Simultaneous but Independent Activation of Adenylate Cyclase and Glycosylphosphatidylinositol-Phospholipase C under Stress Conditions in Trypanosoma brucei*

(Received for publication, December 19, 1995, and in revised form, February 16, 1996)

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Previous observations suggested a concomitant relationship between the release of the variant surface glycoprotein (VSG) and the activation of adenylate cyclase in the bloodstream form of the parasitic protozoan Trypanosoma brucei. In order to evaluate this hypothesis, adenylate cyclase activity was measured in live trypanosomes subjected to different treatments known to induce the shedding of the VSG coat, namely low pH and trypsin digestion. In both cases adenylate cyclase activation occurred in parallel with the release of the VSG. The latter was found to be mediated by the glycosylphosphatidylinositol-specific phospholipase C that cleaves the glycosylphosphatidylinositol anchor of the protein (VSG lipase). Furthermore, both adenylate cyclase and VSG release were activated by the incubation of trypanosomes with specific inhibitors of protein kinase C on both VSG lipase and adenylate cyclase activities. Significantly, in mutant trypanosomes lacking VSG lipase, adenylate cyclase was activated under conditions where VSG release did not occur. Moreover, VSG release was also found to occur in the absence of activation of the cyclase, as observed in the presence of low concentration of the thiol modifying reagent p-chloromercuriphenylsulfonic acid. These observations provide the first demonstration that release of the VSG in response to cellular stress is mediated by the VSG lipase and that activation of adenylate cyclase occurs in response to the same stimuli they are not obligatorily coupled.

Trypanosoma brucei, the parasitic protozoan causative of Nagana in the African cattle, is transmitted between mammals by the tsetse fly. Its life cycle includes several distinct nonreproductive (infertile) and proliferative (noninfertile) stages in both the mammalian host and the insect vector. In addition to biochemical and morphological modifications, the differentiation from the mammalian bloodstream form to the insect procyclic form is accompanied by important changes in protein composition of the cell surface, probably as a protective adaptation to different host defenses. In particular, the major surface antigen of the bloodstream form, the VSG,1 is replaced in procyclic forms by another predominant surface glycoprotein, termed procyclin (1). So far the mechanisms involved in the induction of these transformations are unclear. On the basis of observations made in other eukaryotes, it is probable that activation of cell surface receptors by appropriate ligands may cause the generation of second messengers which trigger changes in the programming of gene expression. Typical in this respect is the generation of CAMP produced as a result of the stimulation of adenylate cyclase (2–4).

In a variety of trypanosomal species (5–8) and particularly in T. brucei (9, 10), changes in CAMP levels seem to be associated with events triggering cell proliferation and differentiation. In T. brucei, adenylate cyclase is located in the plasma membrane (11, 12). This activity is encoded by four gene families, two of which contain at least 10 members (13, 14).2 Genes from one of these families, termed ESAG 4 (for expression site-associated gene 4) belong to the transcription units of the VSG genes and are thus only expressed in the bloodstream form, whereas the genes from the three other families termed GRESAG 4.1, 4.2, and 4.3 (for gene related to ESAG 4), are not linked to the VSG genes and are expressed in both bloodstream and procyclic forms. An adenylate cyclase activity stimulated by calcium was found to be restricted to the bloodstream form and is likely to be the product of the ESAG 4 genes (15, 16). The different adenylate cyclases appear to be transmembrane glycoproteins with a divergent external domain at the N terminus and a conserved catalytic domain located in front of a C-terminal extension (16).

In dividing procyclic and bloodstream forms the activity of adenylate cyclase is down-regulated, since it can be strongly stimulated upon rupturing of the cells (17). However, in both intact cells3 and isolated plasma membranes (11), the adenylate cyclase activity of trypanosomes was found to be insensitive to agents known to activate G-protein-responsive adenylate cyclases, such as GTPγS, Gpp(NH)p, forskolin, cholera, and pertussis toxins. These results are in keeping with the

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* This work was supported by research contracts with the Communauté Française de Belgique (ARC), by the Belgian Fonds de la Recherche Scientifique (FRSM and Crédit aux Chercheurs), and by the International Brachet Stiftung (IBS).

‡ Fellow of the Fonds National de la Recherche Scientifique.

§ Supported by a fellowship from the European Commission (DGXI).

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1 The abbreviations used are: VSG, variant surface glycoprotein; GTPγS, guanosine 5′-O-(3-thiotriphosphate); PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; DMG, 1,2-dimyristyl-rac-glycerol; DAG, diacylglycerol; FCCP, carbonylcyanide p-trifluoromethoxyphenylhydrazone; pCMPS, p-chloromercuriphenylsulfonic acid; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)ethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; GPPi, glycosylphosphatidylinositol; PLC, phospholipase C; PAGE, polyacrylamide gel electrophoresis; CRD, cross-reacting determinant; ESAG, expression site-associated gene.

