RAPID INCREASE IN INTRACELLULAR FREE Ca^{2+} INDUCED BY ANTIBODY PLUS COMPLEMENT

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1. Introduction

The characteristic release of macromolecules during antibody–complement-induced cell lysis may take many minutes or even hours to occur and is preceded by a rapid increase in the permeability of the cell membrane to ions and low molecular weight compounds [1–3]. Relatively little is known of these early events occurring in cells exposed to antibody plus complement compared to present knowledge of the complement pathway itself [4–6].

The aim of this study was to use the Ca^{2+}-activated photoprotein obelin [7] to investigate effects of antibody plus complement on intracellular free Ca^{2+} An increase in intracellular free Ca^{2+} concentration above the resting level of ~0.1 μM [8] mediates the effects of many physiological stimuli [9] and has also been implicated as an early event in cell damage induced by anoxia [10] and in membrane damage by causing vesiculation [11,12]. Such an increase induced by antibody–complement would therefore be expected to cause severe disruption of cell metabolism well before any significant cell lysis occurs.

Pigeon erythrocytes were used as an experimental system since they contain an adenylate cyclase which can be activated by β-adrenergic agonists and inhibited by intracellular Ca^{2+} (1–10 μM) [13,14]. In addition it is possible to prepare sealed pigeon erythrocyte ghosts containing obelin which are relatively impermeable to Ca^{2+} [13,15]. Entry of Ca^{2+} into such ghosts in response to external stimuli allows the Ca^{2+} to bind to the obelin resulting in light emission and consumption of the prosthetic group of the protein.

2. Methods

2.1. *Obelin and luminescence measurements*

The Ca^{2+}-activated photoprotein obelin was prepared from the hydroid *Obelia geniculata* as in [7,15]. Luminescence was measured using a highly sensitive, low dark current photomultiplier tube coupled to a scalar, to record luminescence counts in a defined time interval, and a chart recorder, to record the luminescence counts per second [7,13,15]. Once a molecule of obelin has luminesced it is to all intents and purposes irreversibly inactivated.

2.2. *Preparation of sealed pigeon erythrocyte 'ghosts'*

'Ghosts' were prepared from pigeon erythrocytes by osmotic lysis and resealed in a medium containing 10 mM Tes, 2 mM $MgSO_4$, 150 mM KCl, 2 mM ATP, 10 mM PEP, 20 U pyruvate kinase/ml (pH 7.4) and obelin at 37°C for 30 min, as in [13]. In some experiments ghosts were resealed in the same medium, containing in addition 0.1 mg/ml ^{125}I-labelled bovine serum albumin (spec. act. 140 μCi/mg protein). The protein was radioactively labelled using the chloramine T method [16]. Osmotic lysis of the resealed ghosts resulted in 80% release of the intracellular ^{125}I-labelled protein.

2.3. *Permeability of 'ghosts' to Ca^{2+}*

The permeability of the 'ghosts' to Ca^{2+} in the presence or absence of antibody–complement was assessed by recording the rate of obelin luminescence on a chart recorder or as counts recorded/10 s on a scalar. At the end of the experiment Triton X-100

(final conc. 7%, w/v) and Ca^{2+} (final conc. ~14 mM) were added to stimulate all of the remaining active obelin. The percentage utilisation of obelin was then calculated.

2.4. *Preparation of antisera*

Antisera to pigeon erythrocytes were prepared by injection of pigeon erythrocytes together with Pertussis vaccine subcutaneously into rabbits. After 4 weeks the rabbits were reinjected and serum collected after a further 10 days. All rabbit sera were incubated at 56°C for 30 min before use in order to inactivate the complement pathway. Serum from rabbits which had not been immunised was used as non-immune serum and serum (non-heat treated) from guinea pigs was used as a source of complement.

2.5. *Assay of cyclic AMP*

Cyclic AMP was assayed in perchloric acid treated samples of pigeon erythrocytes, after precipitation of the perchlorate and neutralisation, by radioimmunoassay [13,14].

2.6. *Assay of K^+*

The K^+ content of 'ghosts' was determined after nitric acid digestion of ghost pellets obtained by centrifugation at 8000 × *g* for 10 s, followed by flame photometry as in [13,14].

3. Results

The effect of the antibody–complement reaction on intracellular free Ca^{2+} was investigated in sealed pigeon erythrocyte ghosts containing ATP, a high K^+ concentration (150 mM) and obelin [13,15] (see section 2). The ghosts were incubated with different concentrations of immune (1/10–1/10 000) and non-immune sera for 5–10 min, guinea-pig serum added (dilution 1/10–1/10 000) and the light emission from obelin recorded.

