Anti-VSG antibodies induce an increase in *Trypanosoma evansi* intracellular Ca^{2+} concentration

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SUMMARY

Trypanosoma evansi and *Trypanosoma vivax* have shown a very high immunological cross-reactivity. Anti-*T. vivax* antibodies were used to monitor changes in the *T. evansi* intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by fluorometric ratio imaging from single parasites. A short-time exposure of *T. evansi* parasites to sera from *T. vivax*-infected bovines induced an increase in $[Ca^{2+}]_i$, which generated their complete lysis. The parasite $[Ca^{2+}]_i$ boost was reduced but not eliminated in the absence of extracellular Ca^{2+} or following serum decomplementation. Decomplemented anti-*T. evansi* VSG antibodies also produced an increase in the parasite $[Ca^{2+}]_i$, in the presence of extracellular Ca^{2+}. Furthermore, this Ca^{2+} signal was reduced following blockage with Ni^{2+} or in the absence of extracellular Ca^{2+}, suggesting that this response was a combination of an influx of Ca^{2+} throughout membrane channels and a release of this ion from intracellular stores. The observed Ca^{2+} signal was specific since (i) it was completely eliminated following pre-incubation of the anti-VSG antibodies with the purified soluble VSG, and (ii) affinity-purified anti-VSG antibodies also generated an increase in $[Ca^{2+}]_i$ by measurements on single cells or parasite populations. We also showed that an increase of the *T. evansi* $[Ca^{2+}]_i$ by the calcium A-23187 ionophore led to VSG release from the parasite surface. In addition, *in vivo* immunofluorescence labelling revealed that anti-VSG antibodies induced the formation of raft patches of VSG on the parasite surface. This is the first study to identify a ligand that is coupled to calcium flux in salivarian trypanosomes.

Key words: antibodies, calcium, complement, *Trypanosoma evansi*, *Trypanosoma vivax*, variant surface glycoproteins.

INTRODUCTION

Salivarian trypanosomes are capable of adapting and developing mechanisms to avoid immune destruction (Jokiranta *et al*. 1995). These trypanosomes evade the host immune response by sequentially changing its variant surface glycoprotein (VSG), a strategy-denominated antigenic variation (Zambrano-Villa *et al*. 2002). Each trypanosome carries a large repertoire of VSG genes coding for approximately 1000 VSG variants (Donelson, 2003), but at any one time expresses only a single VSG gene. These parasites keep one step ahead of the host immune system by periodically switching from the expression of one VSG on their surface to the expression of another immunologically distinct VSG. This repeated antigenic change on the trypanosomes' outer coat allows them to evade the host humoral response, resulting in successive surges of parasitaemia, a condition similar to being infected successively by related, but not identical, pathogens.

Due to their intravascular and extracellular location, the prevailing immune reaction of the vertebrate host towards infection by these trypanosomes is mediated by the production of specific antibodies, the activation of the classical complement system, and by phagocytic responses (Taylor, 1998). The complement system consists of a set of serum proteins in charge of many of the cytolytic and inflammatory effects of humoral immunity, and as such acts as a first-line defence mechanism against trypanosomes. The activation of the terminal components of the complement system culminates in the formation of pores in the parasite plasma membrane by the

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membrane attack complex (Benz *et al*. 1986), which allows Ca^{2+} to enter and initiate changes in its cytosolic concentration (Campbell and Luzio, 1981; Campbell *et al*. 1981; Morgan and Campbell, 1985; Laffafian *et al*. 1995; Newsholme *et al*. 1993). This abrupt increase of Ca^{2+} concentration is extremely toxic for the parasite. Additionally, this phenomenon produces an influx of water into the parasites, which leads to death via osmotic lysis.

Increasing antibody titres eventually lead to complement lysis; however, the remaining parasites survive and establish the infection thanks to their antigenic variation strategy. To date, no evidence has been reported to demonstrate that the host immune system directly induces switching of the trypanosome outer layer of VSG, but it is known that it contributes to an environment in which new and temporarily unrecognized serotypes can prosper, giving rise to new waves of parasitaemia (Donelson, 1995). Additionally, antigenic variation in trypanosomatids has been related to Ca^{2+} signalling processes, since the release of the surface coat protein is dependent on the presence of extracellular Ca^{2+} (Voorheis and Martin, 1981; Voorheis *et al*. 1982; Bowles and Voorheis, 1982).

Trypanosoma evansi and *Trypanosoma vivax* are the major aetiological agents of animal trypanosomosis in Venezuela, and generate a significant economic impact in livestock. *T. vivax* affects predominantly bovines while *T. evansi* causes the disease known as derrengadera or surra in equines. Recently, Uzcanga *et al*. (2002, 2004) have demonstrated that sera from *T. evansi*-infected horses and *T. vivax*-infected bovines immunorecognized a purified VSG variant from the TEVA1 Venezuelan isolate of *T. evansi* and suggested the potential use of this VSG variant as a diagnostic reagent. Interestingly, anti-VSG polyclonal antibodies generated against this variant immunolabelled the surface of both *T. evansi* and *T. vivax* (Uzcanga *et al*. 2004). Here, fluorometric ratio imaging from single isolated *T. evansi* parasites (Mendoza *et al*. 2001) were used to explore the effects of sera from trypanosome-infected animals and anti-VSG antibodies on the parasite Ca^{2+} homeostasis. Our results indicated that the interaction of anti-VSG antibodies with the parasite surface promotes a Ca^{2+} signal, which probably is involved in the *T. evansi* response against the host immune system, and most likely regulates the interactions between the parasite and its host. A preliminary report of this study has already been published (Mendoza *et al*. 2004*a*).

MATERIALS AND METHODS

Bovine sera

Two bovines were infected by intravenous injection of a suspension containing approximately 10^6 *T. vivax* parasites from a cryopreserved Venezuelan isolate. Blood samples were taken every day, for a 2-month period, in order to determine parasitaemia by the microhaematocrit method (Woo, 1970). Blood samples were also taken in the absence of anticoagulant to obtain sera containing anti-*T. vivax* antibodies and the complement system proteins. The progress of the infection was followed by indirect enzyme-linked immunoabsorbent assays using a *T. evansi* clarified fraction as antigen, which was prepared as described below (Aray *et al*. 1998; Camargo *et al*. 2004). Animal sera were decomplemented by heating to 58 °C for 30 min or by the addition of 180 μM rosmarinic acid.

Parasite preparation and purification

Cryopreserved samples of *T. evansi* from the Venezuelan TEVA1 isolate (Desquesnes and Tresse, 1996) were expanded in adult albino rats (Sprague-Dawley). When the number of parasites reached 10^6–10^8 trypanosomes/ml, the blood was extracted from the rats by cardiac puncture using EDTA as anti-coagulant. Trypanosomes were purified by anion-exchange chromatography using a fibrous DEAE-cellulose column (Lanham and Godfrey, 1970) equilibrated with PBS-G buffer (57 mM Na_2HPO_4, 3 mM NaH_2PO_4, 43·8 mM NaCl, 1% glucose, pH 8). Parasites eluting from the column were collected by centrifugation at 1475 ***g***, for 20 min, and washed 3 times with PBS-G buffer.

