

Original Article

## **Pertussis toxin inhibits cAMP-induced desensitization of adenylate cyclase in *Dictyostelium discoideum***

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### **Abstract**

cAMP binds to surface receptors of *Dictyostelium discoideum* cells, transducing the signal to adenylate cyclase, guanylate cyclase and to chemotaxis. The activation of adenylate cyclase is maximal after 1 min and then declines to basal levels due to desensitization, which is composed of two components: a rapidly reversible adaptation process, and a slowly reversible down-regulation of cAMP receptors. Adaptation is correlated with receptor phosphorylation.

The chemotactic response and the cAMP-induced cGMP response were not significantly altered in *D. discoideum* cells pretreated with pertussis toxin. The initial increase of cAMP levels was identical in control and toxin treated cells, suggesting that activation of adenylate cyclase was also not affected. However, cAMP synthesis continued in toxin treated cells, due to a strongly diminished desensitization. Pertussis toxin inhibited the adaptation of adenylate cyclase stimulation, but not the down-regulation or phosphorylation of the cAMP receptors. Adenylate cyclase in *D. discoideum* membranes can be stimulated or inhibited by GTP, depending on the conditions used. Pertussis toxin did not affect the stimulation of adenylate cyclase but nullified the inhibition. In membranes from desensitized control cells, stimulation of adenylate cyclase by GTP was lost, whereas inhibition was retained. Stimulation of adenylate cyclase in membranes from desensitized pertussis toxin treated cells was diminished but not absent. These results indicate that receptor phosphorylation is not sufficient for adaptation of adenylate cyclase, and that a pertussis toxin substrate, possibly Gi, is also involved in this process.

**Abbreviations used:** ATP $\gamma$ S – Adenosine 5'-0-(3-Thiotriphosphate), GTP $\gamma$ S – Guanosine 5'-0-(3-thiotriphosphate), (Sp)-cAMPS – Adenosine 3',5'-monophosphorothioate-Sp-isomer, dcAMP – 2'-deoxyadenosine 3',5'-monophosphate, HEPES – N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, DTT – Dithiothreitol, buffer A – 10 mM KH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub>, pH 6.5, buffer B – 40 mM HEPES/NaOH, 0.5 mM EDTA, 250 mM sucrose, pH 7.7

### **Introduction**

In the cellular slime mold *D. discoideum* cAMP has been identified as an extracellular hormone-like

signaling molecule [1]. cAMP is detected by specific cell surface cAMP-receptors, and induces several intracellular responses including the activation of adenylate and guanylate cyclase (see 2–4). In

addition, chemotaxis is induced [1] and repeated pulses of cAMP accelerate differentiation [5]. Intracellular cGMP reaches a peak after 10 s, and has returned to basal levels at about 30 s after stimulation; cGMP is probably involved in the cAMP-induced chemotactic reaction [6, 7]. The cGMP response has identical kinetics when the cAMP stimulus is rapidly degraded or held at constant levels. Desensitization of the cAMP-stimulated guanylate cyclase occurs with a half-time of 4 s [8, 9]. Adenylate cyclase activity increases at about 10 to 30 s after cAMP addition, reaches a maximal level at 60–120 s, and then decreases to prestimulated level, even when the cAMP concentration remains constant [10]. This desensitization process is composed of two components [11, 12], adaptation and down-regulation of surface receptors. Adaptation of adenylate cyclase is completed after several minutes [10]. Cells deadapt after removal of cAMP with a half-time of about 2–4 min [13]. Down-regulation of surface cAMP receptors takes place at a similar time scale as adaptation of adenylate cyclase, but reverses more slowly after stimulus removal with a half-time of about 1 h [11, 14, 15]. The produced cAMP is secreted and relays the chemotactic signal to more distal cells [2]. Although the stimulation of both adenylate and guanylate cyclase are subjected to desensitization, these processes are probably largely independent, because they show very different kinetics and temperature dependencies [9].

cAMP binding to *Dictyostelium* cells is composed of a subclass of fast dissociating sites that are presumably coupled to the adenylate cyclase (A sites), and a subclass of slowly dissociating sites (B sites) that are probably coupled to the guanylate cyclase [15, 16]. Both subclasses show binding heterogeneity with interconversions of binding forms *in vivo* [16, 17] which are promoted by guanine nucleotides *in vitro* [17–20]. This may suggest that guanine nucleotide regulatory proteins (G-proteins) are involved in the transduction pathways of adenylate and guanylate cyclase. Thus, a functional interaction between receptors and G-protein has been demonstrated [17, 20]. In addition, conditions for both stimulation and inhibition of adenylate

cyclase by the guanine nucleotides have been reported [21, 22].

In vertebrates the dual regulation of adenylate cyclase activity by guanine nucleotides and hormones is mediated by the stimulatory (Gs) and inhibitory (Gi) guanine nucleotide binding regulatory proteins [23–25]. Pertussis toxin has been shown to catalyze specifically the ADP-ribosylation of several  $\alpha$ -subunits of GTP-binding proteins, including Gi [26, 27], the retinal transducin [28, 29] and a so far unidentified 39/40 kD protein termed Go, found in brain [30, 31], in fat cells and cardiac cells [32].

In vertebrates, the role of phosphorylation of the signal transducing components in desensitization has been extensively studied [33–36]. These results strongly suggest that receptor phosphorylation is involved in adenylate cyclase desensitization and decreased receptor-Gs coupling [34]. Recently it has been shown that cAMP induces the phosphorylation of surface cAMP receptors in *Dictyostelium*, and a role of receptor phosphorylation in adaptation of adenylate cyclase has been proposed [37–40].

