RELEASE OF THE SURFACE COAT FROM THE PLASMA MEMBRANE OF INTACT BLOODSTREAM FORMS OF *TRYPANOSOMA BRUCEI* REQUIRES Ca^{2+}

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1. Introduction

The continuous synthesis and degradation of cellular proteins, results in their turnover [1]. Those proteins attached to or embedded in membranes must be assembled into and dissembled from their functional positions to effect their turnover. The variable surface coat glycoprotein of the salivarian trypanosomes is such a membrane protein [2–4] and performs a role in the physiology of these cells [5,6].

There appear to be no studies indicating the mechanism by which a variable surface coat glycoprotein molecule might be removed from its attachment site on the plasma membrane. Here, we show that Ca^{2+} and a calcium ionophore together initiate the release of some 80% of the variant surface coat glycoprotein from the plasma membrane within 10 min, without simultaneous rupture of the cell.

2. Materials and methods

2.1. *Source and preparation of cells*

The source of bloodstream forms of *Trypanosoma brucei* (427-12/ICI-060) as well as their storage, growth and isolation has been reported [7].

2.2. *Materials*

Calcium ionophore A-23187 was the kind gift of Lilly Research Centre Ltd. (Windlesham, Surrey GU20 6PH). Initial samples of [^{3}H]isethionylacetimidate were the kind gift of Dr G. A. Smith (Department of Biochemistry, University of Cambridge). All other chemicals were from standard commercial suppliers.

2.3. *Radiolabelling of the surface coat protein*

The surface coat protein was radiolabelled in situ using the non-penetrating reagent, [^{3}H]isethionylacetimidate, synthesised as in [8]. Bloodstream forms of *T. brucei* were suspended (10^9 cells) in 4 ml Krebs–Ringer–phosphate buffer [8], supplemented with 10 mM glucose and 30 mM bicine pre-adjusted to pH 9–9.5. The suspension was added to 8 mg [^{3}H]-isethionylacetimidate (259 μCi/mmol), containing 1 equiv. NaCl/mol imidate, in a 250 ml conical flask and gently agitated. After 30 min at 22°C the suspension was chilled by adding 40 ml ice-cold Krebs–Ringer–phosphate buffer (pH 8.0) and mixing. Cells were centrifuged for 10 s at 9000 × *g* in a microfuge, the supernatant discarded, and the pellet of cells washed twice by resuspending in buffer and recentrifuging. Damaged cells were removed by passage through a DEAE-cellulose column [9]. The undamaged cells in the eluate were then recentrifuged and resuspended in incubation buffer (0°C) containing TES (20 mM), NaCl (140 mM), EGTA (0.1 mM) and glucose (10 mM) adjusted to pH 7.5 to yield a stock suspension of labelled cells (10^9/ml).

2.4. *Cell incubations*

Samples (20 μl) of stock suspension (10^9 cells/ml) of radiolabelled cells were added to 980 μl portions of Tes buffer at 37°C either without further addition or containing final concentrations of Ca^{2+} (1 mM),

Abbreviations: TES, *N*-tris[hydroxymethyl]methyl-2-aminoethane sulphonic acid; EGTA, ethyleneglycol-bis-(β-aminoethylether)*N,N'*-tetraacetic acid

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A-23187 (5 μg/ml) and EGTA (1 mM). Alternatively, samples were added to 980 μl distilled deionised water at 37°C. Incubations were for 10 min followed by centrifugation at 9000 × *g* for 30 s in a minifuge and the supernatants removed.

2.5. *Analysis of the radiolabelled surface coat*

Supernatants from incubations were analysed for the release of the [^{3}H]acetimido-labelled coat protein by liquid scintillation counting [7].

Samples (20 μl) of labelled stock cell suspensions (10^9 cells/ml) were analysed for their content of labelled proteins by treating with an equal volume of 10% (w/v) trichloroacetic acid, mixing, centrifuging at 9000 × *g* for 30 s, then subjecting the pellets of denatured cells to SDS–polyacrylamide gel electrophoresis (9.2% acrylamide, 0.12% bisacrylamide) as in [10] followed by fluorography [11,12].

