Inhibition by Calcium Ions of Adenosine Cyclic Monophosphate Formation in Sealed Pigeon Erythrocyte 'Ghosts'

A STUDY USING THE PHOTOPROTEIN OBELIN

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1. Sealed pigeon erythrocyte 'ghosts' were prepared containing ATP and the Ca^{2+} -activated photoprotein obelin to investigate the relationship between cyclic AMP formation and internal free Ca^{2+} . 2. The 'ghosts' were characterized by (a) morphology (optical and electron microscopy), (b) composition (haemoglobin, K^+ , Na^+ , Mg^{2+} , ATP, obelin), (c) permeability to Ca^{2+} , assessed by obelin luminescence and (d) hormone sensitivity (the effect of β -adrenergic agonists and antagonists on cyclic AMP formation). 3. The effect of osmolarity at haemolysis and ATP at resealing on these parameters was investigated. 4. Sealed 'ghosts', containing approx. 2% of original haemoglobin, 150 mM- K^+ , 0.5 mM-ATP, 10^3 – 10^4 obelin luminescence counts/ 10^6 'ghosts', which were relatively impermeable to Ca^{2+} and in which cyclic AMP formation was stimulated by β -adrenergic agonists over a concentration range similar to that for intact cells, could be prepared after haemolysis in 6 mM- NaCl /3 mM- MgCl_2 /50 mM-Tes, pH 7, and resealing for 30 min at 37°C in the presence of ATP and 150 mM-KCl. 5. The initial rate of adrenaline-stimulated cyclic AMP formation in these 'ghosts' was 30–50% of that in intact cells and was inhibited by the addition of extracellular Ca^{2+} . Addition of Ca^{2+} to the 'ghosts' resulted in a stimulation of obelin luminescence, indicating an increase in internal free Ca^{2+} under these conditions. 6. The ionophore A23187 increased the rate of obelin luminescence in the 'ghosts' and also inhibited the adrenaline-stimulated increase in cyclic AMP. 7. The effect of ionophore A23187 on obelin luminescence and on cyclic AMP formation in the 'ghosts' was markedly decreased by sealing EGTA inside the 'ghosts'. 8. It was concluded that cyclic AMP formation inside sealed pigeon erythrocyte 'ghosts' could be inhibited by more than 50% by free Ca^{2+} concentrations in the range 1–10 μM .

A considerable amount of indirect evidence exists that changes in intracellular Ca^{2+} play an important part in the action of certain hormones (Berridge, 1976; Hales *et al.*, 1977; Rasmussen & Goodman, 1977). Investigations of the effects of Ca^{2+} on adenylate cyclase have mostly been carried out on broken-cell preparations, in the absence of ionic gradients across the cell membrane, and have often involved the use of high non-physiological concentrations of Ca^{2+} (Hales *et al.*, 1977). Although the concentration of intracellular free Ca^{2+} has only been measured in a few large cells such as barnacle muscle (Ashley & Ridgway, 1970) and the squid giant axon (Baker *et al.*, 1971; Baker, 1972), it now seems likely that under physiological conditions the concentration of free Ca^{2+} in cells does not exceed 10 μM . In intact pigeon erythrocytes it has been reported that adenylate cyclase can be inhibited by concentrations of Ca^{2+} less than 10 μM (Campbell & Siddle, 1976). Further progress in the investigation of interactions between

intracellular Ca^{2+} and cyclic nucleotides has been hampered by the lack of suitable preparations where it is possible to study directly changes in free Ca^{2+} , and to correlate these changes with cyclic-nucleotide formation.

Obelin, a Ca^{2+} -activated photoprotein similar to aequorin (Campbell, 1974; Moisescu *et al.*, 1975; Ashley *et al.*, 1976), has been incorporated into pigeon erythrocyte 'ghosts' (Campbell & Dormer, 1975). The light emission from obelin can be used to monitor changes in free Ca^{2+} inside these 'ghosts'. The aim of the present studies was to prepare hormone-sensitive pigeon erythrocyte 'ghosts' containing obelin and ATP in order to study directly the effect of intracellular free Ca^{2+} on cyclic AMP formation in these cells. It has been proposed that sympathomimetic (adrenergic) agonists might act on avian erythrocytes to decrease the concentration of intracellular free Ca^{2+} and hence activate adenylate cyclase (Steer *et al.*, 1975). A further aim of the studies reported in

the present paper was to see if any effects of adrenergic agonists or antagonists on intracellular free Ca^{2+} could be demonstrated. A preliminary account of some of the results has been presented (Campbell & Dormer, 1977).

Experimental

Materials

Chemicals. ATP (disodium salt), phosphoenolpyruvate (potassium salt), pyruvate kinase (EC 2.7.1.40) and ox heart cyclic AMP phosphodiesterase (EC 3.1.4.1) were obtained from Boehringer Corp. (London), London W5 2TZ, U.K. [^3H]Adenosine 3':5'-cyclic monophosphate (27 Ci/mmol), [^3H]inulin (25 Ci/mmol) or inulin-[^{14}C]carboxylic acid (approx. 10 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. L-Adrenaline [(−)-adrenaline], DL-isoproterenol (isoprenaline) sulphate, L-phenylephrine hydrochloride, 5-hydroxytryptamine and DL-propranolol were obtained from Sigma Chemical Co., Kingston upon Thames, Surrey, U.K. Phentolamine hydrochloride was kindly given by CIBA laboratories, Horsham, West Sussex, U.K. D-Adrenaline bitartrate, D-noradrenaline bitartrate and D-isoproterenol (isoprenaline) bitartrate were kindly given by Sterling-Winthrop Research Institute, Rensselaer, NY, U.S.A. The ionophore A23187 was kindly given by the Lilly Research Centre, Windlesham, Surrey, U.K. EGTA and L-noradrenaline bitartrate were obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Spec Pure KCl, NaCl and MgSO_4 were obtained from Johnson Matthey Chemicals, London, U.K. All other reagents were AnalR grade from BDH Chemicals, Poole, Dorset, U.K.

Pigeons. *Columbia livia domesticus* (Homer strain) were used throughout.

Hydroids. Colonies of *Obelia geniculata* attached to fronds of brown seaweed (*Laminaria digitata*, *Laminaria hyperborea*, *Laminaria ochreluca*, *Laminaria saccharina* and *Saccorhiza polyschides*) were collected at a depth of approx. 8 metres by skin divers in Plymouth Sound.

Preparation of obelin

The Ca^{2+} -activated photoprotein obelin was extracted from the hydroids and purified as previously described (Campbell & Dormer, 1975). The obelin was stored as a freeze-dried powder in the presence of gelatin and EDTA. The concentration of gelatin in the suspensions described below was approx. 0.1 mg/ml and the concentration of EDTA was less than $1\ \mu\text{M}$. Since sufficient Mg^{2+} was always present to saturate the EDTA, no significant quantity of Ca^{2+} was bound to EDTA.