In this paper we compared the effects of pertussis toxin on the cAMP receptors, chemotaxis, cGMP and cAMP responses *in vivo* with the effects on adenylate cyclase stimulation and inhibition by guanine nucleotides *in vitro*. Pertussis toxin pretreatment did not alter the cGMP response and chemotaxis. In contrast, adaptation of the cAMP response was inhibited, without effects on receptor phosphorylation or down-regulation. In pertussis toxin treated cells stimulation of adenylate cyclase by guanine nucleotides was as in control cells, whereas inhibition of this enzyme was lost. In the membranes from desensitized pertussis toxin treated cells adenylate cyclase could not be inhibited and showed reduced stimulation by guanine nucleotides.

These results suggest that *D. discoideum* may contain both Gs and Gi regulatory proteins and that a pertussis toxin substrate is involved in adenylate cyclase desensitization.

## Materials and methods

### Materials

[2,8-<sup>3</sup>H]cAMP (1.5 TBq/mmol) and the cGMP radio-immunoassay were obtained from Amersham; [<sup>32</sup>P]NAD (37.0 TBq/mmol) was from New England Nuclear. ATP $\gamma$ S, GTP, GTP $\gamma$ S, and (Sp)-cAMPS were from Boehringer; cAMP, dcAMP, and dithiothreitol were from Sigma; pertussis toxin was purchased from List Biological Laboratories (Campbell, USA). Anti cAMP-receptor antiserum was a generous gift of Dr. P.N. Devreotes. Transducin (holoenzyme and purified  $\beta\gamma$ -complex) were generous gifts of Drs. P. Gierschik and K.H. Jakobs.

### Culture conditions

*D. discoideum* NC4(H) cells were grown as described [8], harvested in the late logarithmic phase with 10 mM KH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub>, pH 6.5 (buffer A), washed, and starved in suspension in buffer A at a density of 10<sup>7</sup> cells/ml for 4–5 hours.

### Pertussis toxin treatment

Cells were starved in buffer A in the absence or presence of 0.1  $\mu$ g/ml pertussis toxin. After 5 hours, cells were washed three times in buffer A, and used for chemotaxis, cGMP response, cAMP response, desensitization of cAMP response and the adenylate cyclase assay. A higher concentration of pertussis toxin (used until 1  $\mu$ g/ml) did not enhance the effect of toxin on any of these responses. However modification of the holotoxin with N-ethylmaleimide, which inactivates the ADP-ribosyltransferase activity of the A-promotor [41], eliminated the effect of toxin on the cAMP response.

### Chemotaxis assay

Chemotaxis was tested with the small population

assay [42]. Small droplets (0.1  $\mu$ l) containing about 500 aggregation competent amoebae were deposited on hydrophobic agar. After 30 min at 22° C, cAMP solutions (0.1  $\mu$ l, 10<sup>-9</sup>–10<sup>-6</sup>M) were deposited close to the small populations of cells. A reaction was considered positive if at least twice as many amoebae were pressed against the edge close to the test solution as against the opposite edge. About 20 populations were observed for each test solution. Maximal responses were observed at about 30–45 min after deposition of the cAMP.

### cAMP mediated cGMP response [8]

Aggregative cells, at a density of 5 × 10<sup>7</sup> cells/ml, were stimulated with 100 nM cAMP at t = 0. Cells were lysed at the times indicated by the addition of 100  $\mu$ l cell suspensions to 100  $\mu$ l perchloric acid (3.5% vol/vol), and cGMP levels were measured in neutralized lysates with a radio-immunoassay.

### cAMP response [43]

A suspension of aggregative cells (5 × 10<sup>7</sup> cells/ml) was stimulated with dcAMP stimulus yielding final concentrations of 2  $\mu$ M dcAMP and 5 mM DTT in buffer A. Total cAMP was determined by transfer of 100  $\mu$ l suspension at the indicated times to tubes containing 100  $\mu$ l perchloric acid (3.5% vol/vol), and cAMP was detected in the neutralized lysates by the isotope-dilution-assay.

### Desensitization of cAMP stimulation in vivo [9]

During the experiment a suspension of aggregative cells (2 × 10<sup>8</sup> cells/ml) was aerated at a flow rate of about 15 ml of air/ml of suspension. Cells were prestimulated at 20° C with 10  $\mu$ M (Sp)-cAMPS. At the indicated times 70  $\mu$ l of the cell suspension was transferred to a tube containing 5 ml ice-cold buffer A and centrifuged at 100 × g for 1 min at 0° C. The pellets were resuspended in 120  $\mu$ l dcAMP stimulus (2  $\mu$ M dcAMP, 5 mM DTT in buffer A). After 5 min at 20° C 20  $\mu$ l of the cell

suspension was transferred to tubes containing 20  $\mu$ l perchloric acid (3.5% vol/vol). After neutralization of the lysates, total cAMP was determined. Control samples were treated in parallel, but not prestimulated with (Sp)-cAMPS.

#### Down-regulation [11]

Cells were incubated for 15 min at 20°C with 10  $\mu$ M (Sp)-cAMPS. During the experiment, the cell suspension was aerated at a flow rate of about 15 ml of air/ml of suspension. Cells were washed three times with ice-cold buffer A, resuspended in this buffer at a density of  $10^8$  cells/ml, and used for the cAMP-binding assay.

#### cAMP binding [16]

The binding of [ $^3$ H]cAMP to *D. discoideum* cells was detected in a volume of 100  $\mu$ l containing buffer A, 10 mM DTT, 5 nM [ $^3$ H]cAMP, and  $8 \times 10^6$  cells. The incubation period was 75 s at 0°C followed by centrifugation of the cells through silicon oil. Nonspecific binding was determined by including 0.1 mM cAMP in the incubation mixture and was subtracted from all data shown. Nonspecific binding is about 0.5% of the input radioactivity.

