content was determined using the Amplex Red cholesterol assay kit (Amundson and Zhou, 1999). Lipid extracts obtained using both methods yielded similar results. In addition, estimation of total phospholipids obtained from the cell lysates after spinning them down and from lipid extracts yielded similar values, thereby ensuring that the extraction process was efficient and complete.

2.2.4. Estimation of inorganic phosphate

Concentration of lipid phosphate of lipid extracts from control and cholesterol-depleted erythrocytes was determined subsequent to total digestion by perchloric acid (McClare, 1971) using Na_2HPO_4 as standard. DMPC was used as an internal standard to assess complete lipid digestion. Samples without perchloric acid digestion produced negligible readings.

3. Results

Extraction of lipids from control and cholesterol-depleted erythrocytes was carried out using widely used extraction procedures (Kates, 1986; Rose and Oklander, 1965), which ensures quantitative extraction. Cholesterol depletion by the water-soluble compound MβCD has earlier been shown to be dependent on the concentration of MβCD used, time of incubation, and cell type. Importantly, MβCD has previously been shown to selectively and efficiently extract cholesterol from erythrocyte membranes (Steck et al., 2002; Samuel et al., 2001; Rivas and Gennaro, 2003). However, it has recently been reported that cholesterol depletion using MβCD can lead to depletion of other classes of lipids under certain conditions (Ottico et al., 2003). It is, therefore, necessary to show that depletion of cholesterol is specific and does not lead to depletion of phospholipids under the conditions used. To ensure this, we estimated phospholipid and cholesterol contents upon MβCD treatment of erythrocytes. The effect of 5 mM MβCD treatment on the lipid composition of rat erythrocytes is shown in Fig. 1. The figure shows that the cholesterol content of MβCD-treated erythrocytes is reduced by ∼55% of the value of control erythrocytes. It is reassuring to note that the change in phospholipid content under identical conditions is negligible (∼2%). This shows that the depletion of cholesterol by MβCD is specific.

The change in cholesterol-to-phospholipid (C/PL) ratio (mol/mol) upon cholesterol depletion in erythrocytes is shown in Table 1. The table shows that control erythrocytes have C/PL ratio of 0.73, whereas

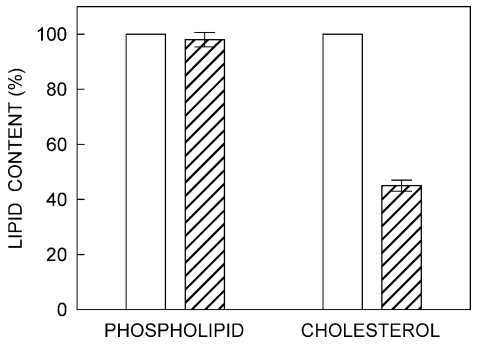

Fig. 1. Effect of 5 mM MβCD treatment (shaded bars) on lipid composition of rat erythrocytes. Values are expressed as percentages of the respective lipid content in control erythrocytes without MβCD treatment (white bars). Phospholipid and cholesterol contents were assayed as described in Section 2. Data shown are the means ± S.E. of at least three independent measurements. The cholesterol-to-phospholipid ratio (mol/mol) for both control and cholesterol-depleted erythrocytes is shown in Table 1.

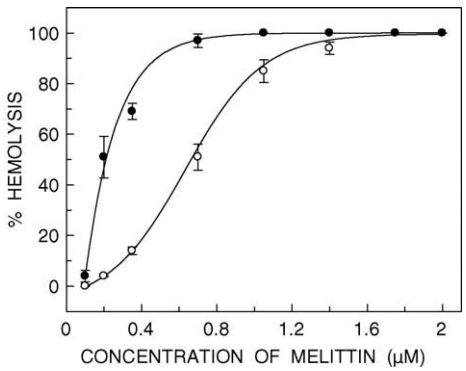

Fig. 2. Hemolysis of untreated (◯) and MβCD-treated (●) rat erythrocytes as a function of increasing concentration of melittin. The hemolytic assay was carried out at room temperature (23 °C). The data points shown are the means ± S.E. of at least three independent measurements. See Section 2 and Table 2 for other details.

cholesterol-depleted erythrocytes have C/PL ratio of 0.32. This implies presence of ∼42 and ∼24 mol% cholesterol in control and cholesterol-depleted erythrocytes, respectively, with respect to their total lipid (phospholipid plus cholesterol) contents. The concentration of MβCD used in this study, therefore, helps in selectively depleting a significant amount of cholesterol without affecting the phospholipid content (see Fig. 1).

Fig. 2 shows the extent of hemolysis in control and cholesterol-depleted erythrocytes as a function of increasing concentration of melittin. As shown in the figure, increasing concentration of melittin drastically increases the release of hemoglobin, which is indicative of cell lysis. The LD_{50} values (the concentration required for 50% lysis) for melittin-induced hemolysis in both cases are given in Table 2. The LD_{50} value for melittin-induced lysis in control erythrocytes is found to be 0.67 μM in overall agreement with previously reported values (Tosteson et al., 1985; Mousli et al., 1990). Interestingly, the LD_{50} value for melittin-induced lysis in cholesterol-depleted erythrocytes is found to be 0.21 μM (see Table 2). This clearly shows that the lytic ability of melittin is more pronounced in erythrocytes with reduced cholesterol content. In other words, cholesterol inhibits the hemolytic activity of melittin since the concentration of melittin required for 50% lysis is ∼3 times lower in cholesterol-depleted erythrocytes than what is required in case of control erythrocytes.