Purification of a VSG from Trypanosoma evansi

T. evansi parasites ($2{\cdot}7 \times 10^{11}$) were extracted on ice, by sonication (2 cycles, 30 sec each, with a 30 sec resting period in between) using 2 ml of a 5 mM Tris-HCl buffer (pH 7·2) containing 1 mM benzamidine, 1 mM PMSF, 5 mM EDTA, and 1 mM iodoacetamide. The resulting homogenate was centrifuged at 15 000 ***g*** for 30 min, at 4 °C, to obtain a supernatant containing the clarified antigenic fraction and the corresponding pellet. The clarified antigenic fraction was loaded onto a 50 ml Q-Sepharose column connected in tandem with a 10 ml S-Sepharose column, which previously had been equilibrated with 50 mM Tris-HCl buffer (pH 7·2). The *T. evansi* VSG eluted in the flow-through fraction as previously described by Uzcanga *et al*. (2002, 2004).

Protein sequencing

Purified VSG was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% polyacrylamide gels (Laemmli, 1970) and electroblotted to polyvinylidene difluoride membranes (Immobilon-P, Millipore) in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS, pH 11), 10% methanol (HPLC grade), and 0·1 mM sodium thioglycolate, for 1 h at 250 mV. Polypeptide

bands were subjected to sequencing according to Matsudaira (1987), using an automated protein microsequencer Prosize 493 cLC (PE-Applied Biosystems).

Preparation of polyclonal antibodies against purified Trypanosoma evansi *VSG*

The purified VSG was used to raise polyclonal antibodies in ascitic fluids of 7 female Balb/c mice, according to the procedure described by Bubis *et al*. (1993). Briefly, mice were pre-immunized twice (at 7 and 9 weeks of age) using lipid A and muramyl dipeptide as adjuvants, and under conditions that did not involve ascites fluid formation. Then, when the mice were 10 weeks old, we followed the standard protocol described by Tung (1983).

Purification of anti-VSG antibodies from ascitic fluids

Purified VSG (0·56 mg) was adsorbed on a nitrocellulose paper (11 cm × 7 cm), which was blocked using TBST buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 0·05% Tween 20) with 5% skimmed milk, and was subsequently incubated for 2 h with mouse ascitic fluids containing anti-VSG polyclonal antibodies (20 ml of a dilution of 1 : 50). Anti-VSG specific antibodies were eluted employing 20 ml of 100 mM glycine-HCl (pH 2·8) by gentle shaking for 20 min. The eluted antibodies were neutralized by adding 670 mg of solid Tris and were then dialysed overnight against 20 mM phosphate buffer (pH 8). We obtained 3·3 mg of purified anti-VSG-specific antibodies.

Western blot analysis

Proteins separated by SDS-PAGE (Laemmli, 1970) were electrotransferred from the gels to nitrocellulose sheets (0·45 μm pore size) according to the procedure described by Towbin *et al*. (1979). The nitrocellulose filters were incubated with either mouse ascitic fluid or affinity-purified anti-VSG antibodies, and developed using alkaline phosphatase-conjugated secondary antibodies against mouse IgG. The protein bands were visualized by the addition of NBT and BCIP. The specificity of the ascitic fluids was evaluated by competiton with increasing concentrations of purified VSG (0·1–30 μg) on blots containing either parasite homogenates or purified VSG.

Fluorescence measurements

Isolated parasites (10^6 cell/ml) were incubated with 8 μM of 1-[2-(5-carboxyoxazol-2-yl)-6aminobenzofuran-5oxyl]-2-(2′amino-5′-methylphenoxy)-ethane-N,N,N′,N′-tetraacetic acid/acetoxy-methyl ester (Fura-2/AM) in PBS-G buffer for 30–45 min, at room temperature (22 °C), with gentle and continuous agitation. Loaded parasites were washed 3 times with PBS-G to remove excess Fura-2/AM. Then, the suspension of Fura-2/AM-loaded parasites was added to an open experimental chamber mounted in an inverted microscope connected to a fluorescence imaging apparatus. The cover-slips of the chamber bottom were treated with poly-L-lysine solution (0·01% v/v) to allow partial adhesion of the parasites. Excitation wavelengths at 340 and 380 nm were used and the emitted light was measured at 510 nm. Fluorescence ratios and images were collected every 500 ms through a steeper-switch light source via a charge-coupled device video-camera coupled to an image intensifier. Single parasites could be discriminated through video-acquisition software. Calibration of the Fura-2 fluorescence ratio in terms of $[Ca^{2+}]_i$ was carried out as described previously by Grynkiewicz *et al*. (1985). Test solutions were delivered to the parasite via pressure ejection from a carbonated pipette using a perfusion system, which is connected to a computer and a temperature control system (Mendoza *et al*. 2001, 2002). Since the solutions can be rapidly changed, the effect of various conditions can be analysed on the same single parasite. The composition of the various solutions employed and the number of parasites in which $[Ca^{2+}]_i$ was measured are indicated in the figure legends. In all cases the temperature was maintained at 30 °C and all solutions contained 300 mM NaCl to prevent non-specific interactions. In order to determine the difference in calcium concentration, the final calcium level was measured when the steady state was reached. The results of fluorescence experiments were expressed as mean values ± standard error of the mean.

Variation of intracellular calcium concentrations induced by affinity-purified anti-VSG antibodies in parasite populations

To evaluate the effect of purified anti-VSG antibodies (66 μg/ml) on cell populations, measurements of intracellular Ca^{2+} concentration were performed in the presence of 1·8 mM extracellular Ca^{2+}. Briefly, a suspension of 2×10^6 cells/ml previously loaded with Fura-2/AM (8 mM) was monitored in a stirred cuvette, at 30 °C, using a ratiometric fluorescence spectrophotometer Perkin-Elmer LS-55 (Colina *et al*. 2005), and $[Ca^{2+}]_i$ was estimated as described by Grynkiewicz *et al*. (1985).

In vivo *direct immunofluorescence*

Purified parasites were incubated at 37 °C, for 30 min, with decomplemented anti-*T. evansi* VSG antibodies, and then with fluorescein-conjugated

anti-mouse IgG secondary antibodies. The suspension of parasites was added to an experimental chamber containing a cover-slip that was treated with a solution of poly-L-lysine (0·01% v/v) to allow partial adhesion of the parasites. The parasites were viewed using a fluorescence microscope directly connected with a photographic camera.

Effect of Ca^{2+} on the release of Trypanosoma evansi *VSG*

Parasites (1×10^6 cells) were incubated with Ca^{2+} (1·8 mM) in the presence or absence of the A-23187 ionophore (2·5 μg/ml). The time-course of VSG release was evaluated by removing an aliquot (150 μl) every 5 min. Following centrifugation at 8000 ***g*** for 1 min, the supernatants were analysed by Western blot using anti-VSG antibodies. Live parasites were quantified on a Neubauer counting chamber.

