Adaptation to chemotactic cyclic AMP signals in Dictyostelium involves the G-protein

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Summary

Amoebae of Dictyostelium discoideum show adaptation towards a chemotactic cyclic AMP signal. Within a few seconds of receipt of the signal they are inhibited for a period of 1–2 min from further chemotactic responses to subsequent cyclic AMP signals of similar or smaller magnitude. The site of this adaptation mechanism in the chemotactic transduction pathway was investigated by addition of components of the transduction chain (GTP analogues, myo-inositol-1,4,5-trisphosphate (InsP3) and Ca2+) to permeabilized cells followed by determination of the amount of cyclic GMP formed as a measure of the chemotactic response. This approach was made possible by finding that permeabilization of amoebae with saponin did not uncouple the cell surface cyclic AMP receptors from stimulation of cyclic GMP formation.

It was found that InsP3 and Ca2+ were ‘downstream’ from the adaptation mechanism: they could trigger a cyclic GMP response in cyclic AMP-adapted amoebae but could not themselves induce adaptation. In contrast, GTPyS was unable to trigger a cyclic GMP response in cyclic AMP-adapted cells, although it could trigger multiple cyclic GMP responses in non-adapted cells. We deduce that the site of adaptation to cyclic AMP stimulation is at the G-protein involved in this signalling pathway. Moreover, as GTPyS was found to be unable to induce adaptation, we conclude that the mechanism of adaptation involves an action of the cyclic AMP receptor on the G-protein that is distinct from its commonly reported action of stimulating G-protein binding of GTP.

Key words: adaptation, chemotaxis, G-protein, Dictyostelium, cyclic GMP, desensitization.

Introduction

Cell signalling in Dictyostelium discoideum involves two distinct signal transduction systems: one involving adenylate cyclase for relay of the cyclic AMP signal and the other involving myo-inositol 1,4,5-trisphosphate (InsP3) and the chemotactic responses of cyclic GMP formation and actin polymerization (Fig. 1). Evidence for the inositol pathway has come from a number of studies using intact and saponin-permeabilized amoebae (Europe-Finner & Newell, 1985, 1986a,b, 1987a,b; Small et al. 1986; Newell et al. 1987).

Both transduction pathways show adaptation to cyclic AMP signals (Devreotes & Steck, 1979; Van Haastert & Van der Heijden, 1983). (In the context of this paper adaptation is defined as having occurred when a response to a second chemotactic signal can be demonstrated only if this signal is greater in magnitude than the original one.) The adaptation and deadaptation to cyclic AMP signals of the pathways, however, are very different in their rates. For the adenylate cyclase pathway the time for half-maximal adaptation (t4) is 110 s at 20°C with a t4 for deadaptation of 3–4 min (Dinauer et al. 1980a,b; Van Haastert et al. 1986; Van Haastert, 1987). For the inositol pathway, adaptation (as measured by cyclic GMP formation) occurs over a concentration range of cyclic AMP between 1 nM and 1 μM, and has been found to be much more rapid, with a t4 of 2–4 s at 20°C and a t4 for deadaptation of 1–2 min (Van Haastert et al. 1986; Van Haastert, 1987).

The kinetics of adaptation of the adenylate cyclase correlate well with cyclic AMP receptor phosphorylation that has been observed to be induced by cyclic ...
AMP (P. Klein et al. 1985; C. Klein et al. 1985). However, the mechanism of adaptation of the inositol pathway to cyclic AMP signals is unknown, but because of the difference in kinetics it is probably distinct from the mechanism operating in the adenylate cyclase pathway.

The purpose of the investigation reported below was to find the site on the inositol transduction pathway at which adaptation was operating. The technique used was that of bypassing the block induced by adaptation by the addition of intermediates of this transduction chain, followed by measurement of their effectiveness by assay of cyclic GMP formation.

