ResearchGate

See discussions, stats, and author profiles for this publication at:
https://www.researchgate.net/publication/20292341

Cyclic AMP activates adenylate and guanylate cyclase of Dictyostelium discoideum cells by binding to different classes of cell-surface receptors A study with extracellular Ca++

ARTICLE *in* BIOCHIMICA ET BIOPHYSICA ACTA · OCTOBER 1985

Impact Factor: 4.66 · DOI: 10.1016/0167-4889(85)90002-3 · Source: PubMed

CITATIONS
32

READS
10

1 AUTHOR:

Peter J M Van Haastert
University of Groningen
245 PUBLICATIONS **8,227** CITATIONS

SEE PROFILE

Available from: Peter J M Van Haastert
Retrieved on: 24 January 2016

Biochimica et Biophysica Acta 846 (1985) 324–333
Elsevier

BBA 11548

cAMP activates adenylate and guanylate cyclase of *Dictyostelium discoideum* cells by binding to different classes of cell-surface receptors. A study with extracellular Ca^{2+}

Peter J.M. van Haastert

Cell Biology and Morphogenesis Unit, Zoological Laboratory, University of Leiden, Kaiserstraat 63, NL-2311 GP Leiden (The Netherlands)

(Received March 27th, 1985)

Key words: cAMP; Adenylate cyclase; Guanylate cyclase; Enzyme activation; Cell-surface receptor; Ca^{2+}; (*D. discoideum*)

cAMP induces a transient increase of cAMP and cGMP levels in *Dictyostelium discoideum* cells. Fast binding experiments reveal three types of cAMP-binding site (S, H and L), which have different off-rates ($t_{0.5}$, 0.7–15 s) and different affinities (K_d, 15–450 nM). A time- and cAMP-concentration-dependent transition of H- to L-sites occurs during the binding reaction (Van Haastert, P.J.M. and De Wit, R.J.W. (1984) J. Biol. Chem. 13321–13328). Extracellular Ca^{2+} had multiple effects on cAMP-binding sites. (i) The number of H + L-sites increased 2.5-fold, while the number of S-sites was not strongly affected. (ii) The K_d of the S-sites was reduced from 16 nM to 5 nM (iii) The conversion of H-sites to L-sites was inhibited up to 80%. The kinetics of the cAMP-induced cAMP accumulation was not strongly altered by Ca^{2+}, but the amount of cAMP produced was inhibited up to 80%. The kinetics of the cAMP-induced cGMP accumulation was strongly altered; maximal levels were obtained sooner, and the K_a was reduced from 15 to 3.5 nM cAMP. Ca^{2+}, Mg^{2+} and Mn^{2+} increased the number of binding sites, all with $EC_{50} = 0.5$ mM. The S-sites and the cGMP response were modified by equal Ca^{2+} concentrations and by higher concentrations of Mg^{2+} and Mn^{2+} (EC_{50} are respectively 0.4 mM, 2.5 mM and about 25 mM). The conversion of H- to L-sites and the cAMP response were specifically inhibited by Ca^{2+} with $EC_{50} = 20$ μM. It is concluded that cAMP activates guanylate cyclase through the S-sites; adenylate cyclase is activated by the H + L-sites, in which the appearance of the L-sites during the binding reaction represents the coupling of occupied surface cAMP receptors to adenylate cyclase.

Introduction

The development of the cellular slime mold, *Dictyostelium discoideum*, to a multicellular organism is mediated by extracellular cAMP signals [1–3]. cAMP is secreted periodically by center cells; neighboring cells react chemotactically and relay the signal [4,5]. Extracellular cAMP is detected by cell-surface receptors [1–3]. This induces several responses, such as the fast activation of guanylate cyclase and the slower activation of adenylate cyclase [6,7]. The increase of intracellular cGMP is assumed to be related to the chemotactic reaction [8], while the cAMP produced intracellularly is secreted into the medium [9].

Binding of cAMP to *D. discoideum* cells is heterogeneous; at least three binding sites can be distinguished in fast binding experiments [10]. A small portin of the total of binding sites (about 4%) slowly releases bound [^{3}H]cAMP with a half-life of about 15 s. This binding site, which has been designated as S, has high affinity for cAMP

with an apparent K_d of 15 nM. The remaining binding sites release bound [^{3}H]cAMP very quickly with a half-life of 0.7–1.5 s. Two types belong to this class, with respectively high (H) and low (L) affinity (the apparent K_d is respectively 60 nM and 450 nM). During the binding reaction there is a time- and cAMP dose-dependent transition of H to L binding sites.

The transition of high-affinity binding to low-affinity binding strongly resembles the binding of agonists to β-adrenergic receptors [11]. There, transition appears to be due to the coupling of the occupied high-affinity receptor with a GTP-binding protein, resulting in the activation of adenylate cyclase [12]. Recently, evidence has been presented for a functional G-protein in *D. discoideum* [13–15]. Guanine nucleotides promote the transition of H- to L-sites; they also alter the affinity and number of S-sites. It might be hypothesized that in *D. discoideum* the different binding types represent the coupling of an occupied cAMP receptor to different effector molecules, such as, adenylate ecyclase, guanylate cyclase or GTP-binding proteins. To investigate this hypothesis we have analysed the effects of several drugs on the different binding types, the activation of adenylate cyclase and on the activation of guanylate cyclase.

Extracellular calcium ions are reported to increase the number of cAMP-binding sites in *D. discoideum* [16], to accelerate development to a multicellular stage [17], and to inhibit the cAMP-mediated activation of adenylate cyclase [18]. Here it is shown that extracellular Ca^{2+} inhibits both the transition of H to L and the cAMP-induced cAMP accumulation with an EC_{50} = 20 μM Ca^{2+}; other divalent cations are not inhibitory. The apparent K_d of S and the apparent K_a of the cAMP-induced cGMP accumulation are both reduced 3–4-fold with EC_{50} = 400 μM Ca^{2+}. Mg^{2+} and Mn^{2+} are active at respectively 6- and about 50-fold higher concentrations. These data are interpreted as evidence that the cAMP signal for the activation of adenylate and guanylate cyclase are detected by different classes of cell-surface cAMP-binding site.

