Reaction Components Influencing CAMP Factor Induced Lysis

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The reaction components and conditions affecting CAMP factor (*Streptococcus agalactiae*) induced lysis of target cells have been investigated. Both the amount of sphingomyelinase used and the time of preincubation with sphingomyelinase directly affected the rate of haemolysis by CAMP factor. The CAMP factor induced lysis was temperature dependent between 15 and 30 °C and was proportional to the amount of CAMP factor added.

INTRODUCTION

The CAMP factor is released by most strains of *Streptococcus agalactiae* and was described first by Christie *et al.* (1944). CAMP factor causes lysis of red blood cells (RBC) that contain at least 45 mol% of sphingomyelin in the cell membrane and which have been pretreated with *Staphylococcus aureus* β -toxin (sphingomyelinase). Thus, cow and sheep RBC are sensitive to CAMP factor lysis whereas RBC from human, horse, rabbit and guinea-pig are not lysed (Christie *et al.*, 1944). The factor has been characterized as an extracellular polypeptide, to which molecular weight values of 15 000 (Esseveld *et al.*, 1975), 23 500 (Bernheimer *et al.*, 1979) and 33 000 (Brown *et al.*, 1972) have been attributed.

The role of sphingomyelinase in sensitizing target RBC is to hydrolyse sphingomyelin, releasing phosphorylcholine and *N*-acylsphingosine (ceramide). The hydrolysis products were identified by Doery *et al.* (1963) and shown by Colley *et al.* (1973) to be generated from the cell membrane without causing lysis. However, lysis can then be initiated by the addition of CAMP factor or by chilling pretreated cells – ‘hot-cold lysis’ (Rogolski, 1979). Although several aspects of the CAMP reaction have been studied in the past (Esseveld *et al.*, 1958, 1975; Brown *et al.*, 1974; Bernheimer *et al.*, 1979) a detailed analysis of the interaction of the individual components of the CAMP reaction has not been published. This investigation was undertaken to study the conditions for the CAMP reaction in more detail.

METHODS

CAMP factor production. *Streptococcus agalactiae* was grown in a fermenter as described by Huser *et al.* (1983) and the CAMP factor purified by the method of Juergens *et al.* (1984). CAMP factor activity was determined as previously described (Huser *et al.*, 1983).

Sphingomyelinase production. Sphingomyelinase (EC 3.1.4.12) was prepared from *Staphylococcus aureus* (T19) cultures as described by Brown *et al.* (1974). In addition, purified sphingomyelinase (specific activity 800 units mg⁻¹) from *Bacillus cereus*, purchased from Boehringer/Mannheim, was used as a standard. Sphingomyelinase activity was determined according to Krug *et al.* (1979).

RESULTS AND DISCUSSION

Dependence of the CAMP reaction on sphingomyelinase. Dependence of the CAMP reaction on the amount of sphingomyelinase was determined with sheep RBC (50 mol% sphingomyelin) as a target. The rate of haemolysis increased directly with an increase in sphingomyelinase

Abbreviation: RBC, red blood cells.

Fig. 1. Effect of CAMP factor concentration on the rate of lysis of sheep RBC preincubated with sphingomyelinase. The RBC (1.5×10^6 cells ml^{-1}) were incubated in 0.01 M-Tris/HCl buffer containing 0.14 M-NaCl and 0.01 M-MgCl₂, pH 7.4, with sphingomyelinase (0.6 units ml^{-1}) for 5 min at 30 °C. Haemolysis was started by adding CAMP factor and the rate was measured as $\Delta\text{OD}_{546} \text{ min}^{-1}$. The bars indicate the standard error calculated from three experiments.