Materials and methods

**Materials**

myo-inositol 1,4,5-trisphosphate (potassium salt; InsP3) and the cyclic GMP radioimmunoassay kit were obtained from Amersham International PLC. GTPγS was obtained from Boehringer-Mannheim. Antimycin A, oligomycin, dinitrophenol, creatine phosphokinase, creatine phosphate, ATP, EGTA, saponin, folic acid and cyclic AMP were obtained from Sigma.

**Harvesting, permeabilization and pretreatment of amoebae**

*Dictyostelium discoideum*, strain NC4, was grown in association with *Klebsiella aerogenes* (strain OXF1) on SM nutrient agar (Sussman, 1966). Amoebae were prepared by growth as lawns on SM agar under conditions permitting uniform clearing of the bacteria by the feeding amoebae. Amoebae were harvested from the bacterial plates in P buffer (17 mM-KH2PO4/Na2HPO4, pH 6.1) and washed free of bacteria by centrifugation at 190g for 2 min. After three such washes the cells were resuspended in P buffer at 2×10⁷ cells ml⁻¹.

**Permeabilization of amoebae**

For permeabilization, 5×10⁷ amoebae ml⁻¹ were incubated at 22°C in a rotary incubator at 170 revs min⁻¹ in the presence of 1 mg ml⁻¹ saponin and 5 mM-ATP, and shaken for 30 min. Permeability was judged by addition of Giemsa stain (Gurr’s improved R66) to a final concentration of 20% (v/v) to samples of cell suspensions, and amoebae were observed after 5 min using a haemocytometer and phase-contrast microscopy. Permeabilized cells stain deep purplish-blue under these conditions while control unpermeabilized cells remain unstained. After treatment with saponin, amoebae were washed three times with P buffer and resuspended at 10⁶ cells ml⁻¹.

**Pretreatment of amoebae before stimulation with chemotactic agents**

In experiments testing the ability of various agents to stimulate cyclic GMP formation in permeabilized cells, the following pretreatment was routinely adopted to ensure that they were reliant on exogenous ATP and that any unpermeabilized cells present were rendered inactive. The following additions were made (final concentrations): antimycin A (10μM) (to prevent endogenous substrate oxidation); ATP (5 mM); an ATP-regenerating system comprising 5 mM-creatine phosphate and 5 units ml⁻¹ creatine phosphokinase; and dinitrophenol (0.5 mM) and oligomycin (10μM) to inhibit mitochondria in whole cells (Europe-Finner & Newell, 1987b). The free Ca²⁺ concentration was set at 180 nM using 1 mM-EGTA–Ca²⁺ buffers (Saito, 1979; Europe-Finner & Newell, 1986b).

**Stimulation of amoebae for cyclic GMP formation**

Permeabilized amoebae were dispensed into a series of 1.5 ml Eppendorf tubes shaken at 22°C on an IKA Vibrax platform shaker at 1400 revs min⁻¹. The amoebae were stimulated in individual tubes by addition of 20μl of stimulating agent (cyclic AMP, InsP3, GTPγS, or Ca²⁺) and the reaction was stopped at carefully measured times from 0 to 56 s by rapid addition of 100μl of 3.5% HClO₄. Controls using addition of 20μl of water in place of the stimulating agent were performed alternately with the stimulated samples, rather than as a separate series at a later or earlier time, to ensure that the background level of cyclic GMP was not changing during the course of the experiment. For assay of cyclic GMP we used a radioimmunoassay kit from Amersham with sample preparation as described by Van Haastert et al. (1981).

**Results**

**The chemotactic signal transduction pathway**

Our current model of the chemotactic signal transduction pathway (Fig. 1) links cyclic AMP receptors on the outside of the amoebae to the intracellular responses of actin polymerization and cyclic GMP formation that lead to pseudopodium production, cell elongation and cell movement. According to this model InsP3 is formed by the action of a plasma membrane-bound phospholipase that is activated by a G-protein coupled to the cyclic AMP receptor. The increased InsP3 production leads to release of Ca²⁺ from (non-mitochondrial) internal stores and this activates the polymerization of actin associated with the cytoskeleton and (directly or indirectly) activates guanylate cyclase with consequent accumulation of cyclic GMP.

