Riva, S., Cline, M. A., & Laipis, P. J. (1978) in *DNA Synthesis. Present and Future* (Molineux, I., & Kohiyama, M., Eds.) pp 641–653, Plenum Press, New York.
Sauer, B., & Lehman, I. R. (1982) *J. Biol. Chem. 257*, 12394–12398.
Schaffner, W., & Weissmann, C. (1973) *Anal. Biochem. 56*, 502–514.
Schwyzer, M., Weil, R., Frank, G., & Zuber, H. (1980) *J. Biol. Chem. 255*, 5627–5634.
Smith, A. E., Smith, R., & Paucha, E. (1978) *J. Virol. 28*, 140–153.
Sons, W., Unsöld, H. J., & Knippers, R. (1976) *Eur. J. Biochem. 65*, 263–269.
Spadari, S., & Weissbach, A. (1974) *J. Mol. Biol. 86*, 11–20.
Spadari, S., Villani, G., & Hardt, N. (1978) *Exp. Cell Res. 113*, 57–62.
Stahl, H., Dröge, P., Zentgraf, H. W., & Knippers, R. (1985) *J. Virol. 54*, 473–482.
Tanabe, K., Karawya, E., Fewell, J. W., Kuff, E. L., & Wilson, S. H. (1984) *Nucleic Acids Res. 11*, 8253–8268.
Tanaka, S., Hu, S., Wang, T. S. F., & Korn, D. (1982) *J. Biol. Chem. 257*, 8386–8390.
Wahl, A. F., Kowalski, S. P., Harwell, L. W., Lord, E. M., & Bambara, R. A. (1984) *Biochemistry 23*, 1895–1899.
Wang, T. S. F., Hu, S. Z., & Korn, D. (1984) *J. Biol. Chem. 259*, 1854–1865.
Wierowski, J. V., Lawton, K. G., Hockensmith, J. W., & Bambara, R. B. (1983) *J. Biol. Chem. 258*, 6250–6254.

# Forms of the Chemotactic Adenosine 3′,5′-Cyclic Phosphate Receptor in Isolated *Dictyostelium discoideum* Membranes and Interconversions Induced by Guanine Nucleotides

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ABSTRACT: Aggregating *Dictyostelium discoideum* cells possess receptors for the chemoattractant cAMP on their cell surface. Membranes enriched in these receptors were isolated. Kinetic studies indicated the same receptor heterogeneity in membranes as found for intact cells [van Haastert, P. J. M., & De Wit, R. J. W. (1984) *J. Biol. Chem. 259*, 13321–13328]. Dissociation kinetics revealed at least three receptor forms: one form, called SS, with $k_{-1} = 0.9 \times 10^{-3}$ s$^{-1}$ and $K_D = 6.5$ nM; one form, called S, with $k_{-1} = 1.3 \times 10^{-2}$ s$^{-1}$ and $K_D = \sim 6$ nM; and one or more forms, called F, with $k_{-1} > 0.1$ s$^{-1}$. The contribution of the SS form to the dissociation process was lower in the presence of millimolar concentrations of cAMP compared to dissociation induced by dilution only. Guanosine di- and triphosphates decreased the affinity of membranes for cAMP by increasing the dissociation rate of the cAMP–receptor complex. This was shown to result from a reduction in the number of sites of the slowly dissociating, high-affinity receptor form SS and probably also the high-affinity form S. Because the total number of cAMP binding sites was not changed by guanine nucleotides, it is inferred that the SS and S receptor forms are converted to other more rapidly dissociating receptor forms with lower affinities than SS and S. We propose that cAMP receptors in *Dictyostelium* membranes interact with G protein which binds guanosine di- and triphosphates. The different complexes between receptor and occupied or unoccupied G protein explain the different receptor forms and their interconversions.

