Chemotactic antagonists of cAMP inhibit Dictyostelium phospholipase C

Anthony A. Bominaar and Peter J. M. Van Haastert*

Department of Biochemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

*Author for correspondence

SUMMARY

In Dictyostelium discoideum extracellular cAMP induces chemotaxis via a transmembrane signal transduction cascade consisting of surface cAMP receptors, G-proteins and effector enzymes including adenylyl cyclase, guanylyl cyclase and phospholipase C. Previously it was demonstrated that some cAMP derivatives such as 3′-deoxy-3′-aminoadenosine 3′:5′-monophosphate (3′NH-cAMP) bind to the receptor and induce normal activation of adenylyl cyclase and guanylyl cyclase. However these analogues do not induce chemotaxis, probably because the signal is transduced in an inappropriate manner.

We have now studied the regulation of phospholipase C by cAMP and these chemotactic antagonists. cAMP induced the two-fold activation of phospholipase C leading to a transient increase of Ins(1,4,5)P3 levels. In contrast, the analogues induced a rapid decrease of intracellular Ins(1,4,5)P3 levels, due to the inhibition of phospholipase C activity. In a transformed cell-line lacking the G-protein that mediates phospholipase C inhibition, 3′NH-cAMP did not decrease phospholipase C activity and was no longer an antagonist of chemotaxis. These results suggest that inhibition of phospholipase C leads to aberrant chemotaxis.

INTRODUCTION

The cellular slime mold Dictyostelium discoideum is a microorganism that grows on bacteria. Exhaustion of the food supply induces a differentiation program that starts with the aggregation of the individual cells. In the multicellular structure cells differentiate to stalk cells and spores. Cell aggregation is mediated by chemotaxis towards cAMP that is secreted by starving cells (for review see Van Haastert, 1990). Extracellular cAMP is detected by surface receptors that have a presumed topography of seven transmembrane domains typical of G-protein-coupled receptors (Klein et al., 1988). The receptor interacts with one or more G-proteins inducing the activation or inhibition of effector enzymes. In vivo extracellular cAMP induces the transient accumulation of intracellular cAMP, cGMP and Ins(1,4,5)P3 (Mato et al., 1977; Europe-Finner and Newell, 1987; Europe-Finner et al., 1989; Van Haastert et al., 1989).

Using Dictyostelium mutants with specific defects the sensory transduction pathway has been partly unravelled. Mutant SYNAG is defective in receptor-stimulated adenylyl cyclase, but shows normal cAMP-induced chemotaxis (Schaap et al., 1986; Theibert and Devreotes, 1986; Van Haastert et al., 1987). In mutant stmF cGMP-phosphodiesterase activity is reduced, resulting in both prolonged cAMP-induced cGMP accumulation and prolonged chemotaxis (Ross and Newell, 1981). Mutant fgdC shows no cAMP-induced Ins(1,4,5)P3 accumulation and has a reduced chemotactic activity (Coukell et al., 1983; Bominaar et al., 1991a). These data suggest that the second messengers cGMP and Ins(1,4,5)P3/DAG play a role in chemotaxis, whereas intracellular cAMP does not.

In Dictyostelium cAMP can be detected by surface cAMP-receptors or by intracellular cAMP-dependent protein kinase. Analogues of cAMP have been extensively used to elucidate which cAMP-binding protein detects the cAMP signal. These proteins have a very different specificity for binding of cAMP analogues (Van Ments-Cohen and Van Haastert, 1989), and all studies with cAMP analogues indicate that the signal for chemotaxis, activation of adenylyl cyclase, guanylyl cyclase and the induction of differentiation is detected by the surface receptor (Van Haastert and Kien, 1983; Van Haastert, 1983a; Theibert et al., 1986; Schaap and Van Driel, 1985). During one of the first studies it was observed that some analogues behaved anomalously. Modification of the axial exocyclic oxygen atom with sulphur led to an analogue that, although it still bound to the receptor, was not able to induce a cGMP response or chemotaxis. This analogue was the first full competitive antagonist of cAMP in Dictyostelium. These full antagonists prevent the transduction of the cAMP signal (Van Haastert and Kien, 1983). Several other analogues, includ-
ing 3’NH-cAMP and cBIMP, show the following characteristics: (i) they bind to the surface receptor; (ii) they induce a cGMP response at a concentration proportional to their binding activity; (iii) they induce a chemotactic response only at extremely high concentration; (iv) they competitively antagonize cAMP-induced chemotaxis at very low analogue concentration. From these results it was concluded that these partial antagonists “do not prevent the transduction of the cAMP signal, but they erase the response evoked by cAMP” (Van Haastert, 1983a). However, we were unable to identify the response that was erased by the partial antagonists.