3 S. Alexandre, P. Paindavoine, F. Paturiaux-Hanocq, J. Hanocq-Quertier, and E. Pays, unpublished data.

S. Rolin, unpublished data.
general structure of the kinetoplastid cyclases (14, 18), which is very similar to that of G-protein-independent single transmembrane span cyclases (19–21). In view of the observations reported for Dictyostelium (20), it may be suggested that the trypanosonal adenylyl cyclases differ in their response to specific ligands and are involved in distinct events of transformation and/or proliferation in the parasite life cycle.

In the bloodstream form, stress conditions that trigger the release of the VSG (osmotic shock, Ca2+, local anesthetics) were also found to activate adenylyl cyclase, whereas zinc inhibited both processes (22–25). In addition, a transient activation of adenylyl cyclase was found to follow VSG release during the cold shock-induced differentiation from bloodstream to procyclic forms (26). These observations suggested that VSG release and adenylyl cyclase activity were dependent on the same stimuli. In order to evaluate this hypothesis we submitted trypanosomes to different experimental conditions known to induce the release of the VSG, and we monitored simultaneously the release of the VSG and the activity of adenylyl cyclase. In wild-type cells, activation of adenylyl cyclase was found to correlate with VSG lipase-dependent VSG release independently of the experimental conditions used to trigger this release (low pH or trypsin). Moreover, specific inhibitors of protein kinase C (PKC) were found to stimulate both processes. Significantly, in a mutant lacking the VSG lipase, stimulation of adenylyl cyclase occurred under conditions where the VSG was not released, indicating that adenylyl cyclase activation is not simply the result of the disruption of the VSG coat. Moreover, the differential sensitivity of VSG lipase and adenylyl cyclase to pCMPS allowed the design of experiments where VSG release still occurred under conditions where adenylyl cyclase was totally inhibited. We conclude that stimulation of VSG lipase and adenylyl cyclase occur independently in response to different stress conditions and that PKC activity may be involved in the control of these responses.

MATERIALS AND METHODS

Chemicals

Phorbol 12-myristate 13-acetate (PMA, 5 mg/ml in MeSO4), 1,2-
dimyristoyl-rac-glycerol (DMG, 0.1 g/ml in chloroform), diaetylcoline (DAG, 0.1 g/ml in chloroform), carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP), pCMPS, peptatin A, cycloheximide, trypsin (l-tosylamide-2-phenyl ethyl chloromethyl ketone type XII), soybean inhibitor, TES, and MES were obtained from Sigma. Creatine kinase, AEBSF (Pefabloc), E64, and leupeptin were from Boehringer Mannheim. Calphastin C (1 mg/ml in MeSO4) was from Calbiochem. Random peptides and PKC- and PKA-pseudopeptide inhibitors were from Promega or synthesized by A. Vandermeers (Faculty of Medicine and Pharmacy, Free University of Brussels) and by C. Sergheraet (URA 1309 CNRS, Institut Pasteur de Lille, Lille, France). [32P]ATP (10–40 Ci/mmol) and [3H]cAMP (30 Ci/mmol) were from Amersham Corp.

Trypanosomes

Three clones of the same T. brucei monoclonal bloodstream cell line (19, 21) were propagated in mice and isolated from the blood 6 days after infection. Established procyclic cell lines originating from the same stock were cultured at 27°C in SDM-79 medium supplemented with 15% inactivated fetal calf serum (28).

**Experimental Treatments Inducing VSG Release**

Mild Acid Treatment (29)—Bloodstream or procyclic trypanosomes were suspended (3 × 10⁷ cells/ml) in pH 5.5 buffer (125 mM sodium phosphate (pH 5.5), containing 1% glucose and 20 μg/ml leupeptin) and incubated for various periods at 4°C.

Trypsin Treatment (30)—Bloodstream and procyclic trypanosomes were, respectively, suspended (3 × 10⁷ cells/ml) in 4 mM KCl, 2 mM MgCl2, 140 mM NaCl, 10 mM glucose, 30 mM TES (pH 7.5) and 57 mM NaCl, 2.7 mM KCl, 10 mM praline, 42.3 mM phosphate buffer (pH 7.5). After first being treated for 30 min at 20°C with cycloheximide (10 μg/ml) in order to avoid de novo synthesis of VSG, they received 50 μg/ml trypsin and were sampled at intervals during 120 min by centrifugation (3,250 rpm for 5 min) after blocking trypsin activity with 290 μg/ml soybean inhibitor.

**Assay of Adenylyl Cyclase**

Adenylyl cyclase was assayed according to Salomon et al. (31) in procyclic and bloodstream forms permeabilized by "swell dialysis" (17). Trypanosomes were harvested by centrifugation (5 min, 3,250 rpm) and counted with a hemocytometer. The pellet was suspended at 4°C in a swelling dialysis medium (55 mM KCl, 1 mM glucose, 1 mM EGTA, 20 μg/ml leupeptin, 13.3 mM TES (pH 7.5) (; 5 × 10⁶ cells/ml). Samples (20 μl) were added to 80 μl of the assay medium containing 1 mM glucose, 1 mM EGTA, 20 μg/ml leupeptin, 0.5 mM cAMP, 10 mM phosphocreatine, 50 μM creatine kinase, 10 mM MgCl2, 20 mM KCl, 0.5 mM ATP, 1 μCi of [32P]cAMP, 25 mM TES (pH 7.5). The assay was usually performed during 5 min at 37°C (27°C for the procyclic forms). Each assay was run in triplicate. Although the absolute amount of cAMP produced was variable depending on the conditions employed, the relative activation of the cyclase compared with the basal activity in each case was usually greater than an order of magnitude. Conditions specific to individual experiments are given in the legends to figures or tables.