2.6. *Enzyme assays*

Samples (20 μl) of stock cell suspensions were incubated as in section 2.4 except for 15 min and at 2 mM Ca^{2+}. Supernatants from these incubations were analysed for glucose-6-phosphate dehydrogenase and malic enzyme by measuring the fluorescence of NADPH produced (345 nm, excitation; 460 nm, emission) under standard assay conditions [13,14]. The enzyme activity of whole untreated cells permeabilised with 1% Triton X-100 was measured to determine the total amount of each enzyme present. Malic enzyme was found to be unstable in the presence of water alone but stable in the presence of buffer alone and buffer + A-23187 + Ca^{2+}. Therefore, malic enzyme was analysed immediately after centrifugation, when cells were treated with water; the Triton-permeabilised control was identically treated with water.

2.7. *Oxygen uptake*

This was measured in a Rank oxygen electrode at 37°C.

3. Results

3.1. *Cell proteins labelled with [^{3}H]isethionylacetimidate*

Analysis of the [^{3}H]acetimido-surface-labelled proteins in *T. brucei* revealed that >79% of the radioactivity incorporated into whole cells was found in the variant surface coat glycoprotein (fig.1), similar

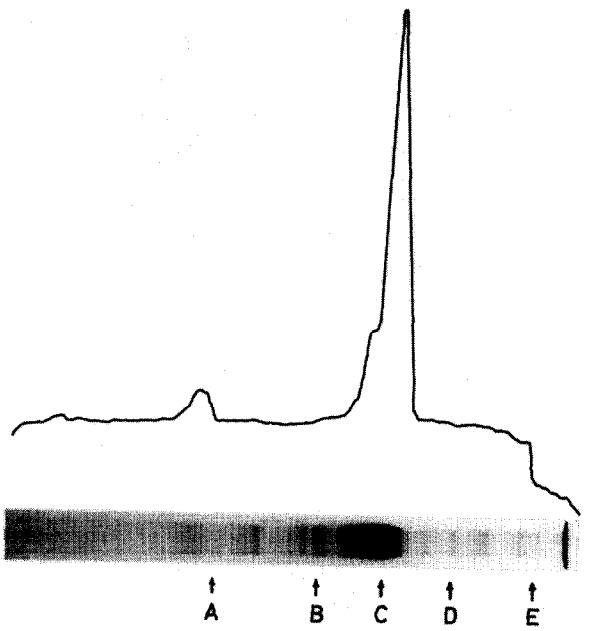

Fig.1. Surface labelling of intact bloodstream forms of *T. brucei*. Bloodstream forms of *T. brucei* were surface-labelled and subjected to SDS–polyacrylamide gel electrophoresis as in section 2. The solid line is a densitometer tracing of the fluorogram, showing the intensity of radioactivity in the gel. A photograph of the Coomassie blue-stained gel is shown below the fluorogram tracing. The arrows show the position of M_r-markers: (A) β-galactosidase, 130 000; (B) bovine serum albumin, 68 000; (D) ovalbumin, 45 000; (E) α-chymotrypsinogen A, 25 700. The arrow at (C) shows the position of the surface coat protein; calc. M_r = 58 000.

to [4,15]. When total cellular radioactivity had appeared in the supernatant medium, the amount of radioactivity released from cells could be taken as a measure of the amount of coat protein released.

3.2. *Effect of Ca^{2+} and A-23187 on surface coat release*

Table 1 shows the amount of surface coat released from bloodstream forms of *T. brucei* in 3 separate expt after 10 min incubation at 37°C. Ca^{2+} at 1 mM and A-23187 at 5 μg/ml stimulated the amount of surface-labelled coat released from cells by 2.4–3-fold over that released by incubation in buffer alone, Ca^{2+} alone or A-23187 alone. EGTA at 1 mM decreased the amount of label released by 50% even when the same amounts of Ca^{2+} and A-23187 were retained in the incubation. With no incubation, ~11% of the label was still present in the suspending medium. If the