#### Covalent modification of the receptor [40]

Control and pertussis toxin treated cells (100  $\mu$ l of  $10^8$  cells/ml) were incubated at 20°C with 5 mM caffeine for 30 min. Subsequently, cells were incubated for 15 min in the absence or presence of 10 mM DTT and 0.5  $\mu$ M cAMP. The incubations were terminated by addition of 1 ml of ice-cold 95% saturated ammonium sulfate. Samples for SDS-PAGE and Western blots were prepared [40] and stained [44].

#### Desensitization of cells for adenylate cyclase assays

Aggregative cells, at a density of  $10^8$  cells/ml were

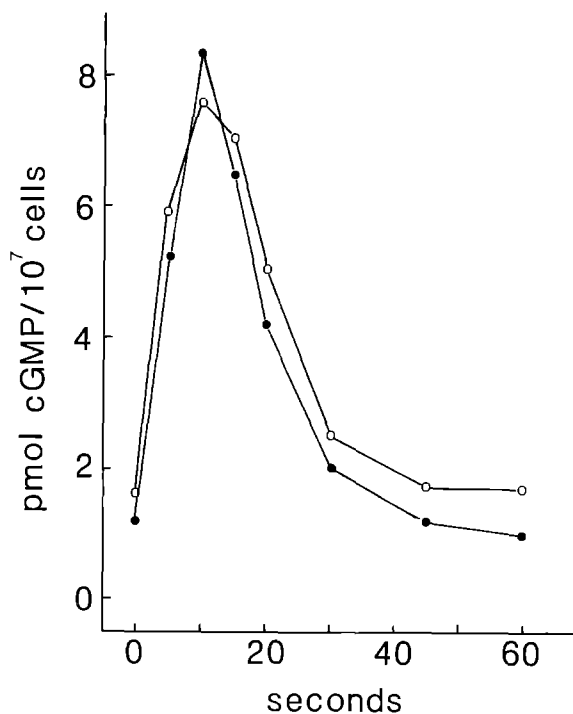


Fig. 1. cAMP mediated cGMP response in control (●) and pertussis toxin treated cells (○). Aggregative *D. discoideum* cells were stimulated with 100 nM cAMP at  $t_0$ . Cells were lysed at the times indicated by the addition of 100  $\mu$ l perchloric acid (3.5% vol/vol), and cGMP levels were measured in the neutralized lysates with a radioimmunoassay. Cells were starved for 5 h in the absence or presence of 0.1  $\mu$ g/ml pertussis toxin. Data shown are the means of three experiments.

incubated for 15 min at 20°C with 10  $\mu$ M (Sp)-cAMPS. The cell suspension was diluted 15-fold in ice-cold buffer A, and washed twice at 0°C with buffer A. The final cell pellet was resuspended in buffer B (40 mM Hepes/NaOH, 0.5 mM EDTA, 250 mM sucrose pH 7.7) and used for cell lysis and adenylate cyclase assays.

#### Membrane isolation

Cells at a density of  $2 \times 10^8$  cells/ml in buffer B were lysed by pressing them through 3  $\mu$ m pores of a Nuclepore filter [45]. The homogenate was centrifuged at  $10,000 \times g$  for 5 min, the supernatant was saved, and the pellet was washed twice and resuspended in buffer B to a concentration equivalent to  $2 \times 10^8$  cells/ml. A portion of the mem-

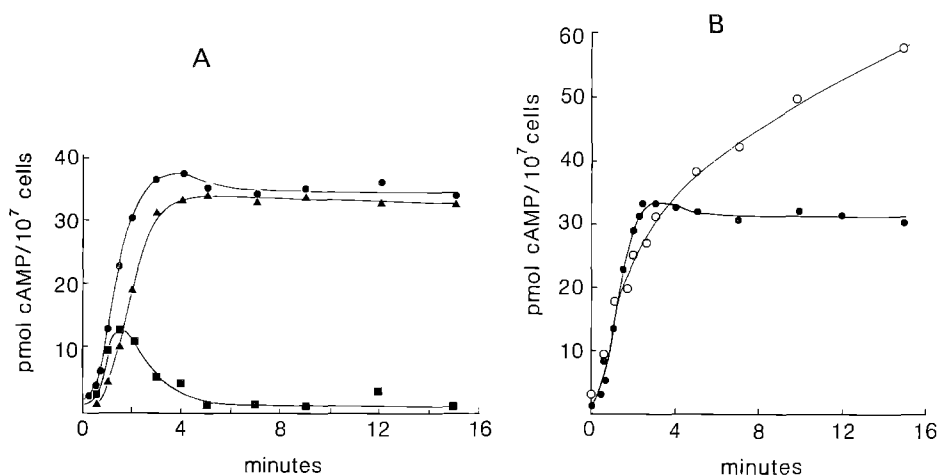


Fig. 2A. cAMP relay in *D. discoideum* at 20°C. Aggregation competent cells were stimulated with 5  $\mu$ M dcAMP and 10 mM DTT in buffer A. Total cAMP was determined by transfer of 100  $\mu$ l suspensions at the indicated times to tubes containing 100  $\mu$ l perchloric acid. Extracellular cAMP was determined by centrifugation of 110  $\mu$ l suspension for 5 s at 8000 g and transfer of 100  $\mu$ l supernatant to 100  $\mu$ l perchloric acid. The cAMP concentration was measured in the neutralized lysates: (●) total cAMP, (▲) extracellular cAMP, (■) intracellular cAMP calculated from total and extracellular levels. The cAMP analog was used as stimulus in order to discriminate between stimulus and produced cAMP [43].

