Developmental regulation and evolution of cAMP signalling in Dictyostelium

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Chapter Two

Pharmacological profiling of the *Dictyostelium* adenylyl cyclases ACA, ACB, and ACG

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Pharmacological profiling of the *Dictyostelium* adenylyl cyclases ACA, ACB and ACG
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Abstract

Intracellular and secreted cAMP play key roles in controlling cell movement and regulation of numerous genes throughout development of the social amoeba *Dictyostelium discoideum.* cAMP is produced by three structurally distinct adenylyl cyclases, ACA, ACG and ACB which have distinctive but overlapping patterns of expression and, as concluded from gene disruption studies, seemingly overlapping functions. In addition to gene disruption, acute pharmacological abrogation of protein activity can be a powerful tool to identify its role in the biology of the organism. I have analysed the effects of a range of compounds on the activity of ACA, ACB and ACG to identify enzyme-specific modulators. Caffeine, which was previously used to specifically block ACA function, also inhibited cAMP accumulation by ACB and ACG. 2'3'-O-methyl isopropylidene adenosine (IPA) specifically inhibits ACA when measured in intact cells, without affecting ACB or ACG. All three enzymes are inhibited by the P-site inhibitor 2'5'dideoxyadenosine (DDA) when assayed in cell lysates, but not in intact cells. Tyrphostin A25 and SQ22536 proved to be effective and specific inhibitors for ACG and ACA respectively. Both compounds acted directly on enzyme activity assayed in cell lysates, but only SQ22536 was also a specific inhibitor when added to intact cells.

Introduction

The evolution of social amoebas or Dictyostelids was accompanied by extensive elaboration of cAMP signalling pathways (1). In *Dictyostelium discoideum*, cAMP acts as a classical second messenger for external stimuli. In this role, cAMP controls the initiation of multicellular development, the maturation of stalk and spore cells and the germination of spores. cAMP is also secreted in a highly regulated manner. As an extracellular signal, it coordinates the aggregation of starving cells and the directional movement of cells in multicellular structures. In addition, extracellular cAMP acts as a trigger for gene regulation at different stages of development (2,3).

D.discoideum has three structurally distinct adenylyl cyclases (ACs), ACA, ACB and ACG, for synthesis of cAMP. ACA produces cAMP for cell aggregation. It is structurally similar to the mammalian adenylyl cyclases with two different catalytic domains that are interspersed by two sets of six transmembrane helices (4). Similar to mammalian adenylyl cyclases, ACA is activated by a serpentine receptor, in this case the cAMP receptor cAR1 that interacts with a heterotrimeric G-protein, G2. However, in contrast to mammalian ACs (5), the G-protein does not interact directly with ACA. Instead the G2 βγ-subunit activates a phospholipid inositol kinase that generates plasma membrane binding sites for the Cytosolic Regulator of ACA, CRAC, which activates ACA upon recruitment to the plasma membrane (6).

ACG has an extracellular sensor domain, one or two transmembrane helices, and a single intracellular catalytic domain. ACG is an osmosensor that controls the germination of spores (7,8), and has an overlapping role with ACA and ACB in triggering prespore differentiation (See Chapter One). The catalytic domain of ACB (9), encoded by the *AcrA* gene (10), is homologous to that of the bicarbonate regulated bacterial adenylyl cyclases (11). Similar to the CyaC adenylyl cyclases from the cyanobacteria *Anabena spirulensis* and *Spirulina platensis*, ACB also harbours a response regulator domain and a histidine kinase domain. ACB is required for the maturation of spores (10).

Information on the role of each of the enzymes in particular aspects of the developmental programme has been derived from studies with null mutants in their respective genes (4,7,10). However, this approach precludes the demonstration of late developmental roles for those enzymes that are essential for an early stage of development. Moreover, the results shown in Chapter One indicate that the *Dictyostelium* adenylyl cyclases negatively regulate each other's expression. As a consequence, gene disruption in each of the genes will lead to overexpression of the others and partial or full restoration of the function of the abrogated gene . The use of enzyme-specific inhibitors with acute effects circumvents such problems.

Similar to the mammalian adenylyl cyclases, the *D.discoideum* enzyme ACG is active as a dimer, potentially creating two binding sites for ATP binding and catalysis (8). For ACA, ran-

dom mutagenesis studies have identified amino acids that are either essential for catalysis or for regulation by upstream components in the signalling pathway (12,13). However, apart from these data no structural information on enzyme regulation is available. In addition to studies of the protein crystal structure, the elucidation of the catalytic mechanism of the mammalian adenylyl cyclases has benefited greatly from pharmacological interference with enzyme activity. Notably the use of some ribose-modified adenosine analogs (known as P-site inhibitors) and of the AC activator forskolin, have contributed considerably to the understanding of how ATP interacts with the catalytic site and how the catalytically active dimer is formed (14,15).