Preparation of pigeon erythrocyte 'ghosts'

Sealed pigeon erythrocyte 'ghosts' were prepared by modification of the method previously described (Campbell & Dormer, 1975). Blood (5–10 ml) was obtained from the wing vein of a pigeon, and clotting was prevented by the addition of lithium heparin (approx. 50 units/ml). The erythrocytes were centrifuged in an MSE bench centrifuge at 2100 g for 5 min, and washed twice in 10 ml of medium A consisting of 10 mM-Tes (2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulphonic acid), 140 mM-NaCl, 5 mM-KCl and 2 mM- MgCl_2 , adjusted to pH 7.4 with NaOH. The cells (approx. 5×10^9 cells) were haemolysed in 30–40 ml of ice-cold haemolysing medium, (a) 6 mM-NaCl/3 mM- MgCl_2 , approx. pH 7, or (b) 6 mM-NaCl/3 mM- MgCl_2 /50 mM-Tes, pH 7. After 15 min the 'ghosts' were centrifuged at 4°C for 5 min in a Sorval RC-5 centrifuge at approx. 10000 g. The 'ghosts' were washed four or five times in haemolysing medium (total volume of washes approx. 100 ml). The 'ghosts' were then resuspended in 5–10 ml of medium containing 10 mM-Tes, 6 mM-NaCl, 2 mM- MgCl_2 or 2 mM- MgSO_4 , obelin (10^8 – 10^{10} recorded luminescence counts, see below), 2 mM-ATP (disodium salt), 10 mM-phosphoenolpyruvate (potassium salt) and pyruvate kinase (approx. 20 units/ml), adjusted to pH 7.4, with KOH. The pyruvate kinase was centrifuged for 5 min in a Beckman microfuge to remove $(\text{NH}_4)_2\text{SO}_4$. After incubation at 0°C for 5–10 min a quantity of 3 M-KCl was added such that the final concentration was 150 mM-KCl. The suspension was incubated for up to 60 min at 37°C. Samples were taken to estimate the loss of ATP and inactivation of obelin that had occurred during the incubation at 37°C. The sealed 'ghosts' were centrifuged for 5 min at 4°C at 10000 g, washed twice in medium A (total volume of washes approx. 20 ml) and resuspended in 1–4 ml of medium A. A sample was taken to estimate the number of 'ghosts'/ μl of suspension by using a haemocytometer.

Assay of obelin

Obelin luminescence is stimulated by Ca^{2+} and requires no other substrates or cofactors. After emission of a photon the protein becomes inactive, hence, on addition of Ca^{2+} the intensity of light emission reaches a maximum within 0.1 s and then decreases exponentially. The rate constant of this decay in the presence of a concentration of calcium sufficient to saturate the protein was approx. 4 s^{-1} at room temperature (approx. 20°C). To estimate the total obelin activity in a solution, a sample was added to 0.5 ml of 200 mM-Tris/0.1 mM-EDTA, pH 7.4, and then placed in front of a highly sensitive photomultiplier tube (P4232B, from Twentieth Century

Electronics, Croydon, Surrey, U.K.; dark current approx. 0.1 nA at 1285 V, overall sensitivity 200 A/lm) in a specially constructed thermostatically controllable light-tight apparatus. The photomultiplier tube was connected to a scalar (M5060A; Ecko Electronics, Maidenhead, Berks., U.K.), and the total counts were recorded during the first 10 s after the addition of 0.5 ml of 50 mM-CaCl₂. The CaCl₂ was added from a syringe while the tube containing the obelin was in front of the photomultiplier tube. The background count in the absence of obelin was 10–20 c.p.s. To estimate the quantity of obelin trapped inside 'ghosts' a portion of 'ghosts' was added to 0.5 ml of 200 mM-Tris/0.1 mM-EDTA, pH 7.4, or 0.5 ml of medium A and the suspension placed in front of the photomultiplier tube. The total luminescence counts were recorded after addition of 0.5 ml of 40 mM-CaCl₂/20% (v/v) Triton X-100, which was sufficient to lyse the 'ghosts'.

The rate of obelin luminescence, expressed as c.p.s., was decreased by lowering the Ca²⁺ concentration or the quantity of obelin present. The rate of luminescence was independent of the concentration of obelin. The rate of luminescence was measured by connecting the output of the photomultiplier, through a rate meter (M5190; Ecko Electronics) to a chart recorder (J. J. Instruments, Southampton, U.K.; CR550 or Bryans, Surrey, U.K.; 26000 X-Y). To measure the decay rate constants of the most rapid exponential decays the output from the rate meter was first stored in a transient recorder (DL901; Datalab, Surrey, U.K.) before transferring the information to the chart recorder. This was done to eliminate any distortion of the luminescence trace resulting from the time constant of the recorder. The time constant of the rate meter was 0.01 s. Results were expressed as total counts recorded in a defined time interval or as c.p.s. The precise efficiency of the luminescence apparatus is not known, although based on the luminol method (Nakamura, 1972) it may be as low as 0.1%. The major factors involved in this relatively low efficiency were the geometry of the tube housing, the efficiency of the photomultiplier tube and the discriminator in the scalar.

Assay of haemoglobin

This was carried out in a modified Drabkins solution (Davie & Lewis, 1970). A portion (10–50 µl) of intact cells or erythrocyte 'ghosts' in medium A was added to 4 ml of a solution containing 0.06 mM-potassium ferricyanide and 0.77 mM-KCN. The suspension was incubated at room temperature for 30 min, centrifuged at 2100g in an MSE bench centrifuge, and the A₅₄₀ of the supernatant determined. Ox blood haemoglobin was used as a standard. Results were expressed as mg of haemoglobin/10⁶ cells or 'ghosts'.

Concentration of K⁺, Na⁺ and Mg²⁺ in sealed erythrocyte 'ghosts'

Portions of 'ghosts' (50 µl) incubated in 1 ml of medium under various conditions were centrifuged for 1 min in an Eppendorf microfuge. The supernatant was removed and the pellet dried overnight, weighed and digested in 0.1 ml of Aristar conc. HNO₃. After addition of 1 ml of water, 0.5 ml samples were diluted with 15 mM-Li₂SO₄ for measurement of K⁺ and Na⁺ by flame photometry or 26.9 mM-LaCl₃ for measurement of Mg²⁺ by atomic absorption spectrometry. The addition of a small quantity of [³H]inulin (approx. 2 µCi) before centrifugation of the 'ghost' suspension enabled the extracellular space in the 'ghost' pellet to be estimated. The calculated values for extracellular K⁺, Na⁺ and Mg²⁺ in the pellet were subtracted from the total ion content. The resulting value for intracellular ion content was converted into a concentration by dividing the value by the intracellular water content of the 'ghosts'. The intracellular water was calculated as the difference between the total and extracellular water.

Assay of cyclic AMP

Portions (50 µl) of 'ghosts' were incubated in 1 ml of various media for up to 30 min at 37°C. Then 1 ml of 10% (w/v) trichloroacetic acid was added and the suspension centrifuged for 5 min at approx. 4000g in an MSE Mistral 6L centrifuge. The trichloroacetic acid in the supernatant was extracted four times with 5 ml of water-saturated diethyl ether. Samples (40 µl) were then used for assay of cyclic AMP by radioimmunoassay (Siddle *et al.*, 1973). In some experiments it was necessary to concentrate the samples by freeze-drying before assaying for cyclic AMP. More than 85% of the substance measured as cyclic AMP in the 'ghosts' could be destroyed by cyclic AMP phosphodiesterase as previously described for intact pigeon erythrocytes (Campbell & Siddle, 1976).

Assay of ATP

Samples (10 µl) were taken from the same extract as that used for assay of cyclic AMP and assayed for ATP by using firefly luciferase (Campbell & Siddle, 1976).

Ca²⁺ contamination in the media

To minimize Ca²⁺ contamination in the solution used to reseal the erythrocyte 'ghosts' all vessels and pipettes were rinsed in 1–10% (v/v) HCl, followed by rinsing in double-glass-distilled water. The 10 mM-Tes was passed down a Chelex 100 column (Campbell & Dormer, 1975) to decrease the free Ca²⁺ to less than 0.1 µM. The NaCl, KCl and MgSO₄ were Spec Pure reagents.

Results and Discussion

Preparation of hormone-sensitive sealed pigeon erythrocyte 'ghosts'

The aim of the initial experiments was to prepare sealed pigeon erythrocyte 'ghosts' containing ATP and obelin, which were relatively impermeable to Ca^{2+} and which were sensitive to sympathomimetic (adrenergic) agonists and antagonists in a similar manner to intact cells. A number of workers using non-nucleated erythrocytes have reported that different conditions at haemolysis and during resealing affect both the enzyme and protein composition as well as the permeability characteristics of the final 'ghost' preparation. These conditions include osmolarity, temperature, the concentration of bivalent cations and pH (Whittam, 1962; Dodge *et al.*, 1963; Bramley & Coleman, 1972; Schwoch & Passow, 1973; Wessels & Verkamp, 1973). Previous studies had shown that pigeon erythrocyte 'ghosts' containing ATP and obelin could be prepared after haemolysis in 6 mM-NaCl/3 mM-MgCl₂, pH 7.0, and resealing for up to 90 min at 37°C (Campbell & Dormer, 1975). Although these 'ghosts' were sensitive to adrenaline, the concentration of cyclic AMP formed was small relative to intact cells and sometimes difficult to detect. These 'ghosts' also appeared to be more permeable to Ca^{2+} than intact cells. Experiments were therefore carried out in an attempt to both increase the formation of cyclic AMP in the 'ghosts' induced by adrenaline and decrease their permeability to Ca^{2+} .