In the course of a developmental program, induced by starvation, cells of the cellular slime mould *Dictyostelium discoideum* become sensitive to extracellular cAMP. Extracellular cAMP is used as a chemoattractant which guides the solitary cells to aggregation sites (Devreotes, 1982). Here, a fruiting body is formed. Furthermore, extracellular cAMP also stimulates cell differentiation (Sussman, 1982; Schaap & van Driel, 1985). Extracellular cAMP is produced by cells during cell aggregation, by means of a signal relay mechanism in which cell-surface receptors specific for cAMP are functionally coupled to adenylate cyclase. Receptor occupation results in a rapid, 10-fold activation of adenylate cyclase. Subsequently, within a few minutes, the enzyme is inactivated by an adaptation process (Devreotes, 1982).

The cAMP receptor–adenylate cyclase system in *Dictyostelium discoideum* is used as a model system for signal transduction in lower eukaryotes. Receptor binding studies with intact cells have demonstrated the occurrence of site heterogeneity (multiple receptor sites with different kinetic constants), ligand-dependent interconversion of binding sites (cooperativity) (van Haastert & De Wit, 1984; van Haastert, 1985), and ligand-induced decrease of the number of binding sites (down-regulation) (Klein & Juliani, 1977). Two fast dissociating types of cAMP binding sites (called H and L; $K_d$'s of 60 and 450 nM, respectively) and one slowly dissociating site (called S, $K_D = 12.5$ nM) have been identified (van Haastert & De Wit, 1984). Studies with intact cells are limited, however, by the fact that the cAMP that is produced

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by the activated adenylate cyclase within minutes after receptor occupation (Devreotes, 1982) interferes with binding experiments by lowering the specific activity of labeled ligand. Also, in intact cells regulatory factors which act on receptors from the cytoplasmic site of the membrane are difficult to manipulate. We have therefore initiated studies with cAMP receptors in isolated membranes.

In a previous report (Janssens et al., 1985), we have presented evidence that the receptor population in isolated *D. discoideum* membranes is heterogeneous. Also, it was shown that guanine nucleotides decrease the affinity of membranes for cAMP without altering the total number of binding sites (Janssens et al., 1985; van Haastert, 1984). By analogy with vertebrate receptors (Stadel et al., 1982), this was interpreted as evidence for the involvement of a guanine nucleotide binding protein (G protein) in *Dictyostelium* signal transduction. In dissociation kinetics of the cAMP–receptor complex, guanine nucleotides decrease the contribution of two apparently different receptor forms which have $k_{-1}$ values of $5 \times 10^{-2}$ $s^{-1}$ (van Haastert, 1984) and $0.9 \times 10^{-3}$ $s^{-1}$ (Janssens et al., 1985). Both forms are characterized by a $K_D$ of about 10 nM.

In this paper, we present a detailed analysis of the association and dissociation kinetics and equilibrium binding of cAMP receptors in isolated membranes. The effect of guanine nucleotides on the different kinetics and equilibrium binding parameters is investigated. The results are interpreted in terms of receptor heterogeneity and cooperativity.

## Materials and Methods

*Materials.* [5′,8-$^3$H]cAMP was purchased from Amersham International (Amersham, U.K.), cAMP was from Serva (Heidelberg, FRG), dithiothreitol was from Calbiochem-Behring (San Diego, CA), 5′-AMP was from Boehringer (Mannheim, FRG), and cGMP, cIMP, and 2′-deoxy-cAMP were from Sigma (St. Louis, MO). Other cAMP derivatives used in this study were kindly provided by Dr. B. Jastorff (University of Bremen, FRG). Polycarbonate filters were obtained from Nuclepore Corp. (Pleasanton, CA). Nitrocellulose filters (type BA 85) were from Schleicher & Schuell (Dassel, FRG).