RESULTS

Since *T. evansi* and *T. vivax* have exhibited a very high immunological cross-reactivity (Aray *et al.* 1998; Uzcanga *et al.* 2002, 2004; Mendoza *et al.* 2004*a*), fluorometric ratio imaging from single *T. evansi* parasites (Mendoza *et al.* 2001) was used here to evaluate changes in the parasite $[Ca^{2+}]_i$ following perfusion with sera from *T. vivax*-infected bovines. In the presence of extracellular Ca^{2+}, a short exposure of *T. evansi* parasites to sera from bovines infected with *T. vivax* yielded a fast and substantial raise on their $[Ca^{2+}]_i$. Fig. 1A shows a representative experiment in which the parasite $[Ca^{2+}]_i$ was increased from 100 to 400 nM following exposure, for 5 sec, to bovine sera withdrawn 25 days after infection. This effect terminated and was reversed when the extracellular Ca^{2+} was eliminated (Fig. 1A). Additionally, cell lysis was generated after an extended exposure (>10 sec) of the parasites to sera from infected bovines in the presence of Ca^{2+} (data not shown). Parasites were also exposed to sera from *T. vivax*-infected bovines in a calcium-free medium that contained 100 μM EGTA. As shown in Fig. 1B, only a slight increase in the parasite $[Ca^{2+}]_i$ (~25 nM) was obtained under these conditions. These results indicated that the main source for the Ca^{2+} that is mobilized during the parasite-serum interaction is extracellular. On the contrary, neither sera from healthy animals (Fig. 1A) nor sera from bovines living in France, which is a trypanosomosis non-endemic area (data not shown), affected the *T. evansi* $[Ca^{2+}]_i$, which was maintained approximately at its basal value of 100 nM. Similar results were obtained with control parasites without any addition (data not included). When *T. evansi* parasites were exposed to decomplemented sera from infected animals, only a small increase of ~50 nM in the parasite $[Ca^{2+}]_i$ was observed (Fig. 1C). Thus, the activation of the

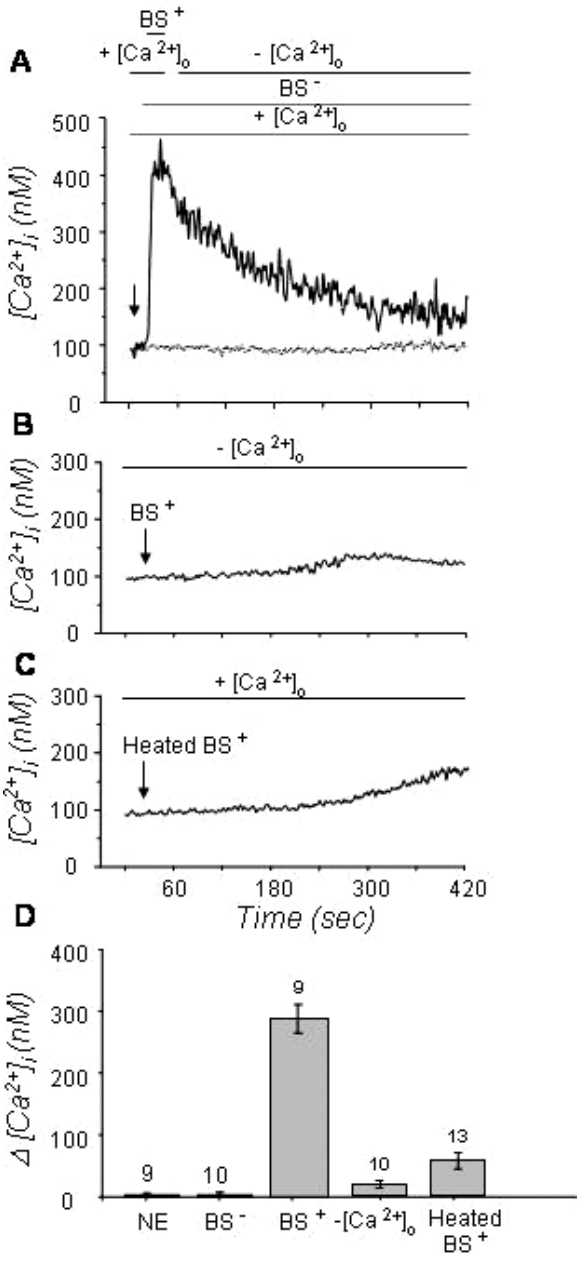

Fig. 1. Effect of sera from infected and non-infected animals on the $[Ca^{2+}]_i$ in single isolated *Trypanosoma evansi* cells loaded with Fura-2/AM. Arrows show the time of addition of the sera. (A) Changes in the parasite $[Ca^{2+}]_i$ following addition of sera from a bovine infected with *T. vivax* (BS^+) or from a healthy bovine (BS^-). Sera (1 : 100) were initially added in the presence of 1·8 mM external Ca^{2+} ($+[Ca^{2+}]_o$) and then this ion was removed using a solution containing no calcium and 100 μM EGTA ($-[Ca^{2+}]_o$). (B) Changes in parasite $[Ca^{2+}]_i$ after addition of BS^+ in a solution containing no calcium and 100 μM EGTA ($-[Ca^{2+}]_o$). (C) Changes in the parasite $[Ca^{2+}]_i$ after addition of BS^+ previously heated at 58 °C, to inactivate the complement, in the presence of 1·8 mM external Ca^{2+} ($+[Ca^{2+}]_o$). (D) Histograms corresponding to the intracellular Ca^{2+} increase (expressed as $\Delta[Ca^{2+}]_i$) observed following sera perfusion under the various conditions. Also shown is the effect of sera from bovines living in a non-endemic area (NE). On top of each bar is indicated the number of parasites in which $[Ca^{2+}]_i$ was measured.

classical complement system pathway is probably responsible for the generation of most of the rise in the $[Ca^{2+}]_i$ reported here. Fig. 1D summarizes the mean values of $[Ca^{2+}]_i$ obtained under the various conditions described. These values were the following: 288 ± 23 nM when individual parasites were exposed to sera from infected bovines in the presence of external Ca^{2+}, 19 ± 6 nM for the exposure of parasites to sera from infected bovines in the absence of Ca^{2+}, and 57 ± 13 nM for the exposure of parasites to decomplemented infected sera in the presence of external Ca^{2+}.

Since sera from infected bovines recognized VSG molecules from *T. evansi* (Uzcanga *et al.* 2004) and the dense surface coat formed by the closely packed monolayer of VSG has been shown to be essential for the parasite's life in the host's bloodstream, we purified the soluble form of a particular *T. evansi* VSG variant to prepare specific polyclonal antibodies and to evaluate their effect on the parasite $[Ca^{2+}]_i$. Fig. 2A illustrates a Coomassie blue-stained gel containing the resulting chromatography fractions (left). As seen in this figure, we obtained a highly pure VSG that migrated as a single polypeptide band. However, in some preparations, 2 polypeptide bands were obtained (right). To demonstrate that these two polypeptides represented isoforms of the same VSG, we determined their partial primary structure by Edman degradation and found that the first 10 amino acid residues were identical (data not shown). This amino acid sequence corresponded to that previously reported by Uzcanga *et al.* (2004), which displayed a significant homology with a putative *T. brucei* VSG gene. The purified VSG shown in Fig. 2A was then employed to raise ascites polyclonal antibodies in mice. Western blot analyses show that these antibodies, at various dilutions, specifically recognized VSG from parasite whole-cell homogenates (Fig. 2B, left). Purified VSG was also recognized by these ascitic fluids (Fig. 2B, right). Additionally, no recognition of purified VSG or VSG from parasite extracts was obtained by anti-VSG antibodies (dilution 1 : 5000) that were pre-incubated with 5 μg or more of purified VSG (Fig. 2C). These results confirmed the specificity of the ascites anti-VSG polyclonal antibodies.