**Cyclic AMP receptors on permeabilized cells remain coupled to cyclic GMP formation**

In order to explore the problem of adaptation in this pathway it was first necessary to determine whether exogenous pulses of cyclic AMP could induce cyclic GMP formation in permeabilized amoebae as it could in amoebae before permeabilization. After harvesting the amoebae from clearing SM agar plates they were permeabilized with saponin and then stimulated at 22°C with 50 nM cyclic AMP. The cyclic GMP that was formed was measured at subsequent time points between 0 and 31 s. The results (Fig. 2A) revealed that cyclic GMP was formed with a peak height that was 60-100% of that normally seen in non-permeabilized

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Fig. 1. Current model of the signal transduction pathways leading to relay of the cyclic AMP signal via adenylate cyclase and to chemotaxis via InsP3. Evidence from Van Haastert (1985) indicates that the cell surface cyclic AMP receptors coupled to adenylate cyclase differ in their kinetic properties from those concerned with InsP3 production and chemotaxis. G, GTP/GDP-binding G-protein; AC, adenylate cyclase; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; 1,2-DAG, 1,2-diacyl glycerol; GC, guanylate cyclase.

cells and at approximately the same timing. For reasons not fully understood, the rates of increase and decrease of the cyclic GMP varied somewhat with different batches of cells. Control experiments with addition of water showed no significant change in the cyclic GMP level.

To determine whether the coupling of cyclic AMP receptors in permeabilized cells was a general phenomenon, cell-surface folate receptors were also tested for their coupling to cyclic GMP formation. The results (Fig. 2B) using 50 μM-folate as stimulant indicated that these receptors also remained coupled.

To ensure that the cyclic GMP responses observed in Fig. 2 were entirely produced by the permeabilized amoebae and not by any intact amoebae that might have been present, experiments were conducted in which intact cells were incubated for 10 min prior to cyclic AMP stimulation in the presence of the mitochondrial inhibitors oligomycin (10 μM) and dinitrophenol (0.5 mM) together with 5 mM-ATP and an ATP-regenerating system (5 mM-creatine phosphate and 5 units ml⁻¹ creatine phosphokinase). As expected, the intact cells (which had lost their endogenous energy supply and were unable to take up the exogenous ATP) did not form cyclic GMP (Fig. 3). When the same experiment was repeated using permeabilized cells it was observed that the mitochondrial inhibitors were unable to inhibit cyclic GMP formation. Subsequently, oligomycin and dinitrophenol were routinely added to all permeabilized cell preparations prior to their use to ensure that any non-permeabilized cells remaining did not influence results obtained.

At what position does adaptation occur in the signal transduction pathway?

The phenomenon of adaptation in permeabilized cells is demonstrated by the results shown in Fig. 4A. Addition of 1 μM-cyclic AMP at zero time produced a peak of cyclic GMP but a second addition of 1 μM-cyclic AMP given at 25 s failed to produce any response. (Addition of 1 μM-cyclic AMP was used in order to ensure that maximum adaptation was
Fig. 2. Time course of cyclic GMP formation (●) in saponin-permeabilized amoebae of D. discoideum at 22°C in response to a pulse of: A, cyclic AMP (50 nM); or B, folate (50 μM). Controls (○) were treated with an equal volume of water. Results are means of three experiments, with error bars representing S.E.M.

achieved. Repetition of this experiment and the others shown in Fig. 4 with 50 nM pulses of cyclic AMP, which would be broken down to inactive 5'AMP within a few seconds of addition, produced essentially identical results.) When the first cyclic AMP signal was followed by addition of Ca²⁺ instead of cyclic AMP (sufficient to raise the free Ca²⁺ concentration from 180 nM to 110 μM), a second cyclic GMP peak was produced and this occurred 10–15 s after adding the Ca²⁺ (Fig. 4B). When 5 μM-InsP₃ was used instead of Ca²⁺ a similar result was obtained (Fig. 4C). However, a similar experiment with 53 μM-GTPyS failed to elicit a significant response.