Materials and Methods

Materials. [2,8-^{3}H]cAMP (1.5 TBq/mmol), the cGMP radioimmunoassay kit and the cAMP binding protein assay kit were obtained from Amersham International (Bucks., U.K.), dithiothreitol was from Sigma, and silicon oils were from Wacker Chemie (München, F.R.G.). NaCl, KCl, $MgCl_2$, $MnCl_2$, $CaCl_2$, KH_2PO_4, Na_2HPO_4 and Tris were of analytical grade obtained from Merck (Darmstadt, F.R.G.).

Culture conditions. All experiments have been performed at 20–22°C. *D. discoideum* NC_4(H) was grown in association with *Escherichia coli* 281 on a solid medium containing 3.3 g peptone 3.3 g glucose, 4.5 g KH_2PO_4, 1.5 g $Na_2HPO_4 \cdot 2H_2O$ and 15 g of agar per liter. Cells were harvested in the late log phase with 10 mM sodium/potassium phosphate buffer (pH 6.5) and freed from bacteria by repeated centrifugation at 100 × *g* for 4 min.

cAMP-binding assay [10]. Cells were starved for 5 h by shaking in phosphate buffer at a density of 10^7 cells/ml. Then cells were collected, washed twice and resuspended in 10 mM phosphate buffer (pH 6.5) at a density of $1 \cdot 10^8$ cells/ml. cAMP-binding was measured in a total volume of 100 μl containing phosphate buffer, 5 mM dithiothreitol, different concentrations [^{3}H]cAMP, 80 μl cells and additives as indicated in the figures. Bound and free [^{3}H]cAMP was separated by centrifugation of the cells through 200 μl silicon oil (AR 200/Ar 20-1/2) in a swing-out rotor at 10000 × *g* for 30 s. The technique for rapid mixing and centrifugation has been described previously [10].

cAMP-relay response [19]. Cells were starved for 4–5 h in suspension, washed twice and resuspended in phosphate buffer at a density of $5 \cdot 10^7$ cells/ml. Cells (90 μl) were stimulated with 10 μl of a mixture containing (final concentrations) phosphate buffer, 5 mM dithiothreitol, 10 μM 2′-deoxyadenosine 3′,5′-monophosphate (cdAMP). The reaction was terminated by the addition of 100 μl of perchloric acid (3.5% v/v). Lysates were neutralized with 50 μl $KHCO_3$ (50% saturated at 20°C), and centrifuged at 8000 × *g* for 2 min. The cAMP concentration was measured in the supernatant by isotope dilution assay using a cAMP-binding protein which has a very low affinity for cdAMP.

cGMP-response [20]. Cells were starved on non-nutrient agar at a density of $1.5 \cdot 10^6$ cells/cm^2. After 4–5 h cells were harvested, washed twice, and resuspended in phosphate buffer at a density

of 10^8 cells/ml. Cells (100 μl) were stimulated with 20 μl containing different cAMP concentrations, and lysed at the indicated times with 100 μl perchloric acid. Lysates were neutrlized as described above, and the cGMP content was measured by radioimmunoassay.

Solubility of cations. The solubility of the divalent cations in 10 mM phosphate buffer or 15 mM Tris-HCl was investigated as follows. $CaCl_2$, $MgCl_2$ or $MnCl_2$ (10 mM final concentration of cations) was added to the buffers in the same way as during experiments with cells. In phosphate buffer, $MnCl_2$ precipitated immediately; $CaCl_2$ was soluble for at least 1 h, but a precipitate was observed after 24 h; no precipitate was found with $MgCl_2$. All ions were soluble in Tris-HCl. The effects of Ca^{2+} on signal transduction was investigated in phosphate buffer to enable a direct comparison with all previous results on signal transduction in *D. discoideum*. A comparison of the divalent cations was made in Tris-HCl, because manganese could not be used in phosphate buffer.

Experiments were performed at least three times with similar results. The standard deviation in cAMP-binding data is 3–4% of the mean. The standard deviation in cAMP and cGMP determinations is about 10% of the mean. Error bars indicating the standard deviations are shown only when they are beyond 3-times the size of the symbols.

Results

Effect of extracellular Ca^{2+} on cAMP-binding to D. discoideum cells

The association of 30 nM [^{3}H]cAMP to aggregative *D. discoideum* cells at 20°C is shown in fig. 1. In the absence of added Ca^{2+}, cAMP-binding increases to a maximum obtained at about 6 s, which is followed by a decline to equilibrium value at 45–60 s. Previously [10], it has been shown that this decline of [^{3}H]cAMP-binding is caused neither by degradation of [^{3}H]cAMP, nor by dilution of added [^{3}H]cAMP by secreted cAMP, nor by a reduction of the number of cAMP-binding sites. The reduction of [^{3}H]cAMP-binding between 6 s and 45 s appears to be due to a transition of high-affinity binding sites to low-affinity binding

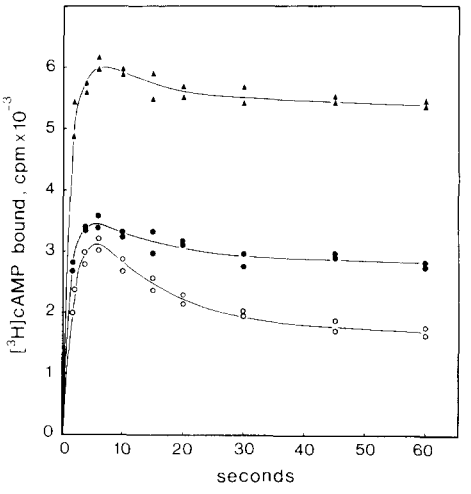

Fig. 1. The effect of Ca^{2+} on the association of cAMP to *D. discoideum* cells. Cells were rapidly mixed with 30 nM [^{3}H]cAMP (about 100000 cpm) in the absence (○) or presence of 30 μM Ca^{2+} (●) or 1 mM Ca^{2+} (▲). At the times indicated, cell-associated [^{3}H]cAMP and free [^{3}H]cAMP were separated by centrifugation of the cells through silicon oil in a small swing-out rotor at $10000 \times g$ for 15 s. Nonspecific binding, which was determined by including 100 μM cAMP in the incubation mixture, was substracted from all data shown.

