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Chemiluminescence monitoring of hemolysis by lysophospholipids

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Abstract

Hemolysis by 1-myristoyl- and 1-palmitoyl-*sn*-glycero-3-phosphocholine was monitored by the chemiluminescence of unsubstituted and long alkyl chain isoluminols. The times required for the appearance of chemiluminescence signals versus lysophospholipid concentrations show an abrupt drop at the critical micelle concentrations of the lysophospholipids, offering evidence that lysophospholipids attack the red cell membrane as micelles. Light integrals and temperature effects are also discussed.

Keywords: Chemiluminescence; Hemolysis; Isosluminol; Lysophospholipids

1. Introduction

Lysophospholipids, present in snake venoms, attack the red blood cell (RBC) membrane, disrupting membrane integrity and causing hemolysis. The mode of attack, however has for years been a matter of controversy [1,2]. One school of thought believes that lysophospholipids attack the RBC membrane as "monomers", homogeneously, while another claims that lysophospholipids are first organised into micelles and it is the aggregates that attack the RBC. The chemiluminescence (CL) of luminol (3-aminophthalhydrazide) and isoluminol (4-aminophthalhydrazide) is catalyzed by Fe^{3+} present in hematin and these light reactions have been employed in numerous analytical applications, the most recent one so far as we know being the work of Tatsu et al. [3].

It was, however, at this point that the issue of lysophospholipid attack on the RBC could be resolved by monitoring the CL of isoluminol and the recently synthesized long alkyl isoluminols [4] as functions of the time required for lysis and lysophospholipid concentrations. Indeed, the time required for leakage of haemoglobin and appearance of the CL signal should depend on the time required for lysis which should in turn depend on the lysophospholipid concentration.

The present work, therefore, mainly deals with the determination of the lysophospholipid concentrations required for fast hemolysis and comparison with the critical micelle concentrations (CMC) of the lysophospholipids employed, namely, 1-palmitoyl- (PAL) and 1-myristoyl-*sn*-glycero-3-phosphocholine (MYR).

2. Experimental techniques

2.1. Reagents

2.1.1. Chemicals

Isoluminol (Aldrich) was recrystallized from methanol, 4-(n-nonylamino) and 4-(di-n-nonylamino)phthalhydrizides ($C_{9\times1}$, $C_{9\times2}$) were synthesized as described earlier [4], and 1-palmitoyl- (PAL) and 1-myristoyl-*sn*-glycero-3-phosphocholine (MYR) were purchased from NovaBiochem A.G., having 2.17% and 2.3% water content respectively. Pyrene (Aldrich) was zone refined and hydrogen peroxide solutions were prepared from Jansen 30% stock.

2.1.2. Preparation of erythrocyte solutions

Blood (human) was obtained from the local blood bank, intended for a 35 day storage. RBCs were isolated from whole blood and were collected in anticoagulant-preservative solution collection bags (Ork, Transfusion Equipment Center, Austrian Red Cross). 5 ml increments of the RBC thus obtained were stored in sealed vials at 4–6 °C, the hematocrit being measured prior to usage. Working suspensions were prepared by diluting 0.5 ml of said RBC in 10 ml of water or saline solutions followed by a second dilution, about 1:70 to 1:90 volume ratio, depending on the hematocrit, to suit result

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reproducibility. These suspensions were freshly prepared and kept at 6–8 °C for up to 6 h.

2.2. Equipment

The UV–Vis spectra were recorded using a Jasco V-560 spectrophotometer. Excitation and fluorescence spectra were obtained on a Jasco FP 777 spectrofluorometer and are uncorrected. CL parameters were measured using a 1250 LKB-Wallac BioOrbit luminometer with the timer circuitry disconnected. The cell's jacket was thermostatically controlled at 25 ± 0.1 °C, unless otherwise stated. Water content was determined with the aid of a 652 KF Coulometer Metrohm (Aa CH-9100 Herisau) Karl Fischer apparatus.