Fig. 2B. cAMP response in control (●) and pertussis toxin preincubated cells (○). Cells were stimulated at 20°C with 2  $\mu$ M dcAMP, 5 mM DTT in buffer A, and lysed at the times indicated. Total cAMP was determined by the isotope dilution assay [33]. Data shown are the means and standard deviations of six independent experiments normalized to plateau values of the control at 5 min.

branes was incubated under phosphorylation conditions [46] in buffer B containing 10 mM MgCl<sub>2</sub>, 10 mM NaF, and 1 mM ATP $\gamma$ S. The reaction was terminated after 5 min at 20°C by addition of 5 volumes of ice-cold buffer B. Membranes were centrifuged, washed twice, and resuspended in buffer B to the original volume.

#### Adenylate cyclase assay [22]

Adenylate cyclase was measured in a total volume of 40  $\mu$ l containing buffer B, 6 mM MgCl<sub>2</sub>, 0.5 mM ATP, 10 mM dithiothreitol, and 20  $\mu$ l enzyme. The incubation period was 40 min at 0°C. The reaction was terminated by the addition of 10  $\mu$ l 0.1 M EDTA. Enzyme activities were destroyed by boiling the samples for 2 min; subsequently, the cAMP content was determined by isotope-dilution-assay.

#### ADP-ribosylation

Pertussis toxin-catalyzed ADP-ribosylation was

carried out in a final volume of 50  $\mu$ l for 45 min at 28°C. The toxin (0.5  $\mu$ g) was preactivated by incubation in 100 mM Tris/HCl, pH 8.0, 50 mM DTT for 1 h at room temperature and added to the reaction mixture. The reaction mixture contained 100 mM Tris/HCl, pH 8, 4 mM ATP, 25  $\mu$ g crude membranes and 20  $\mu$ M [<sup>32</sup>P]-NAD (2  $\mu$ Ci). Control samples were prepared in the absence of toxin or in the presence of 10 mM NAD. The reaction was terminated by addition of sample buffer and samples were subsequently subjected to SDS-PAGE. The gels were autoradiographed at -80°C using Fuji X-ray film. The exposure time used for individual experiments are given in the figure legends.

## Results

### *Effect of pertussis toxin on chemotaxis and the cAMP induced cGMP response*

*D. discoideum* cells were starved for 5 hours in the absence or presence of 0.1  $\mu$ g-1  $\mu$ g/ml pertussis toxin and then washed. Chemotaxis was measured

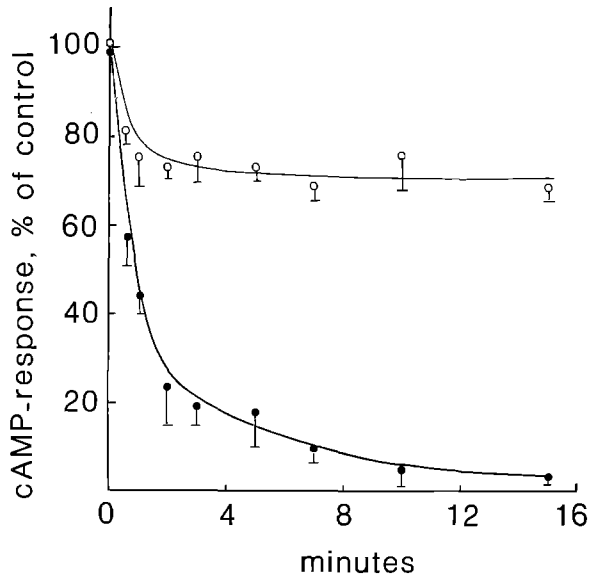


Fig. 3. Desensitization of adenylate cyclase *in vivo* by (Sp)-cAMPS in control (●) and pertussis toxin treated cells (○). Cells were prestimulated at 20°C with 10 μM (Sp)-cAMPS, transferred at the indicated times to a tube containing ice-cold phosphate buffer and centrifuged at 0°C. The supernatant was removed and the pellet was directly resuspended in the 120 μl stimulus (2 μM dcAMP, 5 mM DTT). At 5 min after restimulation cells were lysed, and total cAMP levels were measured with the isotope dilution-assay. The results shown are the means and standard deviations of four independent experiments normalized to control values (100%) of samples which were not prestimulated with (Sp)-cAMPS. Absolute values of cAMP response in control and pertussis toxin treated cells, which were not prestimulated with (Sp)-cAMPS achieved the level of 32 pmol cAMP/10<sup>7</sup> cells and 37 pmol cAMP/10<sup>7</sup> cells respectively.

with the small population assay [42]. In control cells, about 50% of the populations reacted chemotactically to 100 nM cAMP, and more than 90% reacted positively to 1 μM cAMP. In four independent experiments no statistically significant differences in chemotactic response between control and pertussis toxin treated cells were observed (data not shown). This assay can detect 3-fold differences in the cAMP concentration.