sites [10]. The inclusion of 30 μM Ca^{2+} during the binding reaction slightly increases the initial binding of [^{3}H]cAMP at 6 s and strongly increases the equilibrium binding at 45–60 s. Apparently, the transition of high-affinity binding to low-affinity binding is impaired by 30 μM Ca^{2+}. The inclusion of 1 mM Ca^{2+} during the binding reaction also strongly increases the initial binding of [^{3}H]cAMP. As with 30 μM Ca^{2+}, the decrease of [^{3}H]cAMP-binding between 6 s and 60 s is reduced.

The dissociation of bound [^{3}H]cAMP from *D. discoideum* cells is shown in Fig. 2. At 2 nM [^{3}H]cAMP most of the bound [^{3}H]cAMP dissociates with a half-life of less than 2 s, while a small part dissociates more slowly with a half-life of about 15 s. This slowly dissociating binding site has been called S [10]. Extracellular Ca^{2+} increases the binding of 2 nM [^{3}H]cAMP, both before and after the chase with excess cAMP (Fig. 2); the off-rate of S is not altered by Ca^{2+}, neither is the fraction of cell-associated label bound to S (inset Fig. 2). Binding of [^{3}H]cAMP to S is easily measured by association of [^{3}H]cAMP during 45 s (by which all binding sites are occupied) followed by a

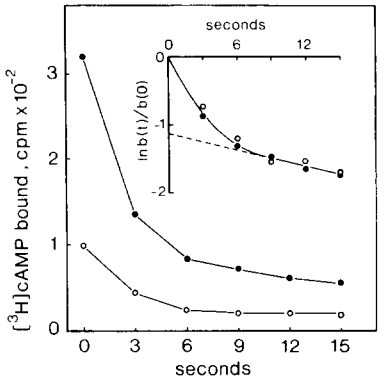

Fig. 2. The effect of Ca^{2+} on the dissociation of the [^{3}H]cAMP-receptor complex. *D. discoideum* cells were incubated for 45 s with 2 nM [^{3}H] cAMP in the absence (○) or presence (●) of 10 mM Ca^{2+}. At $t = 0$ s excess cAMP (100 μM) was added, and cells were centrifuged through silicon oil at the times indicated in the figure. Inset: Semilogarithmic plot of the same data. The slope is the rate constant of dissociation, which yields $k_{-1} = 0.05$ s^{-1} in the absence or presence of Ca^{2+}.

chase with excess cAMP during 6 s (during which all radioactivity bound to H and L is released). A Scatchard plot S in the absence and presence of 10 mM Ca^{2+} is shown in Fig. 3. Both Scatchard plots are approximately linear; Ca^{2+} does not increase the total number of S-binding sites, but reduces the apparent K_d from 16.2 nM to 5.2 nM.

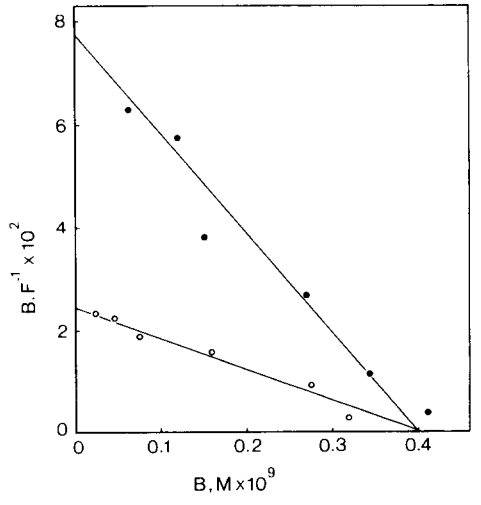

Fig. 3. The effect of Ca^{2+} on the Scatchard plot of S. *D. discoideum* cells were incubated for 45 s with different [^{3}H]cAMP concentrations in the absence (○) or presence (●) of 10 mM Ca^{2+}. At $t = 0$ s excess cAMP was added, and cell-associated [^{3}H]cAMP was determined 6 s later. The apparent K_d is 16.2 nM in the absence and 5.2 nM in the presence of Ca^{2+}. The B_{max} is 0.4 nM, which equals about 4000 binding sites/cell.

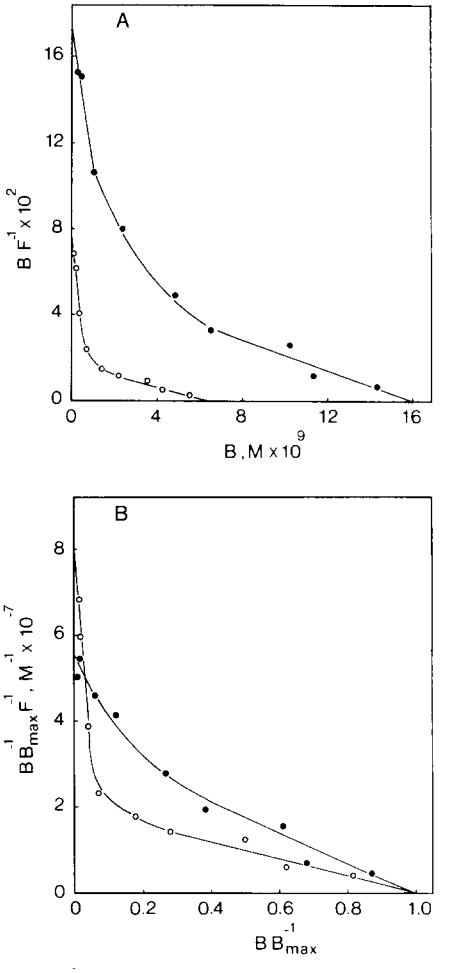

Fig. 4. The effect of Ca^{2+} on the Scatchard plot of H+L. *D. discoideum* cells were incubated with different [^{3}H]cAMP concentrations in the absence (○) or presence (●) of 10 mM Ca^{2+}. Cell-associated radioactivity was determined after an incubation period of 45 s, and is bound to the three cAMP-binding sites, H, L and S (part A) At 6 s after a chase with excess cAMP, essentially all cell-associated cAMP is bound to S. During this 6 s chase, about 25% of the occupied S-sites dissociate; therefore the occupation of S at 45 s equals the occupation after a 6 s chase (Fig. 3) divided by 0.75. This value is subtracted from total binding at 45 s (Fig. A), whereby the binding to H + L is obtained. These data are presented in part B. Data were normalized for differences in B_{max} to obtain a better impression of the differences in linearity of the two curves.