concentration in the range 0.12–0.42 units ml^{-1} . An excess of sphingomyelinase over 0.5 units ml^{-1} did not further increase the rate of lysis. Although the sphingomyelin content of cow and goat RBC (45 mol%) is slightly lower than that of sheep RBC, the former proved to be more sensitive to sphingomyelinase treatment. Complete sensitization of cow and goat RBC was achieved with 0.12 units ml^{-1} whereas sheep RBC needed 0.43 units ml^{-1} . The influence of the time of preincubation of sheep RBC with sphingomyelinase on the CAMP reaction was also investigated. The CAMP factor induced onset of lysis was measured as a function of the preincubation time of sheep RBC at low sphingomyelinase concentration (0.6 units ml^{-1}). The time to onset of lysis decreased from 200 to 10 s as the preincubation time with sphingomyelinase was increased from 0.3 to 6 min (the lower limit for time measurement by this assay was 10 s). Hydrolysis of the accessible membrane sphingomyelin in target cells thus appeared to occur rapidly and to exhibit saturation kinetics. Thus hydrolysis of membrane sphingomyelin in target cells may be achieved either rapidly with an excess of sphingomyelinase, or on prolonged incubation with small amounts of the enzyme. Sphingomyelinases from both *Staphylococcus aureus* and *Bacillus cereus* were equally active in sensitizing target cells in the CAMP reaction. Since, according to the purification protocol of Ikezawa *et al.* (1978), the commercial *B. cereus* sphingomyelinase used in this study contained no protease, lipase or phospholipase activity, the possibility of an interaction of other 'membrane active' components present in crude *S. aureus* sphingomyelinase preparations is excluded.

Effect of temperature, pH and CAMP factor concentration. When the temperature was raised, the rate of CAMP factor induced lysis increased linearly within the range 15–30 °C. The pH optimum for the reaction was between pH 7 and 8, the rate of lysis decreasing both below and above this range. The influence of the concentration of CAMP factor on the rate of haemolysis was studied using sheep RBC. The rate of haemolysis increased linearly as the concentration of CAMP factor was increased from 5 to 40 units ml^{-1} (Fig. 1).

Effect of sphingomyelin content of target cells. Wiseman & Caird (1967) and Bernheimer *et al.* (1974) reported that RBC of different species differed in their sensitivity to *Staphylococcus aureus* β -toxin (sphingomyelinase) as measured in the 'hot-cold lysis' test. These authors found a correlation between the sphingomyelin content of target cells and their sensitivity to 'hot-cold lysis'. It was therefore of interest to compare the rates of CAMP factor lysis of different RBC species in relation to their sphingomyelin content. The relative rates of lysis determined for RBC

of sheep, cow and goat were 20:6:1. Thus, cow RBC were lysed six times more rapidly than goat RBC although their content of membrane sphingomyelin is nearly the same (Nelson, 1967). It is even more striking that sheep RBC were lysed 20 times more rapidly than those of goat although they differ in their sphingomyelin content by 5 mol% only.

The order of sensitivity of RBC of different species to the 'hot-cold' type of haemolysis after exposure of RBC to sphingomyelinase was not the same as the order of sensitivity to CAMP factor induced lysis, which obviously involves a different mechanism of membrane disruption. Lysis results from the interaction of the CAMP protein with the sensitized membrane and is not initiated by the temperature shift applied in 'hot-cold lysis'. We have thus shown that RBC species containing nearly equal amounts of sphingomyelin (i.e. cow or goat) differed considerably in their rates of lysis. Moreover, RBC from human, rabbit and mouse, which contain between 26 and 12 mol% sphingomyelin (Ways & Hanahan, 1964; Montfort & Boere, 1978) were completely insensitive to CAMP factor, although according to Bernheimer *et al.* (1974) they lyse in the 'hot-cold' reaction.

Our findings indicate that sphingomyelinase creates a metastable membrane phase which is destabilized either by chilling and heating or by the interaction with the CAMP factor (Fehrenbach *et al.*, 1984). It can be assumed that the stability of the metastable phase depends on several other factors such as the amount of diglycerides (generated from membrane sphingomyelin), the individual lipid or protein composition of the membrane and the overall fluidity resulting from these factors. The differences in susceptibility of target cells to sphingomyelinase on the other hand may be due to an asymmetric distribution of the substrate (sphingomyelin) between the two leaflets (inside > outside) or to the steric or fluidity influences exerted by other membrane components.

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