*Cells and Membrane Preparations. Dictyostelium discoideum* (strain AX2) cells were grown and developed by 6 h of starvation (aggregation-competent cells) as described before (van Driel, 1981). Membranes enriched in cAMP receptors were isolated by sucrose gradient centrifugation (Janssens & van Driel, 1984). In short, washed cells were homogenized by nitrogen cavitation in 40 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes)[1]/NaOH (pH 7.7), 250 mM sucrose, 1 mM dithiothreitol, and 0.5 mM EDTA containing protease inhibitors (50 units/mL aprotinin, 0.05 mg/mL trypsin inhibitor, and 100 μM phenylmethanesulfonyl fluoride) and centrifuged for 10 min at 3000*g*, at 4 °C. The pellet was resuspended in a 80% (w/v) sucrose solution containing the same Hepes/NaOH (pH 7.7), dithiothreitol, EDTA, and protease inhibitors and was layered at the bottom of a linear 35–60% (w/v) sucrose gradient, buffered with 20 mM Hepes/NaOH (pH 7.7) and containing EDTA, dithiothreitol, and protease inhibitors as above. After centrifugation for 1 or 4 h at 100000*g* (40 °C), membranes containing cAMP receptors were collected, pooled, and stored in liquid nitrogen. Protein was determined according to Peterson (1977).

[1] Abbreviations: Gpp(NH)p, guanyl-5′-yl imidodiphosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

*Cyclic AMP Binding Assays.* Cyclic AMP binding was measured at 0 °C in 20 mM Hepes/NaOH (pH 7.0), 10 mM dithiothreitol [an inhibitor of *Dictyostelium* membrane-bound phosphodiesterase (Green & Newell, 1975)], and 20 μM 5′-AMP (cAMP binding medium) containing [$^3$H]cAMP at concentrations given in the text. Where indicated, guanine nucleotides were present. The binding reaction was initiated by the addition of membranes (20–50 μg of protein and 0.1–1 pmol of cAMP binding sites/100 μL). For equilibrium binding, membranes were equilibrated with [$^3$H]cAMP for 5–7 min. Subsequently, the amount of [$^3$H]cAMP bound to membranes was determined by measuring the radioactivity in the membrane pellet after 2-min centrifugation at 10000*g* in a microcentrifuge (Janssens et al., 1985) or by filtration through a 0.2-μm pore-size polycarbonate filter. Both methods gave the same results. For association kinetics, bound cAMP and free cAMP were separated by filtration through polycarbonate filters. This method had an estimated dead time of 10 s.

The dissociation kinetics of the cAMP–receptor complex after equilibration with [$^3$H]cAMP in the presence or absence of guanine nucleotides (concentrations given in the text) were investigated as follows. When dissociation was induced by adding excess unlabeled cAMP (0.1–0.8 mM), bound cAMP and free cAMP were separated by centrifugation of membranes through a layer of 5% sucrose as described before (Janssens et al., 1985). In a number of experiments, the dissociation kinetics of the cAMP–receptor complex induced by 100-fold dilution were compared to dissociation in the presence of excess unlabeled cAMP. After equilibration with [$^3$H]cAMP, dissociation was initiated by 100-fold dilution in 20 mM potassium phosphate (pH 7.0) containing 20 μM 5′-AMP, 5 mM dithiothreitol, 1 mg/mL ovalbumin, and 5% sucrose (dilution medium), with or without 100 μM unlabeled cAMP. At different time points, samples were filtered through a nitrocellulose filter (pore size 0.45 μm) to separate bound and free cAMP. The estimated dead time of this method was about 30 s.

Nonspecific binding was determined by equilibration of membranes with [$^3$H]cAMP in the presence of 0.1–0.8 mM unlabeled cAMP. However, when the dissociation of the cAMP–receptor complex was brought about by 100-fold dilution, the background (i.e., in this type of experiment defined as the residual binding after infinite dissociation time) was determined by incubating membranes for 20 min in dilution medium containing [$^3$H]cAMP at a concentration 100 times below the concentration used to equilibrate membranes before dilution.