In *Dictyostelium*, ACA is not activated by forskolin, but its activity in intact cells is inhibited by caffeine and by ribose-modified adenosine analogs (16-18). However, it is not clear whether the target for the analogs is ACA itself or the cAMP receptor that activates ACA (19,20). Neither these compounds nor any of the drugs known to directly modulate the activity of the mammalian adenylyl cyclases have yet been tested on ACG or ACB.

In this work we perform a systematic investigation of the effects of caffeine, ribose-modified adenosine analogs and other known regulators of mammalian adenylyl cyclases on the activities of ACA, ACB and ACG. This study identifies two enzyme-specific inhibitors for the *Dictyostelium* adenylyl cyclases and indicates that the effects of caffeine on adenylyl cyclase inhibition are mediated by two different targets.

Material and methods

Materials, cell lines and cell cultures

Guanosine 5'-O-(3-thiotriphosphate) (GTPγS), 2'-deoxyadenosine 3',5'-monophosphate (DcAMP), 2',3'-O-isopropylideneadenosine (IPA), 3-isobutyl-1-methylxanthine (IBMX), dithiothreitol (DTT), sodium pyrophosphate (PPi) and G418 were from Sigma (St.Louis, USA). 2',5'-dideoxyadenosine (DDA), α-cyano-(3,4,5-trihydroxy)cinnamonitrile (tyrphostin A25), 9-(tetrahydro-2'-furyl)adenine (SQ22536), cis-N-(2-phenylcyclopentyl)azacyclotridec-1-en-2-amine, HCl (MDL-12,330A), 2-Amino-7-(furanyl)-7,8-dihydro-5(6H)-quinazolinone (NKY80) were from Calbiochem (San Diego, USA). [2,8-^{3}H]cAMP was from Amersham Pharmacia Biotech (Little Chalfont, United Kingdom). *Naja messambica* snake venom was from SA venom suppliers (Louis Trichardt, South Africa)

Wild type NC4 cells, aca*-/A15::ACG* (4) and *aca-/rdeA*- (9) mutants were grown in standard axenic medium, which was supplemented with 20 µg/ml G418 for *aca-/A15::ACG* cells. A *pdeE-/regA*- double null mutant was created by transforming *pdeE*- cells (21) with the *pRegA*KO construct (22). Null mutants were selected from blasticidin resistant transformed clones by two PCR reactions and Southern analysis of genomic digests. While *pdeE*- cells develop normally (21), the *pdeE-/regA*- cells displayed the rapidly developing phenotype of *regA*- mutants (22). aca-/A15::ACG, *aca-/rdeA*- and *pdeE-/regA*- cells were harvested during exponential growth, washed once with 10 mM sodium/potassium phosphate buffer, pH 6.5 (PB) and resuspended in either PB or lysis buffer (2 mM $MgCl_2$ and 250 mM sucrose in 10 mM Tris, pH 8.0) to 10^8 cells/ml. Wild-type cells were plated on PB agar (1.5% agar in PB) at 2.5 x 10^6 cells/cm^2, starved for 6-8 hr at 22^{o}C until aggregation territories were formed, and subsequently collected and resuspended in PB or lysis buffer to 10^8 cells/ml.

Adenylyl cyclase assays in intact cells

Cells were resuspended in PB and exposed to either 5 mM DTT (ACB, ACG) or stimulated with 5 µM DcAMP in 5 mM DTT (ACA) in a total volume of 30 µl in microtiterplate wells under gentle agitation. After variable time periods, the reaction was terminated by addition of 30 µl of 3.5% (v/v) perchloric acid. Lysates were neutralized by addition of 15 µl of 50% saturated $KHCO_3$ and 75 µl cAMP assay buffer (4 mM EDTA in 150 mM K-phosphate, pH 7.5). Microtiterplates were centrifuged for 5 min at 3000 x g to precipitate protein and perchlorate. cAMP was assayed in 30 µl of the supernatant fraction by isotope dilution assay, using purified PKA

regulatory subunit (PKA-R) from beef muscle as cAMP-binding protein (23) and [2,8-^{3}H]cAMP as competitor. Since several of the compounds used in this work to alter adenylyl cyclase activity have some structural similarity to cAMP, and could potentially compete with [2,8-^{3}H]cAMP for binding to PKA-R, we compared and show t=0 time points for each assay without and with the highest concentration of the compound. No significant interference of any of the compounds with the cAMP assay could be detected.

Adenylyl cyclase assays in cell lysates

Cells were resuspended in ice-cold lysis buffer and lysed through nuclepore filters (pore size, 3 µM), conditionally in the presence of 30 µM GTPγS for NC4 cells. Aliquots of 10 µl cell lysate were added to 5 µl of variables at 4x the desired final concentration and 5 µl of assay mix (2 mM ATP, 0.8 mM IBMX and 40 mM DTT in lysis buffer), which was supplemented with 8 mM $MnCl_2$ for ACG and 38 mM $MgCl_2$ for ACB assays. After 5 min of incubation on ice, reactions were started by transferring the samples to a 22°C water bath. Reactions were terminated by adding 10 µl of 0.4 M EDTA, pH 8.0, followed by boiling for 1 min (23). cAMP was assayed directly in the boiled lysate. For all assays the cAMP levels were standardized on the protein content of the lysate or cell suspension.

cAMP phosphodiesterase assay

Cells were resuspended to 10^8 cells/ml in 10 mM DTT in PB, and incubated for 30 min with 10^{-7} M [2,8-^{3}H]cAMP, caffeine and IBMX as indicated in a total volume of 20 µl. Reactions were stopped by boiling, and the reaction product was converted into [2,8-^{3}H]adenosine by incubation for 30 min with 10 µg *Naja messambica* snake venom (which contains 5'nucleotidase). [2,8-^{3}H]adenosine was separated from [2,8-^{3}H]cAMP by adsorption of the latter to Dowex anion exchange resin and measured by scintillation counting.