It has been reported that osmolarity at haemolysis may be an important factor affecting the properties of the sealed 'ghosts' (Dodge *et al.*, 1963; Schwoch & Passow, 1973). This was investigated with pigeon erythrocytes by comparing the 'ghosts' prepared after haemolysis in 6 mM-NaCl/3 mM-MgCl₂ in the presence or absence of 50 mM-Tes, pH 7. At haemolysis 95–99% of the haemoglobin and ATP were lost from the cells. The 'ghosts' produced by haemolysis in the higher osmolarity medium contained approx. 5 times the content of haemoglobin and ATP of 'ghosts' haemolysed in 6 mM-NaCl/3 mM-MgCl₂ alone.

After haemolysis the 'ghosts' were sealed by incubating them for up to 1 h at 37°C in the presence of ATP, obelin and 150 mM-KCl (see the Experimental section). The final sealed 'ghost' preparations were characterized by using four criteria: (a) morphology (optical and electron microscopy); (b) composition (haemoglobin, K^+ , Na^+ , Mg^{2+} , ATP, obelin and other substances such as inulin added to the resealing solution); (c) permeability to Ca^{2+} , assessed by obelin luminescence, and the permeability to macromolecules, assessed by inulin release (Campbell & Dormer, 1975); (d) hormone sensitivity (the

effect of β -adrenergic agonists and antagonists on cyclic AMP formation).

(a) Morphology

All sealed 'ghosts' retained their nuclei, mitochondria (3–10 per section) and an intact cell membrane. The cytoplasm of the 'ghosts' was considerably clearer than in intact cells, reflecting the loss of more than 95% of the cell haemoglobin. The suspension containing 'ghosts' originally haemolysed in 6 mM-NaCl/3 mM-MgCl₂/50 mM-Tes, pH 7, contained a small proportion (less than 10%) of 'ghosts' with a darker cytoplasm than the rest. This suggested that morphologically only a small degree of heterogeneity existed in the 'ghost' population.

(b) Composition

The haemoglobin content of the final 'ghost' suspension, resealed and suspended in medium A, after initial haemolysis in 6 mM-NaCl/3 mM-MgCl₂, pH 7, was less than 0.5% of that in intact cells, compared with approx. 2% in 'ghosts' originally haemolysed in 6 mM-NaCl/3 mM-MgCl₂/50 mM-Tes, pH 7, some additional haemoglobin and ATP having been lost during the resealing process. The concentration of K^+ in the 'ghosts' increased with time of resealing up to 30 min (Fig. 1) and was greatest in 'ghosts' originally prepared by haemolysis in 6 mM-NaCl/3 mM-MgCl₂/50 mM-Tes, pH 7.0, the K^+ concentration in these 'ghosts' being approx. 70–80% of that in intact cells. In contrast with K^+ , the Na^+ concentration in the 'ghosts' decreased with time of resealing (Fig. 1). The calculated internal Mg^{2+} concentration was approx. 15 mM in 'ghosts' originally haemolysed in 6 mM-NaCl/3 mM-MgCl₂, pH 7, and appeared to change little during the 60 min resealed. However, in 'ghosts' originally haemolysed in 6 mM-NaCl/3 mM-MgCl₂/50 mM-Tes, pH 7.0, the internal Mg^{2+} concentration decreased by approx. 40% during the 60 min resealed at 37°C.

The ATP content was highest in 'ghosts' sealed for only 15 min at 37°C (Fig. 2). The lower ATP content of 'ghosts' sealed for longer times could be accounted for by its hydrolysis, the concentration of ATP in the whole suspension after 60 min being less than 10% at the start of the incubation. 'Ghosts' originally haemolysed in 6 mM-NaCl/3 mM-MgCl₂/50 mM-Tes, pH 7.0, and sealed for 30 min at 37°C contained 5–20% of the ATP of intact cells, the calculated internal ATP concentration of sealed 'ghosts' being up to 1 mM. These 'ghosts' normally contained between 5 and 20 times as much ATP as 'ghosts' originally haemolysed in 6 mM-NaCl/3 mM-MgCl₂, pH 7.0 (Fig. 2).

As with ATP, the obelin content of the 'ghosts' was greatest in 'ghosts' sealed for only 15 min at 37°C. Incubation for a further 45 min decreased the obelin

Fig. 1. K^+ and Na^+ concentrations in sealed pigeon erythrocyte 'ghosts'

Pigeon erythrocytes were haemolysed in 6mM-NaCl/3mM-MgCl₂, pH 7.0 (□, ○), or 6mM-NaCl/3mM-MgCl₂/50mM-Tes, pH 7.0 (■, ●), and sealed 'ghosts' prepared as described in the Experimental section by incubating the 'ghosts' for up to 60 min at 37°C. At various times the 'ghosts' were centrifuged, washed twice and resuspended in medium A. Samples (50 μl) containing 4.6×10^7 – 5.2×10^7 'ghosts' + approx. 2 μCi of [³H]inulin were centrifuged and the supernatant was removed for scintillation counting. The K^+ and Na^+ content of the pellets were measured as described in the Experimental section. The radioactivity in the pellet enabled the extracellular K^+ and Na^+ to be calculated. The intracellular K^+ and Na^+ concentrations were calculated by dividing the intracellular ionic content by the intracellular water (calculated from total water – extracellular water). Results are means of two estimations.

content of the 'ghosts' by approx. 50%. This was in spite of the fact that the total obelin recovered in the medium after 60 min at 37°C was less than 5%. In most experiments 1–5% of the obelin added to the medium at resealing was entrapped within the 'ghosts'. Addition of obelin to 'ghosts' that had already been sealed for 30 min at 37°C resulted in no detectable obelin being associated with the 'ghosts' after centrifugation and washing (see the Experimental section). It therefore appeared that a negligible quantity of obelin was attached to the outside of the cell membrane. Approx. 10^3 – 10^4 obelin counts/ 10^6 'ghosts' were entrapped in 'ghosts' originally prepared by haemolysis in 6M-NaCl/3M-MgCl₂ with or without 50mM-Tes, pH 7.0.

In these experiments the extracellular water was calculated from the amount of [³H]inulin in the 'ghost' pellet after centrifugation in a medium containing [³H]inulin (see the Experimental section). The internal inulin 'space', estimated by measuring [³H]inulin trapped inside the 'ghosts', was approx. $0.1 \mu\text{l}/10^6$ 'ghosts' for 'ghosts' that were originally haemolysed in the higher osmolarity medium. This value was similar to that calculated by subtraction of the extracellular water from the total water content in the centrifuged 'ghost' pellet.

(c) Permeability to Ca^{2+}

Addition of Ca^{2+} to pigeon erythrocyte 'ghosts' containing obelin resulted in an increase in light emission from the 'ghosts' and a gradual consumption

of the photoprotein. The relative permeability to Ca^{2+} of various 'ghost' preparations was assessed by measuring the percentage of entrapped obelin utilized in the 100s period after the addition of Ca^{2+} . The smaller the utilization the less permeable were the 'ghosts' to Ca^{2+} . The relative permeability to Ca^{2+} of the 'ghosts' decreased with increasing time of resealing. The rate of utilization of obelin in 'ghosts' originally haemolysed in 6mM-NaCl/3mM-MgCl₂/50mM-Tes, pH 7.0, and incubated in medium A (see the Experimental section) containing 1mM- Ca^{2+} was 2–3 times lower than that in 'ghosts' originally haemolysed in 6mM-NaCl/3mM-MgCl₂, pH 7, and incubated under the same conditions.