Analysis of dissociation kinetic data was done on the assumption that the curves obtained could be described by $B_t = B_{0,1}e^{-k_{-1,1}t} + B_{0,2}e^{-k_{-1,2}t} \ldots + B_{0,n}e^{-k_{-1,n}t}$ (Boeynaems & Dumont, 1980). Here $B_{0,1}$, $B_{0,2}$, ..., $B_{0,n}$ represent the equilibrium binding to each of the different components at the start of dissociation, and $k_{-1,1}$, $k_{-1,2}$, ..., $k_{-1,n}$ are the dissociation rate constants of the components 1 to $n$.

## Results

*Isolation of Membrane-Bound cAMP Receptors.* Membranes containing cAMP receptors were isolated by differential centrifugation followed by equilibrium sucrose gradient centrifugation (Figure 1). In this way, stable preparations of membrane-bound receptors were obtained, which could be stored frozen. The recovery of cAMP binding after gradient centrifugation was 59 ± 5% (five experiments) compared to the binding to intact *Dictyostelium* cells, as measured at 10 nM [$^3$H]cAMP. The binding activity per milligram of protein

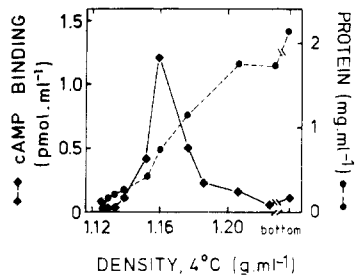


FIGURE 1: Sucrose gradient centrifugation of membrane-bound cAMP receptors. A sediment obtained after 10-min centrifugation at 3000*g* of a homogenate of aggregation-competent *Dictyostelium* cells was layered at the bottom of a 35–60% (w/v) sucrose gradient, which was centrifuged for 4 h at 100000*g*. Fractions with a density of 1.15–1.17 g/mL were pooled, stored frozen, and used to study cAMP receptors.

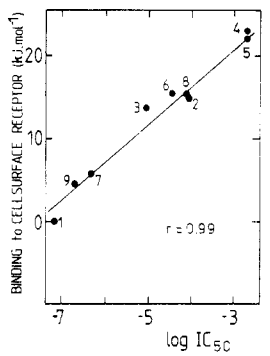


FIGURE 2: Comparison of the nucleotide binding specificity of isolated membrane-bound cAMP receptors with the cell-surface receptor from aggregation-component *Dictyostelium* cells. Binding data for the cAMP receptor on intact cells were taken from van Haastert and Kien (1983). Numbers 1–9 refer to the cAMP derivatives used: (1) adenosine 3′,5′-monophosphate; (2) 6-chloropurine 3′,5′-monophosphate; (3) 7-deazaadenosine 3′,5′-monophosphate; (4) inosine 3′,5′-monophosphate; (5) guanosine 3′,5′-monophosphate; (6) 8-bromoadenosine 3′,5′-monophosphate; (7) 2′-deoxyadenosine 3′,5′-monophosphate; (8) 3′-amino-3′-deoxyadenosine 3′,5′-monophosphate; (9) 5′-amino-5′-deoxyadenosine 3′,5′-monophosphate. $IC_{50}$ values represent the cAMP derivative concentrations at which the binding of [$^3$H]cAMP, present at a concentration of 10 nM, is inhibited by 50%. *r*, correlation coefficient.

(at 10 nM cAMP) was increased 5.8-fold (±2.0) ($n$ = 4) by the isolation procedure. The nucleotide binding specificity of receptors in isolated membranes closely matched that the cell-surface receptor, as determined by the binding of eight cAMP derivatives (Figure 2). From this, we conclude that the cAMP receptor in our membrane preparation is identical with the cell-surface receptor found on intact cells.

*Association Kinetics of cAMP to Isolated Membranes.* The kinetics of equilibration of cAMP with membrane-bound receptors were investigated at cAMP concentrations between 1 and 90 nM (Figure 3). Compared to the rate of binding of cAMP to intact cells (Green & Newell, 1975; van Haastert & De Wit, 1984), the rate of binding of cAMP to isolated membranes was slow. The overshoot in cAMP binding, which characteristically occurs with intact cells (van Haastert & De Wit, 1984), was not apparent with isolated membranes (Figure 3a). The association data were analyzed by assuming first-order kinetics (Figure 3b). This revealed that equilibration at cAMP concentrations lower than 10 nM followed first-order kinetics. At higher cAMP concentrations, association was multiphasic. This suggests that multiple binding sites are present which are more evident at higher cAMP concentrations, i.e., above 10 nM (Figure 3b).

