Activation of a pertussis-toxin-sensitive guanine-nucleotide-binding regulatory protein during desensitization of Dictyostelium discoideum cells to chemotactic signals

Snaar-Jagalska, B. Ewa; Es, Saskia van; Kesbeke, Fanja; Haastert, Peter J.M. van

Published in:
European Journal of Biochemistry

DOI:

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1991

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Activation of a pertussis-toxin-sensitive guanine-nucleotide-binding regulatory protein during desensitization of *Dictyostelium discoideum* cells to chemotactic signals

B. Ewa Snaar-Jagalska¹, Saskia Van ES¹, Fanja Kesbeke¹ and Peter J. M. Van Haastert²

¹ Cell Biology and Genetics Unit, Zoological Laboratory, Leiden University, Leiden, The Netherlands
² Department of Biochemistry, University of Groningen, Groningen, The Netherlands

(Received May 30, 1990) — EJB 90 0620

The chemoattractant cAMP induces the activation of adenylate cyclase in *Dictyostelium discoideum*. Upon prolonged incubation with cAMP, cells become desensitized via two distinct processes: a decrease in the number of available cAMP-binding sites (down regulation) and modification of the receptor (presumably via phosphorylation) correlated with adaptation. These processes occur simultaneously, but differ in the cAMP dose dependency and reversibility. In this study we investigated the mechanism of adaptation; cells were incubated with a cAMP analog to induce desensitization mediated by adaptation. The cells were then washed, lysed and the interaction between cAMP receptor, guanine-nucleotide-binding regulatory proteins (G proteins) and GTP was investigated.

(1) cAMP receptors that are phosphorylated *in vivo* remain phosphorylated for at least 45 min after lysis. (2) Desensitization did not alter basal cAMP binding to the receptor nor the inhibitory effect of guanosine 5'-[y-thio]triphosphate (GTP[S]) on this binding. (3) The stimulatory effect of cAMP on GTP[S] binding was also unchanged, while basal GTP[S] binding and the kinetics of binding were only slightly different. (4) Basal high-affinity GTPase activity was not altered but cAMP stimulation was reduced from 43 ± 7% in control lysates to 14 ± 4% in lysates from desensitized cells. (5) cAMP stimulation of GTPase was decreased by pretreatment of cells with pertussis toxin from 43 ± 7% to 17 ± 8% but this was not further altered in lysates from desensitized pertussis-toxin-treated cells. These observations indicate that during desensitization the phosphorylated receptor can still interact with G proteins. Furthermore, desensitization did not affect cAMP stimulation of GTP[S] binding but strongly reduced cAMP stimulation of GTPase, suggesting that a G protein becomes activated. This G protein is pertussis toxin sensitive and may be the inhibitor G protein (G_i). This would imply that adenylate cyclase desensitizes because G_i becomes activated.

The chemoattractant cAMP induces the activation of adenylate cyclase in *Dictyostelium discoideum*. Upon prolonged incubation with cAMP, cells become desensitized via two distinct processes: a decrease in the number of available cAMP-binding sites (down regulation) and modification of the receptor (presumably via phosphorylation) correlated with adaptation. These processes occur simultaneously, but differ in the cAMP dose dependency and reversibility. In this study we investigated the mechanism of adaptation; cells were incubated with a cAMP analog to induce desensitization mediated by adaptation. The cells were then washed, lysed and the interaction between cAMP receptor, guanine-nucleotide-binding regulatory proteins (G proteins) and GTP was investigated.

(1) cAMP receptors that are phosphorylated *in vivo* remain phosphorylated for at least 45 min after lysis. (2) Desensitization did not alter basal cAMP binding to the receptor nor the inhibitory effect of guanosine 5'-[y-thio]triphosphate (GTP[S]) on this binding. (3) The stimulatory effect of cAMP on GTP[S] binding was also unchanged, while basal GTP[S] binding and the kinetics of binding were only slightly different. (4) Basal high-affinity GTPase activity was not altered but cAMP stimulation was reduced from 43 ± 7% in control lysates to 14 ± 4% in lysates from desensitized cells. (5) cAMP stimulation of GTPase was decreased by pretreatment of cells with pertussis toxin from 43 ± 7% to 17 ± 8% but this was not further altered in lysates from desensitized pertussis-toxin-treated cells. These observations indicate that during desensitization the phosphorylated receptor can still interact with G proteins. Furthermore, desensitization did not affect cAMP stimulation of GTP[S] binding but strongly reduced cAMP stimulation of GTPase, suggesting that a G protein becomes activated. This G protein is pertussis toxin sensitive and may be the inhibitor G protein (G_i). This would imply that adenylate cyclase desensitizes because G_i becomes activated.
loration of surface receptors but does not induce receptor activation or its modification (phosphorylation) [15].