Studies with cAMP in mutant fgdC (Bominaar et al., 1991a) or the analogue 8-CPT-cAMP in wild-type cells (Peters et al., 1991) suggest that phospholipase C can be both stimulated and inhibited by extracellular cAMP. Recently, we have identified the G-proteins that regulate phospholipase C using mutants and transformants that lack specific G-proteins; expression of the α-subunit Go2 is essential for stimulation of phospholipase C, whereas Go1 is required for the inhibition of the enzyme (Bominaar and Van Haastert, unpublished).

Here we report that the chemotactic antagonist 3’NH-cAMP does not increase cellular Ins(1,4,5)P3 levels as observed with cAMP stimulation. In contrast, Ins(1,4,5)P3 levels decrease within 15 s by more than 80% due to the inhibition of phospholipase C. In a transformant with a Go1 gene disruption, 3’NH-cAMP cannot inhibit phospholipase C and is no longer an antagonist for chemotaxis. These results suggest that 3’NH-cAMP is a chemotactic antagonist of cAMP because it counteracts the cAMP-induced activation of phospholipase C.

**MATERIALS AND METHODS**

**Chemicals**
cAMP, 8-CPT-cAMP, EGTA and Hepes were obtained from Sigma (St. Louis). 3’NH-cAMP and cBIMP were kind gifts from Dr. B. Jastorff (University of Bremen, Germany).

**Cell culture and chemotaxis**
*Dictyostelium discoideum* strain NC-4 was grown in co-culture with *Klebsiella aerogaenes* on solid medium containing 3.3 g glucose, 3.3 g peptone, 4.5 g KH2PO4, 1.5 g Na2HPO4 and 15 g agar per liter. Strains AX9, JH130 and JH131 were grown in HL-5 medium (Coccuci and Sussman, 1970) containing 10 g/l of glucose instead of 16 g/l. Cells were harvested just before clearing the bacterial lawn (NC-4) or at the late logarithmic phase (axenic strains) in 10 mM Na/K-phosphate buffer (PB), washed three times by repeated centrifugation at 300 g and starved for 5 hours by shaking in PB to acquire aggregation competence. Chemotactic responses were determined using the small population assay (Konijn, 1970).

**Ins(1,4,5)P3 responses in vivo**
Aggregation-competent cells were washed and resuspended at 5x10^7 cells/ml in 40 mM Hepes, pH 6.5, and aerated for 10 min. Subsequently samples of cells were stimulated with cAMP or analogues as indicated in the figure legends. At the indicated timepoints cells were lysed by addition of an equal volume of 3.5% perchloric acid.

**Assay for phospholipase C activity**
Cells were prepared as described above and stimulated with cAMP or analogues in the presence of 5.9 mM EGTA. After 20 s the cells were lysed by rapid filtration through nucleopore polycarbonate filters (pore size 3 μm). At 10 s after lysis the phospholipase C reaction was started by increasing the free Ca2+ concentration to 10^-5 M with CaCl2. The reaction was quenched after 20 s by the addition of an equal volume of 3.5% perchloric acid. Basal Ins(1,4,5)P3 levels were determined in samples just before addition of CaCl2. Phospholipase C activity was calculated as the increase of Ins(1,4,5)P3 levels in vitro after Ca2+ addition.

**Determination of Ins(1,4,5)P3 levels**
Samples were brought to neutral pH with KHCO3 and centrifuged to remove the insoluble potassium perchlorate and cell debris. Ins(1,4,5)P3 levels in the supernatant were determined using an isotope dilution assay as described (Van Haastert, 1989).