**Analysis of Cytoplasmic Enzyme Release**

In order to assess cell lysis after treatments inducing VSG release (trypsin, acid, peptides), alanine aminotransferase, a cytoplasmic marker enzyme in T. brucei (32) was assayed in the presence of Triton X-100 (0.1%, w/v) essentially as described by Bergmeyer et al. (33). After each experimental treatment the cells were separated from the incubation medium by centrifugation. The cellular pellets were resuspended in the assay buffer in the presence of Triton X-100 (0.1%, w/v) prior to assay of the activity. The percentage of the total cellular activity recovered in the supernatant, and the cellular pellet after each of the treatments was determined.

**Analysis of VSG Release**

**Immunodetection**—Cell pellets and supernatants were electrophoresed in SDS-10% polyacrylamide gels and analyzed by Western blotting as described previously (34, 16). The primary antibodies were the hyperimmune anti-AnTat 1.8 antibodies (dilution 1/100,000) or an anti-CRD antibody (kindly provided by P. Englund, Baltimore; dilution, 1/500). The secondary antibody used was anti-rabbit IgG alkaline-phosphatase conjugate from Promega.

**Surface Radiolabelling**—To assess VSG release quantitatively by a different method, surface iodination of intact trypanosomes was performed using IODO GEN (trademark of Pierce) essentially as described by Fraker and Speck (35). Cells (10⁸/ml) were incubated in phosphate-buffered saline (pH 7.6) in a glass test tube previously coated with IODO GEN, in the presence of carrier-free Na¹²¹ (100–500 μCi) for 10 min at 0°C. The reaction was terminated by the addition of 10 volumes of ice-cold buffer, and the cells were washed twice by centrifugation at 4°C followed by resuspension in a required buffer. The release of iodinated VSG was measured as described by Voorhees et al. (24). At various times samples of 6 × 10⁷ cells were removed from the incubation and separated from the extracellular medium by centrifugation.

Coat release was estimated by scintillation spectrophotometry of samples of the supernatant (600 μl) after adding sufficient carrier protein (RNase A, final concentration 0.05% w/v) to obtain complete precipitation of the release protein after addition of an equal volume of 10% (w/v) ice-cold trichloroacetic acid and centrifugation at 8,000 × g for 2 min. The pellet of precipitated protein was washed with ethyl ether and solubilized with 100 μl urea (8 M) and then subjected to liquid scintillation counting. Analysis of the released material using SDS-PAGE and autoradiography indicated that the acid-precipitated radioactivity was associated only with the VSG (Fig. 3B).

**RESULTS**

**Effect of Mild Acid and Trypsin on Bloodstream and Procydic Adenylyl Cyclase Activities**—Release of the VSG can be induced in vitro by hypotonic lysis (36) or cold, mild acid treat-
Effect of pH 5.5 buffer and trypsin treatments on bloodstream and procyclic adenylate cyclase activities

Bloodstream and procyclic trypanosomes preincubated either at 4°C in pH 5.5 buffer (60 min) or at 20°C in the presence of cycloheximide (30 min) and followed by trypsin (60 min) were assayed for adenylate cyclase for 5 min at 37°C (bloodstream forms) or 27°C (procyclics) as described under “Materials and Method.” Each value represents the mean ± S.E. of more than 10 determinations. The values given in parentheses represent the relative activity compared to the control (1). Incubations with only cycloheximide had no effect on adenylate cyclase activity.

| Experimental conditions | cAMP produced (pmol/min/10⁷ trypanosomes) |
|-------------------------|------------------------------------------|
|                         | Bloodstream variants                     |
|                         | AnTat 1.1      | AnTat 1.3      | AnTat 1.8      |
| pH 7.3 buffer (control) | 2.08 ± 0.24 (1) | 1.50 ± 0.17 (1) | 1.24 ± 0.45 (1) |
| pH 5.5 buffer           | 43.74 ± 6.19 (21.0) | 57.19 ± 2.93 (38.0) | 36.19 ± 2.80 (29.0) |
| pH 7.3 buffer + trypsin | 20.68 ± 4.80 (10.0) | 17.26 ± 1.70 (11.5) | 0.84 ± 0.25 (0.7) |
|                         | 2.93 ± 0.50 (1) | 36.19 ± 2.80 (29.0) | 1.50 ± 0.40 (0.5) |