Guanine nucleotides [GTP, Gpp(NH)p, and GDP] lower the equilibrium level of cAMP binding (van Haastert, 1984; Janssens et al., 1985). They increase the rate of equilibration

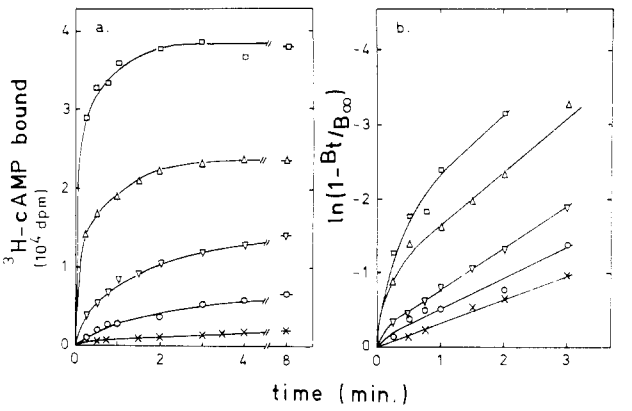


FIGURE 3: Equilibration rate of [$^3$H]cAMP with isolated membranes. At time zero, membranes were mixed with 500 $\mu$L of binding medium containing various concentrations of [$^3$H]cAMP: (×) 1 nM; (○) 3 nM; (▽) 10 nM; (△) 30 nM; and (□) 90 nM. At different times, membrane-bound cAMP and free cAMP were separated by filtration through a 0.2-$\mu$m pore-size polycarbonate filter. (a) Radioactive cAMP bound after subtraction of nonspecific binding. (b) Semilogarithmic representation of the data in (a). $B_t$ is the binding at time $t$ and $B_\infty$ the binding at equilibrium, which was taken after 8-min incubation. Data represent means of duplicate measurements of an experiment reproduced twice.

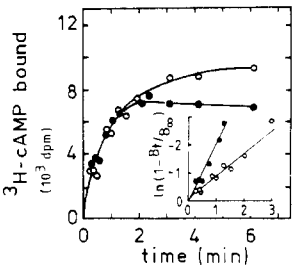


FIGURE 4: Rate of equilibration of [$^3$H]cAMP (10 nM) with membranes in the absence (○) or presence (●) of $10^{-4}$ M GTP. Insert: Semilogarithmic representation of the data. $B_t$, binding at time $t$; $B_\infty$, binding at equilibrium (6-min incubation). The experiment was repeated twice.

of cAMP with isolated membranes (Figure 4). The effect of guanine nucleotides on cAMP binding to isolated membranes was not dependent on the presence of $Mg^{2+}$ or $Ca^{2+}$ ions during the binding reaction. Both $Mg^{2+}$ and $Ca^{2+}$ ions at 5 mM increased the equilibrium cAMP binding (10 nM) to isolated membranes, as reported before (Janssens & van Driel, 1984), but the relative inhibition by guanine nucleotides in the presence or absence of these divalent cations was the same (not shown). The latter result is very similar to results recently obtained with the vertebrate glucagon receptor (Rojas & Birnbaumer, 1985). In *Dictyostelium*, the effects of divalent cations on the cAMP receptor are complex, since the affinity for cAMP as well as the total receptor site number is increased (Janssens & van Driel, 1984; van Haastert, 1985). In the present study, we have chosen to investigate the effects of guanine nucleotides on the cAMP receptor under the simplest conditions, i.e., in the absence of divalent cations.