**RESULTS**
The cAMP analogues 3’NH-cAMP, cBIMP and 8-CPT-cAMP bind to surface receptors with about 100-fold lower affinity than cAMP. However, at least 10,000-fold higher concentrations are required to induce a chemotactic response (Van Haastert, 1983a; Peters et al., 1991; see Fig. 1). Interestingly, at intermediate concentrations these analogues antagonize cAMP-induced chemotaxis. The underlying biochemical cause for this effect is unknown, but a blockade of receptor function is unlikely, because these analogues induce the normal activation of adenyllyl guanyl cyclase.

The Ins(1,4,5)P3 response might be involved in signal-transduction leading to chemotaxis (Europe-Finner and Newell, 1987; Europe-Finner et al., 1989; Van Haastert et al., 1989; Bominaar et al., 1991a,b). Therefore we investigated the effect of these analogues on the Ins(1,4,5)P3 response in vivo, as well as on the activity of phospholipase C in vitro following stimulation of cells in vivo. Fig. 2 shows that where cAMP itself gives a small but significant (20%, P<0.05) transient rise in Ins(1,4,5)P3 levels, all similar responses are observed using 3’NH-cAMP and 8-CPT-cAMP. The 3’NH-cAMP stimulated chemotaxis is not significantly different from that of the non-stimulated controls (Fig. 3). The chemotaxis towards cAMP and 3’NH-cAMP is expressed as a percentage of responding populations in the small population assay. (●●●) chemotaxis towards cAMP; (○○○) Chemotaxis towards 3’NH-cAMP; (▲▲▲) chemotaxis towards 10^-8 M cAMP in the presence of different concentrations of 3’NH-cAMP.

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*Fig. 1.* Dose-response curves for chemotaxis towards cAMP and 3’NH-cAMP. Chemotaxis is expressed as a percentage of responding populations in the small population assay. (●●●) chemotaxis towards cAMP; (○○○) Chemotaxis towards 3’NH-cAMP; (▲▲▲) chemotaxis towards 10^-8 M cAMP in the presence of different concentrations of 3’NH-cAMP.
three analogues induce a reduction of Ins(1,4,5)P3 levels. This effect is strongest for 3′NH-cAMP and cBIMP, leading to a persistent 80-100% reduction of Ins(1,4,5)P3 levels within 15 s. In the case of 8-CPT-cAMP the decrease in Ins(1,4,5)P3 is less pronounced, with a 60% reduction at 15 s, and a partial recovery towards basal levels after 30 s, as was demonstrated previously (Peters et al., 1991). Thus, instead of an increase in Ins(1,4,5)P3 levels the antagonistic analogues induce a decrease.

To discover the molecular mechanism of the reduction of Ins(1,4,5)P3 levels in vivo, the activity of phospholipase C was measured in vitro after stimulation of cells with cAMP or analogues in vivo. Fig. 3 demonstrates that cAMP stimulation of cells leads to a 2-fold increase of phospholipase C activity. In contrast, 3′NH-cAMP and cBIMP induced a 30% decrease of basal phospholipase C activity. The somewhat weaker effect of 8-CPT-cAMP on Ins(1,4,5)P3 levels in cells (Fig. 2) is also reflected in the effect on phospholipase C, where neither activation nor a significant inhibition was observed after 20 s of stimulation (Fig. 3). Cells were stimulated with different concentrations of cAMP and 3′NH-cAMP, lysed and phospholipase C activity was determined. Half-maximal stimulation was induced by 0.1 µM cAMP, and half-maximal inhibition by 10 µM 3′NH-cAMP (Table 1). If cAMP or the analogues were applied after cell lysis, no effects on phospholipase C activity were observed (data not shown).

The data presented above suggest a relation between inhibition of phospholipase C and antagonism of chemotaxis. To obtain further proof, the transformed cell line JH131 lacking the G-protein α-subunit Gt1 was examined (Kumagai et al., 1991); this G-protein has been shown to be essential for phospholipase C inhibition (Bominaar and Van Haastert, unpublished). Fig. 4 reveals that 3′NH-cAMP had no effect on Ins(1,4,5)P3 levels in Gt1-null cells (JH131), whereas a strong decrease of Ins(1,4,5)P3 levels was observed in the control cells (JH130). These cell-lines were then used to assay chemotactic and antagonistic activity of 3′NH-cAMP. Fig. 5A demonstrates that chemotaxis towards cAMP or high concentrations of 3′NH-cAMP in these Gt1-null cells is not different from what is seen in control cells. However, 3′NH-cAMP did not antagonize chemotaxis towards cAMP in the cell-line lacking Gt1 (Fig. 5B).