In vertebrates, communication between hormone-occupied receptors and adenylate cyclase occurs via two distinct regulatory proteins, the stimulatory G protein (Gs) and Gi, that stimulate and inhibit adenylate cyclase activity, respectively [19]. Pertussis toxin catalyzes specifically the ADP ribosylation of several a subunits of GTP-binding proteins, including Gi, the other G protein (Gt) and transducin [20–23]. The role of phosphorylation of the signal-transducing components in desensitization has been extensively studied [24–27]. The experiments suggest that receptor phosphorylation is involved in adenylate-cyclase desensitization, presumably by decreasing the coupling between the receptor and Gi [28].

Genetic and biochemical studies of D. discoideum indicate that the surface cAMP receptors are coupled with effectors via G proteins. The primary structure of the cAMP receptor contains seven putative transmembrane domains, a structure identical to other G-protein-linked receptors [29]. Genes for two G-protein a subunits have been cloned from D. discoideum [30, 31]. Furthermore, the affinity of the cAMP receptor is reduced by guanine nucleotides [32], and the occupied receptor stimulates GTP binding [33] and GTP hydrolysis [34]. In addition, GTP stimulates inositol 1,4,5-trisphosphate formation in permeabilized cells [6] and adenylate cyclase is stimulated in membranes [35, 36] or inhibited by GTP [36] depending on the conditions used.

In this study we have investigated further the adaptation mechanism of cAMP-stimulated adenylate cyclase. Previously, we demonstrated that inhibition of adenylate cyclase by GTP was blocked by treatment of the cells with pertussis toxin [36]. Furthermore, when cells were desensitized by exposure to a cAMP agonist, stimulation by guanine nucleotides was lost, while inhibition was retained. These results suggested that D. discoideum adenylate cyclase may be regulated by a Gt-like and Gi-like activities, and that these activities are modulated by the phosphorylation of the cAMP receptor. This hypothesis was supported by the observation that treatment of cells with pertussis toxin resulted in a strongly reduced desensitization of adenylate cyclase whereas phosphorylation of the receptor was not altered [37]. In the present study we have investigated the interaction between phosphorylated cAMP receptors and G proteins in lysates from desensitized D. discoideum cells. The results show that receptors remain phosphorylated in the lysate and the phosphorylated receptor is still able to interact with the G protein, since the inhibitory effect of GTP[S] on cAMP binding and the stimulatory effect of cAMP on GTP[S] binding were not altered. In contrast, chemoattractant-stimulated GTPase activity was decreased in lysates from desensitized cells; this GTPase is pertussis toxin sensitive. These results suggest that a phosphorylated receptor may interact with a permanently activated pertussis-toxin-sensitive G protein, which leads to desensitization of adenylate cyclase.

MATERIALS AND METHODS

Materials

[2,8-3H]cAMP (44.6 Ci/mmol) was obtained from Amersham. [35S]GTP[S] (1355 Ci/mmol) and [y-32P]GTP (37.9 Ci/mmol) were from New England Nuclear. Adenosine 5'-[gamma-thio]triphosphate, GTP, GTP[S], the (Sp)isomer of adenosine 3',5'-monophosphorothioate, (Sp)-cAMP[S], 2'-deoxyadenosine 3',5'-phosphate (dcAMP), adenosine 5'-(beta, gamma-imino)triphosphate, creatine phosphate and creatine kinase were purchased from Boehringer. Anti-(cAMP receptor)antiserum was a generous gift of Dr P. N. Devreotes.

Culture conditions and cell treatment

D. discoideum cells (strain NC-4) were grown in association with Escherichia coli 281 on a buffered glucose-peptone medium [11]. Cells were harvested in the late log phase with 10 mM sodium/potassium phosphate buffer, pH 6.5 (cAMP buffer), washed and starved in a shaking suspension in phosphate buffer at a density of 10⁷ cells/ml. After 5 h the cells were collected by centrifugation, washed twice and resuspended in phosphate buffer at a density of 10⁸ cells/ml. During the experiment, the cell suspension was aerated at a flow rate of about 15 ml air/ml suspension.