Characteristics of the Trypsin-mediated Activation of Adenylate Cyclase and VSG Release—The effect of trypsin was monitored in clones with VSG sensitive or trypsin-resistant VSGs (respectively, AnTat 1.1 and 1.8). Activation of adenylate cyclase only occurred in the trypsin-sensitive AnTat 1.1 clone (Fig. 1A, squares). This process was detectable after a 15-min delay and reached a maximum value after 60 min (Fig. 1A, 1A). This time course did not parallel the release of the N-terminal domain of the VSG, which was probed with anti-VSG antibodies (Fig. 1B). The release of the N-terminal domain occurred without delay, was half-complete in 15 min (compare lane P with lane labeled P (15 min) in Fig. 1B, upper panel) and complete in 30 min (compare lanes P and S after 30, 60, and 120 min in Fig. 1B, upper panel). This situation was in contrast to that found for the AnTat 1.8 variant. The data in Fig. 1B (bottom panel) confirmed (41, 42) that VSG expressed in this clone was only slowly cleaved by trypsin. Of the total amount of VSG present in cells at the beginning of trypsin treatment (lane P, 0 min, Fig. 1B, bottom), approximately half remained after 120 min of exposure to trypsin (lane P, 120 min, Fig. 1B, bottom). It should be noted that there was always a low amount of VSG present in the supernatant of cells at the beginning of each experiment (Fig. 1B, lane S, 0 min), but the amount of released VSG did not increase during the experiment if trypsin was omitted (Fig. 1B, lane S, 30 min). We also noted that trypsin treatment of whole cells did not produce cleavage of adenylate cyclase, as determined by probing Western blots of SDS-PAGE gels of treated and untreated cells with anti-pESAG 4 antibodies (data not shown). These experiments suggested that the...
time courses of cyclase activation and proteolytic cleavage of VSG in the presence of trypsin were not concurrent.

We examined whether trypsin was required throughout the time course of cyclase activation. AnTat 1.1 trypanosomes were treated with trypsin for 15 min then soybean trypsin inhibitor was added, the cells were centrifuged and resuspended in a buffer supplemented with trypsin inhibitor before incubation at 4 or 20 °C for different periods. Under these conditions adenylate cyclase activation occurred as in the case of a continuous presence of trypsin, in a process dependent on temperature and requiring a lag phase of at least 15 min (Fig. 2A). In accordance with the data of Fig. 1, the 15-min preincubation with trypsin led to a virtually complete removal of the N-terminal domain of the VSG, although some VSG remained refractory to cleavage (data not shown). As shown in Fig. 2B, two distinct components were found to be released after the preincubation period, despite the inactivation of trypsin. In addition to the VSG still uncleaved by trypsin, anti-CRD antibodies, which only allow the detection of the C-terminal domain of the VSG after cleavage of the GPI anchor by the GPI-PLC (41), revealed the presence of a diffuse band with an apparent molecular mass of 30 kDa (Fig. 2B). This component could not be detected in case of a continuous presence of trypsin (conditions used in Fig. 1B), probably due to uninterrupted proteolysis. In contrast with the observations concerning the N-terminal domain of the VSG, the 30-kDa component was found to be released with a kinetics similar to that of the cyclase stimulation, with a maximum after 90 min (Fig. 2, A and B). Under conditions where the surface coat was removed (15-min incubation with trypsin then 60-min incubation without trypsin), the majority of the cells remained physiologically intact as judged by the low percentage of a cytoplasmic enzyme activity found in the supernatant of treated cells (Table IIA). These results are consistent with the view that the removal of the N-terminal domain of the VSG by trypsin is followed by an active process which requires physiological temperatures but not trypsin and which induces the progressive release of the CRD positive C-terminal domain. The kinetics of this process was similar to that of the activation of adenylate cyclase, suggesting a relationship between cyclase stimulation and release of the C-terminal domain of the VSG.

Characteristics of the pH 5.5-mediated Activation of Adenylate Cyclase and VSG Release—The time courses of acid-induced adenylate cyclase activation and VSG release demonstrated that both processes occurred in parallel and that the activity of the cyclase and the release of VSG both reached a maximum after 60 min (Fig. 3, A and C, top panel). The results presented in Fig. 3A and Table IIB show that under these conditions 45–50% of the total releasable VSG was found in the extracellular medium, and this released VSG was recognized by anti-CRD antibodies, indicating a GPI-PLC cleavage of the VSG anchor (29, 41) (Fig. 3C, bottom panel). Interestingly, a low residual level of VSG remained attached to the parasites even after 90 min of treatment (see lanes P in Fig. 3C, bottom panel).