(d) Sympathomimetic (adrenergic) agents on cyclic AMP formation

Pigeon erythrocytes, in common with nucleated erythrocytes from birds, fish, amphibians and reptiles, contain an adenylate cyclase which is activated by β -adrenergic agonists. In order to compare the ability of sealed erythrocyte 'ghosts' to form cyclic AMP in response to adrenaline the 'ghosts' were incubated in medium A at 37°C for 5 min in the presence of a concentration of L-adrenaline (55 μM), which maximally stimulated the 'ghosts'. The formation of cyclic AMP was approximately linear over the 5 min period studied (see Fig. 4). Cyclic AMP formation was greatest in 'ghosts' that had been resealed for 15 min (Fig. 3). As the ATP concentration in the 'ghosts' decreased (Fig. 2) so did

Fig. 2. ATP content in sealed pigeon erythrocyte 'ghosts'

Pigeon erythrocytes were haemolysed in 6mM-NaCl/3mM-MgCl₂, pH 7.0 (□, ○), or 6mM-NaCl/3mM-MgCl₂/50mM-Tes, pH 7.0 (■, ●). Sealed 'ghosts' were prepared as described in the Experimental section. Samples of 'ghosts' were taken at various times, up to 60 min, during the reseal. The 'ghosts' were centrifuged, washed and resuspended in medium A. Portions (50 μl) of the final suspension, containing 4.6×10^7 – 5.2×10^7 'ghosts', were incubated for 5 min at 37°C in medium A + 1 mM-CaCl₂ in the presence (□, ■) or absence (○, ●) of adrenaline (55 μM). ATP and cyclic AMP (Fig. 3) were measured as described in the Experimental section. Results represent means of two determinations.

the formation of cyclic AMP stimulated by adrenaline (Fig. 3). 'Ghosts' originally haemolysed in 6mM-NaCl/3mM-MgCl₂/50mM-Tes consistently formed more cyclic AMP in the presence of adrenaline than 'ghosts' originally prepared in 6mM-NaCl/3mM-MgCl₂, pH 7.0 (Fig. 3). In some experiments the adrenaline-stimulated formation of cyclic AMP in these 'ghosts' was 10 times that in 'ghosts' originally haemolysed in 6mM-NaCl/3mM-MgCl₂, pH 7.0. Although it is not possible to make an accurate estimate of the K_m for ATP of adenylate cyclase in sealed 'ghosts', it appears from the data in Figs. 2 and 3, and from experiments not shown here, that the internal ATP concentration producing a half-maximal response to adrenaline (55 μM) was 0.1–0.5 mM. This estimate can only be made if one assumes a homogeneous 'ghost' population and a similar

Fig. 3. Cyclic AMP formation in sealed pigeon erythrocyte 'ghosts'

Sealed pigeon erythrocyte 'ghosts' were prepared and incubated for 5 min at 37°C with (□, ■) or without (○, ●) 55 μM-adrenaline as described in Fig. 2. The values for cyclic AMP represent means of two determinations.

recovery of adenylate cyclase under all conditions. An experiment sealing different ATP concentrations inside the 'ghosts' prepared from the same cell population has not yet been carried out.

Selection of conditions of preparation of 'ghosts' for further experiments

To be able to study the relationship between intracellular free Ca²⁺ and cyclic AMP formation in sealed 'ghosts' it was necessary that the 'ghosts' should be relatively impermeable to Ca²⁺, contain high internal K⁺ and ATP concentrations and on stimulation by adrenaline form sufficient cyclic AMP, which could be easily measured. These conditions were best satisfied by 'ghosts' prepared after original haemolysis in 6mM-NaCl/3mM-MgCl₂/50mM-Tes, pH 7.0, rather than in 6mM-NaCl/3mM-MgCl₂, pH 7.0. It was more difficult to decide on the most suitable time of reseal, since under no conditions could all the parameters be optimized. 'Ghosts' resealed for 60 min at 37°C, although apparently highly impermeable to Ca²⁺, often contained such low ATP concentrations that it was difficult to

detect the formation of cyclic AMP. On the other hand 'ghosts' sealed for only 15 min contained a high ATP concentration, formed cyclic AMP on stimulation by adrenaline, but appeared to be more permeable to Ca^{2+} than did 'ghosts' sealed for 60 min. It was therefore decided that in all further experiments 'ghosts' that had been prepared by initial haemolysis in 6 mM-NaCl/3 mM-MgCl₂/50 mM-Tes, pH 7, and then sealed at 37°C for 30 min would be used. These 'ghosts' were relatively stable, since they could be stored in ice for at least 2 h, with a loss of active obelin and decrease in adrenaline-stimulated cyclic AMP formation of less than 20%, and a loss of ATP of less than 30%. In most experiments no detectable changes in these parameters occurred while the 'ghosts' were stored in ice. 'Ghosts' were rarely used after longer periods of storage than 2 h, since after this time there appeared to be some increase in the permeability of the 'ghosts' to Ca^{2+} , assessed by measurement of the percentage of obelin utilized on addition of 1 mM- Ca^{2+} to the 'ghosts'.

To demonstrate that the 'ghosts' responded to hormones in a similar manner to intact cells the effect on cyclic AMP formation in the 'ghosts' of a variety of β -adrenergic agents was investigated. Isoprenaline was the most potent β -agonist tested, being approximately five times more potent than adrenaline. The concentration of adrenaline required for a half-maximal response in cyclic AMP formation in intact cells was $2.0 \pm 0.02 \mu\text{M}$ (range 1.3–2.5; mean of eight experiments). Estimates of this parameter in 'ghosts' incubated for 2 or 5 min with L-adrenaline were within the same range as for intact cells. Both D-adrenaline and L-phenylephrine were able to stimulate cyclic AMP formation in sealed erythrocyte 'ghosts'. However, these compounds were approx. 30 and 100 times respectively less potent than L-adrenaline. Propranolol (10 μM) completely abolished the effect of 55 μM -adrenaline, whereas phentolamine (10 μM) was without effect. The effect of L-adrenaline on cyclic AMP formation in intact cells and sealed 'ghosts' was also inhibited by 5-hydroxytryptamine (Campbell & Siddle, 1977) and by replacement of Na^+ by Li^+ in the incubation medium. It was concluded that the sealed 'ghosts' were sensitive to β -adrenergic agonists and antagonists over a concentration range similar to that effective on intact cells.

Formation of pigeon erythrocyte 'ghosts' in the absence of ATP

To demonstrate that the ATP required for cyclic AMP synthesis was not simply a small fraction of endogenous ATP remaining after sealing, 'ghosts' were sealed for 30 or 60 min at 37°C in the absence of an ATP-regenerating system, but in the presence of 150 mM-KCl, obelin and [³H]inulin. The initial

haemolysis in 6 mM-NaCl/3 mM-MgCl₂/50 mM-Tes, pH 7.0, resulted in a loss of more than 90% of the cell ATP content. Incubation of these 'ghosts' in the absence of ATP for 60 min at 37°C resulted in 'ghosts' with an ATP content less than 0.05% of intact cells. 'Ghosts' sealed in the absence of ATP contained 20–100 times less ATP than 'ghosts' sealed in the presence of ATP. Furthermore 'ghosts' prepared in the absence of ATP and sealed for 30 or 60 min produced no detectable cyclic AMP (less than 0.02 pmol/10⁶ 'ghosts') in the presence of L-adrenaline (10–55 μM). 'Ghosts' sealed in the absence of ATP contained an internal K^+ concentration approx. 50% of that of 'ghosts' sealed in the presence of ATP. The 'ghosts' without ATP also contained approx. half as much haemoglobin and entrapped 2–3 times as much [³H]inulin.