Table 1
Change in cholesterol-to-phospholipid ratio (C/PL) in erythrocytes upon cholesterol depletion[a]

Erythrocytes	C/PL ratio[b] (mol/mol)
Control	0.73 ± 0.03
Cholesterol-depleted	0.32 ± 0.01

[a] The amount of cholesterol depleted using 5 mM MβCD was 55% (see Fig. 1). See Section 2 for other details.

[b] Control erythrocytes have ∼42 mol% cholesterol whereas cholesterol-depleted erythrocytes have ∼24% cholesterol with respect to their phospholipid content. Phospholipid and cholesterol contents were determined after lipid extraction. The values shown are the means ± S.E. of at least three independent measurements. See Section 2 for other details.

Table 2
Effect of cholesterol depletion on the lytic activity of melittin in erythrocytes[a]

Erythrocytes	LD_{50} (μM)[b]
Control	0.67 ± 0.03
Cholesterol-depleted	0.21 ± 0.02

[a] The hemolytic activity of melittin was evaluated with $\sim 10^7$ rat erythrocytes. The amount of cholesterol depleted using 5 mM MβCD was 55%. See Section 2 for other details.

[b] The values shown are the means ± S.E. of at least three independent measurements. See Section 2 for other details.

4. Discussion

Although cell lysis by melittin has been extensively studied, the molecular mechanism of its hemolytic activity is still not well understood. In particular, the role of specific lipids on melittin-induced hemolysis is not yet clear. In this paper, we monitored the modulatory role of cholesterol on the hemolytic activity of melittin by depleting cholesterol using MβCD, a well-characterized cholesterol-depleting agent (Pucadyil and Chattopadhyay, 2004). Our results show that the hemolytic ability of melittin is enhanced upon cholesterol depletion. This could be due to increased penetration of melittin caused by a change in membrane packing in cholesterol-depleted erythrocytes since the presence of cholesterol is known to induce tight hydrocarbon chain packing in membranes (Yeagle, 1985; Mitchell and Litman, 1998). This is supported by our recent observation that the membrane penetration of melittin is decreased with increasing amounts of cholesterol in model membranes (Raghuraman and Chattopadhyay, 2004). This is further reinforced by previous results in which it was shown that bacterial lipopolysaccharides act similarly as cholesterol in providing protection to melittin-induced lysis due to tight packing of the lipid acyl chains in membranes (Allende and McIntosh, 2003). Depletion of membrane cholesterol has previously been shown to increase the pore-forming activity of the antifungal agent syringomycin E in erythrocytes (Blasko et al., 1998). To the best of our knowledge, this is the first report demonstrating the inhibition of lytic activity of melittin in erythrocytes by cholesterol.

The existence of membrane domains in human erythrocytes has previously been reported (Rodgers and Glaser, 1991; Samuel et al., 2001). While the exact nature of these domains is not still resolved, high amounts of cholesterol in the erythrocyte membrane have been shown to induce raft-like detergent-resistant membrane microdomains (Samuel et al., 2001). These microdomains have been reported to act as concentration platforms to facilitate entry of pore-forming toxins (Abrami and van der Goot, 1999). This raises the obvious question: does melittin-induced hemolysis involve raft-like membrane domains? We believe this not to be the case for the following reasons. The action of melittin in membranes is believed to be independent of any receptors (Tosteson et al., 1985; Dempsey, 1990; Mousli et al., 1990). Therefore, localization of any possible receptor in cholesterol-rich membrane domains, and hence, the dependence of melittin entry on membrane cholesterol does not arise. More importantly, it has been previously shown, by analysis of binding of melittin to membranes having characteristic lipid compositions resembling raft-like (detergent resistant) and nonraft-like (detergent soluble) domains, that melittin selectively binds to nonraft membranes (Gandhavadi et al., 2002). The role of bilayer structure and elastic properties of membranes in peptide localization has recently attracted a lot of attention (Chen et al., 2003; Allende et al., 2005). The mode of action of melittin could, therefore, be attributed to membrane destabilization caused by cholesterol depletion since it has been shown that cholesterol depletion causes increased acyl chain disorder in erythrocyte membranes (Cassera et al., 2002). This would favor the insertion and pore formation of melittin.

In summary, our results show that depletion of cholesterol increases the hemolytic activity of melittin. This novel result demonstrates that the presence of cholesterol inhibits the lytic activity of melittin in its natural target membrane, i.e., the erythrocyte membrane. Overall, our results are relevant in understanding the role of cholesterol in the mechanism of action of melittin in the erythrocyte membrane in particular, and other cytolytic peptides in general. Future studies in our laboratory will focus on the detailed characterization of cholesterol-depleted erythrocytes with reference to the hemolytic action of melittin.

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