2.3. Solutions and chemiluminescence measurements

In a series of tests, the reagent concentrations required for maximum CL signals combined with maximum stability of the RBC under the experimental conditions required for hydrazide CL were optimized. All reagent solutions with the exception of the RBC suspensions were prepared in NaOH (10^{-2} N) containing NaCl (0.75%) and were stirred for 4 h (C_o), 72 h ($C_{9\times1}$) and 96 h ($C_{9\times2}$). Said solutions were clear, with the exception of the di-nonyl derivative ($C_{9\times2}$), which was turbid.

The hydrazide concentrations employed were 10^{-3} M and 10^{-4} M for C_o and $C_{9\times1}$ and 10^{-5} M for the $C_{9\times2}$ derivative. Lysophospholipid solutions were prepared either by microscale dilution in Wheaton V vials (Aldrich), or by the addition of the appropriate amount of lysophospholipid in ethanol in vials, removal of the solvent by passage of He, addition of the appropriate volume of aqueous NaOH (10^{-2} N) plus NaCl (0.75%) and stirring for 64 h (PAL) or 48 h (MYR). CL intensity–time diagrams were obtained on injection of H_2O_2 (100 μl, 1% aqueous solution) to the hydrazide solutions (500 μl), followed by the RBC suspension (10 μl) into the reaction vessel positioned in the dark chamber of the luminometer.

2.4. CMC determinations

CMCs of the phosholipids were determined by following the ratios of the III and I peaks (excitation λ_{max} 336 nm) in the pyrene fluorescence spectrum versus lysophospholipid concentrations. The solutions employed contained NaOH and NaCl as described above. Pyrene concentration was 5×10^{-6} M.

3. Results

In Fig. 1 the delay in the appearance of the CL signal after injection of the RBC is depicted for various PAL concentrations. A similar situation was observed for MYR. The plot of the delays versus lysophospholipid concentration is shown in Fig. 2; it should be noted that the results above were obtained with unsubstituted isoluminol (C_o). In the case of long alkyl isosuminols the delays are smaller and such a plot is depicted in Fig. 3 for $C_{9\times1}$ isosluminol and PAL, while the same trend exists for MYR as well as with $C_{9\times2}$ isoluminol. Finally, temperature effects were observed and they are shown in Fig. 4.

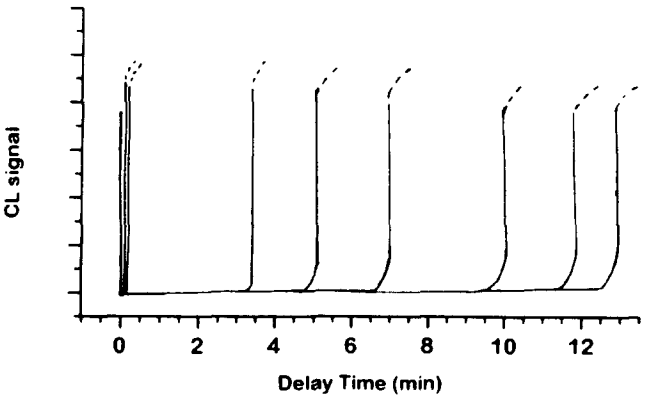

Fig. 1. Delay in the appearance of the CL isoluminol (C_o) signal after injecting RBC ($C_o = 1.0\times10^{-3}$ M). From right to left, 1-palmitoyl-*sn*-glycero-3-phosphocholine (PAL) concentrations depicted: 5.0×10^{-7} M, 1.0×10^{-6} M, 1.5×10^{-6} M, 2.5×10^{-6} M, 3.5×10^{-6} M, 5.0×10^{-6} M, 1.0×10^{-5} M, 5.0×10^{-5} M.