Binding of [^{3}H]cAMP to the total of the three binding sites at equilibrium is shown in Fig. 4. Addition of 10 mM Ca^{2+} results in an approx. 2.5-fold increase of the number of binding sites (Fig. 4A). The binding of [^{3}H]cAMP to S at equilibrium is calculated by dividing the binding at 6 s after a chase with excess cAMP by 0.75 (this figure is obtained from the off-rate of S; $k_{-1} = 0.05$ s^{-1} predicts 25% dissociation during 6 s). The binding of [^{3}H]cAMP to the sum of H + L is then calculated by subtraction of S-specific binding from total binding (Fig. 4B). In the absence of added extracellular Ca^{2+} the Scatchard plot of H + L thus obtained is strongly curved, which is due to the cAMP concentration-dependent transition of H to L [10]. After using several kinetic approaches, and investigating the effects of drugs, it was suggested that the K_d of H is 60 nM, the K_d of L is 450 nM and that the fraction of H-sites is 40% in the absence of cAMP, and 5% in the presence of 100 nM cAMP [10]. The Scatchard plot of H + L is almost linear in the presence of Ca^{2+}, again demonstrating that the transition of H to L is suppressed by Ca^{2+}. The Scatchard plot was resolved into two components with fixed affinities and a fixed ratio (i.e., it is assumed that no transition of H to L occurs in the presence of Ca^{2+}). Such a computer fit shows that 23% of the sites have a K_d of 57 nM and 77% have a K_d of 462 nM. These K_d values are close to those obtained previously for respectively H and L [10]. This would suggest that Ca^{2+} does not alter the affinities of H and L, but that it interferes with the interconversion of H to L.

The results obtained so far, show that extracellular calcium ions have at least three effects: (i) The total number of binding sites is increased about 2.5-fold; (ii) the affinity of S is increased about 3-fold, while the number of S-binding sites and its off-rate are not altered; (iii) the transition of high-affinity binding to low-affinity binding is suppressed by Ca^{2+}.

The Ca^{2+} concentration-dependences of these processes are shown in Fig. 5. The effect on B_{max} was detected by measuring binding at high [^{3}H]cAMP concentrations (200 nM). Ca^{2+} induces approx. 2.5-fold stimulation, the concentration which induces a half-maximal effect (EC_{50}) is about 500 μM Ca^{2+}. The effect of Ca^{2+} on the

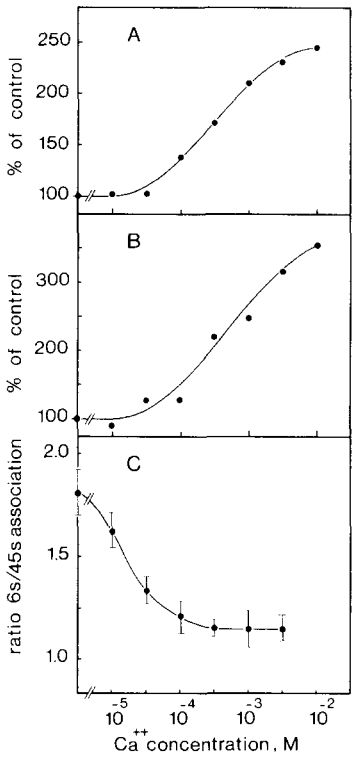

Fig. 5. Dose-response curves of the effect of Ca^{2+} on B_{max} (A); the affinity of S (B); and the transition of H to L (C). (A) cells were incubated with 200 nM [^{3}H]cAMP in the presence of different Ca^{2+} concentrations for 45 s. Binding in the absence of added Ca^{2+} was 400 ± 26 fmol/10^7 cells ($n = 4$). (B) cells were incubated with 1 nM [^{3}H]cAMP in the presence of different Ca^{2+} concentrations for 45 s. Then excess cAMP was added, and cell-associated [^{3}H]cAMP was measured 6 s later. Binding in the absence of added Ca^{2+} was 3 ± 0.1 fmol/10^7 cells, ($n = 4$). (C) Cells were incubated with 30 nM [^{3}H]cAMP in the presence of different Ca^{2+} concentrations for 6 s and for 45 s. The ratio of cell-associated [^{3}H]cAMP after these incubation periods is presented.

affinity of S was detected by measuring the binding of low [^{3}H]cAMP concentrations (1 nM) 6 s after a chase with excess cAMP. Ca^{2+} induces approx. 3.5-fold increase of the affinity of S with $EC_{50} = 400$ μM Ca^{2+}. The transition of high-affinity binding to low-affinity binding is given by the reduction of binding of moderate [^{3}H]cAMP concentrations (30 nM) between 6 s and 45 s after the onset of association. The ratio of binding at 6 s and at 45 s decreases from about 1.8 to about 1.2 with $EC_{50} = 20$ μM Ca^{2+}. The effect of extracellular calcium ions thus appears to be rather specific:

the effect on the transition of H to L occurs at about 25-fold lower concentrations than the effect on S.