The acid-induced adenylate cyclase activation was characterized further by varying the experimental conditions (Fig. 4). First, this stimulation was only transient and decreased with increasing incubation time in the swell medium used for the assay of activity (Fig. 4A). Second, the stimulation was reversed by a subsequent incubation at neutral pH only at physiological temperatures (compare the effect of 30 and 4 °C in Fig. 4B). Third, the addition of the protonophore FCCP, which causes an acidification of the cytosol (43), allowed adenylate cyclase activation at neutral pH (Fig. 4C). Taken together, these data indicated that the stimulation of adenylate cyclase by acid treatment is a transient process which occurs in response to a slight acidification of the cytosol. Moreover this process is reversed when cytoplasmic pH is returned to physiological values. These results strongly suggest that the stimulation of the cyclase does not result from cell breakage and are consistent with the observation that only 3.7% of a cytoplasmic marker is found in the supernatant of cells incubated for 60 min at 4 °C in pH 5.5 buffer (Table IIA).
The release of a cytoplasmic marker enzyme (A) and VSG (B) by AnTat 1.1 trypanosomes was measured. Trypanosomes were either preincubated at 4°C for 60 min in pH 5.5 buffer or at 20°C for 15 min with trypsin. In the latter case trypsin was subsequently inactivated with soybean inhibitor, the cells were centrifuged, resuspended in a buffer supplemented with protease inhibitors (soybean and leupeptin), and incubated at 20°C for a further 60 min. After both treatments, the cells were centrifuged and incubated in the swell dialysis assay medium for 5 min at 37°C. For control and treatment with myristoylated PKC inhibitor (100 μM Myr-αPKC-α), trypanosomes were incubated as above in the assay medium. After swell dialysis, the osmotic strength of the incubation medium was adjusted to 340 mOsm by the addition of KCl prior to centrifugation. The amount of alanine aminotransferase activity present in the supernatant and cellular pellets of control and treated cells was determined as described under “Materials and Methods.” Each value represents the mean ± S.E. of three determinations. B, measurement of VSG release. Surface-labeled trypanosomes were either preincubated at 4°C for 60 min in pH 5.5 buffer prior to incubation in swell dialysis assay medium (5 min, 37°C) or directly incubated in the assay medium supplemented with 100 μM myristoylated PKC inhibitor (Myr-αPKC-α), alone or in combination with 1.5 or 30 μM pCMPS. Control cells were incubated for 5 min at 37°C in the assay medium. After swell dialysis, the osmotic strength of the incubation medium was adjusted to 340 mOsm by the addition of KCl prior to centrifugation. The supernatants were analyzed for the release of the VSG as described under “Materials and Methods.” 100% release was defined as the amount present in the supernatant of cells lysed by osmotic shock. In this case the cells were resuspended in distilled water and incubated for 10 min at 37°C. Only VSG was detected in the labeled material released in the medium (Fig. 3B). Each value represents the mean of three determinations ± S.E.

| Treatments | Recovery of enzyme activity | | % | Pellet | % |
|---|---|---|---|---|---|
| Triton (0.1%) | 79.1 ± 4.1 | 105.0 ± 1.2 |
| pH 5.5 buffer | 3.7 ± 0.1 | 88.3 ± 0.8 |
| Trypsin | 3.5 ± 0.1 | 93.0 ± 1.0 |
| 100 μM Myr-αPKC-α | 4.9 ± 1.0 | 88.6 ± 3.5 |
| Control | 0.7 ± 0.1 | 97.0 ± 4.8 |

| Treatments | Labeled coat release | | % | Pellet | % |
|---|---|---|---|---|---|
| Hypotonic lysis | 100 |
| pH 5.5 buffer | 44.6 ± 0.3 |
| 100 μM Myr-αPKC-α | 79.0 ± 1.0 |
| Idem + 1.5 μM pCMPS | 77.6 ± 0.6 |
| Idem + 30 μM pCMPS | 82.6 ± 3.4 |
| Control | 1.7 ± 0.5 |

Adenylate Cyclase Activation Can be Uncoupled from VSG Release—All treatments and agents that activated adenylate cyclase also released CRD-positive VSG, indicating cleavage by a GPI-PLC (Figs. 2B, 3C, and 5, B and C). Therefore, we speculated that the activation of adenylate cyclase was dependent on GPI-PLC. In order to evaluate this hypothesis blood stream forms of a GPI-PLC null mutant cell line were subjected to acid treatment (4°C, 60 min) and then assayed for adenylate cyclase activity and VSG release. As shown in Fig. 6, in this mutant acid treatment stimulated adenylate cyclase activity (A), whereas no VSG was detected in the medium (B) under conditions where it was readily detectable in wild type AnTat 1.1 cells. The lack of VSG release was verified by DEAE-cellulose chromatography of the trypanosomes. Previous observations have revealed that bloodstream forms of T. brucei have their external surface covered with VSG appear in the void volume of DEAE columns, whereas those trypanosomes that have released their VSG remained attached to these columns. It was found that all of the acid-treated null mutant cells were recovered after elution on DEAE cellulose, whereas in the case of the wild type about 83% of the acid-treated cells remained adsorbed to the column. These observations indicated that adenylate cyclase activation can occur in the absence of any detectable VSG release.