Cells were incubated for 15 min at 20°C with 3 μM (Sp)-cAMP[S], then washed by one of two methods. In the first method, cells were washed three times with phosphate buffer at 0°C, resuspended in this buffer at a density of 10⁹ cells/ml and used for the measurement of cAMP binding to cells or activation of adenylate cyclase in vivo. In the second method, cells were washed at 0°C twice with phosphate buffer and once with buffer A (40 mM Hepes/NaOH, 0.5 mM EDTA, 250 mM sucrose, pH 7.7). Cells were lysed at a density of 10⁶ cells/ml by pressing them through a Nuclepore filter (pore size 3 μM) at 0°C. The lysates were used immediately for the detection of cAMP binding, GTP[S] binding, GTPase and adenylate cyclase activity.

Down regulation of cAMP receptors and desensitization of adenylate cyclase stimulation in vivo

The binding of [3H]cAMP to cells treated with (Sp)-cAMP[S] was detected in a volume of 0.1 ml containing phosphate buffer, 10 mM dithiothreitol, 5 mM [3H]cAMP and 8×10⁶ cells. The incubation period was 75 s at 0°C followed by centrifugation of the cells through silicon oil as described [38]. Non-specific binding was determined by including 0.1 mM cAMP in the incubation mixture and was subtracted from all data shown.

Desensitization of adenylate cyclase was determined as follows. After desensitization with (Sp)-cAMP[S], cells were washed and re-stimulated with 10 mM dithiothreitol and 10 μM cAMP. The reaction was stopped after 0 min and 5 min by adding 0.1 ml 3.5% perchloric acid. Lysates were neutralized with 50 μl KHCO₃ (50% saturated at 20°C) and the cAMP content was measured using the isotope-dilution assay [39].

cAMP binding in lysates

cAMP binding was measured in a volume of 0.1 ml containing buffer A, 5 mM [3H]cAMP, 5 mM dithiothreitol, 0.1 mM GTP[S] and 80 μl lysate. The incubation time was 5 min at 0°C. Samples were centrifuged for 3 min at 10000 x g, the supernatant was aspirated and the pellet was dissolved in 80 μl 1% SDS; 1 ml Emulsifier (Packard) was added and radioactivity was determined. Non-specific binding was measured by including 0.1 mM cAMP in the incubation mixture and subtracted from all data.

GTP[S] binding in lysates

The binding of [35S]GTP[S] to lysates was detected in 0.1 ml containing buffer A, 0.1 nM [35S]GTP[S], 1 mM MgCl₂...
and 80 μl lysate. Stimulation of GTP[S] binding was measured in the presence of 10 μM cAMP. The incubation time was 30 min at 0°C. Samples were centrifuged for 3 min at 10000 × g, the supernatant was aspirated and the radioactivity of the pellet was determined as described for the cAMP-binding assay. Non-specific binding was measured by including 0.1 mM GTP in the incubation mixture and subtracted from all data.

GTPase assay

GTPase activity in the lysates from control and desensitized cells was determined in a reaction mixture of 0.1 ml containing [γ-32P]GTP (0.1 μCi/assay), 2 mM MgCl2, 0.1 mM EGTA, 0.2 mM adenosine 5'-[β,γ-imino]triphosphate, 0.1 mM ATP[S], 10 mM dithiothreitol, 5 mM creatine phosphate, 0.4 mg/ml creatine kinase, 2 mg/ml bovine serum albumin (purified) and 30 μl lysate in 40 mM Hepes/NaOH, pH 7.7 as described [34]. The reaction was stopped after 3 min by the addition of 0.5 ml phosphate buffer, pH 2.0, containing 5% (mass/vol.) activated charcoal. The reaction tubes were centrifuged for 5 min at 10000 × g at 4°C and the radioactivity of the supernatant was determined using Cerenkov radiation.