Initial experiments attempting to incorporate obelin into 'ghosts' in the absence of ATP were unsuccessful because of very low recoveries of obelin after the incubation at 37°C (Campbell & Dormer, 1975). The use of Tes buffer passed through a Chelex 100 column (Campbell & Dormer, 1975) together with the use of Spec Pure reagents, decreased Ca^{2+} contamination sufficiently to allow some active obelin to be entrapped within the 'ghosts' without ATP. However, the obelin recovered in the whole suspension after a 30 min reseal at 37°C was less than 20% of that with 'ghosts' in the presence of ATP and the obelin entrapped was approx. 10 times less in the 'ghosts' without ATP. Addition of 1 mM- Ca^{2+} to 'ghosts' prepared with ATP resulted in approx. 40% of the obelin being utilized during the first 2 min at 37°C, compared with approx. 70% in 'ghosts' without ATP.

It was therefore concluded that either the 'ghosts' with ATP were less permeable to Ca^{2+} or were able to maintain a lower intracellular Ca^{2+} in the presence of 1 mM-extracellular Ca^{2+} . Also the 'ghosts' without ATP were more fragile than 'ghosts' with ATP, since on resuspension in medium A the 'ghosts' without ATP released up to twice as much of their entrapped inulin as 'ghosts' prepared with ATP. Although extensive work has been carried out on the ATP-requiring Ca^{2+} pump of non-nucleated erythrocytes (Schatzman, 1975), very little evidence exists at present for a similar system in nucleated erythrocytes.

Effect of extracellular Ca^{2+} on cyclic AMP and obelin luminescence in sealed 'ghosts'

It has been reported that increases in intracellular Ca^{2+} inhibit adenylate cyclase both in intact pigeon (Campbell & Siddle, 1976) and turkey erythrocytes (Steer & Levitzki, 1975a,b). Studies were therefore carried out to compare the effect of extracellular Ca^{2+} (1 mM) on cyclic AMP formation in intact

Fig. 4. Effect of Ca^{2+} on cyclic AMP formation in intact pigeon erythrocytes and erythrocyte 'ghosts' (a) Intact pigeon erythrocytes (10.5×10^7 cells) or (b) sealed pigeon erythrocyte 'ghosts' (9.6×10^7 cells) were incubated for up to 30 min at 37°C in the presence (■, ●) or absence (□, ○) of 1 mM-Ca^{2+} and with (□, ■) or without (○, ●) $10 \mu\text{M}$ -adrenaline. The erythrocyte 'ghosts' were prepared as described in the Experimental section after initial haemolysis in $6 \text{ mM-NaCl}/3 \text{ mM-MgCl}_2/50 \text{ mM-Tes}$, pH 7.0 and resealing for 30 min at 37°C . Cyclic AMP and ATP were measured as described in the Experimental section. Results are means of two determinations. The erythrocyte 'ghosts' and intact cells were from the same original blood sample.

pigeon erythrocytes and sealed erythrocyte 'ghosts', and to try to relate any effect, in the 'ghosts', to intracellular free Ca^{2+} , assessed by obelin luminescence.

Pigeon erythrocytes and sealed erythrocyte 'ghosts' prepared from the same batch of cells were incubated for up to 30 min at 37°C in the presence and absence of 1 mM-Ca^{2+} and $10 \mu\text{M}$ -adrenaline (Figs. 4b and 4a). The formation of cyclic AMP in the 'ghosts' increased almost linearly for approx. 5 min at a rate that was 30–50% of that in intact cells. After 5 min the rate of increase in cyclic AMP content was markedly decreased (Fig. 4b). This was in contrast with the continuous formation of cyclic AMP in intact cells (Fig. 4a). The presence of extracellular Ca^{2+} (1 mM) resulted in a 25–50% inhibition in cyclic AMP formed in the 'ghosts', but was without effect on intact erythrocytes.

Sealed 'ghosts' incubated at 37°C in the absence of added extracellular Ca^{2+} exhibited a low rate of obelin luminescence (Fig. 5), approx. 3% of the obelin being utilized during the 2 min period shown. The addition of 1 mM-Ca^{2+} to the 'ghosts' resulted in an initial spike of obelin luminescence, due to consumption of extracellular obelin and obelin in highly permeable 'ghosts', followed by a slower rise and then fall in light emission (Fig. 5). After 2 min the light emission was still detectable, but was only slightly greater than the light emission from 'ghosts' incubated without Ca^{2+} . The addition of Triton X-100 after 2 min showed that, in the presence of 1 mM -extracellular Ca^{2+} , the 'ghosts' still retained approx. 60%

of their active obelin. Although the decrease in the rate of obelin luminescence could partly be accounted for by the consumption of a significant fraction of the obelin, it appeared that the fractional consumption of obelin also decreased. This implied that the mean intracellular Ca^{2+} concentration was decreasing, assuming a homogeneous 'ghost' population. The initial spike in obelin luminescence (Fig. 5) was equivalent to less than 3% of the obelin associated with the 'ghosts'. It was unlikely that this obelin represented protein attached to the outside of the cell membrane, since no detectable obelin was found associated with 'ghosts' first resealed and then incubated with obelin.

It was concluded that the addition of Ca^{2+} (1 mM) to sealed 'ghosts' resulted in a rapid increase, followed by a decrease, in the mean intracellular free Ca^{2+} . The raised intracellular Ca^{2+} concentration caused an inhibition of the adrenaline-stimulated cyclic AMP formation.

Effect of ionophore A23187

In an attempt to increase the concentration of intracellular Ca^{2+} in sealed 'ghosts' for longer periods of time than in the presence of extracellular Ca^{2+} alone, the 'ghosts' were also incubated with ionophore A23187 (Reed & Lardy, 1972). It has previously been shown in intact pigeon erythrocytes (Campbell & Siddle, 1976) and in pigeon erythrocyte 'ghosts' that the efficacy of the ionophore A23187 was dependent on cell concentration, incubation

Fig. 5. Obelin luminescence in pigeon erythrocyte 'ghosts'. Sealed pigeon erythrocyte 'ghosts' containing ATP and obelin were prepared as described in the Experimental section, after initial haemolysis in 6mM-NaCl/3mM-MgCl₂/50mM-Tes, pH 7.0, and resealing at 37°C for 30 min. Portions (50 μ l) of the final suspension containing 9.6×10^7 'ghosts' and 480 000 obelin luminescence counts were added to 0.5 ml medium A (see the Experimental section). The tube containing this was then placed in front of a photomultiplier tube and 0.5 ml medium A (a) with or (b) without 2mM-CaCl₂ added. The rate of obelin luminescence (c.p.s.) was recorded on a chart recorder or as total counts/10s on a scalar. After approx. 2 min 0.5 ml of 40mM-CaCl₂ + 20% (v/v) Triton X-100 was added, enabling the percentage of active obelin remaining to be estimated. The temperature was 37°C.

time and the concentration of Mg²⁺ and Ca²⁺ to which the ionophore was exposed both before and after addition of cells (Campbell & Dormer, 1975; Dormer *et al.*, 1978).

Initial experiments were carried out in the absence of Mg²⁺, since the ionophore A23187 was most effective at inhibiting cyclic AMP formation and stimulating obelin luminescence in the 'ghosts' under these conditions. However, the interpretation of the results of these experiments was made difficult, since in the absence of extracellular Mg²⁺, ionophore A23187 decreased the Mg²⁺ content of the 'ghosts' by 50–90% within a few minutes. Further experiments were therefore carried out in the presence of Mg²⁺ (2mM), when the ionophore A23187 had no de-

tectable effect on 'ghost' Mg²⁺ or K⁺ during the time studied.

Cyclic AMP in the 'ghosts' was measured after a 2 min incubation with adrenaline (10 μ M) at 37°C. The 'ghosts' were incubated with ionophore A23187 (5 μ M) either for 2 min with adrenaline or for a 10 min preincubation without adrenaline, the adrenaline then being added for a further 2 min. The extracellular medium contained EGTA (1 mM) or Ca²⁺ (10 μ M–1 mM). The increase in intracellular Ca²⁺ was assessed by measuring the percentage of obelin utilized during the incubation, and also by following the rate of obelin luminescence on a chart recorder.