Thus, while it was clear that activation of adenylate cyclase does not require VSG release, an alternative model involving cyclase-dependent activation of GPI-PLC remained possible. To test this idea, we exploited the observation that 100 μM pCMPS prevents adenylate cyclase activation under all treatments tested (acid, trypsin, and PKC inhibitors, Fig. 7A). This concentration of pCMPS is 50-fold lower than that reported to be necessary for inhibition of the trypanosomal GPI-PLC (50, 51).
Fig. 3. Mild acid treatment of cells also initiates concurrent activation of adenylate cyclase and release of the VSG. Bloodstream form (AnTat 1.1) trypanosomes (3 × 10⁷ cells/ml) were incubated in isosmotic phosphate buffer (pH 5.5, 4°C) for 90 min. At various times, samples (2 ml, 6 × 10⁷ cells) were removed from the incubation and centrifuged. Following separation of the pellets and supernatants, the pellets were resuspended in swell dialysis medium (0.1 ml, 4°C). Three samples (20 μl each) were analyzed for adenylate cyclase activity under conditions of swell dialysis (5 min, 37°C). The remaining portion (40 μl) was incubated under the same conditions. The cells were separated from the assay medium by centrifugation after adjustment of the osmotic strength of the medium to 340 mOsm by the addition of KCl. The pellets and supernatants from 10⁵ cells were analyzed for the presence of VSG as well as for the presence of the CRD found on the GPI anchor of the VSG. In addition, trypanosomes that had been previously surface radioiodinated were subjected to a parallel incubation that was conducted with the same protocol as that used with unlabeled cells. In this case the ¹²⁵I-labeled VSG present in the supernatants of cells that had been removed at various times and centrifuged was processed and subjected to liquid scintillation spectrophotometry as described under “Materials and Methods.” A shows the time course of activation of adenylate cyclase (●) and the release of the ¹²⁵I-labeled VSG (□). Each value represents the mean ± S.E. of three separate determinations. The measurements of VSG release are expressed as percentage of the total releasable VSG, i.e. the amount released by hypotonic lysis of an equivalent number of cells. ○, cAMP synthesis in control cells incubated at neutral pH. B shows that the labeled material released in the medium essentially contains VSG, as determined by Coomassie Blue staining (lanes 1-3) and autoradiography (lanes 4-6) of equivalent samples of the supernatants after hypotonic lysis (lanes 1 and 4) (pH 5.5) treatment for 20 min (lanes 2 and 5) and in control cells (lanes 3 and 6). The two lower bands present in lanes 1-3 are due to components present in the swell dialysis assay medium (see legend to Fig. 6). C shows the time course of release of the VSG and the cross-reacting determinant as assessed by antibody probing of Western blots of the supernatants that had been subjected to SDS-PAGE, following withdrawal from the incubation at the times indicated at the top of each lane. Lanes labeled C0 and C90 contained the pellets and supernatants of cells incubated at pH 7.5 for 0 and 90 min, respectively. In the upper portion of panel (C) the probe was conducted with polyclonal antibodies raised against the VSG, and in the lower portion of panel (C) the probe was conducted with the polyclonal anti-CRD antibodies.

Fig. 4. Characterization of the acid-induced activation of adenylate cyclase in AnTat 1.1 bloodstream forms. A shows the inhibition of adenylate cyclase stimulation as a function of the incubation time in swell dialysis medium prior to assay of the activity. Adenylate cyclase activity was first stimulated by a 30-min preincubation in pH 5.5 buffer at 4°C. The cells were then centrifuged, resuspended in swell dialysis medium, and incubated at 37°C. Samples taken at various times were assayed as described under “Materials and Methods.” The curves show the results obtained from two separate experiments. B presents the reversibility of acid-induced adenylate cyclase activation. The cells were treated at 4°C in pH 5.5 buffer (●). After 30 min, they were centrifuged, resuspended in a pH 7.5 buffer and incubated at either 30°C (○) or 4°C (□) for various periods prior to assay of adenylate cyclase activity. C shows the effect of H⁺ concentration and of the presence of a protonophore (FCCP) on the activity of adenylate cyclase. Cells were incubated at 4°C at various concentrations of H⁺ as indicated, in the presence (●) or absence (○) of the ionophore FCCP (1.2 μM). After 60 min, the cells were centrifuged and assayed for adenylate cyclase activity. Each value represents the mean ± S.E. of three separate determinations. Where no error bar is shown, the S.E. was less than the representation of the point. The strong stimulation at pH 5.0 is probably due to cellular toxicity.
The adenylate cyclase activity of bloodstream cells was assayed for 5 min at 37 °C in the presence of various inhibitors and activators at the indicated final concentrations. The results are expressed as relative activity (control = 1). The final concentration of protease inhibitors was 20 μg/ml leupeptin, 1 μM pepstatin A, 50 μM AEBSF, 10 μM E64, and 1 mM EDTA.

| Reagents                               | Relative activity |
|----------------------------------------|-------------------|
| None (control)                         | 1.0               |
| Me2SO (0.1%)                           | 1.7               |
| Protease inhibitors                    | 1.5               |
| PMA (40 μM)                            | 1.3               |
| DAG (125 μM)                           | 1.4               |
| PKC-Inhibiting agents                  |                   |
| PMA                                    |                   |
| DAG                                    |                   |
| PKC-stimulating agents                 |                   |
| PMA                                    |                   |
| DAG                                    |                   |