We also explored the effect of specific anti-VSG polyclonal antibodies on the parasite $[Ca^{2+}]_i$ employing fluorometric ratio imaging. Fig. 3A illustrates that a exposure (600 sec) of a single *T. evansi* parasite to mouse ascitic fluids (dilution 1 : 100) containing anti-VSG antibodies generated an increase from 100 to 500 nM in its $[Ca^{2+}]_i$. Cell lysis was also produced following an extended exposure of parasites to these antibodies (data not included). In contrast, control parasites without any addition maintained their $[Ca^{2+}]_i$ at about 100 nM (data not shown). Fig. 3B clearly shows that this effect is dependent on the presence of extracellular Ca^{2+}. In this

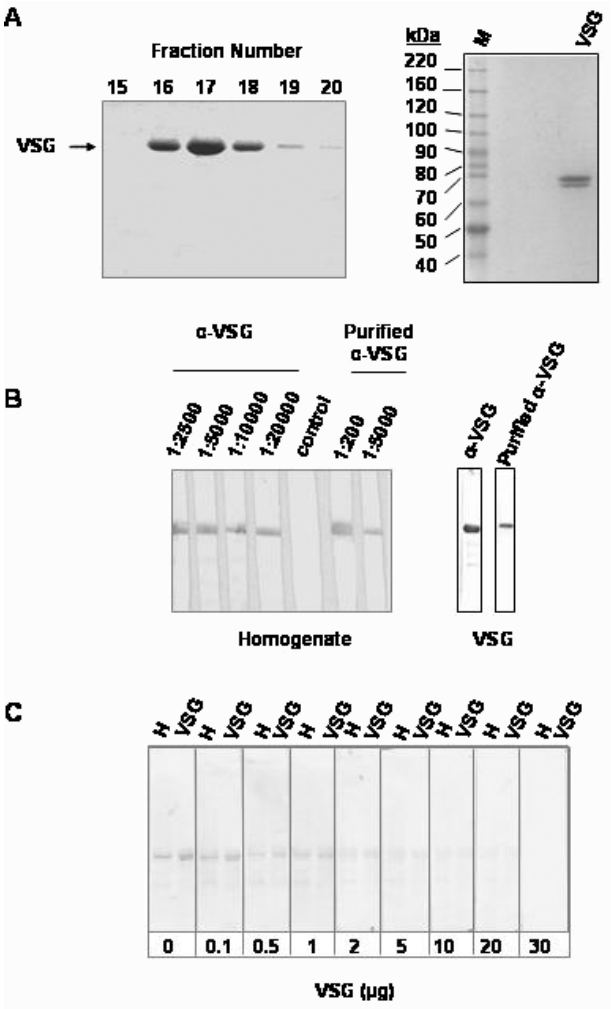

Fig. 2. Purification of VSG and evaluation of the specificity of the anti-VSG polyclonal antibodies. (A) *Trypanosoma evansi* VSG eluted in the flow-through fraction as described by Uzcanga *et al.* (2002, 2004). Left: SDS-PAGE separation of the fractions containing the chromatographically purified VSG (fractions 15–20). Right: In some cases, the purified VSG appeared as 2 polypeptide bands on gels. (B) Specific recognition of VSG by ascitic fluids (α-VSG) and affinity-purified anti-VSG antibodies (purified α-VSG) on both, parasite homogenates (Left) and purified VSG (Right). (C) Blots containing parasite homogenates (H, 25 μg of total protein) and purified VSG (VSG, 5 μg) were developed using ascitic fluids pre-incubated with increasing amounts of purified VSG (0–30 μg).

particular experiment, exposure of a single parasite to anti-VSG antibodies produced an increase of about 400 nM in its $[Ca^{2+}]_i$ in 420 sec, when extracellular calcium was present in the medium. This effect was substantially reduced to 30 nM when Ca^{2+} was eliminated from the extracellular medium by adding EGTA, and was reversed when Ca^{2+} was reloaded in the extracellular medium. Interestingly, when calcium was recharged in the extracellular medium, the increase on the parasite $[Ca^{2+}]_i$ was much faster, generating cell lysis after only 200 sec of exposure (Fig. 3B). Fig. 3C sums up the mean values of the

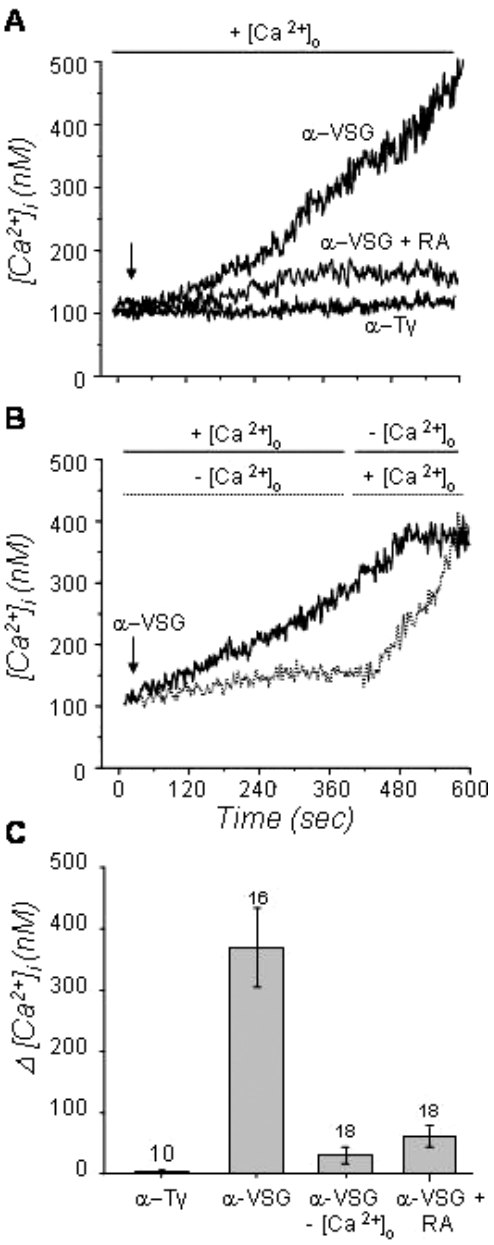

Fig. 3. Effect of anti-VSG polyclonal antibodies (α-VSG) on the $[Ca^{2+}]_i$ in single isolated parasites loaded with Fura-2/AM. Arrows show the time of addition of the antibodies. (A) Changes in the $[Ca^{2+}]_i$ after addition of ascitic fluid containing α-VSG or antibodies against the γ-subunit of bovine transducin (α-T_γ), a non-related protein. Also shown is the effect of adding α-VSG in the presence of 180 μM rosmarinic acid (RA). In all cases, ascites were added in the presence of 1·8 mM external Ca^{2+} ($+[Ca^{2+}]_o$). (B) Changes in the $[Ca^{2+}]_i$ after addition of α-VSG in the presence of 1·8 mM external Ca^{2+} ($+[Ca^{2+}]_o$). Following 6 min incubation, this ion was removed using a calcium-free solution ($-[Ca^{2+}]_o$). In addition, α-VSG were added initially in a Ca^{2+}-free solution ($-[Ca^{2+}]_o$), and subsequently the $[Ca^{2+}]_o$ was raised to 1·8 mM ($+[Ca^{2+}]_o$). (C) Histograms corresponding to the intracellular Ca^{2+} increase (expressed as $\Delta[Ca^{2+}]_i$) observed following ascites perfusion under the various conditions. On top of each bar is indicated the number of parasites in which $[Ca^{2+}]_i$ was measured.