We deduce that Ca²⁺ and InsP₃ are ‘downstream’ from the signal block caused by adaptation while GTPyS is (in this terminology) ‘upstream’ from the block.

Can InsP₃ or Ca²⁺ induce adaptation?

Instead of using two consecutive additions of cyclic AMP as in Fig. 4A, two additions of InsP₃ were made at 0 and 25 s to see if adaptation to the first InsP₃ signal occurred. The results (Fig. 5A) indicated that no significant adaptation could be seen. In order to ensure that the signals (of 5 μM) were saturating for the response, a dose–response curve was run for InsP₃ (shown as an inset in Fig. 5A), which indicated a concentration of approximately 0.6 μM. An interesting, but unexplained, observation was that the response to the second addition of InsP₃ was very rapid (occurring consistently within 3 s). When similar experiments were performed with two consecutive additions of Ca²⁺, again two peaks of cyclic GMP were formed. The level of cyclic GMP failed to return to the baseline level between pulses, possibly because of the high level of free Ca²⁺ remaining after the first pulse (approximately 0.9 mM was calculated to be present at the instant of the first pulse) although the rate of sequestration of Ca²⁺ in such cells is unknown.

A single pulse of GTPyS causes more than one cyclic GMP peak but does not cause adaptation

Because GTPyS is not hydrolysed its effects should be long-lasting. Previous work (Europe-Finner & Newell, 1987b) had indicated that it could cause an oscillation of inositol phosphate formation in synchronized amoebae. The pattern of cyclic GMP formation observed in response to addition of GTPyS was very similar (Fig. 6A). Peaks were observable at 9 s and around 28 s, with a subsequent raised baseline due possibly to asynchronous later peaks.

From the finding of more than one peak of cyclic GMP being produced by the GTPyS within 30 s we conclude that GTPyS is unable to induce adaptation.
Fig. 4. Amoebae adapted to a cyclic AMP signal can respond to Ca\textsuperscript{2+} and InsP\textsubscript{3} signals but not to GTP\textgamma{}S. Cyclic GMP formation was assayed for 56 s in permeabilized amoebae at 22°C after stimulation at time zero with a saturating pulse of cyclic AMP (1 µM) followed at 25 s by a pulse of: A, cyclic AMP (1 µM); B, Ca\textsuperscript{2+} (1 mM); C, InsP\textsubscript{3} (5 µM); D, GTP\textgamma{}S (53 µM). From the concentration of EGTA present (1 mM) and Ca\textsuperscript{2+} added, the free Ca\textsuperscript{2+} concentration in B was calculated by the method of Saito (1979) to be 110 µM. Results are the means of three experiments, with error bars representing S.E.M.
Fig. 5. Two pulses of InsP₃ or two pulses of Ca²⁺ produce two peaks of cyclic GMP formation. Cyclic GMP was assayed for 56 s in permeabilized amoebae at 22°C that were pulsed both at 0 and 25 s with: A, InsP₃ (5 μM); or B, Ca²⁺ 1 mM or 2 mM (results were identical and have been amalgamated). Controls (O) pulsed at the same times with water gave no response. Results are the means of six experiments, with error bars representing the S.E.M. Inset: the dose–response curve for InsP₃ stimulation of cyclic GMP formation (showing means of four experiments).

Discussion

None of the intermediates of the chemotactic signal transduction chain that were tested (InsP₃, Ca²⁺ or the GTP analogue GTPyS) was capable of inducing adaptation. Only the primary signal, cyclic AMP, was able to induce this effect. Chemotactic signals ‘downstream’ from the G-protein (InsP₃ and Ca²⁺) could trigger the formation of cyclic GMP in amoebae that had been fully adapted to cyclic AMP, but GTPyS could not, although capable of eliciting cyclic GMP formation in non-adapted cells. We deduce that adaptation of the chemotactic system to cyclic AMP signals occurs by interaction of the cyclic AMP receptor with the G-protein of this pathway.

