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STIMULATION OF SUPEROXIDE RELEASE IN NEUTROPHILS BY 1-OLEOYL-2-ACETYLGLYCEROL INCORPORATED INTO pH-SENSITIVE LIPOSOMES

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Summary: Incorporation of 1-oleoyl-2-acetylglycerol (OAG) into multilamellar liposomes composed of egg phosphatidylethanolamine (PE) and arachidonic acid (AA) resulted in a significant enhancement of superoxide release by guinea pig neutrophils when compared to free OAG. OAG incorporated into liposomes containing phosphatidylcholine and arachidonic acid were generally less effective than free OAG. The potency of the liposomes correlates well with the ability of the liposomes to undergo lipid mixing at acidic pH. The enhanced effect of liposome-associated OAG could be related to exposure to an acidic environment in the endosomes/lysosomes once liposomes are endocytosed by neutrophils.
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The synthetic diacylglycerol 1-oleoyl-2-acetylglycerol (OAG) (1), phorbol derivatives such as phorbol 12-myristate 13-acetate (PMA) (2,3) and arachidonate (4) stimulate superoxide (O_2^-) release by guinea pig neutrophils following a concentration dependent lag period (2). Recent evidence suggests that a Ca^{2+} -and phospholipid-dependent enzyme, protein kinase C, is activated at the membrane level in intact cell systems by either diacylglycerol or phorbol ester which becomes intercalated into the lipid bilayer (5-7). Kanda and Huang (8) have recently reported that PMA incorporated into liposomes composed of egg phosphatidylethanolamine (PE) and arachidonic acid (AA) can significantly reduce the ED_{50} of O_2^- release by guinea pig neutrophils. The

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Abbreviations : phorbol 12-myristate 13-acetate, PMA; 1-oleoyl-2-acetylglycerol, OAG; egg phosphatidylethanolamine, PE; egg phosphatidylcholine, PC.

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purpose of the present study is to determine the effect of incorporation of a synthetic diacylglycerol, 1-oleoyl-2-acetylglycerol into liposomes, on O_2^- release from guinea pig neutrophils relative to the free form of the diacylglycerol.

MATERIALS AND METHODS

Neutrophils were isolated from the peritoneal cavity of guinea pigs (Charles River Breeding Labs; Wilmington, MA) 15 hr after the intraperitoneal injection of 20 ml of casein in 0.15M NaCl (6% w/v) (9). Neutrophils were harvested, centrifuged at 1200 rpm for 7 min and contaminating red blood cells were removed by subjecting the cells to hypotonic lysis in 0.83% NH_4Cl (pH 7.2) for 30 min (10). The cells were then washed in a modified Dulbecco's (11) medium (138mM NaCl, 2.7mM KCl, 16.2mM Na_2HPO_4, 0.0315mM $CaCl_2$, 1.47mM KH_2PO_4, 0.50 mM $MgCl_2$ and 7.5mM D-glucose; pH 7.4). The pellet was then suspended in the modified Dulbecco's medium and placed on ice until use. Viability of the cells was always >90% as determined by exclusion of trypan blue and >95% of the cells were polymorphonuclear leukocytes.

Liposome composition was 70 mol% egg phosphatidylethanolamine prepared by transphosphatidylation of egg phosphatidylcholine (PE), 20 mol% arachidonic acid (AA), and 10 mol% OAG or 70 mol% egg phosphatidylcholine (PC), 20 mol% arachidonic acid, and 10 mol% OAG. Chloroform was removed by streaming under nitrogen followed by vacuum dessication for 1 hr. The lipid films were then suspended in 10mM Tris base/0.15M NaCl (pH 9.5) or 0.15M NaCl/10mM HEPES (pH 7.4) for PE/AA/OAG and PC/AA/OAG liposomes, respectively, and vigorously vortexed for approximately 1 min.

Superoxide release was measured by the method of Hyslop and Sklar (12). The standard assay mixture (2.0 ml) consisted of 10 microliters of horseradish peroxidase (8 mg/ml), 5 microliters of superoxide dismutase (8 mg/ml), 40 microliters of p-hydroxyphenylacetate (10 mg/ml) and 1.2×10^6 cells in the modified Dulbecco's medium. The assay was initiated by adding the diacylglycerol either in the free form or incorporated into liposomes as the last component. Following incubation of the cells with diacylglycerol for 30 min at 37°C, the reaction was stopped by placing the samples on ice. The fluorescence of the samples were measured with a Perkin-Elmer LS-5 spectrofluorometer with excitation and emission wavelengths of 323 and 410 nm, respectively.