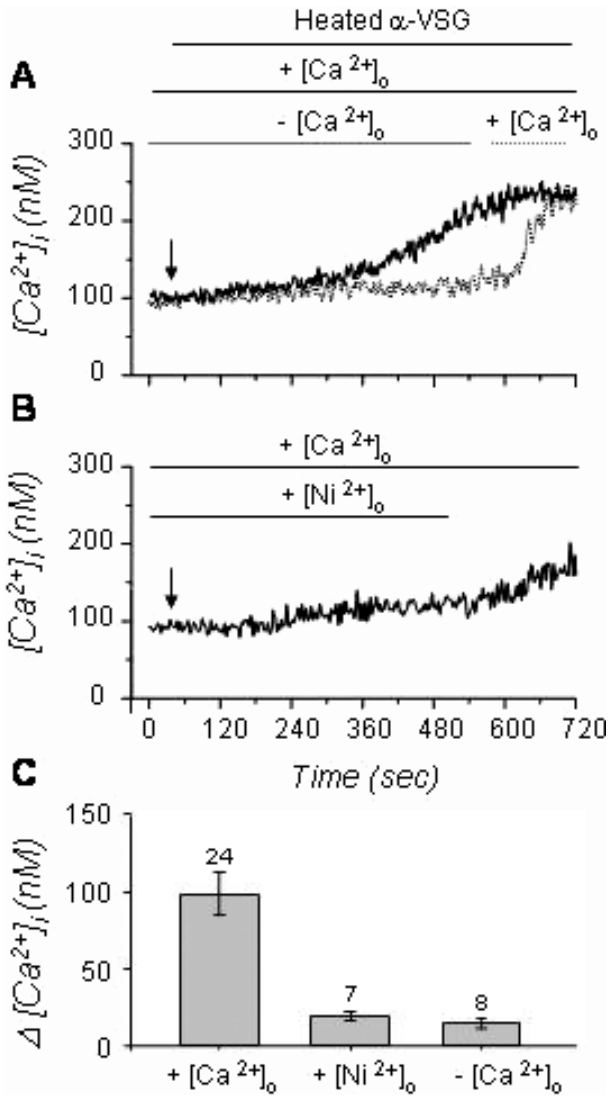

Fig. 4. The effect of Ni^{2+} on the intracellular Ca^{2+} increase induced by decomplemented anti-VSG antibodies (α-VSG) in single isolated *Trypanosoma evansi* parasites loaded with Fura-2/AM. (A) Variations in the $[Ca^{2+}]_i$ after addition of specific antibodies against the parasite VSG protein previously heated at 58 °C, in the presence or absence of 1·8 mM external Ca^{2+} ($+/-[Ca^{2+}]_o$). (B) Changes in the parasite $[Ca^{2+}]_i$ following addition of α-VSG, previously heated at 58 °C, in the presence of 5 mM Ni^{2+} ($+[Ni^{2+}]_o$). Following 8 min incubation, Ni^{2+} was removed and parasites were reincubated with 1·8 mM external calcium ($+[Ca^{2+}]_o$). (C) Histograms corresponding to the intracellular Ca^{2+} variation (expressed as $\Delta[Ca^{2+}]_i$) observed after perfusion with decomplemented α-VSG under the various conditions. On top of each bar is indicated the number of parasites in which $[Ca^{2+}]_i$ was measured.

increases observed on the parasite $[Ca^{2+}]_i$ attained under these conditions. These values were 369 ± 65 and 30 ± 6 nM when individual parasites were exposed to anti-VSG antibodies in the presence or absence of external Ca^{2+}, respectively.

When decomplemented ascitic fluid was employed in the presence of extracellular calcium, a decrease of

approximately 300% in the parasite $[Ca^{2+}]_i$ influx was obtained (Fig. 4A). A similar effect was acquired when 180 μM rosmarinic acid was added to these antibodies (Fig. 3A), obtaining a mean value of 60 ± 7 nM in the parasite $[Ca^{2+}]_i$ (Fig. 3C). These results suggested that most of the increase in the *T. evansi* $[Ca^{2+}]_i$ produced by the ascites containing the anti-VSG antibodies is mediated by the activation of the classical complement pathway. However, a small increase in the parasite $[Ca^{2+}]_i$ of about 100 nM was still observed after decomplementation of the anti-VSG antibodies, which must be directly caused by the specific anti-VSG antibodies present in the ascites. Fig. 4A also compares the effect produced by the decomplemented ascitic fluid containing the anti-VSG antibodies in the presence and absence of extracellular Ca^{2+}. Similar experiments were performed in the presence of Ca^{2+} and Ni^{2+} (Fig. 4B). The rise in parasite $[Ca^{2+}]_i$ was substantially reduced in the absence of Ca^{2+} and in the presence of Ni^{2+}. In addition, the inhibitory effect of Ni^{2+} was reversed when Ni^{2+} was eliminated from the extracellular medium and Ca^{2+} was re-added (Fig. 4B). Either with Ni^{2+} or in the absence of external calcium, an increase of about 19 nM in the parasite $[Ca^{2+}]_i$ was obtained. Fig. 4C presents the main values of the parasite $[Ca^{2+}]_i$ variations attained under these various conditions. These values were 98 ± 14 nM in the presence of extracellular Ca^{2+}, 19 ± 3 nM when both Ca^{2+} and Ni^{2+} were present in the incubation medium, and 15 ± 3 nM in the presence of 100 μM EGTA (Fig. 4C); and were measured after the steady state was reached. These results indicated that approximately 80% of the effect generated by the anti-VSG antibodies was produced by the influx of extracellular Ca^{2+}, probably through some Ca^{2+}-specific channels located at the parasite membrane, while a less significant percentage of the effect ($\sim$20%) was produced by the release of this ion from parasite intracellular storage organelles.

In contrast, decomplemented mouse ascitic fluids containing antibodies generated against the γ-subunit of bovine visual transducin, a non-related protein, did not affect the *T. evansi* $[Ca^{2+}]_i$ (Fig. 3A). Values obtained of the parasite $[Ca^{2+}]_i$ following exposure to ascitic fluids containing anti-bovine γ-subunit of transducin, did not statistically differ from basal concentration values acquired under control conditions (Fig. 3C). Therefore, the presence of specific anti-VSG antibodies is necessary to produce the effect on the parasite $[Ca^{2+}]_i$ attained by these ascites. This was corroborated by comparing the effects on the parasite $[Ca^{2+}]_i$ when the anti-VSG antibodies were applied in the presence or absence of the corresponding purified VSG (Fig. 5A). The purified *T. evansi* VSG completely inhibited the effect of the antibodies because no variation in the levels of the parasite $[Ca^{2+}]_i$ was observed under this condition. Mean values acquired of the parasite