Results

Effects of caffeine on ACA, ACB and ACG

The modified purine caffeine acts as an antagonist for adenosine A1 and A2A receptors in human brain (24), and also inhibits some mammalian cAMP phosphodiesterases (PDEs) (25). In *D.discoideum,* caffeine is commonly used to inhibit ligand-induced ACA activation (17). Its mode of action is not clear. It was suggested that caffeine could act by increasing cytosolic Ca^{2+} levels (17), but later studies showed that ACA activity was not inhibited by Ca^{2+} (26). To study whether caffeine is a specific inhibitor of ACA, we compared its effect on the activities of ACA, ACB and ACG in intact cells.

To measure each enzyme separately, we chose the following conditions: ACA was measured in wild-type NC4 cells that were starved for 6 hours to induce maximal expression of *ACA*. Cells were stimulated with the cAR agonist DcAMP in the presence of dithiothreitol (DTT) that acts here as an inhibitor of the extracellular phosphodiesterase PdsA (27). In *Dictyostelium,* cAMP is rapidly secreted after synthesis, and its extracellular accumulation can therefore be readily measured when PdsA is inhibited.

ACB is closely associated with the intracellular cAMP phosphodiesterase RegA. It appears to be constitutively active, but its activity can only be measured when RegA or the RegA activator RdeA is absent (9). ACB shows significant activity in vegetative *aca-/rdeA-* cells, which is not obscured by the presence of ACA. ACG is maximally expressed in spores, which are virtually inaccessible for measurement of adenylyl cyclase activity. This enzyme was therefore measured in vegetative *aca-* cells that express ACG from the constitutive A15 promoter (*aca-/A15::ACG*) (4). Although ACB is also present in vegetative cells, its activity (0.83 pmol/min.mg protein) is negligable compared to that of ACG (38 pmol/min.mg protein). cAMP production by both ACB or ACG can be measured during exposure of intact cells to DTT. Figure 1A shows that caffeine inhibits ACB, ACG and DcAMP stimulated ACA activity

equally effectively with an IC_{50} (effective concentration that produces halfmaximal inhibition) of 0.2-0.4 mM. Inhibition is complete at 10 mM, except for ACG where the higher caffeine concentrations become less effective. We next measured whether caffeine inhibited the three enzymes directly by testing its effect on the conversion of ATP into cAMP in cell lysates. Figure 1B shows that under these conditions caffeine does not inhibit ACB or ACG activity, nor the basal activity of ACA and even slightly stimulates the three enzymes at 3-10 mM. However, caffeine does inhibit GTPγS induced activation of ACA. These data indicate that neither ACA, ACB nor ACG are direct targets for the inhibitory effects of caffeine. They further more suggest that there are at least two different caffeine targets that mediate either its effect on GTPγS stimulation of ACA in lysates, or on cAMP production by at least two of the three ACs in intact cells.