The addition of 100 nM cAMP to a suspension of cells induced a strong cGMP response which peaked after 10 s; basal levels were recovered in about 30–40 s. The cGMP response was virtually identical in control and pertussis toxin treated cells (Fig. 1). The standard deviation of the cGMP response was less than 10% [3]. When cells were

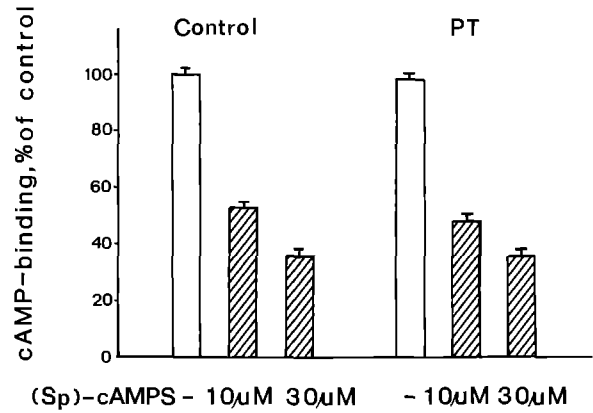


Fig. 4. Down-regulation of surface receptors in control and pertussis toxin treated cells. Control and pertussis toxin treated cells were incubated for 15 min at 20°C in the absence (open bars) or presence (shaded bars) of 10 μM, or 30 μM (Sp)-cAMPS. Then cells were washed extensively at 0°C and [<sup>3</sup>H]cAMP-binding to cell surface receptors was measured.

restimulated with cAMP at 30 s, a new response was not observed in both cell types, indicating that cAMP induced desensitization of guanylate cyclase in control and pertussis toxin treated cells (data not shown). Thus, it was observed that pertussis toxin did not alter chemotaxis, cGMP response and desensitization of the cAMP mediated cGMP response.

#### *cAMP response in control and pertussis toxin treated cells*

Adenylate cyclase activity increases after stimulation reaching a maximum activity at 1–2 min; then the adenylate cyclase activity declines to basal levels due to desensitization [9–12]. The major part of the produced cAMP is secreted and remains constant in the presence of phosphodiesterase inhibitor dithiothreitol (Fig. 2A). Control and pertussis toxin treated cells were stimulated at 20°C with dcAMP, a potent agonist of cAMP [43]. The total cAMP production was estimated at different time points after stimulation with an assay error of less than 10%. The cAMP level of control cells reached a maximal concentration at 3–4 min after stimulation and then remained constant (Fig. 2B). A small decrease of total cAMP between 4 and 6 min may

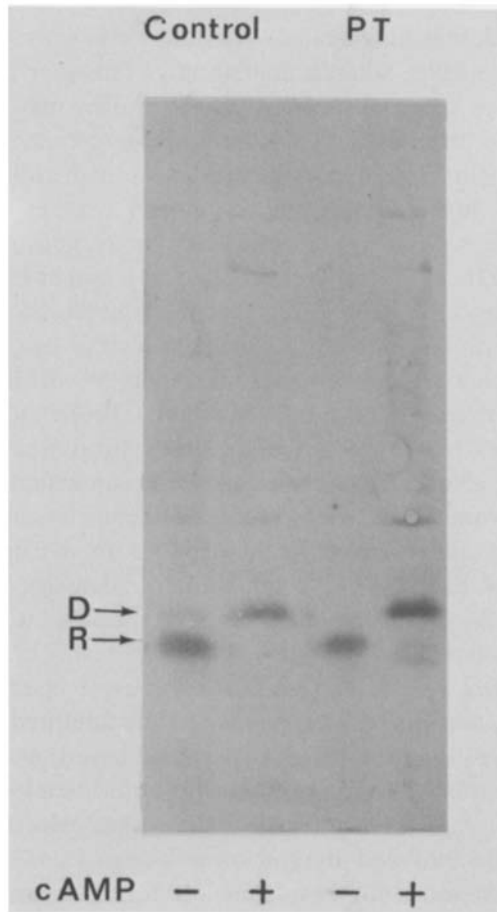


Fig. 5. Receptor modification in control and pertussis toxin treated cells. Cells were incubated with 5 mM caffeine for 30 min and 15 min in the presence (+) or absence (-) of 10 mM DTT and 0.5  $\mu$ M cAMP. Proteins were separated by SDS-PAGE and Western blot analysis with an antiserum directed against the purified receptor. R ( $M_r = 40,000$ ) unmodified form of the receptor, D ( $M_r = 43,000$ ) modified form.

represent intracellular degradation of cAMP [13]. The initial increase of cAMP synthesis was identical in control and toxin treated cells; however, cAMP synthesis continued in cells treated with pertussis toxin (Fig. 2B). This may indicate that pertussis toxin does not affect the activation of adenylate cyclase, but that it may inhibit desensitization. Desensitization of adenylate cyclase was measured by preincubating cells with the cAMP agonist (Sp)-cAMPS, removal of the stimulus, and restimulation with dcAMP [9]. In control cells, 3 min of prestimulation with 10  $\mu$ M (Sp)-cAMPS induced 80% desensitization and after the next 7 min of

incubation the response was reduced 95% (Fig. 3). The level of desensitization in pertussis toxin treated cells was only 30% after preincubation with 10  $\mu$ M (Sp)-cAMPS for 3 min and did not increase further after longer preincubation times with (Sp)-cAMPS. This strongly suggests that pertussis toxin inhibits desensitization of cAMP relay.

Recent results suggest that cAMP-induced desensitization of adenylate cyclase is composed of multiple components [11, 12], a rapidly reversible adaptation and a slowly reversible component related to down-regulation of surface receptors [11, 12, 15]. To examine which step of desensitization was altered by pertussis toxin, control and toxin treated cells were incubated with (Sp)-cAMPS for 15 min at 20°C, washed extensively at 0°C and binding of 5 nM [ $^3$ H]cAMP was detected (Fig. 4). The loss of cAMP binding by 10 and 30  $\mu$ M (Sp)-cAMPS was the same in the control and toxin treated cells. These results indicate that pertussis toxin alters desensitization of adenylate cyclase due to adaptation and not to down-regulation of surface cAMP receptors.