The effect of extracellular Ca^{2+} on cAMP relay

The accumulation of intra- and extracellular cAMP levels after chemotactic stimulation of aggregative *D. discoideum* cells can be measured by using a potent cAMP agonist which shows minimal cross-inhibition in the isotope dilution assay for the determination of cAMP concentrations. 2'-Deoxyadenosine 3',5'-monophosphate (cdAMP) is only 7-times less active than cAMP for *D. discoideum* cell-surface receptors, while it is about 1500-times less active than cAMP in the isotope dilution assay [19]. Dithiothreitol, an inhibitor of phosphodiesterase in *D. discoideum* [21], is used to protect cdAMP and extracellular cAMP.

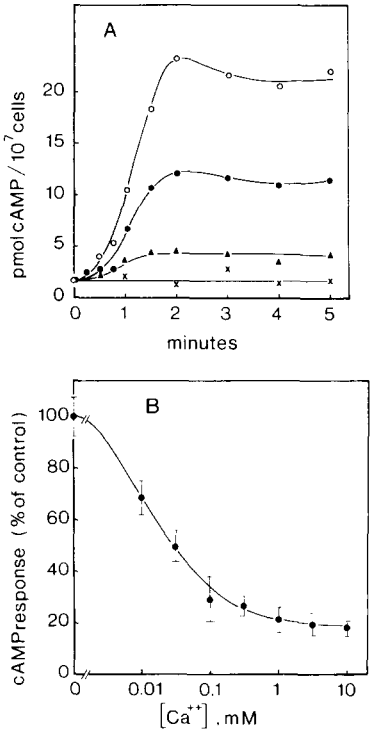

Fig. 6. The effect of Ca^{2+} on cAMP relay. (A) Aggregative *D. discoideum* celals were stimulated with 10 μM cdAMP and 5 mM dithiothreitol in the absence (○) or presence of 30 μM (●) or 1 mM (▲) Ca^{2+}. In a control (×), cells were not stimulated with cdAMP or dithiothreitol; to these cells was added 1 mM Ca^{2+}. At the times indicated, cells were lysed and the cAMP content was measured by isotope dilution assay in which cdAMP did not show cross-inhibition: (B) cAMP content at 3 min after stimulation of aggregative cells with cdAMP and dithiothreitol in the presence of different Ca^{2+} concentrations.

cAMP levels slowly increase after stimulation with cdAMP; a plateau value is reached after 2–3 min, and half maximal values are obtained in aboaut 1.5 min (Fig. 6A.) The addition of 1 mM Ca^{2+} without the cdAMP stimulus does not affect basal cAMP levels. However, 1 mM Ca^{2+} strongly inhibits the cdAMP-induced accumulation of cAMP levels. The pace of the cAMP accumulation is almost identical in the absence or presence of 30 μM added Ca^{2+}, although the cAMP accumulation might level off slightly earlier in the presence of extracellular Ca^{2+}. Small amounts of added extracellular Ca^{2+} are already sufficient to inhibit the cAMP accumulation, but the inhibition is never more than about 80%. A half-maximal effect is induced by about 16 μM added Ca^{2+} (Fig. 6B).

The effect of extracellular Ca^{2+} on the cGMP-response

cAMP induces a fast transient accumulation of cGMP levels in *D. discoideum*. Maximal cGMP concentrations are obtained after about 10 s, and basal levels are recovered in 30–45 s (Fig. 7A, and B). The addition of 10 mM Ca^{2+} without stimulus does not alter basal cGMP levels (Fig. 7A). Preincubation of cells with 10 mM Ca^{2+} during 30 s and subsequent stimulation with 100 nM cAMP strongly alters the dynamics of the cGMP-response. The initial rate of the cGMP accumulation is slightly reduced by Ca^{2+}; maximal cGMP levels are obtained earlier (at about 6 s), and cGMP levels decline faster (Fig. 7A). The addition of 10 mM Ca^{2+} 5 s after stimulation with 100 nM cAMP results in the almost immediate decline of cGMP levels (Fig. 7A). This suggests that added Ca^{2+} affects the cAMP-mediated cGMP-response almost instantaneously. In Fig. 7B, cells were stimulated with 10 nM cAMP, which induces approx. a half-maximal response in control cells. As with higher cAMP concentrations, maximal cGMP levels are obtained after about 10 s in the absence and after about 6 s in the presence of 10 mM Ca^{2+}. In contrast to higher cAMP concentrations, the initial rate of cGMP accumulation is enhanced by Ca. In the presence of 10 mM Ca^{2+}, 10 nM and 100 nM cAMP induced almost the same cGMP accumulation. The experiments of Figure 7A and