RESULTS

Prolonged exposure of D. discoideum cells to the chemotactic attractant cAMP, leads to desensitization of adenylate cyclase by adaptation and down regulation of surface receptors [14, 15]. These processes occur simultaneously, but can be distinguished since they differ is the cAMP dose dependency and reversibility after removal of cAMP [4, 13–15]. Adaptation of adenylate cyclase is correlated with receptor phosphorylation [8, 9]. The aim of the present study is to analyse the interactions between the phosphorylated cAMP receptor and GTP-binding proteins in lysates from desensitized D. discoideum cells. Therefore conditions for maximal adaptation and minimal down regulation were established. Subsequently we investigated the fate of the receptor in vivo after phosphorylation in vivo.

Desensitization of adenylate-cyclase stimulation and down regulation of cAMP receptors

The difference in dose dependency of adaptation and down regulation were used to obtain desensitization of adenylate cyclase caused mainly by adaptation. Cells were incubated for 15 min with low concentrations (3 μM) of the non-hydrolyzable cAMP analog (Sp)-cAMP[S]. Cells were washed extensively at 0°C and dcAMP-stimulated production of cAMP (Fig. 1A) and binding of 5 nM [3H]cAMP to cell surface receptors (Fig. 1B) was measured. Treatment of cells with (Sp)-cAMP[S] induced ≈ 20% loss of binding activity and ≈ 80% desensitization. As was shown previously [37], pertussis toxin did not affect the loss of binding activity but strongly diminished desensitization. The results of Fig. 1 indicate that 3 μM (Sp)-cAMP[S] induces 80% desensitization of adenylate cyclase which is composed of 20% pertussis-toxin-insensitive down regulation of surface receptors and 60% pertussis-toxin-sensitive adaptation.

Kinetics of receptor dephosphorylation in vitro

During desensitization of Dictyostelium cells, the surface receptor becomes phosphorylated; this phosphorylation is associated with a shift in the electrophoretic mobility of the receptor as determined by SDS/PAGE [8, 9]. Upon removal of cAMP the receptor becomes dephosphorylated and the responsiveness of the cells recover; these processes do not occur at 0°C [8, 9]. However, little is known about the kinetics of receptor dephosphorylation in vitro. For our purpose it is necessary to know whether the receptor stays phosphorylated in vitro during our experimental conditions. Therefore, D. discoideum cells were incubated at 20°C for 15 min with 3 μM (Sp)-cAMP[S] or buffer, washed and lysed at 0°C. At the indicated times (Fig. 2) samples of the lysates were taken and added to SDS/PAGE sample buffer. Proteins were separated by SDS/PAGE and the receptor was detected by immunoblotting. In the lysates from control cells more than 90% of the receptors were in the R form (40 kDa). The incubation of cells with (Sp)-cAMP[S] induced a transition of the receptor from the R to the D form (43 kDa). In the homogenates from desensitized cells, 50–60% of the receptor reached the D form and remained in the D form during 60 min after lysis. A slight reduction of the amount of the receptor protein during the experiment was observed. All subsequent experiments were performed within 30–45 min after lysis.
Fig. 2. Kinetics of the cAMP receptor dephosphorylation in lysates from control and desensitized cells. Cells were incubated at 20°C for 15 min in the absence or presence of 3 μM (Sp)-cAMP[S], washed and lysed at 0°C. At the indicated times, samples of lysate were taken and added to sample buffer. Proteins were separated by SDS/PAGE, transferred to nitrocellulose, stained with the anti-receptor antiserum followed by 125I-labelled protein A and autoradiography. Lanes 9 and 10 represent standard samples of the unmodified form of the receptor (R) and the modified form (D) obtained from cells stimulated with 0.5 μM cAMP + 10 mM dithiothreitol.

Fig. 3. Effect of cAMP on GTP[S] binding to lysates from control and desensitized cells. (A) Cells were incubated at 20°C for 15 min in the absence or presence of 3 μM (Sp)-cAMP[S], washed and lysed at 0°C. Association of 0.1 nM [35S]GTP[S] to lysates from control and desensitized cells in the absence (○, △) or presence (●, ▲) of 10 μM cAMP was determined. (B) A semi-logarithmic plot of the data from Fig. 3A; b(x) equals the specific binding at equilibrium (30 min) and b(t) at t min. The results shown are the means of three experiments performed in triplicate.

cAMP binding in lysates from control and desensitized cells

The lysates from control and desensitized cells were incubated with 5 nM [3H]cAMP to reach binding equilibrium in the absence and presence of 0.1 mM GTP[S] (Table 1). Binding of cAMP was reduced ~70% in the presence of GTP[S]. The inhibition by GTP[S] was essentially identical in both lysates; this was observed when the binding assay was performed directly after lysis or 30 min later. Furthermore, shorter desensitization times with (Sp)-cAMP[S] (1.5 and 3 min) yielded similar results (data not shown).