Addition of guinea-pig serum to the antibody coated ghosts caused a rapid increase in obelin luminescence after a lag of 30–40 s (fig.1). Reduction in the concentration of antiserum used decreased the height of the peak and the rate of luminescence but seemed to have little effect on the time of onset of the increase in intracellular free Ca^{2+} as detected by

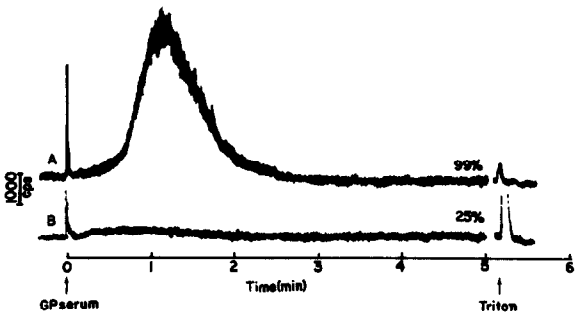


Fig.1. Effect of antibody plus complement on free Ca^{2+} in sealed pigeon erythrocyte 'ghosts'. Sealed erythrocyte 'ghosts' (~10^8 ghosts) were incubated in 0.5 ml medium I (10 mM Tes, 140 mM NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$ (pH 7.4)) containing 5 μl immune (A) or 5 μl non-immune (B) rabbit serum. The suspension was then placed in front of a photomultiplier tube and 0.5 ml medium I plus 0.1 ml guinea pig serum added (GP serum (↑)). The Ca^{2+}-induced luminescence of obelin was recorded on a chart recorder calibrated in luminescence counts per second (cps). At the end of the experiment 0.5 ml Triton X-100 (20%, w/v) plus $CaCl_2$ (40 mM) was added (Triton (↑)). The percentages represent the amount of obelin consumed before addition of Triton. The temperature was 37°C.

obelin (fig.2). In contrast decreasing the concentration of guinea-pig serum not only reduced the height of the luminescence peak, but also increased the lag time before onset of the increase in light emission (fig.3B,C). No significant effect of non-immune rabbit sera, (fig.1) nor of antisera to rat erythrocytes could be demonstrated on obelin luminescence in the ghosts. Incubation of the guinea-pig serum at 56°C for 30 min prior to addition to the ghosts virtually abolished the effect on obelin luminescence (fig.3A). Confirmation that the effect was due to an increase in free Ca^{2+} inside the ghosts was obtained from the reduction in luminescence when EGTA was also sealed within them [13] (data not shown).

The possibility that a significant increase in intracellular free Ca^{2+} concentration occurred before any significant change in the intracellular concentration of other ions or macromolecules was investigated. The data in fig.4. shows that a rapid rise in intracellular free Ca^{2+} indicated by increased utilisation of obelin could be detected using conditions of antibody and complement which resulted only in a slow and incomplete release of intracellular K^+ and of the

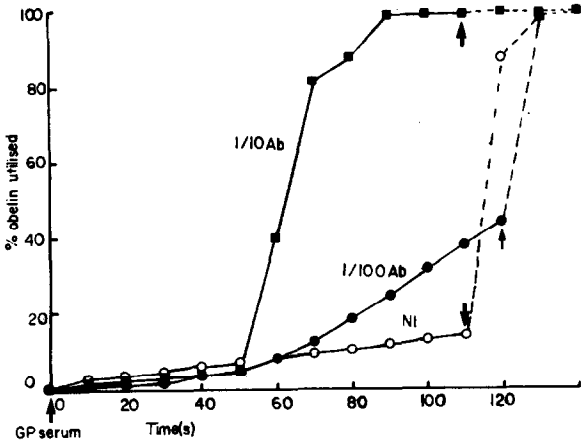


Fig.2. Effect of different antibody concentrations plus complement on free Ca^{2+} in sealed pigeon erythrocyte ghosts. The sealed erythrocyte ghosts (1.2 X 10^8 ghosts) in 0.5 ml medium I (see fig.1) were incubated with rabbit serum at 37°C for 10 min: 100 μl non-immune serum (NI (○)); 100 μl pigeon erythrocyte antiserum (1/10 Ab (■)); 10 μl pigeon erythrocyte antiserum (1/100 Ab (●)). This suspension was then placed in front of a photomultiplier tube and 0.5 ml medium I containing 100 μl normal guinea pig serum added. The Ca^{2+}-induced luminescence of obelin was recorded as total counts/10 s on a scalar, or as cps on a chart recorder. The total active obelin remaining in the ghosts at the end of the experiment was assayed by addition of 0.5 ml Triton (20%, v/v) and 40 mM $CaCl_2$, sufficient to saturate the protein with Ca^{2+} (↑). Results are expressed as the percentage of obelin utilised against time.