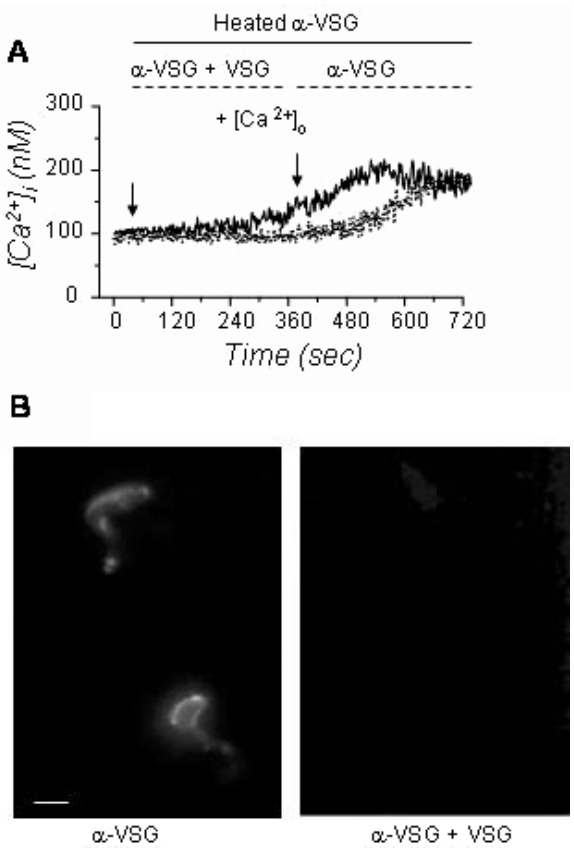

Fig. 5. Effect of decomplemented anti-VSG antibodies (α-VSG) pre-incubated with the purified soluble VSG protein on the $[Ca^{2+}]_i$ in single parasites loaded with Fura-2/AM. (A) No change in the parasite $[Ca^{2+}]_i$ was obtained after addition of decomplemented α-VSG pre-incubated with the purified soluble *Trypanosoma evansi* VSG protein. Following 6 min incubation, parasites were washed and re-incubated with decomplemented α-VSG. The increase in $[Ca^{2+}]_i$ was measured on 14 individual parasites. All experiments were performed in the presence of 1·8 mM external Ca^{2+} ($+[Ca^{2+}]_o$). (B) *In vivo* immunolabelling showing anti-VSG antibodies-induced raft patching of VSG on the parasite surface (left). Raft patching of VSG was hindered when anti-VSG antibodies were pre-incubated with the soluble VSG protein (right). Scale bar = 6 μm.

$[Ca^{2+}]_i$ following exposure to ascitic fluids pre-incubated with the purified VSG variant did not statistically differ from basal concentration values obtained under control conditions, demonstrating the specific responsibility of the anti-VSG antibodies on the effect initially seen. Fig. 5B shows the *in vivo* immunofluorescent recognition of the parasite surface by these antibodies. Trypanosomes displayed fluorescent patches on the flagellum and the anterior face of the cell. As expected, anti-VSG antibodies pre-incubated with the purified VSG did not label the parasites. Therefore, the interaction of anti-VSG antibodies with VSG molecules located on the parasite surface eventually caused an increase in its

$[Ca^{2+}]_i$ as a signal that is probably involved in the parasite response against the host immune system.

Anti-VSG antibodies were affinity purified from mouse ascitic fluids using the purified VSG antigen immobilized on nitrocellulose. As illustrated in Fig. 2B, analyses by Western blot showed that affinity-purified anti-VSG antibodies, at various dilutions, specifically recognized VSG from whole-cell *T. evansi* lysates (left). Purified VSG was also recognized by these antibodies (right). Affinity-purified anti-VSG antibodies were then used to perform direct measurements of their effect on the parasite $[Ca^{2+}]_i$. Fig. 6A is a representative experiment showing that exposure of a single *T. evansi* parasite to a concentration of 66 μg/ml of affinity-purified anti-VSG antibodies generated an increase from 100 to $\sim$190 nM in its $[Ca^{2+}]_i$. The mean value of the parasite $[Ca^{2+}]_i$ variations attained under this condition was 74 ± 9 nM in the presence of extracellular Ca^{2+}, after the steady state was reached (Fig. 6C). No response was obtained using 6·6 or 33 μg/ml of the purified antibodies (data not included). Since in the single cell approach only a single parasite partially immobilized with poly-L-lysine can be assayed, we repeated these measurements using the more conventional bulk cuvette setup. This fluorescence spectrophotometric arrangement allows us to determine the effect of the affinity-purified anti-VSG antibodies on a parasite cell population, where the signals from a suspension containing 2×10^6 cells/ml are averaged. Fig. 6B shows a representative experiment in which the $[Ca^{2+}]_i$ of a population of parasites ($0{\cdot}8 \times 10^6$ cells) was increased from 105 to $\sim$200 nM following exposure to 33 μg of affinity-purified anti-VSG antibodies, in the presence of 1·8 mM extracellular Ca^{2+}. We obtained a mean value for the $[Ca^{2+}]_i$ variation of 122 ± 15 nM, when populations of *T. evansi* parasites were exposed to purified anti-VSG antibodies in the presence of external Ca^{2+} (Fig. 6C). In conclusion, affinity-purified antibodies against VSG were able to elicit comparable calcium fluxes in *T. evansi* using both the single cell and the bulk cuvette set ups.

VSG has been shown to be involved in antigenic variation and shedding, which cause release of this protein from the parasite surface coat. Since the Ca^{2+} signal reported here is generated by the interaction between VSG molecules and specific antibodies, we evaluated the direct effect on the VSG release of the increase of $[Ca^{2+}]_i$ caused by the Ca^{2+} ionophore A-23187. Western blot analyses using anti-VSG antibodies were conducted to detect the time-course of the release of VSG from bloodstream forms of *T. evansi*, in the presence of Ca^{2+} (1·8 mM) and A-23187 (2·5 μg/ml). As shown in Fig. 7A, VSG was released at 15 and 20 min. However, no free VSG was observed in the absence of A-23187 (data not shown). No changes in the number of motile parasites (Fig. 7B) and on the parasite morphology (data not included) occurred during the time-period of the experiment.

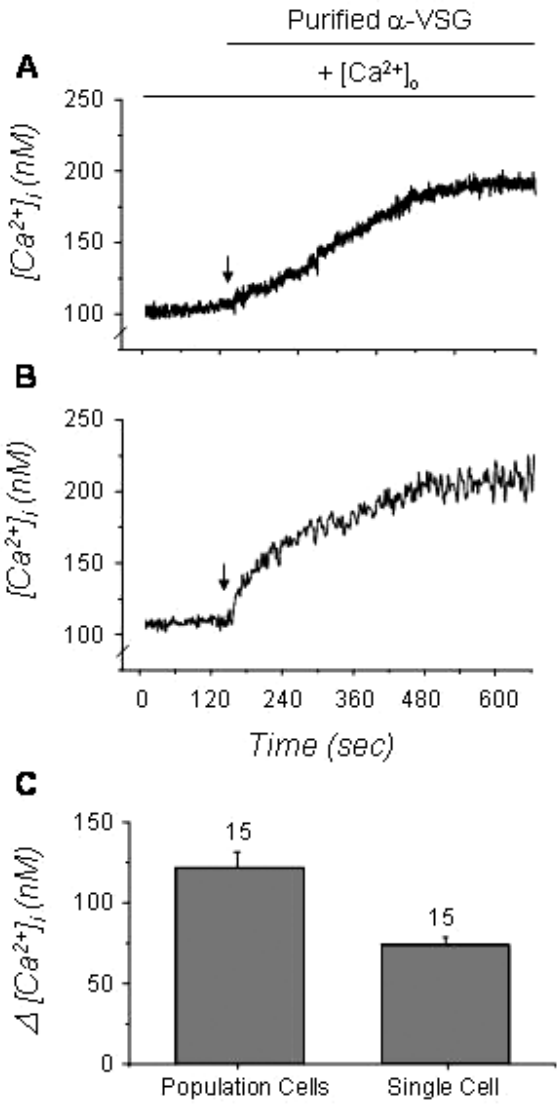

Fig. 6. Effect of affinity-purified anti-VSG antibodies (purified α-VSG) on the parasite $[Ca^{2+}]_i$. Purified α-VSG antibodies were added to single cells (A) or cell populations (B), which were loaded with Fura-2/AM (8 mM) in the presence of extracellular Ca^{2+} (1·8 mM). Arrows indicate the time of α-VSG antibody addition. Shown is a representative trace of 5 independent experiments that were conducted on 3 separate preparations. (C) Histograms corresponding to the intracellular Ca^{2+} variation (expressed as $\Delta[Ca^{2+}]_i$) observed after the addition of the purified α-VSG antibodies when the $[Ca^{2+}]_i$ reached a stable state. All the experiments were carried out at 30 °C. On top of each bar is indicated the number of parasites in which $[Ca^{2+}]_i$ was measured.