*Dissociation Kinetics in the Absence of Guanine Nucleotides.* When after equilibration of the receptor with [$^3$H]-cAMP dissociation of the complex was initiated, either by adding excess unlabeled cAMP or by 100-fold dilution, multiphasic dissociation was observed (Figure 5). After dissociation induced by chase with excess unlabeled cAMP, the slowest dissociation process could be described by a rate constant $k_{-1}$ = 0.9 (±0.3) × $10^{-3}$ $s^{-1}$ ($n$ = 11). The slowest dissociation process after dissociation by dilution could be described with $k_{-1}$ = 0.8 (±0.3) × $10^{-3}$ $s^{-1}$ ($n$ = 3). Thus, with

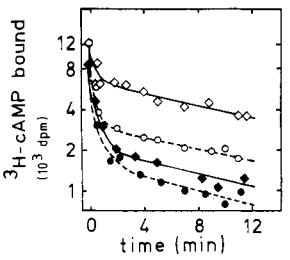


FIGURE 5: Semilogarithmic plot of the dissociation kinetics after equilibration of membranes with 10 nM [$^3$H]cAMP in the absence (open symbols) or presence (closed symbols) of $10^{-4}$ M Gpp(NH)p. Membranes were equilibrated with [$^3$H]cAMP in 40 $\mu$L of binding medium for 5 min. Subsequently, at $t$ = 0 min, 4 mL of dilution medium was added containing 0.1 mM unlabeled cAMP (circles) or no cAMP (diamonds). At different times, this mixture was forced through nitrocellulose filters (pore size 0.45 $\mu$m). [$^3$H]cAMP bound at equilibrium was 12060 dpm in the absence of guanine nucleotides and 8350 dpm in the presence of $10^{-4}$ M Gpp(NH)p. The residual binding at various time points after initiation of dissociation is corrected for the background, which after 100-fold dilution in the presence and absence of 0.1 mM unlabeled cAMP was 940 and 1500 dpm, respectively. Data are the means of duplicates of a representative experiment reproduced 3 times.

both methods the same dissociation process is observed, which we will call SS ("super slow") in the following. The contribution of SS to the dissociation process was 2–3-fold higher after dissociation induced by 100-fold dilution than after dissociation in the presence of excess unlabeled cAMP: 56% and 26% after equilibrium at 10 nM [$^3$H]cAMP, respectively (Figure 5).

After dissociation induced by excess unlabeled cAMP following equilibration with [$^3$H]cAMP at concentrations between 1 and 250 nM, another first-order dissociation process could be resolved in addition to SS. This became clear after subtraction of the contribution of the binding to SS at the earlier time points (Figure 6c,f, open symbols). This dissociation process [called S ("slow") in the following] was described by a first-order rate constant $k_{-1}$ = 1.3 (±0.4) × $10^{-2}$ $s^{-1}$ ($n$ = 4). Finally, a third dissociation process, faster than S and SS, was present which was impossible to evaluate in view of the dead time of our methods, indicating a $k_{-1}$ greater than 0.1 $s^{-1}$ (Figure 6c,f). This process will be called F ("fast") in the following.

*Dissociation Kinetics after Equilibrium in the Presence of Guanine Nucleotides.* As shown in Figures 5–7, after equilibration of membranes with [$^3$H]cAMP in the presence of guanosine di- and triphosphates ($10^{-4}$–$10^{-3}$ M), dissociation of the [$^3$H]cAMP–receptor complex was faster than after equilibration in the absence of guanine nucleotides. This was observed to be independent of the way by which dissociation was induced (Figures 5–7). As shown in Figure 7, an increased dissociation rate of [$^3$H]cAMP from membranes was observed after equilibration in the presence of GTP, Gpp(NH)p, and GDP, the same guanine nucleotides that before were found to reduce the equilibrium binding of cAMP to membranes (van Haastert, 1984; Janssens et al., 1985).