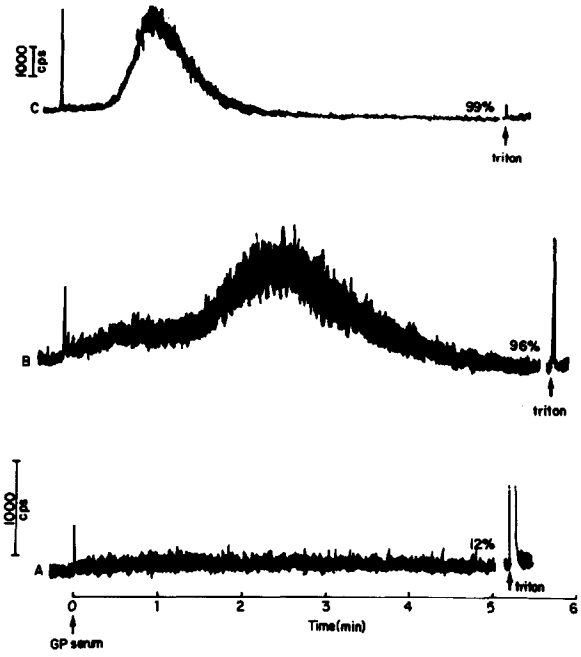
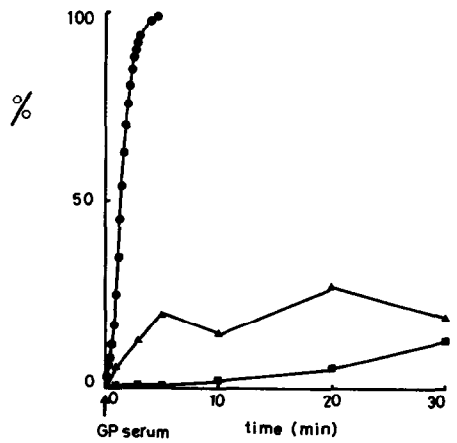


Fig.3. Effect of different concentrations of guinea pig serum on free Ca^{2+} in ghosts. Sealed pigeon erythrocyte ghosts were prepared containing obelin as in fig.1. The ghosts (0.92 X 10^8 ghosts) were incubated for 10 min at 37°C with 5 μl pigeon erythrocyte antiserum. The ghost suspension was then placed in front of the photomultipler tube and 0.5 ml medium I (see fig.1) added containing: (A) 10 μl guinea pig serum, pre-heated at 56°C for 30 min; (B) 10 μl normal guinea pig serum; (C) 50 μl normal guinea pig serum. Luminescence was recorded as luminescent cps. The % obelin utilized is shown at the end of each trace, before addition of Triton.

Fig.4. Effect of antibody plus complement on obelin utilisation and release of intracellular K^+ and ^{125}I-labelled bovine serum albumin from sealed pigeon erythrocyte ghosts. Sealed pigeon erythrocyte ghosts (1.4 X 10^8 ghosts) containing obelin and ^{125}I-labelled bovine serum albumin (0.01 μCi) were incubated in 0.5 ml medium I plus 5 μl pigeon erythrocyte antiserum for 10 min at 37°C. To this suspension 0.5 ml medium I containing 50 μl guinea pig serum was added. The Ca^{2+}-induced luminescence of obelin was determined as in section 2. Separate samples were centrifuged at 8000 X *g* for 10 s, the ghost pellet used for determination of intracellular K^+ and the supernatant for determination of ^{125}I-labelled albumin release. Results are expressed as % utilisation of obelin (●), % release of intracellular K^+ (▲) and % release of intracellular ^{125}I-labelled bovine serum albumin (■), plotted against time after addition of guinea pig serum. All points are the mean of 2 separate determinations.



macromolecule ^{125}I-labelled bovine serum albumin. Within 2 min after addition of the complement source 85% of the obelin was utilised, yet <15% of intracellular K^+ and <1% of ^{125}I-labelled bovine serum albumin released. Even after 30 min the K^+ and ^{125}I content of the ghosts was still >80% of that of the initial ghosts (time 0, fig.4). The results indicated that the increase in intracellular free Ca^{2+} concentration was not due to lysis of the ghosts since if this were the case all the intracellular K^+ and ^{125}I-labelled albumin would have been released.