#### *Modification of the receptor in control and pertussis toxin treated cells*

Adaptation of adenylate cyclase has been correlated with the cAMP-induced modification of the electrophoretic mobility of the cAMP receptor, which is presumably caused by receptor phosphorylation [37–40]. In vertebrates it has been shown that receptor phosphorylation may lead to desensitization of adenylate cyclase [33–36]. The effect of pertussis toxin on receptor modification in *Dictyostelium* is shown in Fig. 5. The incubation of control and pertussis toxin treated cells with 0.5  $\mu$ M cAMP induced identical transitions from the R form ( $M_r = 40,000$ ) to the D form ( $M_r = 43,000$ ) of the cAMP receptor (Fig. 5). The cAMP concentration dependency and kinetics of the receptor modification were essentially identical in control and toxin treated cells (data not shown).

These results indicate that pertussis toxin inhibits cAMP-induced adaptation of adenylate cyclase without affecting receptor modification that is pre-

sumably caused by receptor phosphorylation, suggesting that receptor phosphorylation is not sufficient for desensitization of adenylate cyclase.

*Effect of pertussis toxin treatment in vivo on the regulation of adenylate cyclase by GTP $\gamma$ S in vitro*

Recently, conditions for both stimulation and inhibition of adenylate cyclase by the guanine nucleotides in membranes have been found [21, 22]. Stimulation and inhibition of the adenylate cyclase is specific for guanosine triphosphates and antagonized by guanosine diphosphates. Adenylate cyclase stimulation by guanine nucleotides was maximally 2-fold and required a cytosolic factor [22]. Preincubation of membranes under phosphorylation conditions has been shown to alter the interaction between cAMP receptor and putative G-proteins [46], and to convert stimulation of adenylate cyclase to inhibition of adenylate cyclase by guanine nucleotides [22]. The effect of pertussis toxin treatment *in vivo* on the stimulation and inhibition of adenylate cyclase in the membranes obtained from control and desensitized cells are shown in Table 1. Adenylate cyclase from control cells was stimulated by GTP $\gamma$ S in the presence of the cytosolic factor. The inhibition by GTP $\gamma$ S was obtained after treatment of membranes under phosphorylation conditions. After pertussis toxin treatment the

stimulation by guanine nucleotides was as in the control cells, whereas inhibition of the adenylate cyclase activity by GTP $\gamma$ S was lost. This may indicate that inhibition of adenylate cyclase by GTP $\gamma$ S is regulated as in vertebrates by the inhibitory guanine nucleotide regulatory protein Gi, which is specifically inactivated by pertussis toxin [26, 27]. Adenylate cyclase in the membranes from desensitized cells could not be stimulated by GTP $\gamma$ S. The inhibition of adenylate cyclase by guanine nucleotides is slightly larger in desensitized cells than in control cells ( $P < 0.05$ ). In the membranes from desensitized pertussis toxin treated cells, adenylate cyclase could still be stimulated by guanosine nucleotides, although stimulation was significantly reduced if compared with the non-desensitized cells. The inhibition of adenylate cyclase was also absent in desensitized pertussis toxin treated cells.

These results indicate that adenylate cyclase of *D. discoideum* can be stimulated and inhibited by guanosine nucleotides, and that stimulation but not inhibition is lost during desensitization. The adenylate cyclase from pertussis toxin treated cells cannot be inhibited by guanosine nucleotides, and stimulation is not completely lost during desensitization.

Table 1. Adenylate cyclase activity in membranes

Condition	Activity control	Ratio $\pm$ GTP $\gamma$ S	Activity desensitized	Ratio $\pm$ GTP $\gamma$ S
	pmol min <sup>-1</sup> mg protein <sup>-1</sup>		pmol min <sup>-1</sup> mg protein <sup>-1</sup>	
control	0.53 $\pm$ 0.04	1.73 $\pm$ 0.11*	0.60 $\pm$ 0.06	1.00 $\pm$ 0.09
PT	0.62 $\pm$ 0.04	1.93 $\pm$ 0.24*	0.48 $\pm$ 0.06	1.30 $\pm$ 0.09*
control/ATP $\gamma$ S	0.48 $\pm$ 0.03	0.71 $\pm$ 0.05**	0.54 $\pm$ 0.08	0.67 $\pm$ 0.03**
PT/ATP $\gamma$ S	0.56 $\pm$ 0.06	1.06 $\pm$ 0.07	0.61 $\pm$ 0.07	1.09 $\pm$ 0.07

*D. discoideum* cells were starved for 5 hours in the absence or presence of 0.1  $\mu$ g/ml pertussis toxin (PT) and washed. One part of the cell suspensions was used directly for membrane isolation, another part was first incubated at 20° C for 15 min with 10  $\mu$ M (Sp)-cAMPS to induce desensitization of adenylate cyclase stimulation *in vivo*. The homogenates of control and desensitized cells were centrifuged. Washed pellets were or were not incubated with Mg/ATP $\gamma$ S for 5 min at 20° C, washed and resuspended to the original membrane density. Adenylate cyclase was measured at 0° C in the absence or presence of 100  $\mu$ M GTP $\gamma$ S. The absolute enzyme activities are shown for the incubations without GTP $\gamma$ S (ratio = 1). The ratios of enzyme activities with and without GTP $\gamma$ S are presented. The results shown are the means and standard deviations of 4 independent experiments. \*, The ratio is significantly above 1.0 (stimulation); \*\*, the ratio is significantly below 1.0 (inhibition).