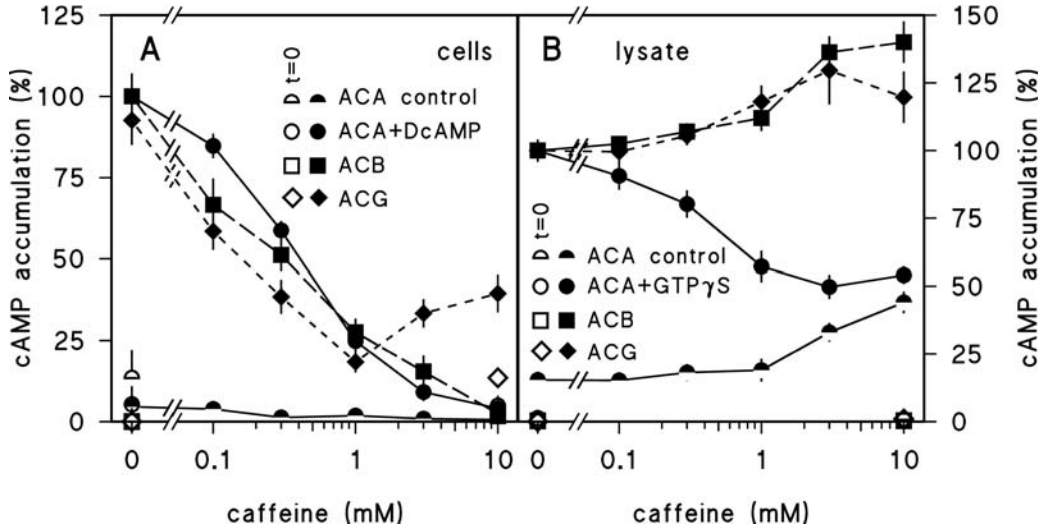

Figure 1. Effects of caffeine on ACA, ACB and ACG activity in intact cells and lysates
(A) *Intact cells.* For assay of ACA mediated cAMP accumulation, 6 hour starved NC4 cells were incubated for 0 and 3 min with 5 mM DTT in the presence and absence of 5 µM DcAMP. For assay of ACG, vegetative *aca-/A15::ACG* cells were incubated for 0 and 5 min with 5 mM DTT and for assay of ACB, vegetative *aca-/rdeA-* cells were incubated for 0 and 30 min with 5 mM DTT. Caffeine was present during incubation at the indicated concentrations. Reactions were terminated by addition of perchloric acid to 1.75% and cAMP was assayed in the neutralized cell lysates. Data are expressed as percentage of cAMP accumulation measured at the maximal incubation time for each enzyme in the absence of caffeine, and for ACA in the presence of DcAMP. This was 43.3±15.5, 24.6±5.1 and 190±53 pmoles cAMP/mg protein for ACA+DcAMP, ACB and ACG respectively.
(B) *Cell lysates.* Six hour starved NC4 cells were filter-lysed in the presence and absence 30 µM GTPγS and incubated for 0 and 5 min with 0.5 mM ATP, 2 mM $MgCl_2$ and 10 mM DTT for assay of ACA. Vegetative *aca-/A15::ACG* cells were lysed and incubated for 0 and 5 min with 0.5 mM ATP, 2 mM $MnCl_2$ and 10 mM DTT for assay of ACG. Vegetative *aca-/rdeA-* cell lysates were assayed for 0 and 30 min with 0.5 mM ATP, 10 mM $MgCl_2$ and 10 mM DTT for assay of ACB. Caffeine was present during incubation at the indicated concentrations. Reactions were terminated by addition of EDTA to 0.13 M and boiling, and cAMP was assayed in the boiled lysate. Data are expressed as percentage of cAMP accumulation measured at the maximal incubation time for each enzyme in the absence of caffeine, and for ACA in the presence of GTPγS. This was 34.0±6.0, 27.1±4.5 and 193±51 pmoles cAMP/mg protein for ACA+GTPγS, ACB and ACG respectively. Means and s.e.m. of at least three experiments performed in triplicate are presented.

A global effect of caffeine on cAMP accumulation could occur if caffeine inhibited cAMP secretion, in which case cAMP would be degraded by intracellular cAMP-PDEs, or if caffeine strongly stimulated a cAMP-PDE. Figure 2A shows that when ACB activity is measured in cells and medium separately, caffeine does not increase the amount of cAMP associated with the cell fraction. The small amount of cAMP that is produced in the presence of caffeine is fully secreted. Therefore caffeine is unlikely to inhibit cAMP secretion. *D.discoideum* has two intracellular cAMP-PDEs, *RegA* (22) and *PdeE* (21), and two cell surface associated enzymes, *PdsA* and *PDE4* (28). PdsA is in our assays inhibited by DTT, while RegA requires RdeA for activity (9) and should not be active in the *aca-/rdeA-* mutants that are used to assay ACB. PDE4 can be inhibited by the common PDE inhibitor IBMX (28). To test whether caffeine inhibition of cAMP accumulation is due to activation any of the four cAMP-PDEs, we measured its effect on cAMP accumulation by ACB in *PdeE-/RegA-* double null mutants with DTT to

inhibit PdsA and IBMX to inhibit PDE4. Figure 2B shows that in the absence of IBMX, cAMP accumulation is effectively inhibited by caffeine, which rules out PdeE, RegA and PdsA as caffeine targets. However in the presence of IBMX, caffeine inhibition is reduced. This suggests that PDE4 could be activated by caffeine. To test this directly we measured the effect of caffeine on ^{3}H-cAMP hydrolysis by intact cells, with DTT added to inhibit PdsA. Figure 2C shows that the IBMX-sensitive PDE activity, which is most likely PDE4, is inhibited instead of stimulated by caffeine. This indicates that caffeine does not inhibit cAMP accumulation by activating PDE4 either. Inhibition by IBMX of the effects of caffeine on cAMP accumulation as observed in figure 2B is perhaps due to true antagonism. IBMX (3-isobutyl-1-methylxanthine) and caffeine (1,3,7-trimethylxanthine) are similar in structure and may both bind to the target of caffeine, which, for the time being, remains obscure.