The enhanced dissociation of [$^3$H]cAMP from membranes was primarily caused by a strong reduction of the contribution of the slowest dissociation process SS ($k_{-1}$ = 0.9 × $10^{-3}$ $s^{-1}$). We reported before that the reduction in equilibrium cAMP binding is maximal with GTP and GDP concentrations between $10^{-4}$ and $10^{-3}$ M, while maximal effects of Gpp(NH)p are reached at $10^{-3}$ M (van Haastert, 1984; Janssens et al.,

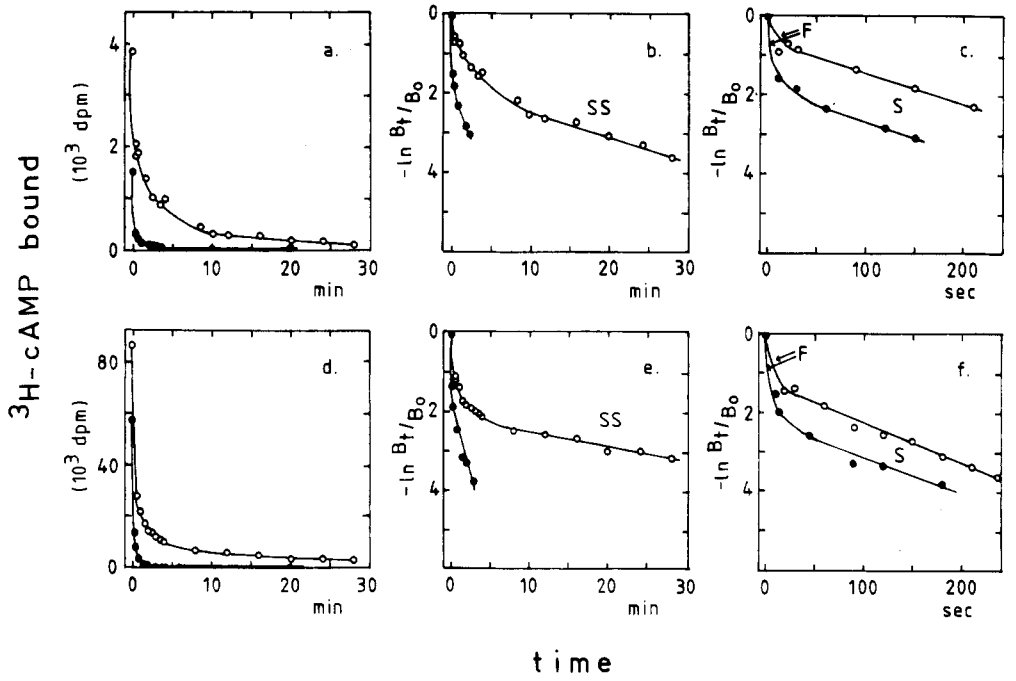


FIGURE 6: Dissociation kinetics after equilibration of membranes with 1 nM (a–c) or 100 nM (d–f) [$^3$H]cAMP. Membranes were equilibrated for 5 min in 100 $\mu$L with [$^3$H]cAMP in the presence (●) or absence (○) of $10^{-4}$ M GTP. Dissociation was initiated by adding excess (0.8 mM) unlabeled cAMP. Subsequently, the residual binding after various times of dissociation was determined by centrifugation of membranes through a layer of 5% sucrose. All points are corrected for aspecific binding. The same results were obtained when bound cAMP and free cAMP were separated by filtration through 0.2-$\mu$m pore-size polycarbonate filters (data not shown). (a and d) Direct representation of the data; (b, c, e, and f) semilogarithmic plots of the data. The slowest first-order process discriminated after equilibrium in the absence of GTP was SS ($k_{-1}$ = 0.9 × $10^{-3}$ $s^{-1}$) (b and e). After subtraction of the binding contributed by SS, the curves in (c) and (f) were obtained (open symbols). In this way, a dissociation process with $k_{-1}$ = 1.3 × $10^{-2}$ $s^{-1}$ was resolved which is called S. The curves in (b) and (e) obtained from the dissociation after equilibration in the presence of GTP are reproduced on a smaller time scale in (c) and (f). Thus, the similarity of the slowest first-order dissociation process in the presence of GTP [$k_{-1}$ = 1.1 (±0.3) × $10^{-2}$ $s^{-1}$ ($n$ = 7)] with the "S process" observed in the absence of GTP is apparent. The dissociation process during the first 15 s of the curves, called F ($k_{-1}$ > 0.1 $s^{-1}$), was insufficiently resolved with our methods. $B_t$ is the binding at time $t$; $B_0$ is the binding at equilibrium. The experiments shown are representative for four different experiments.