GTP[S] binding in lysates from control and desensitized cells

The association kinetics of [35S]GTP[S] binding to lysates from control and desensitized cells are presented in Fig. 3. Binding equilibrium in both lysates was reached within 15 min (Fig. 3A). Analysis of the association rate of GTP[S] binding (Fig. 3B) indicates one binding type with a half-time of association of about 2.3 min and 3.3 min for lysates from control and desensitized cells, respectively. Equilibrium GTP[S] binding to lysates from desensitized cells was about 25% lower than to control lysates. However stimulation of GTP[S] binding by 10 μM cAMP had the same relative level for both lysates (Fig. 3A); the stimulation by cAMP was 101 ± 7% and 128 ± 14% above basal levels for lysates from control and desensitized cells, respectively. The kinetics of GTP[S] binding were also slightly changed by cAMP. The half-time of association in the presence of cAMP was 4.1 and 4.2 min for lysates from control and desensitized cells, respectively.

These observation suggest that desensitization conditions affect total GTP[S] binding and its kinetics but does not disturb the signal-transduction pathway from phosphorylated CAMP receptor to a putative G protein, since cAMP binding to lysates from desensitized cells is still altered by guanine nucleotides and alternatively, cAMP still increases GTP[S] binding to these lysates.

Characteristics of GTPase activity

It was shown previously that GTP hydrolysis in D. discoideum membranes is caused by at least two enzymes with high (K_m = 6.5 μM) and low (K_m > 1 mM) affinity. The high-affinity GTPase is stimulated by cAMP, with half-maximal effects at a cAMP concentration of 3 μM. Treatment of wild-type cells with pertussis toxin decreased the cAMP-induced stimulation of GTPase activity [34].
Table 2. GTPase activity in lysates from control, desensitized and pertussis-toxin-treated cells

Cells were starved for 5 h in the absence or presence of 0.1 μg/ml pertussis toxin (PT), washed and incubated for 15 min with or without 3 μM (Sp)-cAMP[S] at 20°C. Cells were washed and lysed at 0°C. GTP hydrolysis by high-affinity GTPase was determined in the absence and presence of 3 μM cAMP at a GTP concentration of 10 nM. GTP hydrolysis by low-affinity GTPase was determined in the presence of 0.1 mM GTP. Means ± SD of three experiments are presented with 100% = 3.1 pmol Pi hydrolyzed·min⁻¹·mg protein⁻¹ for high-affinity GTPase and 100% = 6.1 nmol Pi·min⁻¹·mg protein⁻¹ for low-affinity GTPase.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>GTPase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>high affinity</td>
</tr>
<tr>
<td></td>
<td>−cAMP</td>
</tr>
<tr>
<td>Control</td>
<td>100 ± 3</td>
</tr>
<tr>
<td>PT</td>
<td>102 ± 5</td>
</tr>
<tr>
<td>Desensitized</td>
<td>100 ± 6</td>
</tr>
<tr>
<td>PT desensitized</td>
<td>104 ± 6</td>
</tr>
</tbody>
</table>

Table 3. Effects of temperature on cAMP-induced adaptation of adenylate cyclase, receptor phosphorylation and decrease of GTPase stimulation in lysates from desensitized cells

<table>
<thead>
<tr>
<th>Response</th>
<th>t₁/₂ at 20°C</th>
<th>Ratio 20°C/0°C</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adaptation guanylate cyclase</td>
<td>≈0.067</td>
<td>&gt;30</td>
<td>[35]</td>
</tr>
<tr>
<td>Adaptation adenylate cyclase</td>
<td>≈2</td>
<td>2</td>
<td>[7, 35, 39]</td>
</tr>
<tr>
<td>Down regulation</td>
<td>≈2</td>
<td>2</td>
<td>[40, 41]</td>
</tr>
<tr>
<td>Receptor phosphorylation</td>
<td>≈2</td>
<td>≈3</td>
<td>[19, 43]</td>
</tr>
<tr>
<td>Decrease of GTPase stimulation</td>
<td>≈2</td>
<td>≈2</td>
<td>this report</td>
</tr>
</tbody>
</table>