The mechanism of adaptation cannot, however, be induction of binding of GTP to the G-protein. If this were the mechanism, then GTPyS should induce adaptation, but clearly it cannot, since multiple peaks of cyclic GMP were formed in response to a single stimulation with GTPyS. We conclude that the adaptation mechanism is an interaction of the cyclic AMP
Fig. 6. A single pulse of GTPyS produces more than one peak of cyclic GMP formation. Cyclic GMP was assayed for 56 s in permeabilized amoebae at 22°C stimulated with a single pulse of GTPyS at time zero. Results are the means of four experiments, with error bars representing s.e.m.

receptor with the G-protein such that the G-protein is modified in some way that is different from the modification that enhances binding of GTP.

In some vertebrate systems (Stadel et al. 1983; Kelleher et al. 1984; Sibley et al. 1984) adaptation has been postulated to involve phosphorylation of the cell surface receptor after activation of protein kinase C by 1,2-diacyl glycerol (produced as the other product, besides InsP$_3$, of activated phospholipase C). This mechanism seems unlikely for *Dictyostelium*, since if this were the case it would be predicted that GTPyS would be capable of causing adaptation.

The interpretation of the results would also be affected if GTP were releasing Ca$^{2+}$ from the microsomes rather than affecting the G-protein at the plasma membrane. Such an effect of GTP on the microsomes has been reported in permeabilized islet B-cells (Wolf et al. 1987). However, that effect was shown to require GTP hydrolysis for its action and was not influenced by non-hydrolysable analogues such as GTPyS, as used in this study.

Although it is deduced that GTPyS, InsP$_3$ and Ca$^{2+}$ cannot cause adaptation, it is also apparent from the results that they do not trigger continuous synthesis of cyclic GMP but elicit peaks of formation similar to those triggered by cyclic AMP. We deduce that another form of adaptive response must be operating that is distinct from that covered by the definition of adaptation used in this paper and that causes the cyclic GMP formation to be transient, whatever the stimulus. This distinction can also be seen in the data of Van Haastert (1987), which demonstrates that at 0°C no adaptation (measured by sequential cyclic AMP additions) is observed, but nevertheless a peak of cyclic GMP is formed at this temperature rather than continuous synthesis. The transient nature of the cyclic GMP may be unrelated to the adaptive mechanism described in this paper. The response appears to be ‘downstream’ from Ca$^{2+}$ and InsP$_3$, and may therefore be involved with inactivation of the guanylate cyclase enzyme more directly.

Ideas about adaptation in the chemotactic signal transduction pathway are relevant to our understanding of the possible role of this pathway in the control of gene expression during development. As this transduction pathway shows adaptation to cyclic AMP, and guanylate cyclase is activated only transiently, how can the use of large (mM) amounts of cyclic AMP bring about induction of gene activity, as has been reported (Sampson et al. 1978; Schaap & Van Driel, 1985)? In the present study, after stimulation of cyclic GMP formation the cyclic GMP concentration rapidly returned to basal levels. However, the literature seems less consistent about the extent of this inhibition of cyclic GMP synthesis. Some reports indicate that the cyclic GMP level returns approximately to its baseline value after stimulation for a few seconds with cyclic AMP, as we report here (de Gunzburg & Brachet, 1982; Van Haastert & Van der Heijden, 1983), while other reports indicate a return only to an intermediate level (50–70 % of peak) in the continued presence of cyclic AMP signal (Lappano & Coukell, 1982; Wurster & Butz, 1983). If the latter finding were more representative of the *in vivo* response, the intermediately elevated level of cyclic GMP might be capable of setting in train a signal that induced gene activation or repression. Unfortunately no direct comparison can be made between the different reports, as different workers used different conditions for the delivery of the cyclic AMP to the cells (continuous feed or a single large dose) and different strains were used (phosphodiesterase deficient or wild type). As the amoebae on solid surfaces would normally receive a complex wave form as the cell-derived cyclic AMP signal passed over them, it is possible that the shape of the wave is important for determining the characteristics of the cyclic GMP response. Comparative studies in amoebae given signals of cyclic AMP with various rates of...
increase or decrease might help to elucidate this aspect of the adaptive mechanism.

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References


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