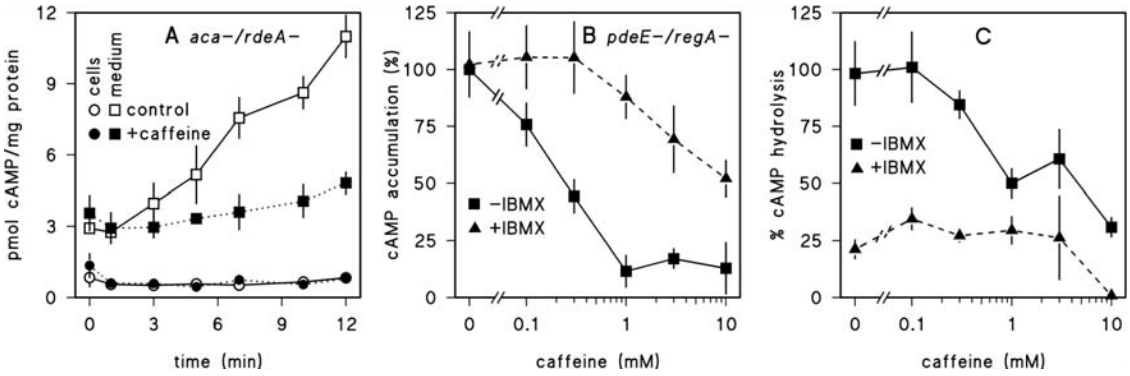

Figure 2. Effects of caffeine on cAMP secretion and cAMP-PDE activity.
(A) *cAMP secretion.* Vegetative *aca-/rdeA-* cells were incubated at 10^{8} cells/ml with 5 mM DTT in PB in the presence and absence of 2 mM caffeine. At the indicated time periods 50 µl aliquots of cell suspension were centrifuged for 5 s at 10.000 *g*. Supernatant (medium) and pellet (cells) fractions were rapidly separated and boiled for 30s. cAMP was assayed in both fractions. (B) *cAMP production in PDE null mutants.* Vegetative *pdeE-/regA-* cells were incubated for 30 min with 10 mM DTT in PB in the presence and absence of 3 mM IBMX and the indicated caffeine concentrations and assayed for cAMP. Data are expressed as percentage of cAMP accumulation in the absence of IBMX and caffeine. (C) *PDE activity.* Vegetative wild-type cells were incubated with 10 mM DTT and 10^{-7} M ^{3}H-cAMP in the presence and absence of IBMX and caffeine as indicated. After 30 min the samples were assayed for ^{3}H-5'AMP production. Data are presented as percentage of ^{3}H-cAMP hydrolysis obtained in the absence of IBMX and caffeine. Means and s.e.m. of two experiments performed in duplicate for (A) and triplicate for (B,C) are presented.

Effects of P-site inhibitors on AC activity in intact cells and cell lysates

In *Dictyostelium*, most if not all cAMP-induced responses that are mediated by the cAMP receptor cAR1, including the activation of ACA, are inhibited by adenosine (18,20,29,30). Adenosine analogs with modifications in the purine moiety are generally less effective than adenosine. Ribose-modified adenosine analogs are more effective and this is particularly the case for 2'3'-O-isopropylidene adenosine (IPA) (18,20,30-32). These effects were attributed to inhibition by adenosine of cAMP binding to cAR1, which shows a similar adenosine analog specificity (20,29).

Many mammalian adenylyl cyclases are directly inhibited by adenosine. Also here modification of the purine moiety reduces efficacy, while some ribose modified analogs, such as 2'-deoxyadenosine (DA) and particularly 2'5'-dideoxyadenosine (DDA) and 2'5'dideoxy-3'AMP are more active than adenosine (33). Due to its dependence on an intact purine moiety, this type of adenylyl cyclase inhibition is known as P-site inhibition (34). Co-crystallization of the C1 and C2 catalytic domains of mammalian AC with P-site inhibitors and pyrophosphate (PPi) showed that the complex occupied the ATP binding pocket of the enzyme, mimicking the enzyme-product complex in the transition state. Apart from this direct interaction, adenosine also has both stimulatory and inhibitory indirect effects on mammalian adenylyl cyclases that are mediated by a large class of G-protein coupled adenosine receptors (35). These receptors require an intact ribose-moiety and were traditionally called R-sites (34). However, this class of adenosine receptors has never been detected in *Dictyostelium*.