Table 1
Effect of Ca^{2+} and A-23187 on surface coat release in *T. brucei*

Experimental condition	Labelled coat released (%)
Buffer alone	27 ± 10
Buffer + Ca^{2+}	31 ± 18
Buffer + A-23187	34 ± 12
Buffer + A-23187 + Ca^{2+}	80 ± 11
Buffer + A-23187 + Ca^{2+} + EGTA	40 ± 15
H_2O alone	100 ± 3
No incubation	11 ± 4

Surface-labelled cells were incubated and processed as in section 2 with additions to the incubations as indicated. Each value shown represents the mean ± SEM of the % of the total cellular radioactivity released in 3 separate expt. The total amount of radioactivity in a 20 μl portion of each stock cell suspension was: 24 555 cpm in expt 1; 9254 cpm in expt 2; and 43 555 cpm in expt 3. The difference in absolute number of counts in stock cells reflects the exact pH of the labelling reaction which is highly dependent on $[H^+]$ between pH 9.0–9.5

results in table 1 are corrected for this zero-time value the degree of stimulation of coat release would become even greater. The levels of Ca^{2+} and A-23187 used were high so that complete release of the surface coat could be accomplished within 10–15 min. Lower levels of both agents stimulated release but incubation times required for completion were increased (unpublished).

3.3. *Effect of osmotic rupture on surface coat release*

We confirm the observation [4,16] that the surface coat of *T. brucei* may be released completely by rupturing the plasma membrane (table 1) and further

Table 2
Effect of temperature and Ca^{2+} on the release of the surface coat of *T. brucei* during osmotic lysis

Experimental condition	Labelled coat released (%)	
	0°C	37°C
Buffer	9	31
H_2O	36	108
H_2O + Ca^{2+}	35	95

Samples (20 μl) of stock suspension of labelled cells (10^9/ml) in Tes buffer were added with mixing to 980 μl buffer alone or to 980 μl distilled deionised water at either 37°C or 0°C and with or without 1 mM Ca^{2+} added. Incubations and analyses were performed as in section 2. The total amount of radioactivity in a 20 μl suspension was 56 332 cpm. Each value shown represents the % of total cellular radioactivity released

show that release of the surface coat by osmotic lysis of cells was complete under these conditions only at 37°C (table 2). Release under these conditions did not require added Ca^{2+}, although sufficient free Ca^{2+} may have been present in cells and the media to trigger release upon rupture of the plasma membrane.

3.4. *Effect of Ca^{2+} + A-23187 and osmotic lysis on the release of cytoplasmic enzymes*

If Ca^{2+} + ionophore simply ruptured cells to produce the release of the surface coat protein, then the release of cytoplasmic enzymes should be similar to that found during osmotic lysis of cells. We have examined the release of alanine aminotransferase, aspartate aminotransferase, glucose-6-phosphate

Table 3
Effect of Ca^{2+} + A-23187 and of osmotic lysis on the release of cytoplasmic enzymes from *T. brucei*

Enzyme measured	Amount of enzyme released (% of total)		
	Buffer alone	Buffer + A-23187 + Ca^{2+}	H_2O alone
Glucose-6-phosphate dehydrogenase	2.9 ± 2.4	11.0 ± 5.1	85.9 ± 0.3
Malic enzyme	9.9 ± 6.0	34.0 ± 5.1	91.0 ± 4.9

Cells were incubated and after centrifugaion the supernatants assayed for their enzyme content as in section 2. Incubation conditions were as indicated above and in section 2. Each value shown represents the mean ± SEM of the % of the total cellular enzyme that was released in 5 separate expt for the case of glucose-6-phosphate dehydrogenase and 4 separate expt for malic enzyme

dehydrogenase, pyruvate kinase and malic enzyme. None of these enzymes was released to the extent of coat protein in the presence of Ca^{2+} and A-23187 but a considerable difference was found to exist between their relative extents of release. Table 3 shows the amount of release found for 2 of these enzymes, glucose-6-phosphate dehydrogenase, which was released to the smallest extent (11%) of any tested and malic enzyme which was released to the greatest extent (34%) of any tested. Under these conditions 92% of the surface coat protein was released. In contrast to these findings, water alone released 86% of the cellular glucose-6-phosphate dehydrogenase and 91% of the total malic enzyme. Clearly, cell rupture cannot account for the Ca^{2+} + A-23187-stimulated release of the surface coat protein.