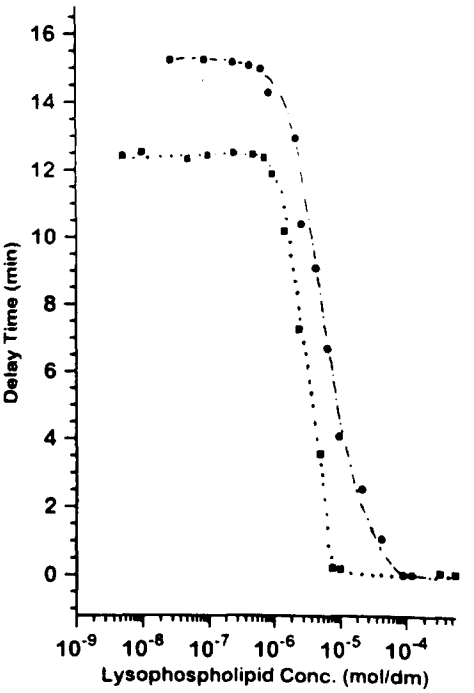

Fig. 2. Lysospholipid concentrations versus the delay in the appearence of the isoluminol (C_o) CL signal, after injecting the RBC; $C_o = 1.0\times10^{-3}$ M. ■, 1-palmitoyl-*sn*-glycero-3-phosphocholine (PAL); ●, 1-myristoyl-*sn*-glycero-3-phospho-choline (MYR).

4. Discussion

Isoluminol was employed in this work instead of luminol so that long alkyl *N*-isoluminols could be also used. Indeed,

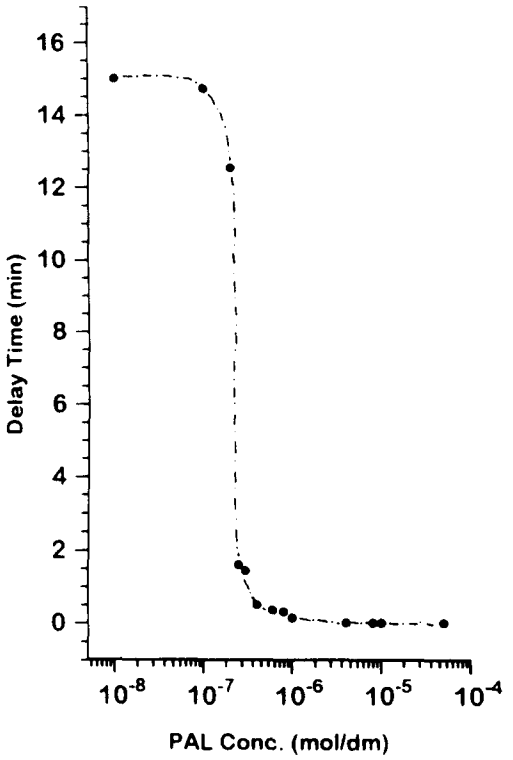

Fig. 3. 1-palmitoyl-*sn*-glycero-3-phosphocholine (PAL) concentrations versus the delay in the appearence of the 4-(n-nonylamino)phthalhydrazide ($C_{9\times1}$) CL signal, after injecting the RBC; $C_{9\times1} = 1.0\times10^{-4}$ M.

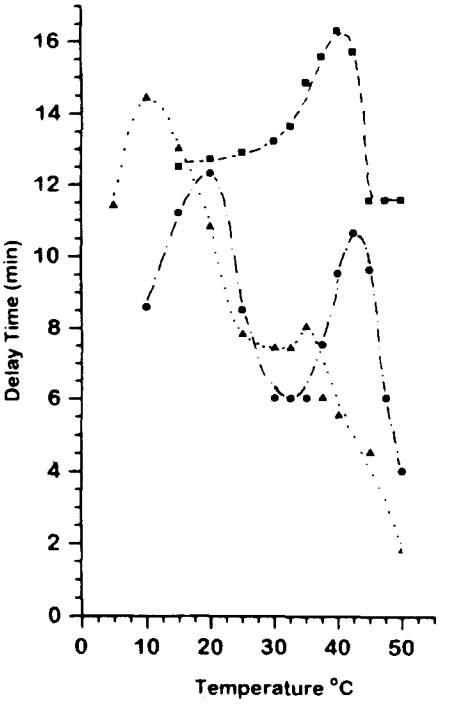

Fig. 4. Temperature versus the delay in the appearence of the isoluminol (C_o) CL signal, after injecting the RBC; $C_o = 1.0\times10^{-3}$ M. ■, absence of lysophospholipid; ●, 2.5×10^{-6} M 1-palmitoyl-*sn*-glycero-3-phosphocholine (PAL); ▲, 2.5×10^{-6} M 1-myristoyl-*sn*-glycero-3-phosphocholine (MYR).