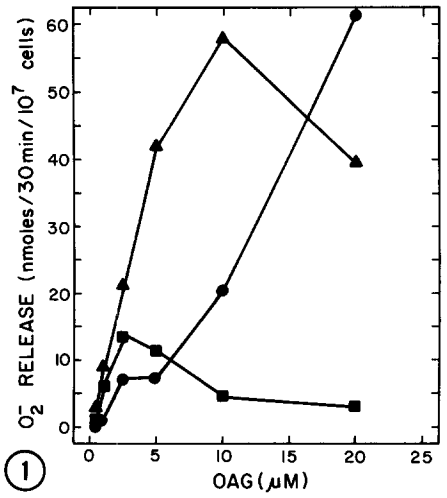
Liposomes for the acid-induced lipid mixing assay were prepared by the method of Connor et al. (13). Lipid films (10 μ-mole) were suspended in phosphate-buffered saline and sonicated under nitrogen for 15 min in a bath type sonicator (Laboratory Supplies; Hicksville, NY). Fluorescence-labeled liposomes containing 1 mol% of N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phosphatidylethanolamine and 1 mol% of N-(lissamine rhodamine B-sulfonyl)-phosphatidylethanolamine were prepared identically as the unlabeled liposomes. The lipid mixing of liposomes was measured by the method of Struck et al. (14). Three parts of unlabeled liposomes and one part of fluorescence-labeled liposomes were mixed and acidification of the medium to the desired pH was

done by adding appropriate amounts of HCl to the mixture. NaOH was then added to return the pH to 7.4. The fluorescence of the liposomes was measured before and after the acid-base cycle with a Perkin-Elmer LS-5 spectrofluorometer. The percent of lipid mixing was calculated according to Connor et al. (13).

RESULTS AND DISCUSSION

Recently Fujita et al. reported that the synthetic diacylglycerol, 1-oleoyl-2-acetylglycerol (OAG) induced superoxide release by neutrophils (1). It has also recently been shown that arachidonate stimulates O_2^- release in guinea pig neutrophils (15). The mechanism of activation may involve intercalation and a resulting membrane perturbation since bovine serum albumin inhibits O_2^- release by neutrophils in a stoichiometric manner (16). Kanda and Huang have shown that incorporation of AA into liposomes abolishes its ability to stimulate O_2^- release by guinea pig neutrophils (15). However, liposomes containing AA serve as an excellent carrier for a phorbol ester, phorbol 12-myristate 13-acetate, for the stimulation of neutrophils. We have thus included AA in our liposome preparation.

Figure 1 shows the superoxide release by guinea pig neutrophils stimulated with PE/AA/OAG (molar ratio 7:2:1) liposomes, PC/AA/OAG (molar ratio 7:2:1) liposomes and free OAG. OAG incorporated into PE/AA liposomes stimulated superoxide release more effectively than free OAG up to 10 μM of OAG. The reason for the decreased effectiveness of OAG in PE/AA liposomes relative to free OAG at high concentration (20 μM) of the diacylglycerol is not clear. Stimulation by free arachidonate also showed a bell-shaped dose-response curve (data not shown). Boni and Rando showed that the stimulation of purified protein kinase C by various sn-1,2-diacylglycerols was enhanced by incorporation into several types of lipid vesicles (17). They also showed that further addition of phospholipid resulted in a decrease in protein kinase C activity and suggested that this was



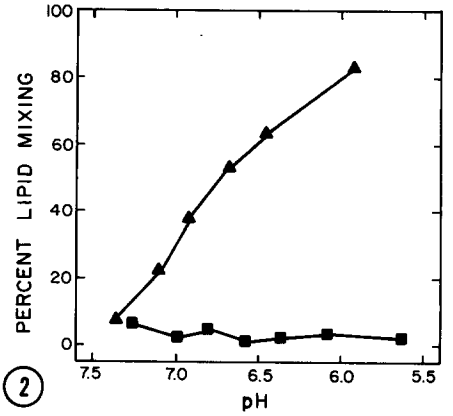


Figure 1. Superoxide release from guinea pig neutrophils stimulated with free OAG or OAG-incorporated liposomes.
Neutrophils (6.3×10^5 cells/ml) were incubated at 37°C with free OAG (●), PE/AA/OAG liposomes (molar ratio 7:2:1) (▲) and PC/AA/OAG liposomes (molar ratio 7:2:1) (■). Superoxide release in 30 min was measured by the method described under "Materials and Methods."

Figure 2. pH-dependence of lipid mixing of liposomes.
Lipid mixing of PE/AA/OAG liposomes (▲) and PC/AA/OAG liposomes (■) were measured at different pH.

possibly due to dilution of available phosphatidylserine which is required for the maximal activity of protein kinase C. A similar dilution effect could take place at high liposome concentrations. The data for superoxide release by free OAG agree with the findings of Fujita et al. (1).

PE/AA/OAG liposomes are pH-sensitive and undergo a lipid mixing with fluorescence-labeled liposomes at acidic pH as measured by the resonance energy transfer method (Fig. 2). Under the same conditions, PC/AA/OAG liposomes did not show any lipid mixing (pH 7.2-5.6). pH-sensitive liposomes are rapidly endocytosed by neutrophils (15) and therefore a possible mechanism for an enhancement of O_2^- release by liposomal OAG could involve an enhanced uptake of PE/AA/OAG liposomes by endocytosis. Liposomes composed of phosphatidylethanolamine and fatty acid form

the hexagonal phase (H_{II}) when they are exposed to the acidic pH of the endosome (18). This could result in the release of liposome-associated OAG into the cytoplasm and delivery to intracellular targets.

In summary we have shown that O_2^- release induced in guinea pig neutrophils by a lipophilic stimulator of protein kinase C can be enhanced by incorporation into liposomes that are acid sensitive, but not with liposomes which are acid-insensitive.

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