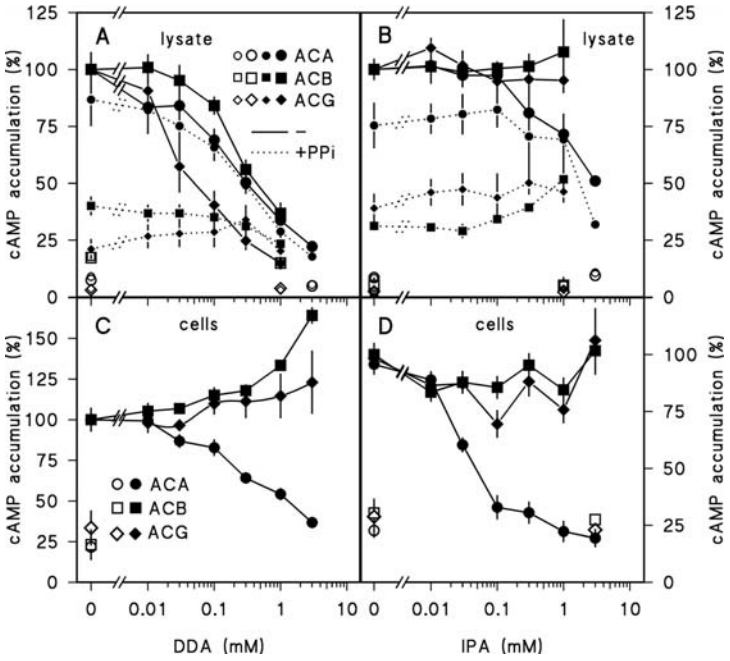

Figure 3. Effects of IPA and DDA on adenylyl cyclase activities
(A, B) *Cell lysates.* For assay of ACB, ACG or ACA activity, *aca-/rdeA-, aca-/A15::ACG* or NC4 cells were lysed without or with 30 µM GTPγS (NC4). Lysates were incubated in the presence (small symbols, dotted lines) and absence (large symbols, solid lines) of 1 mM Ppi and the indicated concentrations of IPA or DDA. Accumulated cAMP levels were determined after 0 (open symbols), 5 (closed symbols - ACA, ACG) or 30 minutes (closed symbols, ACB). C,D. *Intact cells. aca-/rdeA-, aca-/A15::ACG* and NC4 cells were incubated with DTT or DcAMP/DTT (NC4) in the presence of the indicated concentrations of DDA and IPA. Accumulated cAMP levels were determined after 0 (open symbols), 3 (circles, ACA), 5 (diamonds, ACG) or 30 minutes (squares, ACB). Data are standardized on cAMP levels obtained in the absence of DDA, IPA or PPi. Means and s.e.m of two to three experiments performed in triplicate are presented.

In *Dictyostelium*, ACA inhibition by DA and DDA was previously reported (19), but effects of P-site inhibitors on ACB or ACG were never investigated. It is also not known whether IPA inhibits *Dictyostelium* ACs directly. We therefore tested the effect of both DDA and IPA on the three *Dictyostelium* ACs. PPi was added conditionally at 1 mM. Figure 3A shows that DDA inhibits ACG activity in cell lysates most effectively (IC_{50}~75 µM), and inhibits ACA and ACB at 3-5 fold higher concentrations. In crude lysates the inhibitory effects of DDA are not dependent on added PPi, although the compound itself markedly inhibits both ACG and ACB activity. IPA has no effect on ACG and ACB and only slightly inhibits ACA activation in lysates (Fig. 3B). When the three enzymes are measured in intact cells, the effects of DDA and IPA are quite different. Neither of the two analogs inhibits ACG or ACB activity (Figs. 3C,D). IPA strongly inhibits DcAMP-induced ACA activation (IC_{50}~30 µM), while at least 15-fold higher concentrations of DDA are required to inhibit this response. These data indicate that the effects of IPA on ACA activation in intact cells are due to inhibition of cAMP binding to cAR1 as previously proposed. ACG, ACB and ACA are inhibited by the “classical” P-site inhibitor DDA when assayed in lysates, but DDA is apparently not sufficiently membrane-permeable to inhibit the enzymes when added to intact cells.

Searching for compounds that specificaly inhibit ACA, ACB or ACG

To identify inhibitors that act specifically on ACA, ACB or ACG, we tested a range of compounds that inhibit adenylyl cyclases in other organisms, such as NKY80 (36), MDL-12,330A (37) and SQ22536 (38). In addition, we tested tyrphostin A25, a compound that was initially identified as a tyrosine kinase inhibitor, but also proved to inhibit a variety of mammalian guanylyl- and adenylyl cyclases (39). NKY80 and tyrphostin A25 both act directly at the adenylyl cyclase catalytic core (36,39). We first tested the effects of the four inhibitors on ACA, ACB and ACG activity measured in cell lysates. Figure 4A shows that NKY80 does not inhibit ACG and has only a partial inhibitory effect on ACA and ACB. On the other hand, MDL-12,330A inhibits all three enzymes with IC_{50}'s that vary between 0.3 and 0.9 mM (Fig. 4B, Table 1). Tyrphostin A25 did not alter ACA or ACB activity (Fig. 4C), but was an effective ACG inhibitor with an IC_{50} of ~16 µM in cell lysates. However when added to intact cells, ACG inhibition required a 40-fold higher concentration. These concentrations also start to inhibit ACA and ACB, suggesting that this inhibition is due to a pleiotropic effect (Fig. 4E). SQ22536 effectively inhibited both GTPγS stimulated ACA activity in cell lysates and DcAMP stimulated ACA activity in intact cells (Figs. 4D,F). Remarkably, SQ22536 stimulated ACG activity

strongly and ACB activity weakly, both when assayed in lysates and intact cells. To conclude, both tyrphostin A25 and SQ22536 are specific inhibitors for ACG and ACA, respectively, when assayed in lysates. However, only SQ22536 can be used to inhibit ACA specifically in intact cells.