In the absence of extracellular Ca²⁺ (1 mM-EGTA), there was a slow rate of obelin utilization inside the 'ghosts', less than 20% being consumed during the 12 min incubation at 37°C (Table 1). Somewhat surprisingly, under these conditions, ionophore A23187 caused a small transient stimulation in obelin luminescence, as well as an inhibition of cyclic AMP formation (Table 1). This apparent transient increase in free Ca²⁺ might explain the inhibition of cyclic AMP formation by ionophore A23187 observed in intact cells when incubated in medium containing EGTA (Campbell & Siddle, 1976).

Addition of Ca²⁺ to the incubation medium increased the rate of obelin utilisation (Fig. 6) as well as the inhibition of cyclic AMP formation (Table 1). Ionophore A23187 increased the free intracellular Ca²⁺ concentration, as demonstrated by an increase in obelin luminescence (Fig. 6), and increased the inhibition of cyclic AMP formation (Table 1). This inhibition of cyclic AMP formation was greatest when 'ghosts' were preincubated for 10 min with ionophore A23187 + Ca²⁺ before the addition of adrenaline for 2 min, compared with the condition where 'ghosts' were incubated for 2 min with ionophore A23187, Ca²⁺ and adrenaline without a preincubation period (Table 2).

The maximum rate of obelin consumption occurred during the first 2–3 min of incubation of 'ghosts' with Ca²⁺ (Fig. 6). This conclusion was based on an examination of the steepness of the luminescence traces (Fig. 6), and the manner in which this changed with time. This was interpreted as showing that the mean intracellular Ca²⁺ concentration in the 'ghosts' reached a maximum very soon after addition of extracellular Ca²⁺, and then decreased.

It was concluded that an increase in free Ca²⁺ inside pigeon erythrocyte 'ghosts' resulted in an inhibition in cyclic AMP formation within the 'ghosts'. The greater the increase in free Ca²⁺, the greater the inhibition of cyclic AMP formation. Under some conditions the adrenaline stimulation of cyclic AMP formation could be inhibited by more than 90%. However, there did not appear to be a simple, direct correlation between intracellular free Ca²⁺ and the percentage inhibition of cyclic AMP

Table 1. *Effect of ionophore A23187 on cyclic AMP formation and obelin luminescence in sealed 'ghosts' at different extracellular Ca^{2+} concentrations*

Sealed pigeon erythrocyte 'ghosts' were prepared as described in the Experimental section after initial haemolysis in 6 mM-NaCl/3 mM-MgCl₂/50 mM-Tes, pH 7.0, and a 30 min reseal at 37°C in the presence of ATP, obelin and 150 mM-KCl. Portions (50 μ l) of the final suspension containing 5.3×10^7 'ghosts' were incubated for 10 min in medium A + 1 mM-EGTA or 10 μ M-, 0.1 mM- or 1 mM- Ca^{2+} at 37°C. Adrenaline (10 μ M) was then added and after 2 min the cyclic AMP, ATP and active obelin in the 'ghosts' measured (see the Experimental section). The rate of obelin luminescence was also followed on a chart recorder. The obelin counts remaining after the 12 min incubation were expressed as a percentage of the total obelin luminescence counts recorded during the experiment. Results for cyclic AMP are means $\pm$ S.E.M. for three observations. Basal cyclic AMP formation (in the absence of adrenaline) was 0.11 ± 0.01 pmol of cyclic AMP/10⁶ 'ghosts'.

Medium	Cyclic AMP formation after 10 min then 2 min + adrenaline (10 μ M) (pmol of cyclic AMP/10 ⁶ 'ghosts')		Obelin remaining after 12 min incubation at 37°C (%)	
	Ethanol	A23187 (5 μ M)	Ethanol	A23187 (5 μ M)
A + 1 mM-EGTA	0.52 ± 0.02	0.39 ± 0.01	81.3	47.2
A + 10 μ M- Ca^{2+}	0.29 ± 0.08	0.12 ± 0.04	61.2	13.0
A + 0.1 mM- Ca^{2+}	0.24 ± 0.04	0.11 ± 0.01	41.8	1.2
A + 1 mM- Ca^{2+}	0.20 ± 0.06	0.06 ± 0.03	11.5	1.9

formation. Whereas the mean free Ca^{2+} concentration within the 'ghosts' decreased with time, the percentage inhibition of cyclic AMP formation increased. These results suggested that this inhibition may have been partly time-dependent.

Abolition of the effect of ionophore A23187 in 'ghosts' containing EGTA

To confirm that the effects on obelin luminescence and cyclic AMP formation in 'ghosts' that have been described here were due to an increase in intracellular free Ca^{2+} , attempts were made to abolish the effects of Ca^{2+} by incorporating EGTA, a strong chelator of Ca^{2+} , into the 'ghosts'.

Sealed 'ghosts' were prepared as previously described in the presence and absence of EGTA (10 mM). Although the presence of EGTA increased the recovery of obelin in the solution after 30 min at 37°C, it did not appear to have much effect on the obelin entrapped within the 'ghosts'. This suggested that even in the absence of EGTA the concentration of free Ca^{2+} in the 'ghosts' was less than 1 μ M. The rate of obelin luminescence in 'ghosts', prepared with EGTA and incubated with Ca^{2+} (1 mM), was considerably decreased compared with that in 'ghosts' prepared without EGTA and then incubated with Ca^{2+} , both in the presence and absence of ionophore A23187 (Fig. 7). After 2 min incubation with ionophore A23187 and Ca^{2+} (1 mM) approx. 30% of the obelin was consumed in 'ghosts' with EGTA inside, compared with more than 99% in 'ghosts' prepared in the absence of EGTA.

The incorporation of EGTA inside the 'ghosts' abolished the effect of ionophore A23187 in the presence of 0.1 mM- or 1 mM- Ca^{2+} on cyclic AMP

formation over the first 2 min period (Table 2). Also the inhibition by ionophore A23187 with 0.1 mM- Ca^{2+} , after a preincubation of 10 min without adrenaline, was decreased in 'ghosts' prepared with EGTA (Table 2).

These results show that sealing a Ca^{2+} chelator, EGTA, inside the 'ghosts' markedly decreased the ionophore A23187-induced increase in free Ca^{2+} and ionophore A23187-induced inhibition of adrenaline-stimulated cyclic AMP formation within the 'ghosts'. The time taken for ionophore A23187 to transport into the 'ghosts' sufficient Ca^{2+} to saturate the intracellular EGTA appeared to be 2–5 min. It was concluded that the inhibition of cyclic AMP formation caused by addition of extracellular Ca^{2+} or ionophore A23187 could be explained by the increase in intracellular free Ca^{2+} which occurred under these conditions.

To rule out the possibility that the effect of Ca^{2+} in the presence or absence of ionophore A23187 might be explained by a decrease in the substrate for adenylate cyclase, MgATP, the ATP content of the 'ghosts' was measured. Under all conditions there was a decrease in ATP content with increasing time of incubation (Table 3). After a 2 min incubation with 0.1 mM- or 1 mM- Ca^{2+} and ionophore A23187 the ATP content of 'ghosts' prepared without EGTA was 10–20% less than that in the same 'ghosts' incubated with ethanol. After 12 min at 37°C, the ATP content of 'ghosts' prepared without EGTA, and then incubated with ionophore A23187 and Ca^{2+} , was 30–40% less than the control 'ghosts' incubated with ethanol (Table 3). However, after this 12 min incubation, the ATP contents of 'ghosts' prepared with or without EGTA were within 10% of each other. Yet the adrenaline-stimulated cyclic

Fig. 6. Effect of ionophore A23187 on obelin luminescence in 'ghosts'

Sealed erythrocyte 'ghosts' from the same experiment as that described in Table 1 were used. Portions (50 μ l) of the final suspension containing 5.4×10^7 'ghosts' were incubated for 20 s in 0.5 ml of medium A containing 2.6 μ l of ethanol (control; a) or 2.6 μ l of 5 μ M-ionophore A23187 (b). An equal volume of medium A + 0.2 mM- CaCl_2 was added ($\uparrow$) and the rate of obelin luminescence expressed as c.p.s. plotted on a chart recorder. After 10 min, adrenaline was added ($\uparrow$) at a final concentration of 10 μ M. To measure the active obelin remaining at the end of the experiment 0.5 ml of 20% (v/v) Triton X-100 + 40 mM- CaCl_2 was added ($\uparrow$) and the total counts were recorded on a scalar. Total active obelin present at the start of the experiment was 120 000 luminescence counts. The temperature was 37°C. The time constant of the rate meter was 10 ms.

interpretation of the results, they were not able to explain the Ca^{2+} -dependent inhibition of adrenaline-stimulated cyclic AMP formation.