To see whether the antibody–complement-induced increase in intracellular free Ca^{2+} concentration might have any metabolic effects in intact erythrocytes, adrenalin stimulated cyclic AMP concentrations were measured and compared with the extent of haemolysis (fig.5). The results show that the antibody–complement reaction caused an inhibition of cyclic AMP formation of 40–80% within 2–5 min, using conditions which resulted in <5% of haemoglobin released. The rapid inhibition of cyclic AMP formation detectable within 5 min could not be explained by a loss of ATP in the cells. However within 15 min a significant decrease in ATP content in the cells had occurred which was greatest at high concentrations of antiserum and guinea pig serum.

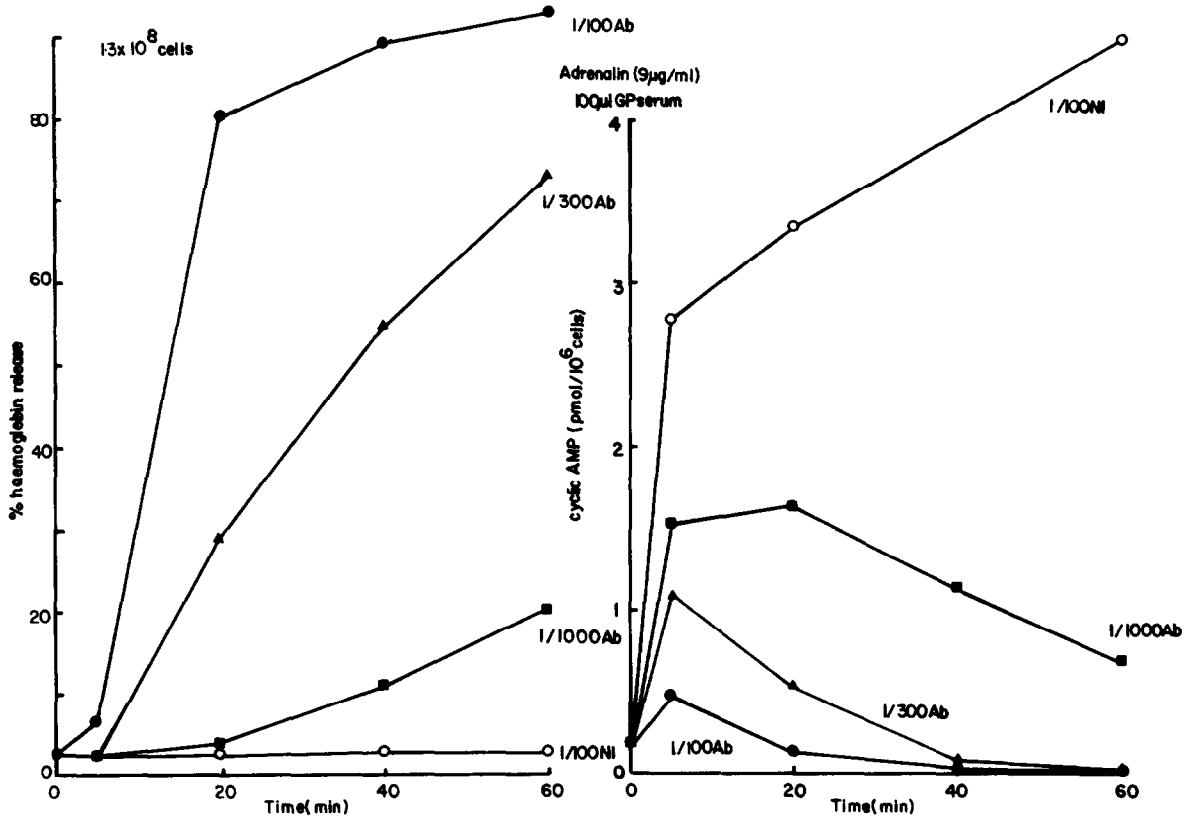


Fig.5. Effect of antibody plus complement on haemoglobin release and cyclic AMP formation in pigeon erythrocytes. Intact pigeon erythrocytes (1.3×10^8 cells) were incubated in 1 ml medium I (see fig.1) with rabbit antisera for 10 min at 37°C; (○) 10 µl non-immune serum; (●) 10 µl pigeon erythrocyte antiserum; (▲) 3 µl pigeon erythrocyte antiserum; (■) 1 µl pigeon erythrocyte antiserum. Adrenaline (9 µg/ml) and guinea pig serum (100 µl) were then added and cells incubated for up to 60 min at 37°C. At defined intervals duplicate samples were centrifuged and A_{440} measured to assess haemoglobin release. Samples were also taken and perchloric acid added to final conc. 0.28 M. After precipitation of the perchlorate the cyclic AMP in the supernatant was assayed by radioimmunoassay [14].