DISCUSSION

Here, we showed that anti-*T. vivax* antibodies and anti-VSG ascitic fluid reacted with *T. evansi ex vivo* and initiated an increase in its $[Ca^{2+}]_i$. This effect was abrogated by the addition of EGTA, showing

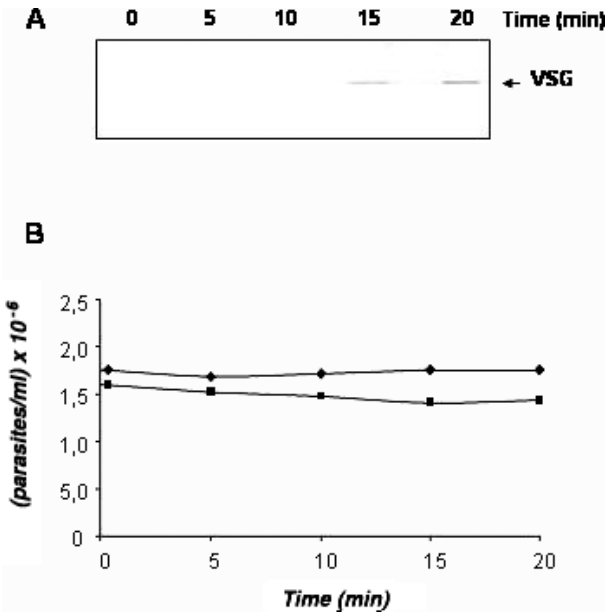

Fig. 7. Induction of VSG release from *Trypanosoma evansi* by Ca^{2+} influx. (A) Release of VSG was detected by Western blot at various times (0–20 min), in the presence of the A-23187 ionophore (2·5 μg/ml). (B) During the time-course, live parasites were quantified in the presence (■) or absence (◆) of A-23187 (2·5 μg/ml). All these experiments included 1·8 mM extracellular Ca^{2+}.

that it is dependent on the presence of extracellular calcium. Accordingly, sera from healthy animals were not capable of increasing the parasite intracellular Ca^{2+} levels. Parasite lysis produced by infected sera and anti-VSG antibodies was sensitive to both, heat and rosmarinic acid, indicating that this effect was mediated by the complement system (Fig. 8a). Physiological evidence has demonstrated that the action of the complement system is dependent on the presence of extracellular Ca^{2+} since the activation of this system culminates in the formation of pores in the plasma membrane, which permits the entry of Ca^{2+} (Campbell *et al*. 1979; Newsholme *et al*. 1993; Laffafian *et al*. 1995). Destruction of *T. vivax* parasites by the complement system has been previously reported in bovines (Murray and Urquhart, 1977; De Gee *et al*. 1982; Mahan *et al*. 1986). However, it has been demonstrated that salivarian trypanosomes have mechanisms for complement evasion. During infection, *T. congolense* releases factors that directly activate the bovine complement cascade at the C1 level resulting in depletion of complement components (Nielsen *et al*. 1978). It has also been proposed that the direct activation of the classical complement pathway observed in humans by a *T. brucei* VSG could lead to considerable depletion of complement components (Musoke and Barbet, 1977). Additionally, reductions in sera of the levels of total haemolytic complement from the alternative pathway and C3 from the classical route have been also reported in *T. congolense*- and *T. vivax*-infected cattle (Nielsen, 1985). Thus, under hypocomplementaemia conditions, a small proportion of trypanosomes expressing the predominant VSG variant might survive once the complement system has decreased (Fig. 8b).

When the complement system was completely inactivated by either heat treatment or addition of rosmarinic acid, in order to simulate hypocomplementaemia conditions, an increase in the $[Ca^{2+}]_i$ was still obtained after exposure of parasites to either animal infected sera or anti-VSG antibodies (Fig. 8c). This effect was dependent on specific anti-VSG antibodies since pre-incubation of these antibodies with the purified VSG completely abolished the increase in the parasite $[Ca^{2+}]_i$. Moreover, similar effects were generated by affinity-purified anti-VSG antibodies using either single cell or parasite population measurements. Since addition of Ni^{2+}, a common inactivator of membrane calcium channels, only partially inhibited the increase of $[Ca^{2+}]_i$, two sources must be responsible for the Ca^{2+} signal reported here: (1) a release of Ca^{2+} from parasite intracellular stores, such as the endoplasmic reticulum, the mitochondria or the acidocalcisomes (Fig. 8d) (Moreno and Docampo, 2003; Mendoza *et al*. 2002, 2004*b*), and (2) an influx of this ion through channels located on its plasma membrane (Fig. 8e). In 2005, the genome sequence of *T. brucei* was published (Berriman *et al*. 2005) and a putative sequence for a calcium channel protein has been reported [GeneDB website (http://www.genedb.org), sequence number Tb10.70.4750]. Accordingly, calcium entry into *T. brucei* bloodstream trypomastigotes has been reported to be apparently regulated via a novel signalling pathway involving phospholipase A_2-mediated generation of arachidonic acid and stimulation of a plasma membrane-located calcium channel (Eintracht *et al*. 1998; Catisti *et al*. 2000).

In higher eukaryotes and kinetoplastid parasitic protozoa, several plasma membrane proteins, particularly those anchored by a glycosyl-phosphatidylinositol (GPI) moiety, are associated with sphingolipid/esterol-rich microdomains termed lipid rafts (Brown and London, 1998; Simons and Ikonen, 1997; Nolan *et al*. 2000; Denny *et al*. 2001). VSG like other GPI-anchored proteins have been reported to be located in these rafts (Denny *et al*. 2001; Denny and Smith, 2004). The presence of membrane protein clusters has been extensively used for lipid raft characterization (Harder *et al*. 1998). CD59, a GPI-anchored protein, was still able to mediate Ca^{2+} intracellular signalling events following antibody-induced ligation, and subsequently was clustered in these microdomains (Hiscox *et al*. 2002). Pizzo *et al*. (2002) also demonstrated in Jurkat cells that anti-CD59 antibodies induced Ca^{2+} signalling and that the CD59-induced Ca^{2+} response was sensitive to treatment with methyl-β-cyclodextrin. These results suggested that the signalling pathway initiated