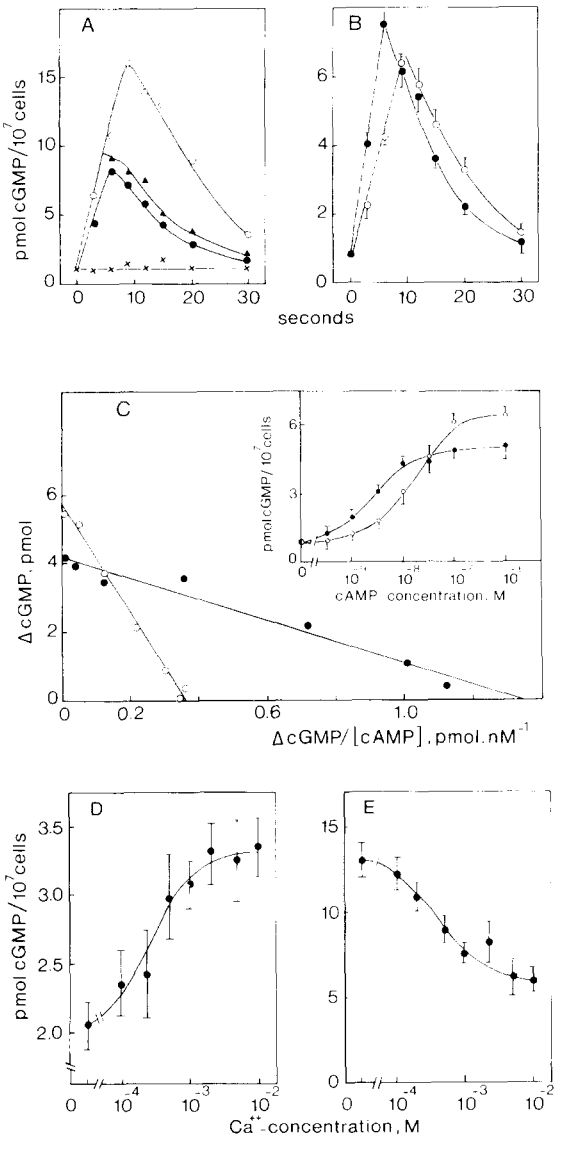

Fig. 7. The effect of Ca^{2+} on the cGMP-response. (A) Aggregative *D. discoideum* cells were stimulated with 100 nM cAMP at $t = 0$ s in the absence (○) or presence (●, ▲) of 10 mM Ca^{2+}. Calcium ions were added at $t = 5$ s (▲), or at $t = -30$ s (●). In a control (×), cells were not stimulated with cAMP, and Ca^{2+} was added at $t = 0$ s. (B) Cells were stimulated with 10 nM cAMP at $t = 0$ s in the absence (○) or presence of 10 mM Ca^{2+} (●). Ca^{2+} was added at $t = -30$ s. (C) Cells were lysed at 5 s after stimulation with different cAMP concentrations. (○), control cells; (●), cells preincubated with 10 mM Ca^{2+} for 30 s. The inset is a dose response-curve. The main figure is an Eadie-Hofstee plot of the same data; ΔcGMP represents the increase in cGMP levels. (D) Cells were preincubated with different Ca^{2+} concentrations for 30 s, then stimulated with 5 nM cAMP and lysed at 5 s after stimulation. (E) Cells were preincubated with different Ca^{2+} concentrations for 30 s, then stimulated with 100 nM cAMP and lysed at 10 s after stimulation. The data are the means of duplicate determinations in (A), and the means ± S.D. of triplicate determinations in the other panels.

B suggest that extracellular Ca^{2+} has at least two effects on the cGMP-response: The response is induced at lower cAMP concentrations, and maximal cGMP levels are obtained earlier after stimulation.

Fig. 7C shows the cGMP-response induced by different cAMP concentrations in the absence or presence of 10 mM Ca^{2+}, cGMP levels were measured 5 s after stimulation; thus, before maximal cGMP levels were obtained, by which the effect of Ca^{2+} on the time parameter of the cGMP peak did not interfere with the dose-response curve (inset Fig. 7C). These data are presented as an Eadie-Hofstee plot (Fig. 7C); the intercept with the ordinate represents the maximal response. Extracellular Ca^{2+} inhibits the maximal rate of cGMP accumulation by about 25%. The slope of the Eadie-Hofstee plot represents the activation constant, K_a (cAMP concentration which induces a half-maximal cGMP response). Extracellular Ca^{2+} reduces the K_a from 15 nM in control cells to about 3.5 nM in cells preincubated with 10 mM Ca^{2+}.

The effect of different Ca^{2+} concentrations on the modulation of the cGMP-response is shown in Fig. 7D, E. The increase of affinity for cAMP was measured by stimulation of cells in the presence of different Ca^{2+} concentratiaons with a low cAMP concentration (5 nM). Extracellular Ca^{2+} induces an about 60% increase of cGMP levels 5 s after stimulation with cAMP; a half-maximal effect is obtained at 200–500 μM Ca^{2+} (Fig. 7D). The effect of different Ca^{2+}-concentrations on the pace of the cGMP-response was measured by recording the response 10 s after stimulation with a high cAMP concentration(100 nM). Extracellular Ca^{2+} induces an approx. 60% decrease of cGMP levels; A half-maximal effect is induced by 300–500 μM Ca^{2+} (fig. 7E).

Specificity of cations

The effects of the monovalent cations K^+ and Na^+, and the divalent cations Mg^{2+}, Mn^{2+} and Ca^{2+} on cAMP binding and on the cAMP and

cGMP accumulation induced by cAMP are shown in Table I. The monovalent cations at concentrations between 0.1 and 10 mM do not have any effect on cAMP-binding or cAMP-induced responses. The divalent cations incrase the number of cAMP-binding sites to the same extent, without differences in concentration-dependency. Mg^{2+}, Mn^{2+} and Ca^{2+} increase the binding of cAMP to the S-sites; Mg^{2+} and Mn^{2+} are active, respectively, at approx. 5- and approx. 50-fold higher concentrations than Ca^{2+}. The transition of high-affinity binding sites H to low-affinity binding sites L is specifically inhibited by Ca^{2+}. Mg^{2+} at a 1000-fold higher concentration is inactive, and even Mn^{2+} only slightly but significantly enhances the transition of H to L.

The cAMP-induced accumulation of cAMP levels is inhibited by Ca^{2+}, but not by Mg^{2+}; Mn^{2+} induces a small but significant increase of the cAMP response. The cation-specificity on the modulation of the cGMP-response was recorded only for the effect on the reduction of the response 10 s after stimulation with 100 nM cAMP (Fig. 7E). The effect of Ca^{2+} in these experiments is statistically more significant than the effect of Ca^{2+} on the response to low cAMP concentrations (Fig. 7D). The effect of cations on the cGMP-response is not specific for Ca^{2+}; Mg^{2+} and Mn^{2+} also inhibit the cGMP-response, but higher concentrations are required. If we assume that Mg^{2+}, Mn^{2+} and Ca^{2+} induce the same maximal inhibition of the cGMP-response, then Mg^{2+} and Mn^{2+} are respectively approx. 6- and approx. 60-fold less active then Ca^{2+} (Table II).