In Table 2, results are shown of GTPase activity in lysates from wild-type and pertussis-toxin-treated cells before and after desensitization with 3 μM (Sp)-cAMP[S]. GTPase activity was measured at 10 μM GTP; at this concentration the high-affinity enzyme is detected more accurately and stimulation by cAMP is optimal. Basal high- and low-affinity GTPase were not significantly changed by the treatment of cells with pertussis toxin or (Sp)-cAMP[S]. About two-thirds of the cAMP-stimulated GTPase activity was blocked in lysates prepared from pertussis-toxin-treated cells (from 43 ± 7% to 17 ± 8% stimulation) suggesting that at least half of the cAMP-stimulated GTP hydrolysis resulted from pertussis-toxin-sensitive G protein(s). Chemoattractant stimulated GTPase activity was also decreased from 43 ± 7% to 14 ± 4% in lysates prepared from desensitized cells. In the lysates from desensitized pertussis-toxin-treated cells, GTPase activity was stimulated by cAMP to the same level as in lysates prepared from cells that were treated with either pertussis toxin or (Sp)-cAMP[S]. This suggests that pertussis toxin treatment and desensitization by (Sp)-cAMP[S] in vivo lead to the alteration of the same signal-transduction component, by which stimulation of GTPase activity by cAMP is decreased.

The decrease of GTPase stimulation by chemoattractant in lysates from desensitized cells was dependent on the duration of the incubation with (Sp)-cAMP[S]. About half of the total loss occurred after ≈2 min incubation at 20°C with 3 μM (Sp)-cAMP[S] (Fig. 4). The temperature dependency was determined by incubating D. discoideum cells for 3 min with 3 μM (Sp)-cAMP[S] at 0°C and 20°C. Cells were washed extensively at 0°C, lysed and GTPase stimulation by cAMP was determined. Desensitization of cells at 0°C caused a decrease of cAMP stimulation of GTPase activity from 46 ± 3% to 33 ± 6%, while desensitization at 20°C reduced cAMP stimulation of GTPase to 16 ± 4%.

DISCUSSION

In D. discoideum, cAMP binds to cell surface receptors and induces the transient activation of adenylate cyclase, which is followed by desensitization that is due to down regulation and adaptation. These processes may occur simultaneously, show similar kinetics but differ in the cAMP dose dependency and reversibility after removal of cAMP [14, 15]. Down regulation
has been correlated with cAMP-induced reduction of the number of detectable cAMP-binding sites [4, 13–15], while adaptation is associated with phosphorylation of the cAMP receptor [8, 9]. Pertussis toxin alters adaptation of adenylate cyclase without having an effect on receptor down regulation and receptor modification (37, Fig. 1). These observations indicate that pertussis toxin specifically inhibits adaptation of adenylate cyclase and that receptor phosphorylation is not sufficient for adaptation. It is likely that a pertussis toxin substrate is directly involved in the regulation of the adaptation process in D. discoideum. Previously we have observed that in D. discoideum, adenylate cyclase may be regulated by G_{i}-like and G_{i}-like activities. The action of G_{i}, but not G_{i}, was lost during desensitization in vivo [36]. This suggests a change in the coupling between receptor, putative G proteins and adenylate cyclase during desensitization.

In the present report we investigated the interaction between phosphorylated-cAMP receptors and GTP-binding proteins in lysates from desensitized cells. The major findings are the following: (1) the cAMP receptor remains phosphorylated in lysates for at least 45 min after lysis of desensitized cells. (2) The phosphorylated receptor is still capable of cAMP binding and interaction with G proteins. (3) Stimulation of GTPS binding by cAMP is not changed, while GTP[S] binding and kinetics are slightly different; (4) basal and low-affinity GTPases are not reduced; (5) GTPase activity stimulated by cAMP is reduced to one-third in the lysates from either desensitized or pertussis-toxin-treated cells (the effects of desensitization and pertussis toxin were not additive. (6) The decrease of chemoattractant-stimulated GTPase during desensitization is rapid (t_{1/2} \approx 2 min) at 20°C and about two-fold slower at 0°C. Similar kinetics, dose and temperature dependence have been reported for adaptation of adenylate cyclase and not for adaptation of guanylate cyclase (Table 3). These data suggest that adaptation of adenylate cyclase and the decrease of cAMP-stimulated GTPase are correlated and may have a causal relationship.