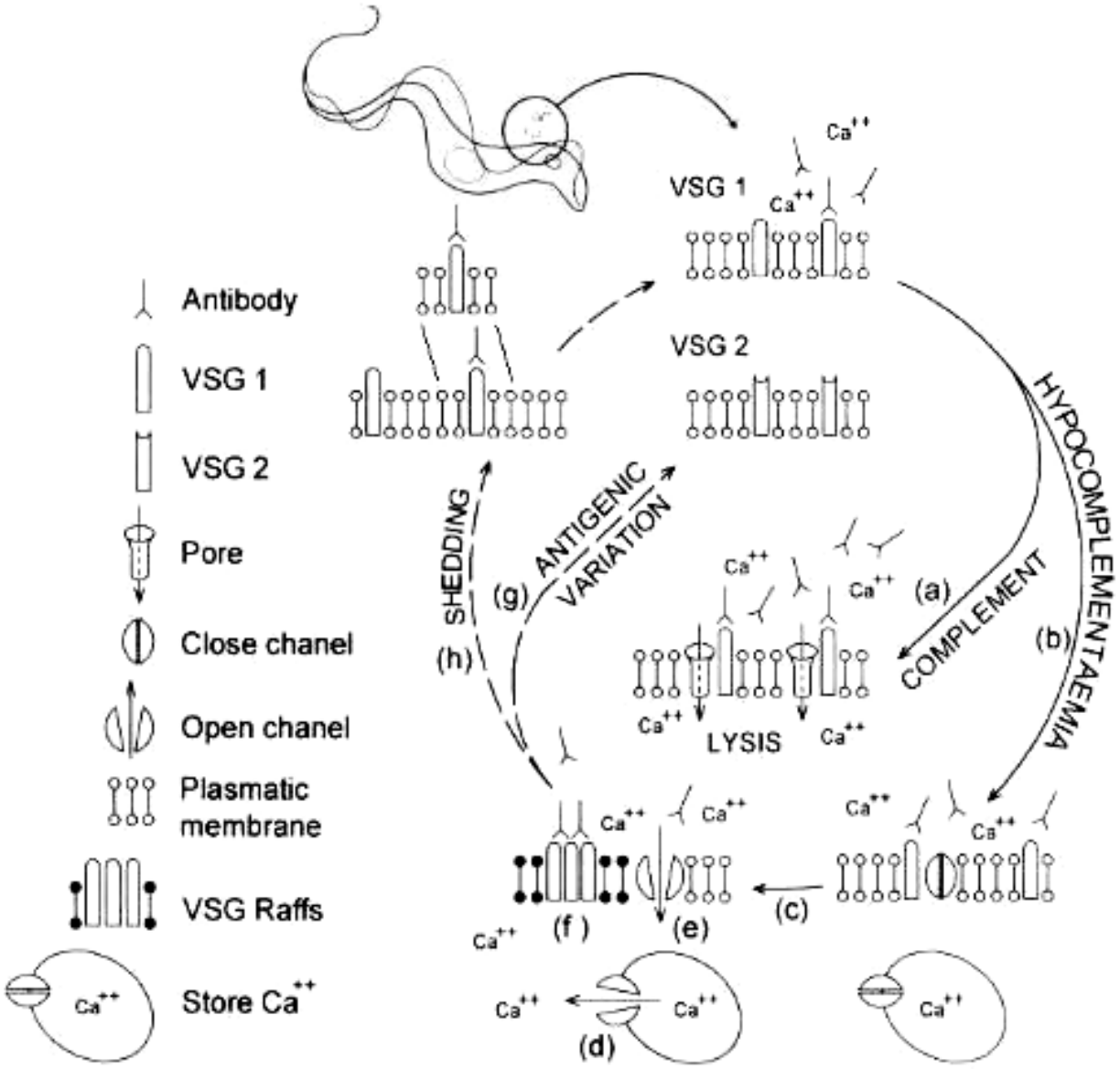

Fig. 8. Scheme of possible Ca^{2+} signal transduction routes induced by anti-VSG antibodies. (a) Parasite lysis by the complement classical pathway. (b) Under hypocomplementaemia conditions, α-VSG did not produce parasite lysis but (c) were capable of inducing a calcium signal. Both, a release of this ion from parasite intracellular stores (d) and an influx of Ca^{2+} through ion channels located on its plasmatic membrane (e) were observed. These Ca^{2+} responses might be a consequence of lipid raft-associated VSG clusters induced by specific α-VSG (f). The antigenic variation strategy has been correlated with a Ca^{2+} signal in trypanosomatids (g). Additionally, VSGs are naturally released by a process known as VSG shedding (h), which could be associated with calcium signalling.

by cross-linking of this raft-associated protein was altered specifically by disruption of the integrity of these rafts. Interestingly, on application of homologous antiserum to a given *T. brucei* VSG, the VSG antigen moves to the flagellar pocket region that overlays the Golgi apparatus (Barry, 1979; Webster, 1990). This redistribution, known as capping, might be related to the presence of lipid rafts in these parasites. Interactions between lipid raft resident molecules, such as VSG, can also be studied by patching, a process in which these molecules are cross-linked by specific antibodies, facilitating association of rafts into a complex that is visible by microscopy (Denny and Smith, 2004). This phenomenon has been observed in various protozoa (Doyle *et al.* 1974; Silva *et al.* 1975; Dwyer, 1976). Here, we demonstrated by *in vivo* immunofluorescence labelling that anti-VSG antibodies induced the formation of patches of VSG on the parasite surface. Similar to the results reported by Hiscox *et al.* (2002), the Ca^{2+} signal we observed might be a consequence of lipid raft-associated VSG clusters induced by specific anti-VSG antibodies (Fig. 8f).

Which processes may be regulated by these Ca^{2+} pathways?. To date, the only stage that has been described for *T. evansi* is the trypomastigote form, and

consequently this parasite does not undergo differentiation. Therefore, the Ca^{2+} signal reported here could be associated with (i) VSG endocytosis and recycling, (ii) antigenic variation, or (iii) shedding. Removal of anti-VSG antibodies from the parasite cell surface might be mediated by endocytosis, as a mechanism of evasion of the host immune response. O'Beirne *et al*. (1998) demonstrated that bloodstream forms of *T. brucei*, suffered a cycle of aggregation-disaggregation in the presence of anti-VSG antibodies. The disaggregation required energy and was dependent upon normal endosomal activity. Also, endocytosis and recycling of the GPI-anchored VSG in *T. brucei* has been described via large clathrin-coated vesicles (Engstler *et al*. 2004). Since the mechanism of disaggregation described by O'Beirne *et al*. (1998) was found to be independent of Ca^{2+}, the endosomal pathway is probably not correlated with the calcium signal we reported here. On the other hand, the antigenic variation strategy has been correlated with a Ca^{2+} signal in trypanosomatids, because the release of their surface coat protein is dependent on the presence of extracellular Ca^{2+} (Fig. 8g). Voorheis *et al*. (1982) detected the release of [^{3}H]acetimido-labelled VSG from bloodstream forms of *T. brucei* in the presence of calcium ions together with the calcium ionophore A-23187. We also showed that the $[Ca^{2+}]_i$ increase by ionophores led to VSG release. All these results suggested that VSG release requires an increase in the parasite $[Ca^{2+}]_i$, presumably by the opening of controlled Ca^{2+}-channels located on the parasite plasma membrane. Additionally, it has been reported that VSG molecules are naturally released during culture of *T. brucei* bloodstream forms (Bulow *et al*. 1989). This process is known as VSG shedding (Seyfang *et al*. 1990). In our model, released VSG may compete with live trypanosomes for antibody binding and consequently avoid parasite lysis (Fig. 8h).

In conclusion, a new methodological approach has been employed to determine parasite signalling routes induced by the host immunological response. This study is quite significant since it is the first to identify a ligand coupled to a calcium signal, which leads to a cellular response in salivarian trypanosomes. More experiments need to be performed in order to understand the physiological and immunological intracellular mechanisms, which are responsible for the activation, modulation and control of various aspects of host innate and acquired immunity during infection.

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