D. discoideum cells resuspended in Ca^{2+} free buffer, secrete Ca^{2+} ions up to a concentration of about 6 μM [22]. Since the cAMP-induced cAMP accumulation and the transition of H to L are very sensitive to Ca^{2+}, they might be slightly inhibited in control cells. The small potentiation of these responses by Mn^{2+} could be due to an antagonistic effect of Mn^{2+} on the inhibition by Ca^{2+}. Indeed, it was observed that 100 μM Mn^{2+} completely reverted the inhibition of the cAMP accumulation

TABLE I

SPECIFICITY OF CATIONS ON cAMP-BINDING AND cAMP-INDUCED RESPONSES

After starvation, cells were washed twice with 15 mM Tris-HCl (pH 6.5) and resuspended in this buffer. The effect of the cations on cAMP-binding was measured as described in the legend of Fig. 5 and are presented as percentages of the binding in control cells for B_{max} and S. H → L represents the reduction of cAMP-binding between 6 and 45 s. The ratio of cAMP-binding at these times in control cells is 1.77 ± 0.12 ($n = 4$). The effect of the cations on the cAMP-induced elevation of cAMP and cGMP levels was measured as described in the legends of Fig. 6B and 7E, respectively. Data are presented as percentages of the response in control cells which are 25 ± 2.05 ($n = 3$) pmol cAMP/10^7 cells and 9.4 ± 0.7 ($n = 3$) pmol cGMP/10^7 cells.

Cation	mM	cAMP-binding			Responses	
		B_{max} (%)	S (%)	H → L ratio	cAMP (%)	cGMP (%)
K^+	0.1	97	101	1.76	97	100
	1	102	95	1.60	98	100
	10	104	106	1.71	102	98
Na^+	0.1	97	120	1.81	98	99
	1	106	102	1.80	98	91
	10	101	106	1.94	98	108
Mg^{2+}	0.1	127	110	1.82	109	100
	1	189	170	1.91	102	85
	10	228	280	1.82	97	63
Mn^{2+}	0.1	124	112	2.05	113	104
	1	208	112	2.09	122	94
	10	242	184	2.15	122	80
Ca^{2+}	0.01	107	97	1.55	63	102
	0.1	130	155	1.28	29	94
	1	212	265	1.10	24	54
	10	245	330	1.14	25	44

TABLE II

CONCENTRATIONS OF CATIONS WHICH INDUCE A HALF-MAXIMAL EFFECT (EC_{50}) ON cAMP-BINDING AND cAMP-INDUCED RESPONSES

Values are based on the data of Table I. It is assumed that the cations, when active, induce the same maximal effect as Ca^{2+}.

Property	Mg^{2+} (EC_{50})	Mn^{2+} (mM)	Ca^{2+} (mM)
B_{max}	0.6	0.5	0.5
S	2.0	≈ 20	0.4
H → L	–[a]	–[b]	0.016
cAMP-response	–[a]	–[b]	0.020
cGMP-response	2.5	≈ 25	0.4

[a] No effect up to 10 mM.

[b] Mn^{2+} enhances the transition of H to L and the cAMP-response.

by 30 μM Ca^{2+}. Mg^{2+} (tested upto 10 mM) had no such effect. The inhibition of the cGMP response by 1 mM Ca^{2+} was not reverted by either 0.2 mM Mg^{2+} or 2 mM Mn^{2+} (data not shown).

Discussion

The addition of Ca^{2+} to the extracellular medium of *D. discoideum* cells has a variety of effects on cell-surface cAMP receptors, on cAMP-induced activation of adenylate and guanylate cyclase, and on the rate of development to the multicellular state (Refs. 16–18 and this report). Some of these effects are also induced by Mg^{2+} and Mn^{2+} (Table II). Ca^{2+} induces an approx. 2.5-fold increase of the total number of binding sites, as has been shown previously [16]. Since the number of slowly dissociating cAMP-binding sites (S) is not altered by Ca^{2+}, the increase of total binding is due to an increase of the number of quickly dissociating sites (H + L). Mg^{2+} and Mn^{2+} induce the same effect at virtually identical concentrations (Table II).

The affinity of the S-binding sites is increased about 3-fold by Ca^{2+}, without an alteration of the off-rate. Therefore, Ca^{2+} increases only the apparent on-rate of the S-binding-sites. Mg^{2+} and Mn^{2+} also affect the S-sites, but higher concentrations are required. A half-maximal effect is induced by 0.4 mM Ca^{2+}, 2 mM Mg^{2+} and about 20 mM Mn^{2+}. The effect of Ca^{2+} on the S-binding-sites parallels the effect on the cAMP-mediated cGMP response: (i) The dose-response curve for a cAMP-induced cGMP response is shifted by Ca^{2+} to about 4-fold lower cAMP concentrations. (ii) The pace of the cGMP response is faster in the presence of Ca^{2+}, suggesting that cAMP is detected more quickly (higher on-rate). (iii) The cGMP response is modified by Ca^{2+}, Mg^{2+} and Mn^{2+} with half-maximal effect at 0.4 mM Ca^{2+}, 2.5 mM Mg^{2+} and about 25 mM Mn^{2+}.

The effect of extracellular Ca^{2+} on the transition of the fast-dissociation cAMP-binding sites and on the cAMP response occurs at much lower Ca^{2+} concentrations and is Ca^{2+}-specific. In the absence of extracellular added Ca^{2+}, a time and cAMP concentration-dependent transition of high-affinity binding H to low-affinity binding L occurs. This transition is largely inhibited by Ca^{2+} with an $EC_{50} = 20$ μM. The cAMP-mediated accumulation of cAMP levels is inhibited by Ca^{2+} at the same low Ca^{2+} concentrations ($EC_{50} = 16$ μM).

These results strongly suggest that the cAMP-mediated activation of adenylate and guanylate cyclase proceed by activation of different binding classes of surface cAMP receptors. However, the data do not discriminate between different binding classes of one protein (one receptor protein with different environments, e.g., transducers) and two receptor proteins coded by two genes.