3.5. *Microscopic appearance of cells*

With the exception of a few dead cells (5–10% produced almost instantly) there was no change in either motility or shape, when treated with Ca^{2+} and A-23187, until ~5 min after release of the surface coat had been completed. At 15–30 min after adding Ca^{2+} and A-23187 the cells began to swell gradually and assume a rounded appearance that was completed by 45 min. During the change in shape, individual cells retained their motility until the final rounded forms had been produced. Even at this final stage many cells remained motile but lost the ability to move directionally; slower periodic waves then passed over the surface of the cell.

3.6. *Effect of Ca^{2+} and A-23187 on aerobic respiration in the presence and absence of added Mg^{2+}*

Studies of the O_2 consumption of control and treated cells revealed that the addition of 2 mM Ca^{2+} and 5 μg A-23187/ml did not alter the rate of aerobic glucose-supported respiration as long as 5 mM Mg^{2+} was present (fig.2). In the absence of added Mg^{2+}, respiration was severely inhibited, suggesting that A-23187 allows Mg^{2+} to leave cells as well as allowing Ca^{2+} to enter. All of our experiments were conducted in the presence of 5 mM Mg^{2+} where respiration was unaffected.

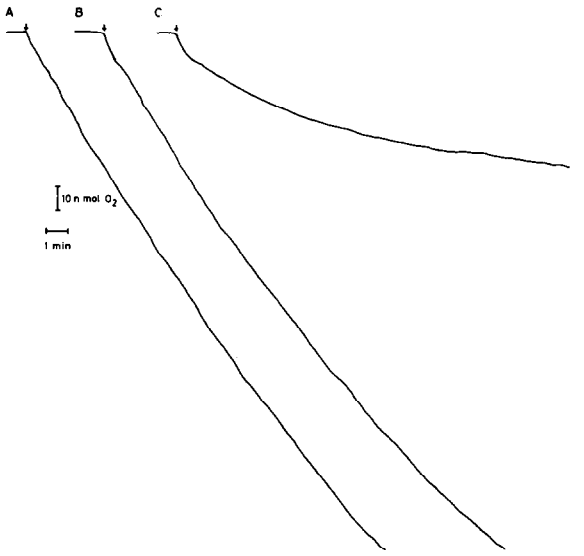

Fig.2. Effect of Ca^{2+} + A-23187 in the presence and absence of added Mg^{2+} on the glucose-supported aerobic respiration of bloodstream forms of *T. brucei*. Measurements of the O_2 consumption of cells were made as in section 2 in TES buffer + glucose (10 mM) with the following additions: (A) none; (B) Ca^{2+} (2 mM) + A-23187 (5 μg/ml); (C) as in (B) but without $MgCl_2$. Each experiment was begun by adding cells (arrows).

4. Discussion

We have provided evidence that the variant surface coat glycoprotein of *T. brucei* is released to the suspending medium when stimulated by Ca^{2+} and A-23187 under conditions where cell rupture cannot account for the release observed and the respiration of these cells is unaffected. Since both treating cells with Ca^{2+} + ionophore under non-lysing conditions and subjecting cells to osmotic lysis results in a temperature-dependent release of the surface coat, we conclude that either of these manipulations is capable of stimulating a previously unidentified coat-releasing mechanism in these cells. Local anaesthetics such as benzyl alcohol also stimulate coat release and Zn^{2+} is a powerful inhibitor of coat release even when release is obtained by osmotic lysis (in preparation).

The conditions required for coat release are also those required for the Ca^{2+}-dependent activation of adenylate cyclase in *T. brucei* [9,17,18]. Both coat release and cyclase activation may occur as a result of stimulation by the same membrane-located mechanism. A search for their physiological role must consider both transformation of bloodstream forms to uncoated insect midgut forms and the transformation of bloodstream forms from one antigenic type to another.

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