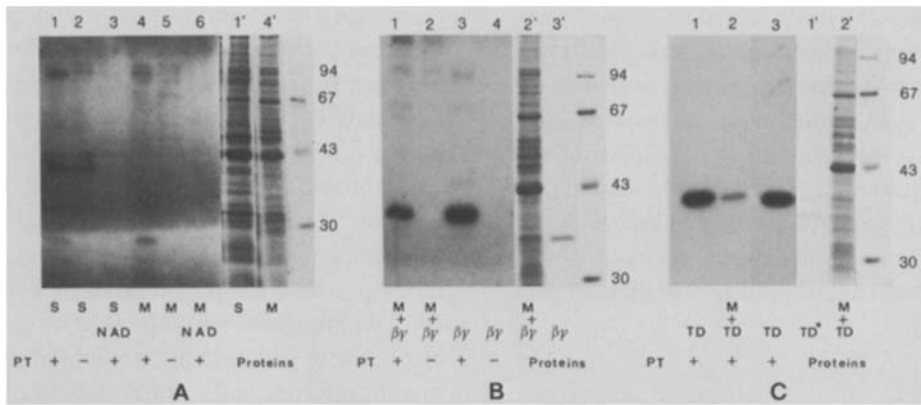


Fig. 6. Pertussis toxin-catalyzed ADP-ribosylation. Proteins were ADP-ribosylated with [ $^{32}$ P]-NAD and pertussis toxin, resolved by electrophoresis, stained with Coomassie blue and autoradiographed. A, *D. discoideum* supernatant (S) and membranes (M) were used for ADP-ribosylation. The gel was exposed for 7 days. B, *D. discoideum* membranes in the presence of  $\beta\gamma$ -complex (lane 1, 2) and  $\beta\gamma$ -complex alone (lane 3, 4) were used for ADP-ribosylation and the gel was exposed for 1 day. C, ADP-ribosylation of transducin (TD) in the absence (lane 1), and presence of membranes (lane 2) and preincubated with *D. discoideum* membranes (lane 3). The exposure time was 2 hours. The samples were prepared in the absence (-) or presence (+) of toxin and some of them in the presence of 10 mM NAD.

### ADP-ribosylation

To determine whether the pertussis toxin substrate in *D. discoideum* represents the  $\alpha$ -subunit of a G protein the pertussis toxin catalyzed ADP-ribosylation was studied at varying experimental conditions. Membrane isolations and reactions were carried out in the presence of a mixture of protease inhibitors [37] to reduce the possible degradation of the pertussis toxin substrate. Also the effect of detergent extraction with 1% Lubrol PX [47] and preincubation of membranes with 0.75 M ammonium sulphate prior ADP-ribosylation [48] were investigated, as well as the presence or absence of 10 mM  $Mg^{2+}$ , 10  $\mu$ M GTP+  $Mg^{2+}$  or 10  $\mu$ M GTP $\gamma$ S +  $Mg^{2+}$  [24], and 0.75 mM NADP during the ADP-ribosylation reaction [49]. A specific pertussis toxin substrate of about 40 kD was not found in *D. discoideum* membranes or supernatant under any of these conditions (see Fig. 6A, lanes 1–6). After very long exposure of the autoradiograms, few bands were detectable in the molecular range higher than 94 kD, between 43–39 kD, and in the range lower than 30 kD. However, they were also present in the absence of toxin. Only the band with apparent  $M_r = 28$  kD seems to be specifically present in membranes and supernatant. Recent re-

ports [31, 50] suggest that the  $\alpha$ -subunit in association with the  $\beta\gamma$ -complex is the preferred substrate for pertussis toxin. One of the characteristics of GTP-binding proteins is that upon treatment with non-hydrolyzable GTP analogs and  $Mg^{2+}$ , the  $\alpha$ -subunit dissociates from the  $\beta\gamma$ -complex [23, 24]. The dissociation of G protein may happen during the preparation of *D. discoideum* membranes. Therefore we tested the effect of additional  $\beta\gamma$ -complex (from transducin) on the pertussis toxin mediated ADP-ribosylation in *D. discoideum* membranes (Fig. 6B, lanes 1–4). Addition of 500 ng  $\beta\gamma$ -complex to *D. discoideum* membranes produced strong pertussis toxin mediated ADP-ribosylation of an approximately 39 kD protein (Fig. 6B, lane 1). The control experiment, where  $\beta\gamma$ -complex was used in the absence of *D. discoideum* membranes, yielded an even stronger labelling of approximately the same band (Fig. 6B, lane 3), suggesting that the purified  $\beta\gamma$ -complex contains a small trace of the  $\alpha$ -subunit of transducin. The inhibition of the ADP-ribosylation reaction by *D. discoideum* membranes was also investigated with transducin holoenzyme, showing a 85% inhibition (Fig. 6C, lane 1, 2). When the soluble transducin was preincubated with *D. discoideum* membranes, separated by centrifugation,

and then used as a substrate for ADP-ribosylation, inhibition of transducin labeling was not longer observed, indicating that transducin was not degraded by *D. discoideum* membranes (Fig. 6C, lane 3). These results suggest that *D. discoideum* membranes contain a factor which inhibits the pertussis toxin-mediated ADP-ribosylation of transducin and thus may conceal any pertussis toxin-mediated ADP-ribosylation of *D. discoideum* proteins *in vitro*.