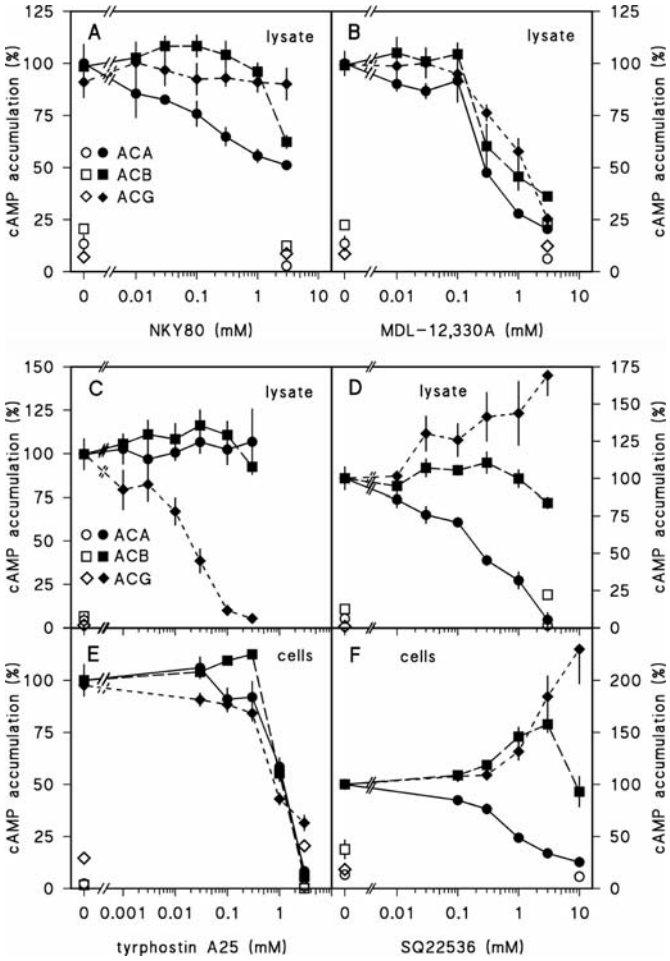

Figure 4. Effects of NKY80, MDL-12,330A, tyrphostin A25 and SQ22536 on ACA, ACB and ACG activities
(A-D) *Cell lysates.* For assay of ACB, ACG or ACA activity, *aca-/rdeA-, aca-/A15::ACG* or NC4 cells were lysed without or with 30 µM GTPγS (NC4) and incubated with the indicated concentrations of NKY80 (A), MDL-12,330A (B), tyrphostin A25 (C) or SQ22536 (D). Accumulated cAMP levels were determined after 0 (open symbols), 5 (closed symbols - ACA, ACG) or 30 minutes (closed symbols, ACB). Data were standardized on control cAMP levels. (E,F) *Intact cells. aca-/rdeA-*, *aca-/A15::ACG* or NC4 cells were incubated with DTT and/or DcAMP/DTT in the presence of the indicated concentrations of tyrphostin A25 or SQ22536. Accumulated cAMP levels were determined after 0 (open symbols), 3 (ACA), 5 (ACG) or 30 min (ACB). Data were standardized on levels obtained in the absence of inhibitor, and in case of ACA in the presence of DcAMP. Means and s.e.m of two to three experiments performed in triplicate are presented.

Discussion

This work was initiated to identify specific inhibitors for the three *Dictyostelium* adenylyl cyclases in order to identify and study specific roles of each of the enzymes during the life cycle of the organism. We first studied the effects of caffeine, which has long been used to study specific roles of ACA in cell aggregation and development (17,40-42). Our experiments showed that in intact cells caffeine inhibited ACB and ACG as efficiently as ACA (Fig. 1). The effects of caffeine on the three enzymes must be indirect, since it does not directly inhibit their basal activities when measured in cell lysates. In case of ACA, it does prevent activation of the enzyme by GTPγS, which activates the G-protein G2, that is part of the signal transduction cascade that normally activates ACA (2,3). However, neither ACB nor ACG are activated by G-proteins (4,9). It therefore appears that there is one target for caffeine that is at or downstream from the G-protein G2, and another that acts globally to prevent cAMP accumulation by either ACB or ACG and possibly also ACA.

We explored the possibility that caffeine could inhibit cAMP accumulation by either activating a cAMP-PDE activity or by inhibiting cAMP secretion, causing cAMP to be degraded intracellularly. However, no effects of caffeine on cAMP secretion were observed (Fig. 2A), and caffeine still inhibited cAMP accumulation when three out of the four cAMP-PDEs were inactivated (Fig. 2B).

Table 1. Pharmacological profiles of the three *Dictyostelium* ACs

ND: not determined; NA: not applicable: 0: no effect; -: inhibition; --: strong inhibition; +: stimulation; ++: strong stimulation; EC_{50}: effective concentration that yields half-maximal stimulation; *Cm*: concentration that yields maximal effect. IC_{50} values were determined by non-linear curve fitting of the data presented in figures 1-4 to $y = 100-[I] \times 100/(IC_{50}+[I])$, where *y* denotes % cAMP accumulation, with *t=0* cAMP levels subtracted, and [I] the inhibitor concentration. IC_{50} values are presented as means ± 95% confidence interval. The goodness-of-fit (r^2) is indicated between brackets.