It is proposed that activation of adenylate cyclase is mediated by the fast-dissociating cAMP receptors. Apparently, the transition of high-affinity binding to low-affinity binding represents the coupling of the occupied receptor to adenylate cyclase. It is interesting to notice that the kinetics of the transition of H to L is are approx. equal to the kinetics of activation of adenylatee cyclase in vivo [9]. Recently we observed that in membranes from *D. discoideum* cells guanyl nucleotides promote the conversion of high-affinity cAMP-binding to low-affinity binding [14], which suggests that activation of adenylate cyclase in *D. discoideum* may proceed via a GTP-binding protein in a way similar to the hormone mediated activation of adenylate cyclase in mammalian cells. Activation of guanylate cyclase is supposed to be mediated by the slow dissociating cAMP receptors (S). Although we have observed an alteration of these sites by guanyl nucleotides, it has not yet been firmly established whether a GTP-binding protein is involved in this activation process.

The mechanism of action of Ca^{2+} is not yet understood, because of its complexity. Ca^{2+} probably acts at the cell surface by different mechanisms, which is suggested by the following experiments (partly unpublished). (i) The Ca^{2+}-uptake blockers La^{3+} and Ruthenium Red (both 100 μM) do not affect cAMP-binding and cAMP-induced responses, neither do they reverse the action of Ca^{2+}. Furthermore, the Ca^{2+}-ionophore, A23187, does not potentiate the effect of Ca^{2+}. These data suggest that Ca^{2+} does not have to enter the cell to become effective. It has not been demonstrated that these drugs actually interfere with the localization of Ca^{2+} in *D. discoideum*; they do alter, however, the behaviour and differentiation of this organism [22,23]. (ii) The effects of Ca^{2+} take

place with different specificity and sensitivity: (a) the effect on B_{max} is not specific for Ca^{2+} and occurs at high concentrations. Such effects are also induced by polyvalent anions [24]; (b) the effects on the S-sites and on the cGMP-response are moderately specific for Ca^{2+} and also occur at high concentrations. The effect of 1 mM Ca^{2+} is not influenced by the simultaneous presence of 0.2 mM Mg^{2+} or 2 mM Mn^{2+}; (c) The effects on the fast sites and on the cAMP accumulation are Ca^{2+}-specific, and occur at low concentrations. Mg^{2+} (upto 10 mM) does not influence the inhibtion by 10–100 μM Ca^{2+}. In contrast, low Mn^{2+} concentrations (100 μM) completely annul the inhibition by 30 μM Ca^{2+}, suggesting that Mn^{2+} is an antagonist of Ca^{2+} in this transduction pathway.

All these observations suggest that Ca^{2+} acts at the cell surface, and that the effects on B_{max}, the fast sites and the slow sites have different mechanisms of action which have to be investigated further. Especially the target of Ca^{2+} which induces inhibition of cAMP relay is potentially interesting. cAMP relay is essential during cell aggregation, morphogenesis, and cell differentiation in *D. discoideum* [1–5,25,26]. The low Ca^{2+} concentrations needed to inhibit this response may be present during development in *D. discoideum*.

Acknowledgements

I gratefully acknowledge Theo Konijn, Aren van Waarde and René de Wit for stimulating discussiosn. This work was supported by the Foundation for Fundamental Biological Research (BION) and the C. and C. Huygens Fund, which are subsidized by the Netherlands Organization for the Advancement of Pure Scientific Research (ZWO).

References

1 Gerisch, G. (1982) Annu. Rev. Physiol. 44, 535–552
2 Devreotes, P.N. (1982) Ad. Cyclic. Nucl. Res. 15, 55–96
3 Van Haastert, P.J.M. and Konijn, T.M. (1982) Mol. Cell. Endocrinol. 26, 1–17
4 Konijn, T.M., Barkley, D.S., Chang, Y.Y. and Bonner, J.T. (1968) Am. Nat. 102, 225–233
5 Shaffer, B.M. (1975) Nature 255, 549–552
6 Mato, J.M. and Malchow, D. (1978) FEBS Lett. 90, 119–122
7 Roos, W. and Gerisch, G. (1976) FEBS Lett. 68, 170–172
8 Van Haastert, P.J.M., Van Lookeren Campagne, M.M. and Ross, F.M. (1982) FEBS Lett. 147, 149–152
9 Dinauer, M.C., Mackay, S.A. and Devreotes, P.N. (1980) J. Cell Biol. 86, 537–544
10 Van Haastert, P.J.M. and De Wit, R.J.W. (1984) J. Biol. Chem. 259, 13321–13328
11 Toews, M.L., Harden, T.K. and Perkins, J.P. (1983) Proc. Natl. Acad. Sci. USA 80, 3553–3557
12 Stadel, J.M., Delcan, A. and Lefkowitz, R.J. (1982) Ad. Enzymol. 53, 1–43
13 Leichtling, B.H., Coffman, D.S., Yeger, E.S. and Rickenberger, H.V. (1981) Biochem. Biophys. Res. Commun. 102, 1187–1195
14 Van Haastert, P.J.M. (1984) Biochem. Biophys. Res. Commun. 124, 597–604
15 De Wit, R.J.W. and Bulgakov, R. (1985) FEBS Lett. 179, 257–261
16 Juliani, M.H. and Klein, C. (1977) Biochim. Biophys. Acta 497, 369–376
17 Marin, F.T. and Rothman, F.G. (1980) J. Cell Biol. 87, 823–827
18 Gerisch, G., Malchow, D., Roos, W. and Wick, U. (1979) J. Exp. Biol. 81, 33–47
19 Van Haastert, P.J.M. (1984) J. Gen. Microbiol. 130, 2559–2564
20 Van Haastert, P.J.M. and Van der Heijden, P.R. (1983) J. Cell Biol. 96, 347–353
21 Pannbacker, R.G. and Bravard, L.J. (1972) Science 175, 1014–1015
22 Bumann, J., Wurster, B. and Malchow, D. (1984) J. Cell Biol. 98, 173–178
23 Brachet, P. and Klein, C. (1977) Differentiation 8, 1–8.
24 Janssens, P.M.W. and Van Driel, R. (1984) FEBS Lett. 176, 245–249
25 Schaap, P., Konijn, T.M. and Van Haastert, P.J.M. (1984) Proc. Natl. Acad. Sci. USA. 81, 2122–2126
26 Kay, R.R. (1982) Proc. Natl. Acad. Sci. USA. 79, 3228–3231