Treatment of cells with pertussis toxin decreased the cAMP-induced stimulation of GTPase from 43 ± 7% to 17 ± 8%, suggesting that at least half of the GTP hydrolysis resulted from the action of a pertussis-toxin-sensitive G protein. The presence of a pertussis toxin substrate in D. discoideum has been suggested [37, 40], but not identified so far. During ADP ribosylation, catalysed by pertussis toxin, one specific polypeptide (28 kDa) becomes ADP ribosylated [40]. This band can be an unusual pertussis toxin substrate. cAMP-stimulated GTPase activity is also decreased to the same level in lysates prepared from desensitized cells and in lysates from desensitized pertussis-toxin-treated cells. This suggests that pertussis toxin treatment and desensitization of adenylate cyclase by (Sp)-cAMP[S] in vivo resulted in the alteration of the same G protein. In D. discoideum, adenylate cyclase can be stimulated and inhibited by GTP[S]. Pertussis toxin did not affect the stimulation of adenylate cyclase but nullified the inhibition by GTP[S], consistent with the hypothesis that this toxin probably catalyses the ADP ribosylation of a specific G_{i} protein and blocks the stimulation of GTPase. Furthermore, the action of G_{i}, but not G_{i}, was lost during desensitization in vivo [36, 37].

Previous and present observations are combined in a model as shown in Fig. 5. Occupation of the surface cAMP receptor leads to the activation of a G_{i}-like G protein and the activation of adenylate cyclase. Prolonged occupation of the receptor induces phosphorylation of the receptor. This phosphorylation of the receptor does not prevent its interaction with G proteins, but it induces the preferential interaction with a G_{i}-like G protein. In addition, the GTPase activity of this G_{i}-like G protein is inhibited, thus this pertussis toxin substrate becomes more permanently activated thereby counteracting the action of G_{i} and therefore inducing desensitization. The model predicts that desensitization will not occur in the absence of receptor phosphorylation or in the absence of the activation of G_{i} (e.g. by treatment with pertussis toxin as was shown previously [37]). The molecular mechanisms of the inhibition of cAMP-stimulated GTPase during desensitization is presently unknown. However, the phosphorylation of the G_{i}-like G protein cannot be excluded.

In vertebrates, the role of phosphorylation of the signal-transducing components in desensitization has been extensively studied, suggesting that receptor phosphorylation is involved in adenylate-cyclase desensitization, presumably by decreasing receptor-G_{i} coupling [28]. The β2-adrenergic receptor that is phosphorylated by protein kinase A has a reduced ability to activate G_{i} in a reconstitution system [24]. Also protein kinase C and adrenergic receptor kinase phosphorylate the pure β2-adrenergic receptor [25]. Furthermore desensitization involves functional alteration of the G_{i} protein [41] and an increase of the apparent level of G_{i} by altering the G_{i}/G_{i} ratio [42]. A purified G_{i} subunit from liver can be phosphorylated by protein kinase C in vitro which suppresses the ability of G_{i} to inhibit adenylate cyclase [43]. The phosphorylation occurs on the xi subunit of G_{i} and is promoted by factors that dissociate xi from β2 subunits, suggesting that activation of G_{i} in vivo might be essential for covariant modification to occur. No evidence for the phosphorylation of G_{i} in intact cells has been reported. There is as yet no evidence documenting the physiological relevance of the phosphorylation of the purified catalytic unit of adenylate cyclase [44]. These findings indicate that in vertebrates ligand-induced receptor phosphorylation is involved in adenylate cyclase desensitization and decreased receptor—G-protein coupling. This mechanism is not observed in D. discoideum. The phosphorylated receptor remains available for interaction with the GTP-binding proteins and probably preferentially couple to and permanently activate a pertussis-toxin-sensitive G protein, which causes desensitization of adenylate cyclase.

We gratefully acknowledge Peter Devreotes for the generous gift of anti-receptor antisera and Theo Konijn for critical reading of the manuscript. We thank Peter Devreotes and Robert Gundersen for discussions and suggestions. This work was supported by the Organization for Fundamental Medical Research (FUNGO) and the C. and C. Huygens Fund, which are subsidized by the Netherlands Organization for the Advancement of Pure Scientific Research (NWO).

REFERENCES