**DISCUSSION**

**Evidence for a Possible Relationship between VSG Release and Adenylate Cyclase Activation**—The results obtained with wild type bloodstream form trypanosomes were consistent with the view that processes that trigger the GPI-PLC-mediated release of the VSG also lead to an activation of adenylate cyclase. First, a series of kinetic studies demonstrated that the release of the VSG by treatments as different as mild acid or trypsin digestion led to a strong activation of adenylate cyclase, confirming similar observations made under other experimental approaches (23–25). Second, adenylate cyclase was not stimulated by trypsin in a clone whose coat was trypsin-resistant, indicating that trypsin-dependent activation of adenylate cyclase involves proteolysis of VSG. Third, this proteolysis-mediated activation of the cyclase was restricted to VSG coated bloodstream forms, since the same treatments did not activate adenylate cyclase in procyclic forms. Significantly these processes occurred in physiologically intact cells and were not the result of cellular breakage as evidenced by the low release of a cytoplasmic marker enzyme under the different experimental conditions employed and from direct morphological examination of the cells. It is unlikely that the 3–5% release of cytoplasmic proteins occurring in these experiments accounts for the depeolysis of the VSG on intact cells, since exogonously added VSG lipase does not seem to have access to the GPI on the surface of the plasma membrane. Moreover, the detailed analysis of the activation of adenylate cyclase by mild acid treatment showed this process to be transient, reversible, and linked to intracellular acidification, all characteristics unlikely to result from lysis of the cells. Interestingly, in the case of trypsin there was a lag period of 30–60 min between proteolytic cleavage of the VSG and the GPI-PLC-mediated release of the C-terminal domain and concomitant activation of the cyclase. This delay is similar to that observed during synchronous differentiation of bloodstream trypomastigotes into procyclic forms. During this process, the VSG also appears to undergo proteolytic cleavage prior to transient activation of adenylate cyclase (26, 42).

The biological significance of the relationship between VSG release and activation of the cyclase is unclear, but may relate to the triggering of a cell signaling process under stress conditions. In this regard it is interesting to note that trypanosomes from highly infected mice (around 10^9 parasites/ml of blood),

**Fig. 5. Inhibitors of PKC induce adenylate cyclase activation and VSG release in AnTat 1.1 bloodstream forms.** Bloodstream form trypanosomes were incubated for 5 min at 37 °C under swelling dialysis conditions in the presence or absence of the indicated compounds and assayed for cAMP production (A) and for VSG release (B and C). A shows the dose response curve of adenylate cyclase assayed in the presence of the indicated concentrations of Myr-γ PKC-α (○), Myr-γ PKC-ζ (△), or Myr-random peptide (▲). Each value represents the mean of three separate determinations and in all cases the S.E. was less than the replication of the point. B shows a Western blot analysis of supernatants of cells incubated under the same experimental conditions with the indicated inhibitor concentrations and probed with either anti-AnTat 1.1 VSG (top panels) or anti-CRD (bottom panels) antibodies. C shows Western blots of proteins present in the supernatant of cells incubated as indicated above without peptide (lane 1) or with Myr-γ PKC-α (lane 2), Myr-random peptide (lane 3), calphostin C (lane 4), and Myr-γ PKA (lane 5), and probed with either anti-AnTat 1.1 VSG (left panel) or anti-CRD (right panel) antibodies.

S. Rolin and P. Voorheis, unpublished data.
thus presumably subjected to growth limiting stress, reproducibly exhibiting higher basal levels of adenylate cyclase activity than cells isolated during lower parasitaemia. Stress-dependent VSG release and activation of adenylate cyclase may initiate a cascade that results in either a metabolic change or an altered pattern of gene expression that enables bloodstream forms of the parasite to adapt to environmental conditions.

Activation of Adenylate Cyclase and VSG Release Can Occur Independently—The GPI-PLC termed VSG lipase is expressed in bloodstream, but not in procyclic trypanosomes. Although there is considerable evidence to suggest that the membrane form of VSG is the biological substrate for the VSG lipase, direct evidence for a role of this enzyme in VSG release has remained elusive (52). In the present study we demonstrate that the release of the VSG does not occur in mutant cells that lack the gene for the VSG lipase. Thus, it is clear that in wild type cells VSG release is mediated through the action of the VSG lipase. The crucial observation that adenylate cyclase activation still occurred in VSG lipase null mutant cells despite the lack of VSG release leads to the inevitable conclusion that activation of adenylate cyclase does not require the release of the VSG. Conversely, VSG release can also occur in the absence of activation of the cyclase, as observed in the presence of low concentrations of pCMPS, which only affect the cyclase. These findings clearly demonstrate that VSG release and activation of adenylate cyclase are independent processes that normally occur in response to the same stimuli.