Summarizing, the partial chemotactic antagonists 3′NH-cAMP, cBIMP and 8-CPT-cAMP induce a decrease in Ins(1,4,5)P3 levels in vivo, which is due to an inhibition of phospholipase C activity. In cells without Gt1 neither inhibition of phospholipase C nor antagonism of chemotaxis are observed.

![Image](http://example.com/image1.png)

**Fig. 2.** cAMP and analogue induced in vivo Ins(1,4,5)P3 responses. Samples (100 µl) of cells were stimulated with 10⁻⁶ M cAMP (A), 10⁻⁵ M 3′NH-cAMP (B), 10⁻⁵ M cBIMP (C) and 10⁻⁴ M 8-CPT-cAMP (D). At the indicated time-points 100 µ1 PCA was added and Ins(1,4,5)P3 levels were determined in the neutralized lysates. Responses are given as a percentage of the basal level. * Significantly above basal with P<0.05; ** significantly below basal with P<0.01 (data are expressed as the mean ± s.e.m. of 3 independent experiments in triplicate).

![Image](http://example.com/image2.png)

**Fig. 3.** Phospholipase C activity in response to 10⁻⁶ M cAMP, 10⁻⁵ M cBIMP, 10⁻⁴ M 3′NH-cAMP and 10⁻³ M 8-CPT-cAMP. Activity is expressed as pmole min⁻¹ 10⁻⁶ cells⁻¹. ** Significantly above control with P<0.001; * significantly below control with P<0.01 (data are expressed as the mean ± s.e.m. of 3 independent experiments in triplicate).

| Table 1. Characteristics of 3′NH-cAMP and cBIMP |
|-----------------|-----------------|-----------------|
| cAMP (µM) | 3′NH-cAMP (µM) | cBIMP (µM) |
| Binding receptor | Chemotaxis | Antagonism |
| 0.03 | 0.003 | – |
| 14.7 | 40 | 1.0 |
| 490 | 13300 | 340⁺ |
| 3.4 | 120 | 0.31 |
| 110 | 40000 | 105⁺ |
| Antagonism | Activation AC | Activation GC |
| 0.004 | 0.015 | 0.1 |
| 6.0 | 4.0 | 10.0 |
| 1500 | 270 | 100† |
| n.d. | n.d. | n.d. |
| Activation PLC | Inhibition PLC | n.d. |
| 0.1 | – | 10.0 |
| – | – | n.d. |
| – | – | n.d. |
| *Antagonism of 3′NH-cAMP and cBIMP relative to chemotaxis of cAMP. |
| †Inhibition of PLC by 3′NH-cAMP relative to activation by cAMP. |
| n.d., not determined. |

Summary of the characteristics of 3′NH-cAMP and cBIMP as compared to cAMP. Concentrations given are those for the half-maximal effect. All data, except those for phospholipase C, were derived from the literature (see text). AC, adenylyl cyclase; GC, guanylyl cyclase; PLC, phospholipase C.
DISCUSSION

Several methods can be used in Dictyostelium to dissect signal transduction pathways of cAMP-induced chemotaxis, including mutants with well-defined defects and drugs that interfere with a specific pathway. In this report we used cAMP derivatives that activate adenylyl cyclase and guanylyl cyclase, but not phospholipase C. The more potent of these analogues, 3′-NH-cAMP and cBIMP nearly completely inhibit basal phospholipase C. The recently reported derivative 8-CPT-cAMP (Peters et al., 1991) is available commercially, but is less potent both in inhibiting phospholipase C and in antagonizing chemotaxis.