substitution on the luminol amino-group ruins CL efficiency owing to steric factors, while the same substitution on isoluminol has the opposite effect. In any case, the requirements for efficient CL are harmful towards RBC membrane stability and although the concentrations of the reaction mixture components were optimized for maximum RBC stability, rapture of the RBC membrane and appearance of the CL signal occurred after ca. 15 min, even in the absence of lysophospholipid.

The response of the system, however, was excellent and a small sharp peak appearing at $t = 0$ (injection of the RBCs to the CL reaction mixture (Fig. 1)) is due to a minute fraction of the RBCs, already hemolyzed. The presence of various concentrations of PAL in the CL reaction mixture, further reduces the times required for RBC rapture and hematin leakage resulting in the CL signals shown in Fig. 1 for PAL.

The delays of Fig. 1 plotted versus lysophospholipid concentrations are shown in Fig. 2 for PAL and MYR. Instead of a progressive shortening of the delays on increasing the lysophospholipid concentration expected if said species attacked as a monomer, here we observe abrupt slope changes reminiscent of micellar effects. Indeed, it appears that effective lysis occurs at lysophospholipid concentrations in the region of the CMCs. A literature search, however for, the CMC of PAL and MYR revealed an unexpected controversy. The reported CMC values being 2×10^{-4} and 2×10^{-5} M for MYR and PAL respectively [5], referred from earlier works [6–8], do not reflect exhaustive experimental results. Additionally, in earlier literature [9,10] the said CMC values are assigned for the respective ether-deoxy lysolecithins.

In any case, the CMCs of PAL and MYR had to be determined under the experimental conditions of this work as strongly ionic media like the ones here are known to lower considerably the CMCs. Attempts to determine the CMCs by the hydrazide fluorescence were not very reliable and this was eventually achieved by following the ratio of peaks III and I of the pyrene fluorescence spectrum as a function of lysopholipid concentration [11]. The values of 5×10^{-6} and 8×10^{-6} M thus obtained were in good agreement with the slope changes of Fig. 2 in the region of 2×10^{-6} and 5×10^{-6} M for PAL and MYR respectively. Employment of $C_{9\times1}$ instead of C_o (Fig. 3) further intensifies the effect as in this case we are apparently dealing with mixed micelles between the lysophospholipid and the long alkyl isoluminol, probably requiring less lysophospholipid. A similar trend was obtained with disubstituted hydrazide ($C_{9\times2}$), but the low solubility of the hydrazide resulted in poor reproducibility.

Unlike the delays versus lysophospholipid concentration, where sharp slope changes are observed, the total light integrals versus lysophospholipid concentration were not equally rewarding. Small slope changes were observed at the CMCs of PAL and MYR with isoluminol (C_o), but nothing so impressive as the former case. This is not surprising as the lysophospholipid does not affect the mechanism of the light reaction and any effect observed should only reflect the degree of isoluminol micellization as in the case of luminol

[12,13]. In this respect larger slope changes were obtained with $C_{9\times1}$ through better micellization [4], but contrary to the plots of delay versus lysophospholipid concentration, such effects are associated with the degree of micellization of the CL probe and not the effect under study.

The effect of temperature on the RBC stability is critical and had to be determined under the particularly severe experimental conditions of the present work. Such a plot is shown in Fig. 4. RBC relative stability is observed in the region of the human body temperature while the large differences between PAL and MYR are difficult to interpret. In any case, the measurements of the present work at room temperature (25 °C) have been obtained in regions of low, yet reasonably constant RBC stability.

5. Conclusions

In conclusion, the CL delays recorded in the present work are far more suitable than any other CL parameter in hemolysis studies. Plotting said delays versus lysophospholipid concentrations and comparison of the slope changes with the CMCs of the lysophospholipids employed, provides strong evidence that hemolysis by lysophospholipids is associated with micelle formation of said species.

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