Determination of free Ca^{2+} concentrations in sealed 'ghosts'

Ca^{2+} -activated photoproteins such as obelin and aequorin can only be used to determine free Ca^{2+} concentrations accurately under conditions where either the fraction of photoprotein utilized during the experiment is a very small portion of the total, for example in giant muscle fibres (Ashley & Ridgway, 1970; Ashley *et al.*, 1976) or the squid giant axon (Baker, 1972), or, if a significant amount of the photoprotein is utilized, the free Ca^{2+} should remain constant. It is important to remember that the rate of luminescence, although being related to the free Ca^{2+} concentration by a cube law relationship (Moiescu *et al.*, 1975; Moiescu & Ashley, 1977) is related to the absolute quantity of photoprotein present and not to its molar concentration. This is because, unlike normal measurements of enzyme activity where one measures an increase in concentration as the product accumulates, in luminescence reactions the product (photons) is measured as the total light intensity ($dh\nu/dt$) from the tube being studied. The rate of obelin luminescence ($dh\nu/dt$) is related to the concentration of free Ca^{2+} by the following equation (Moiescu *et al.*, 1975):

$$dh\nu/dt = Q \cdot O \cdot k_{\text{app}} \cdot e^{-k_{\text{app}} \cdot T}$$

where k_{app} , the apparent rate constant is:

$$\frac{k_{\text{sat}} \cdot K_1 \cdot K_2 \cdot K_3 [\text{Ca}^{2+}]^3}{1 + K_1 [\text{Ca}^{2+}] + K_1 K_2 [\text{Ca}^{2+}]^2 + K_1 K_2 K_3 [\text{Ca}^{2+}]^3}$$

K_1 , K_2 , K_3 are the dissociation constants for the three proposed Ca^{2+} -binding sites, $[\text{Ca}^{2+}]$ is the concentration of free Ca^{2+} and k_{sat} is the rate constant when the photoprotein is saturated with Ca^{2+} ($k_{\text{sat}} = 4 \text{ s}^{-1}$ at 20°C for obelin), O is the total quantity of obelin present before the addition of Ca^{2+} and Q is the quantum yield which is the number of photons emitted/number of molecules reacting.

In the experiments described in this paper, it appeared that a significant consumption of obelin occurred as well as changes in free Ca^{2+} concentration. It was therefore not possible to make precise measurements of free Ca^{2+} inside the 'ghosts'. However, it was possible to make an estimate of the free Ca^{2+} on the basis of the percentage utilization of obelin in a defined time interval. Obelin luminescence is inhibited by univalent cations and Mg^{2+} (Campbell, 1974; Moiescu *et al.*, 1975; Ashley *et al.*, 1976), since these cations lower the apparent affinity of obelin for Ca^{2+} . It was therefore necessary to carry out kinetic studies on obelin in a medium of approximately the

AMP content of 'ghosts' with EGTA inside, and incubated with ionophore A23187 and 0.1 mM- Ca^{2+} , was five times that of 'ghosts' prepared without EGTA and incubated under the same conditions. It was concluded that, although these decreases in ATP content caused some complication in the

Table 2. Cyclic AMP formation in 'ghosts' containing EGTA

Pigeon erythrocytes were haemolysed in 6mM-NaCl/3mM-MgCl₂/50mM-Tes, pH 7.0, and sealed 'ghosts' containing ATP prepared as described in the Experimental section. The resealing medium contained either 10mM-EGTA (+ EGTA) or an equivalent concentration of Na⁺ (- EGTA). Portions (50 μ l) of sealed 'ghosts' (8.4×10^7 'ghosts') were incubated (i) for 2 min with adrenaline (10 μ M) and either 2.6 μ l of ethanol or 2.6 μ l of A23187 (1 mg/ml; final concn. 5 μ M) (ii) for 10 min with ethanol or ionophore A23187 followed by a further 2 min with adrenaline (10 μ M) at 37°C. The extracellular Ca²⁺ concentration was 0.1 or 1 mM. Basal cyclic AMP formation in the absence of adrenaline was 0.016 ± 0.008 pmol of cyclic AMP/10⁶ 'ghosts' and 0.026 ± 0.007 pmol of cyclic AMP/10⁶ 'ghosts' for 'ghosts' prepared in the presence or absence of EGTA respectively. Results are means $\pm$ S.E.M. for three determinations.

'Ghosts' medium	Time without adrenaline (min)	Time with adrenaline (min)	Cyclic AMP formation (pmol of cyclic AMP/10 ⁶ 'ghosts')			
			+ EGTA		- EGTA	
			Ethanol	A23187 (5 μ M)	Ethanol	A23187 (5 μ M)
A + 0.1 mM-Ca ²⁺	(i) 0	2	0.67 $\pm$ 0.03	0.64 $\pm$ 0.01	0.56 $\pm$ 0.01	0.32 $\pm$ 0.05
	(ii) 10	2	0.58 $\pm$ 0.01	0.080 $\pm$ 0.010	0.42 $\pm$ 0.01	0.018 $\pm$ 0.010
A + 1 mM-Ca ²⁺	(i) 0	2	0.65 $\pm$ 0.02	0.65 $\pm$ 0.02	0.51 $\pm$ 0.03	0.24 $\pm$ 0.02
	(ii) 10	2	0.60 $\pm$ 0.02	< 0.001	0.44 $\pm$ 0.02	< 0.001

Fig. 7. Effect of ionophore A23187 on obelin utilization in 'ghosts' prepared with and without EGTA

Pigeon erythrocytes were haemolysed in 6mM-NaCl/3mM-MgCl₂/50mM-Tes, pH 7.0. Sealed 'ghosts' were prepared, as described in the Experimental section containing ATP and obelin. The 'ghosts' were resealed for 30 min at 37°C in the presence (○, ●) or absence (□, ■) of 10mM-EGTA. Portions (50 μ l) of the final suspension containing 6.2×10^7 'ghosts' were added to 0.5 ml of medium A containing 5 μ l of ethanol (○, □) or 5 μ l of A23187 (1 mg/ml) (●, ■) (final concn. 9.5 μ M) and then placed in front of a photomultiplier tube. Then 0.5 ml of medium A + 2mM-CaCl₂ was added and the rate of obelin luminescence followed on a chart recorder or by measuring the counts recorded/10s on a scalar. After 100s 0.5 ml of 40mM-CaCl₂ + 20% (v/v) Triton X-100 was added in order to estimate the remaining active obelin. The total obelin utilized after a given time was expressed as a percentage of the initial entrapped obelin. Results are means $\pm$ S.E.M. of three observations. The temperature was 37°C.

same ionic composition as found inside the 'ghosts' (10mM-Tes/150mM-KCl/50mM-NaCl/2mM-MgSO₄, pH 7.4). Although obelin was stable for long periods in a solution of EGTA at room temperature, at 37°C the photoprotein was rapidly inactivated. The obelin was stabilized by addition of protein carrier (albumin or gelatin at a concentration of 0.1 mg/ml). The studies showed that after 2 min incubation with 10 μ M-Ca²⁺, under these conditions, at 37°C approx. 70% of the obelin was utilized compared with approx. 20 and 95% in 1 μ M- and 25 μ M-Ca²⁺ respectively. Comparison of the percentage utilization within the 'ghosts' (Figs. 5, 6 and 7, Table 1) suggested that cyclic AMP formation was inhibited by more than 50% by concentrations of Ca²⁺ in the range 1–10 μ M.