Dictyostelium cells are very sensitive to chemotactic signals and can detect a cAMP gradient of a few % difference over their cell length with a mean cAMP concentration of about 3 × 10^{-10} M (Van Haastert, 1983b). The dissociation constant of the surface cAMP receptor is about 3 × 10^{-8} M, indicating that at threshold chemotaxis only a few per cent of the receptors are occupied with cAMP (see Table 1 for a quantitative summary of the data). During analysis of the chemotactic activity of cAMP analogues it was observed that some analogues did not induce chemotaxis, although they bound to the receptor and activated adenylyl and guanylyl cyclase. These analogues antagonized cAMP-induced chemotaxis at analogue concentrations at which only a few per cent of the cAMP receptors were occupied (see Table 1). Hence most receptors were unoccupied and still capable of transducing the cAMP signal. The implication of these observations is that these analogues do not prevent cAMP signal transduction by blocking receptor function, but that they somehow erase a cAMP-induced response that is essential for chemotaxis. Neither activation nor desensitization of adenylyl or guanylyl cyclase appears to be involved, since the compounds showed normal stimulation of these effector enzymes, and pretreatment of cells with the analogues induced the correct level of desensitization (Van Haastert, 1983a; unpublished observations).

The present results show that, unlike cAMP, which stimulates Ins(1,4,5)P_{3} formation, the analogues inhibit phospholipase C, resulting in a significant decrease of Ins(1,4,5)P_{3} levels within 15 s. The data suggest a correlation between the antagonizing effect on chemotaxis and the reduction of Ins(1,4,5)P_{3} levels. Both 3′-NH-cAMP and cBIMP show a Ins(1,4,5)P_{3} reduction of up to 80% and are strong chemotactic antagonists, whereas 8-CPT-cAMP shows less reduction of Ins(1,4,5)P_{3} levels and is less potent as an antagonist as well.

Half-maximal stimulation and inhibition of phospholipase C are induced by 100 nM cAMP and 10 μM 3′-NH-cAMP, respectively; these concentrations are approximately equal to the dissociation constants of the surface cAMP receptor for these ligands (see Table 1). These results suggest that 3′-NH-cAMP antagonizes cAMP-induced chemotaxis because it inhibits phospholipase C and thereby counteracts the cAMP-induced stimulation of this enzyme. This hypothesis is strongly supported by experiments using the transformed cell line JH131, lacking the α-subunit of the G-protein G1 (Kumagai et al., 1991). This G-protein appears to be essential for receptor-mediated inhibition of
phospholipase C (Bominaar and Van Haastert, unpublished). Thus, in this cell line 3’NH-cAMP can not inhibit phospholipase C; the observation that 3’NH-cAMP is also not an antagonist of chemotaxis provides strong genetic evidence for the hypothesis that inhibition of phospholipase C is the primary cause of chemotactic antagonism.

Several mechanisms can explain how a reduction of the Ins(1,4,5)P$_3$ concentration may inhibit chemotaxis. First, the cAMP-stimulated formation of Ins(1,4,5)P$_3$ may be essential for chemotaxis; then, inhibition of Ins(1,4,5)P$_3$ formation directly inhibits chemotaxis. Second, Ins(1,4,5)P$_3$ and phospholipase C are not directly involved in chemotaxis, but participate in chemotaxis through the regulation of the intracellular Ca$^{2+}$ concentration (Europe-Finner and Newell, 1986; Van Duijn and Van Haastert, 1992). A sudden decrease of the Ins(1,4,5)P$_3$ levels interferes with Ca$^{2+}$ homeostasis, leading to inhibition of chemotaxis. Since intracellular Ca$^{2+}$ is also regulated by other processes such as release from Ins(1,4,5)P$_3$-insensitive stores or Ca$^{2+}$ uptake, activation of phospholipase C by cAMP may not be essential for chemotaxis. A third possibility is suggested by recent work of Shariff and Luna (1992), who show that DAG is involved in the formation of actin nucleation sites. Inhibition of phospholipase C would lower the DAG concentrations and thereby reduce actin polymerization. To discriminate between these models, we are currently constructing cell lines with a disrupted phospholipase C gene using the recently obtained cDNA encoding the Dictostelium phospholipase C enzyme (Drayer and Van Haastert, unpublished), and cell lines over-expressing DAG-kinase.

We thank Jeff Hadwiger and Richard Firtel for strain JH130 and JH131, and Bernd Jastorff for kindly providing the cAMP analogues. Part of this work was funded from a grant by the NWO Council for Medical Research.

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(Received 21 July 1992 - Accepted 18 September 1992)