A Possible Role for PKC in Both VSG Release and Regulation of Adenylate Cyclase Activity—In T. brucei, protein kinase ac-

FIG. 6. VSG release is not required for activation of adenylate cyclase in bloodstream form trypanosomes. Bloodstream form trypanosomes of either the wild type (AnTat 1.1) or GPI-PLC null mutant were incubated at 4°C for 60 min either in isosmotic TES buffer (pH 7.5) or in isosmotic phosphate buffer (pH 5.5). At the end of the incubation a sample (2 ml, 6 x 10^7 cells) was removed and centrifuged. The cells were resuspended in swell dialysis medium (0.1 ml). Three samples (20 μl each) of the resuspended cells were analyzed for adenylate cyclase activity under conditions of swell dialysis (37°C, 5 min). The supernatants of 10^7 cells were analyzed by SDS-PAGE. A shows the relative activity of wild type and mutant cells. B shows the Coomassie Blue staining of proteins present in the supernatants of 10^7 cells incubated at either pH 7.5 (lanes c) or pH 5.5 (lanes a). The 40- and 35-kDa proteins present in all lanes represent components of the incubation assay medium and disappear if creatine kinase was omitted (data not shown). VSG is indicated by the arrow.

FIG. 7. VSG release can occur in the absence of activation of adenylate cyclase in the AnTat 1.1 bloodstream variant. A shows the effect of 100 μM pCMPS on adenylate cyclase activity. Bloodstream form trypanosomes (3 x 10^7 cells/ml) were preincubated either in isosmotic TES buffer with or without trypsin (30 min, 20°C) or in isosmotic pH 5.5 phosphate buffer (60 min, 4°C) as described in the legends to Figs. 1 and 3 and under “Materials and Methods.” The cells were then centrifuged and assayed for adenylate cyclase activity under swell dialysis conditions (5 min, 37°C) in the absence or presence of 100 μM pCMPS. The activity was also assayed in the presence of 100 μM Myr-ϕ PKC-α with or without pCMPS. The results are expressed as relative activity (control = 1). B shows the effect of the concentration of pCMPS on Myr-ϕ PKC-α-induced adenylate cyclase activation. Trypanosomes (3 x 10^7 cells/ml) were assayed under swell dialysis conditions in the presence of 100 μM Myr-ϕ PKC-α at various concentrations of pCMPS. Each value represents the mean ± S.E. of three separate determinations. Where no error bar is shown the S.E. was less than the representation of the point. C shows the effect of pCMPS on PKC inhibitor-induced VSG release. Bloodstream form trypanosomes were incubated under swell dialysis conditions (37°C, 5 min) in the presence or absence of 100 μM Myr-ϕ PKC-α and with or without 100 μM pCMPS as indicated. The cells were separated from the incubation medium by centrifugation after the osmotic strength was adjusted to 340 mOsM with KCl and the supernatants from 10^7 cells were subjected to Western blot analysis using either anti-AnTat 1.1 VSG (left panel) or anti-CRD (right panel) antibodies.
activated adenylate cyclase and induced VSG release. Third, random peptides or a pseudosubstrate inhibitor of PKA were without effect, even if myristoylated or palmitoylated. This last observation strongly suggests that the effect of the myristoylated PKC inhibitors was not simply due to perturbation of the plasma membrane by the nonspecific insertion of fatty acyl groups. Additional support for this conclusion can be found in the effect of NaF (10 mM), which inhibited the trypanosome adenylate cyclase activity both in isolated plasma membranes (11) and in osmotically permeabilized cells recovering from stimulation by mild acid treatment. Indeed, NaF has been reported to stimulate phosphorylation and inhibit protein phosphatases (58). Finally local anesthetics, which are indirect inhibitors of PKC in other cell types (59), also activated adenylate cyclase and VSG release in bloodstream trypanomastigotes (24, 25). A possible role for protein phosphorylation in the regulation of adenylate cyclase activity in T. brucei is consistent with the results obtained with other eukaryotes. For example, in the alga Chlamydomonas, Snell and collaborators (60, 61) presented evidence for inhibition of flagellar adenylate cyclase by phosphorylation, whereas several mammalian adenylate cyclases appear to be regulated by protein kinase (62–65). In the case of bloodstream forms of T. brucei it is tempting to speculate that PKC plays a role in a common signaling pathway that is involved in both adenylate cyclase activation and GPI-PLC mediated VSG release in response to cellular stress. Studies are currently under way to evaluate this hypothesis.

Acknowledgments—We thank Dr. A. Vandermeers (Faculty of Medicine and Pharmacy, Free University of Brussels), I. Graff and L. B. Beghin for help in the realization of these experiments, S. Van Assel for invaluable help in the preparation of the manuscript, D. Franckx for photography, P. Englund (Baltimore) for anti-CRD antibodies, and Pr. C. Sergheraert (Institut Pasteur de Lille, Lille, France) for PKC inhibitors.

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Simultaneous but Independent Activation of Adenylate Cyclase and Glycosylphosphatidylinositol-Phospholipase C under Stress Conditions in *Trypanosoma brucei*

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*J. Biol. Chem.* 1996, 271:10844-10852. doi: 10.1074/jbc.271.18.10844

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