Conclusions

The studies reported here have shown that it is possible to prepare hormone-sensitive sealed pigeon erythrocyte 'ghosts' that can maintain a K⁺ gradient across the cell membrane. The incorporation of obelin into these 'ghosts' provides for the first time a system in which changes in free Ca²⁺, measured directly by obelin luminescence, can be correlated with effects on cyclic AMP formation. There appear to be some differences between the 'ghost' preparation reported here and that described for turkey erythrocyte 'ghosts' (Steer *et al.*, 1976). These may be accounted for by differences in the conditions at haemolysis or resealing of the 'ghosts'.

It has been reported that the sensitivity to β -adrenergic agonists of turkey erythrocyte cyclase in broken membranes may be 5–10-fold less than in intact cells (Gardner *et al.*, 1976). The present studies emphasize the importance of studying hormone

Table 3. *Effect of ionophore A23187 on 'ghost' ATP content*

Sealed pigeon erythrocyte 'ghosts' were prepared containing ATP and obelin. The resealing medium contained either 10 mM-EGTA (+ EGTA) or an equivalent concentration of Na⁺ (– EGTA), the 'ghosts' being the same as those described in Table 2. Portions (50 µl) of sealed 'ghosts' (8.4×10^7 'ghosts') were incubated (i) for 2 min with adrenaline (10 µM) and either 2.6 µl of ethanol or 2.6 µl of ionophore A23187 (1 mg/ml; final concentration 5 µM), (ii) for 10 min with ethanol or ionophore A23187 followed by a further 2 min with adrenaline (10 µM) at 37°C. The extracellular Ca²⁺ concentration was 0.1 or 1 mM. Cyclic AMP and ATP were measured as described in the Experimental section. Results are means $\pm$ S.E.M. for three determinations.

'Ghosts' medium	Time without adrenaline (min)	Time with adrenaline (min)	ATP content (pmol of ATP/10 ⁶ 'ghosts')			
			+ EGTA		– EGTA	
			Ethanol	A23187 (5 µM)	Ethanol	A23187 (5 µM)
A + 0.1 mM-Ca ²⁺	(i) 0	2	133 $\pm$ 7	139 $\pm$ 5	106 $\pm$ 17	85 $\pm$ 3
	(ii) 10	2	69 $\pm$ 6	43 $\pm$ 2	62 $\pm$ 5	43 $\pm$ 4
A + 1 mM-Ca ²⁺	(i) 0	2	117 $\pm$ 6	100 $\pm$ 12	96 $\pm$ 8	86 $\pm$ 11
	(ii) 10	2	77 $\pm$ 1	61 $\pm$ 7	69 $\pm$ 2	53 $\pm$ 7

adenylate cyclase coupling in a sealed membrane preparation in which both uni- and bi-valent cation gradients can be maintained. In sealed pigeon erythrocyte 'ghosts' the sensitivity of adenylate cyclase to β -adrenergic agonists was in the same concentration range as that for intact cells and this enzyme appeared to be inhibited by more than 50% by Ca²⁺ concentrations in the range 1–10 µM. This inhibition is most likely to be related to direct effects on membrane proteins, since the 'ghosts' contained less than 2% of the protein of intact cells. The effect of Ca²⁺ could not be explained by an activation of cyclic AMP phosphodiesterase, since it has previously been shown (Campbell & Siddle, 1976) that an increase in free Ca²⁺ in intact erythrocytes did not enhance cyclic AMP degradation.

It has been proposed that β -adrenergic agonists might act to decrease free Ca²⁺ in turkey erythrocytes and thus activate adenylate cyclase (Steer *et al.*, 1975). Under a variety of conditions where adrenaline can be shown to increase the activity of adenylate cyclase no detectable effect on obelin luminescence in the 'ghosts' could be demonstrated. However, in certain 'ghost' preparations, namely those prepared by haemolysis in media of low osmolarity, adrenaline did affect the luminescence of obelin in the 'ghosts'. The significance of this effect is not yet understood, since both D- and L-adrenaline were equally effective and the recovery of total obelin in these experiments was decreased by up to 80%.

The possible time-dependency of the inhibition by Ca²⁺ of adenylate cyclase (Tables 1 and 2) suggests that further studies on the time course of inhibition by Ca²⁺ of this enzyme as well as the rapidity of reversal of this inhibition might be a fruitful line of investigation.

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References

- Ashley, C. C. & Ridgway, E. B. (1970) *J. Physiol. (London)* **209**, 105–130
- Ashley, C. C., Caldwell, P. C., Campbell, A. K., Lea, T. J. & Moisesu, D. G. (1976) *Symp. Soc. Exp. Biol.* **30**, 397–422
- Baker, P. F. (1972) *Prog. Biophys. Mol. Biol.* **24**, 177–223
- Baker, P. F., Hodgkin, A. L. & Ridgway, E. B. (1971) *J. Physiol. (London)* **218**, 709–755
- Berridge, M. J. (1976) *Adv. Cyclic Nucleotide Res.* **6**, 1–96
- Bramley, T. A. & Coleman, R. (1972) *Biochim. Biophys. Acta* **290**, 219–228
- Campbell, A. K. (1974) *Biochem. J.* **143**, 411–418
- Campbell, A. K. & Dormer, R. L. (1975) *Biochem. J.* **152**, 255–265
- Campbell, A. K. & Dormer, R. L. (1977) *Biochem. Soc. Trans.* **5**, 962–965
- Campbell, A. K. & Siddle, K. (1976) *Biochem. J.* **158**, 211–221
- Campbell, A. K. & Siddle, K. (1977) *Biochim. Biophys. Acta* **497**, 62–74
- Davie, J. V. & Lewis, S. M. (1970) *Practical Haematology*, pp. 37–39, J. and A. Churchill, London

- Dodge, J. T., Mitchell, C. & Hanahan, D. J. (1963) *Arch. Biochem. Biophys.* **100**, 119–130
- Dormer, R. L., Newman, G. R. & Campbell, A. K. (1978) *Biochim. Biophys. Acta* **538**, 87–105
- Gardner, J. D., Aurbach, G. D., Spiegel, A. M. & Brown, E. M. (1976) *Recent Prog. Horm. Res.* **32**, 567–595
- Hales, C. N., Campbell, A. K., Luzio, J. P. & Siddle, K. (1977) *Biochem. Soc. Trans.* **5**, 866–872
- Moiescu, D. G. & Ashley, C. C. (1977) *Biochim. Biophys. Acta* **460**, 189–205
- Moiescu, D. G., Ashley, C. C. & Campbell, A. K. (1975) *Biochim. Biophys. Acta* **396**, 133–140
- Nakamura, T. (1972) *J. Biochem. (Tokyo)* **72**, 173–177
- Rasmussen, H. & Goodman, D. B. P. (1977) *Physiol. Rev.* **57**, 421–509
- Reed, P. N. & Lardy, H. A. (1972) *J. Biol. Chem.* **247**, 6970–6977
- Schatzman, H. J. (1975) *Curr. Top. Membr. Trans.* **6**, 125–188
- Schwoch, G. & Passow, H. (1973) *Mol. Cell. Biochem.* **2**, 197–218
- Siddle, K., Kane-Maguire, B. & Campbell, A. K. (1973) *Biochem. J.* **132**, 765–773
- Steer, M. L. & Levitzki, A. (1975a) *Arch. Biochem. Biophys.* **167**, 371–376
- Steer, M. L. & Levitzki, A. (1975b) *J. Biol. Chem.* **250**, 2080–2084
- Steer, M. L., Atlas, D. & Levitzki, A. (1975) *N. Engl. J. Med.* **292**, 409–414
- Steer, M. L., Baldwin, C. & Levitzki, A. (1976) *J. Biol. Chem.* **251**, 4930–4935
- Wessels, J. M. C. & Verkamp, J. H. (1973) *Biochim. Biophys. Acta* **291**, 178–189
- Whittam, R. (1962) *Biochem J.* **84**, 110–118