## Discussion

In vertebrate cells, the surface receptor-linked adenylate cyclase system involves three classes of membrane proteins: surface receptors (R), a catalytic component (C) and heterotrimeric guanylnucleotide-binding regulatory proteins (G proteins). Pertussis toxin causes in vertebrate cells the ADP-ribosylation of several GTP binding proteins [26–32]. The function of the G protein is not limited to the control of one process, therefore, pretreatment of intact cells with pertussis toxin can alter multiple pathways of cellular regulation. The inhibition of chemotaxis by pertussis toxin was observed in human neutrophils [51], macrophages, and human-mouse hybrid cell lines [52]. In this report we investigated the effect of pertussis toxin on the transmembrane signal transduction in *D. discoideum*. These cells react chemotactically to cAMP. Binding of cAMP to isolated membranes is modified by guanine nucleotides, suggesting that the cell surface cAMP receptor may interact with a G protein.

A pertussis toxin substrate could not be identified in *D. discoideum* membranes using ADP-ribosylation experiments. However, we found that *D. discoideum* membranes inhibit ADP-ribosylation of transducin. This suggests the presence of a membrane-associated factor which probably masks the ADP-ribosylation of a pertussis toxin substrate during *in vitro* experiments with *D. discoideum* membranes.

The major findings of the present report are 1) chemotaxis was not affected by pertussis toxin; 2) excitation and desensitization of cAMP stimulated

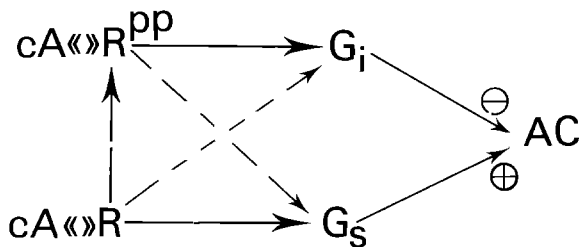
guanylate cyclase was not altered by pertussis toxin; 3) activation of adenylate cyclase *in vivo* was not changed by pertussis toxin; 4) desensitization of adenylate cyclase *in vivo* was strongly reduced in pertussis toxin treated cells; 5) pertussis toxin alters adaptation of adenylate cyclase without effect on receptor down-regulation; 6) receptor modification (presumably phosphorylation) was not affected by pertussis toxin; 7) adenylate cyclase *in vitro* can be stimulated and inhibited by guanosine nucleotides; stimulation but not inhibition was lost in membranes derived from desensitized cells. Adenylate cyclase from pertussis toxin treated cells could be stimulated but not inhibited by guanosine nucleotides and stimulation was not completely lost in desensitized pertussis toxin treated cells; 8) although a pertussis toxin substrate could not be identified in *D. discoideum*, the presence of a substrate is not only suggested by the present observations, but also by the loss of receptor stimulated GTPase activity in membranes from pertussis toxin treated cells [53]. After long exposure of the autoradiograms one specific band was detected in membranes and supernatant with apparent  $M_r = 28$  kD. This band can be the consequence of an auto-ADP-ribosylation of the major subunit of the pertussis toxin. A-promotor [41] or maybe an unusual pertussis toxin substrate.

Recently two G-protein  $\alpha$  subunits  $G\alpha_1$  and  $G\alpha_2$  have been cloned and sequenced [54, 55]. Both described genes lack the consensus site for ADP-ribosylation by pertussis toxin [54, 55]. Following analysis should identify other G-proteins whose function has been postulated in biochemical studies. The anti-peptide antibodies specific for  $G\alpha_1$  and  $G\alpha_2$  have been used to identify the proteins encoded by the  $G\alpha_1$  and  $G\alpha_2$  genes. The  $G\alpha_1$  antibody detects 38 kD band, while  $G\alpha_2$  antibody labels the 40 kD band. Both the  $G\alpha_1$  and  $G\alpha_2$  antibodies also label additional bands of roughly 24 kD and 30 kD (55, Gundersen and Devreotes, personal communication). Additional study should show if pertussis toxin substrate (28 kD) could be immunoprecipitated by the  $G\alpha_1$  and  $G\alpha_2$  antibody.

In vertebrates it has been proposed that phosphorylation of the receptor is the mechanism of the

adenylate cyclase desensitization [33, 34]. In *D. discoideum*, cAMP induced phosphorylation of the receptor has been established and was associated with the cAMP induced desensitization of adenylylate cyclase [37–40]. The surface cAMP receptor appears as a doublet when analyzed by SDS-PAGE. The lower mobility band (43 kD, designated D), appears to be a highly phosphorylated form of the higher mobility band (40 kD, designated R) [40, 44]. The effects of pertussis toxin on the kinetics and cAMP concentration dependency of receptor modification were presently investigated. Pertussis toxin did not prevent the cAMP induced shift of the receptor from 40 kD to 43 kD which has been related to phosphorylation of the receptor [40].

The present observations indicate that pertussis toxin specifically inhibits adaptation of adenylylate cyclase in *D. discoideum*, and that receptor phosphorylation is not sufficient for adaptation. We propose a ‘preference’ model (see below) for the stimulation and desensitization of adenylylate cyclase in *D. discoideum* in which both Gs and Gi are involved.



The occupation of the cAMP-receptor stimulates G<sub>s</sub>, which leads to the activation of adenylylate cyclase. Phosphorylation of the cAMP receptor causes a stimulation of G<sub>i</sub> by which stimulation of adenylylate cyclase terminates. Pertussis toxin inactivates G<sub>i</sub> and therefore G<sub>s</sub>-mediated stimulation of adenylylate cyclase is no longer antagonized and desensitization of adenylylate cyclase is inhibited. The differential activation of G<sub>s</sub> and G<sub>i</sub> depending on receptor occupancy and receptor phosphorylation could be a mechanism of adenylylate cyclase stimulation and desensitization in *D. discoideum* and other organisms.

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