4. Discussion

It has been known for some time that the earliest event induced in the cell membrane by antibody–complement reactions is an increase in the permeability to ions and other small molecules [1,2]. Since the cell membrane contains active ion pumps it is not possible to estimate intracellular ion concentrations by measuring fluxes alone. The approach of the present study was to measure changes in the concentration of intracellular free Ca^{2+} in response to complement action, using the Ca^{2+}-activated photoprotein obelin. The results of this study show that a large increase in intracellular free Ca^{2+} concentration can start to occur 30 s after addition of complement to antibody-coated cells. This rise in intracellular free Ca^{2+} concentration is the earliest change in the intracellular concentration of an ion or metabolite in response to complement yet demonstrated.

During the study the question arose of whether the observed increase in intracellular free Ca^{2+} concentration in response to complement was simply the result of cell lysis. By measuring the % utilisation of obelin in solutions of similar composition to those inside resealed 'ghosts' it is possible to relate obelin utilisation to the concentration of free Ca^{2+} [13]. Under these conditions it has been shown that within 2 min at 1 μM Ca^{2+}, 20% of obelin is utilised; with 10 μM Ca^{2+}, 70%; with 25 μM Ca^{2+}, 95%; and at 0.1–1 mM Ca^{2+}, >99% [7,13]. Although when using conditions where significant obelin utilisation occurs and where the free Ca^{2+} concentration is also changing it is difficult to be precise about the absolute free Ca^{2+} concentration, nevertheless it is possible to estimate this value from the % utilisation of obelin within a defined time interval [13]. In the experiments reported here the % utilisation of obelin after the addition of antibody and complement is consistent with a rise in the intracellular free Ca^{2+} to ~25 μM. Certainly, this is well below the concentration of Ca^{2+} in the extracellular medium (1 mM). This observation together with the fact that the rise in intracellular free Ca^{2+} could be obtained under conditions which resulted in <20% release of cell K^+ and macromolecules (fig.4) shows that the rise in intracellular free Ca^{2+} concentration in response to complement cannot be explained by cell lysis which would cause rapid equilibration of the intracellular compartment with the extracellular fluid.

The stimulation of Ca^{2+} influx by antibody–complement is not restricted to erythrocytes since a similar increase in Ca^{2+}-induced luminescence has been observed in membrane vesicles containing obelin from rat liver and adipose tissue (Hallett, J.P.L., A.K.C., unpublished). An important consequence of this complement-induced rise in intracellular Ca^{2+} concentration may be the disruption of intracellular metabolism. Here it was shown that the antibody–complement reaction can cause a rapid inhibition of adrenalin-stimulated cyclic AMP formation in intact pigeon erythrocytes using conditions which result in little lysis. Experiments from our laboratory have previously shown that >50% inhibition of cyclic AMP formation in pigeon erythrocyte ghosts can be achieved by raising the intracellular Ca^{2+} concentration to 1–10 μM [13,14].

It is possible that the reaction of antibodies and complement with cells could be an important means of manipulating intracellular Ca^{2+} both in vitro and in vivo. An example of an experimental application is the possibility of using this effect to determine whether liposomes containing obelin fuse with isolated mammalian cells and then release obelin into the cytoplasm [17]. The incorporation of obelin into the cytoplasm of small cells after liposome–cell fusion would provide, for the first time, a method for studying directly the effect of hormones and pharmacological substances on intracellular free Ca^{2+}.

In recent experiments, using reactive lysis induced by purified complement components in the absence of antibody [18,19] we have obtained evidence that the complete complement pathway (up to C9) is required for the rapid increase in Ca^{2+} permeability to occur (A.K.C., R.A.D., J.P.L., unpublished). Whether the complement induced increase in intracellular free Ca^{2+} concentration mediates other cellular changes including membrane depolarisation [20] and eventual cell lysis is unknown. However, if it can be shown in other cell systems in vivo that antibodies and complement interact to disrupt intracellular metabolism through an increase in free Ca^{2+} without necessarily causing lysis, then this mechanism could have important implications in understanding the aetiology of many diseases in which the immune system is thought to play a part.

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