	ACA (6h starvation)		ACB (vegetative)		ACG (vegetative)	
	Cells	Lysates	Cells	Lysates	Cells	Lysates
GTPγS	NA		NA		NA	
Mg^{+2}	NA		NA		NA	
Mn^{+2}	NA		NA		NA	
Caffeine	-- IC_{50}:361±113 µM (0.99)(17)	-* IC_{50}:825±114 µM (0.99)	-- IC_{50}:300±107 µM (0.99)	+ EC_{50}: 1-2 mM	-- IC_{50}:207±312 µM (0.79)	+ EC_{50}: 1-2 mM
IPA	-- IC_{50}:31±13 µM (0.98)	0/- IC_{50}:2.18±0.65 mM (0.96)	0	0	0	0
DDA	- IC_{50}:465±217 µM (0.97)	- IC_{50}: 274±153 µM (0.98)	+ EC_{50}:> 1 mM	- IC_{50}: 336±82 µM (0.99)	0/+	-- IC_{50}: 76±25 µM (0.99)
PP_i	NA	0/-	NA	-	NA	-
NKY80	ND	0/- IC_{50}:1.31±1.36 mM (0.78)	ND	0/- IC_{50}:5.78±4.97 mM (0.89)	ND	0
MDL-12,330A	ND	- IC_{50}: 329±174 µM (0.95)	ND	- IC_{50}: 497±383 µM (0.93)	ND	- IC_{50}: 885±258 µM (0.99)
Tyrphostin A25	0/- 1119±879 µM (0.94)	0	0/- 1336±715 µM (0.89)	0	0/- IC_{50}: 602±336 µM (0.95)	-- IC_{50}:16.6 ± 7.4 µM (0.97)
SQ22536	- IC_{50}: 812±280 µM (0.99)	-* IC_{50}: 247±134 µM (0.96)	+	0	++	+

* GTPγS-stimulated activity

The fourth cAMP-PDE was inhibited rather than activated by caffeine (Fig. 2C), in agreement with the known effect of caffeine on mammalian cAMP-PDEs (25). The observation that caffeine still inhibits cAMP accumulation in the *pdeE-/regA-* mutant that lacks intracellular cAMP-PDEs, confirms that it does not act on cAMP secretion, since in this mutant cAMP should then accumulate intracellularly.

We next analyzed the potential of ribose-modified adenosine analogs as specific AC inhibitors. 2'5'dideoxyadenosine (DDA) is a characteristic P-site inhibitor for mammalian adenylyl cyclases that acts directly at the catalytic core of the enzymes (15). It also inhibited the three *Dictyostelium* adenylyl cyclases, and particularly ACG, when measured in cell lysates indicating that also here it may interact directly with the catalytic region of the enzymes. However, DDA did not inhibit ACB nor ACG activity when added to intact cells, which implies that there is insufficient uptake of the compound. There was an inhibitory effect of DDA on ligand-induced ACA activation, but this, as discussed below, could be due to interference with ligand binding. 2'3'-O-isopropylideneadenosine (IPA) is another ribose-modified adenosine analog that is very effective in inhibiting cAR1-mediated responses in *Dictyostelium* (30-32). However, it has no reported merits as P-site inhibitor for mammalian adenylyl

cyclases and had no effects on ACB and ACG, and only modest effects on ACA activity measured in cell lysates (Fig 3C). IPA was however a very effective inhibitor of cAR1 mediated ACA activation in intact cells (Fig. 3D). Other ribose modified cAMP analogs, such as 2'-chloroadenosine and 2'O-methyladenosine also inhibit cAMP induced ACA activation in intact cells (18). These compounds as well as IPA and 2'deoxyadenosine are also effective inhibitors of cAMP binding to cAR1 (20). This strongly suggests that all inhibitory effects of ribose-modified analogs on cAMP-induced responses in intact cells are due to inhibition of cAMP binding to cAR1.

Lastly we tested four known inhibitors of adenylyl cyclases in other organisms for effects on ACA, ACB and ACG. One compound, NKY80, was not effective, while another, MDL-12,330A, was effective, but not specific for either of the three enzymes. The third compound tyrphostinA25 proved to be a specific and effective inhibitor of ACG in lysates. However, in intact cells it required much higher concentrations to have an effect, which was then no longer specific for ACG. The fourth inhibitor SQ22536, was a specific ACA inhibitor, also acting both in lysates and in intact cells. SQ22536 is highly lipophilic and may prove to be a useful agent for investigation of specific roles of ACA in *Dictyostelium* chemotaxis and development.

This work added a large number of traits that distinguish the adenylyl cyclases from each other. The novel traits are summarized with previously reported distinguishing features in Table 1. They will prove useful for enzyme identification in specific cell types or during specific stages in development of *Dictyostelium discoideum* or to recognize similar activities in other organisms.

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