Table I: Summary of Kinetic Data of cAMP Receptors in Isolated Membranes[a]

| condn | SS $k_{-1}$ ($s^{-1}$) | SS app $K_D$ (nM) | SS rel no. of sites (%) | S + SS app $K_D$ (nM) | S + SS rel no. of sites (%) | S $k_{-1}$ ($s^{-1}$) | S rel no. of sites (%)[b] | F $k_{-1}$ ($s^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| no guanine nucleotides | | | | | | | | |
| chase dissociation | $0.9\ (\pm0.3) \times 10^{-3}$ | 6.5 (±2.6) | 100 | 6.5 (±3.6) | 100 | $1.3\ (\pm0.4) \times 10^{-2}$ | 100 | >0.1 |
| dilution dissociation | $0.8\ (\pm0.3) \times 10^{-3}$ | 4.3 | 300 | nd | nd | nd | | nd |
| $10^{-4}$ M Gpp(NH)p | | | | | | | | |
| chase dissociation | $0.9 \times 10^{-3}$ | 5.5 | 30 | 6.9 (±3.0) | 40 | $1.3 \times 10^{-2}$ | 40 | >0.1 |
| dilution dissociation | $1.0 \times 10^{-3}$ | 4.5 | 90 | nd | nd | nd | nd | nd |
| $10^{-4}$ M GTP | | | | | | | | |
| chase dissociation | | not detectable | | ~4 | 15 | $1.1\ (\pm0.3) \times 10^{-2}$ | 15 | >0.1 |

[a] The number of sites, detected after dissociation by chase in the absence of guanine nucleotides, has arbitrarily been taken as 100% for each column. The number of sites under each condition as determined after dissociation for 8 min (SS) and for 30 s (S + SS and S) is given relative to this number. [b] The binding curve for S was obtained indirectly, by subtracting from S + SS the binding curve of SS. The latter was calculated for 30-s dissociation from the data (in the absence and presence of guanine nucleotides) after 8-min dissociation, using $B_{t30} = B_{t480}/e^{-k_{-1}(480-30)}$ with $k_{-1} = 0.9 \times 10^{-3}\ s^{-1}$. This gave an estimate of the apparent $K_D$ of S and the relative site number in the absence and presence of guanine nucleotides. [c] nd, not determined.

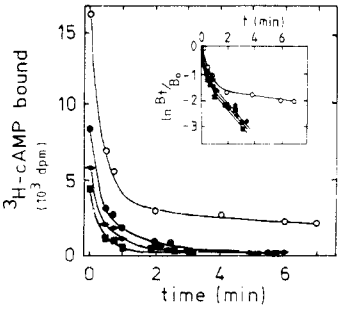


FIGURE 7: Effect of GTP, Gpp(NH)p, and GDP on the dissociation of [$^3$H]cAMP from membrane-bound receptors. Membranes were equilibrated for 5 min with 10 nM [$^3$H]cAMP in 100 $\mu$L of binding medium containing 1 mM GTP (■), Gpp(NH)p (●), or GDP (◆) or no guanine nucleotides (○). Subsequently, dissociation was induced by adding 0.1 mM unlabeled cAMP, and bound cAMP and free cAMP were separated at different times by centrifugation though a layer of 5% sucrose. Insert: Semilogarithmic representation of the data; $B_t$, binding after $t$ minutes of dissociation; $B_0$, binding at equilibrium for each condition. The experiment was reproduced twice.

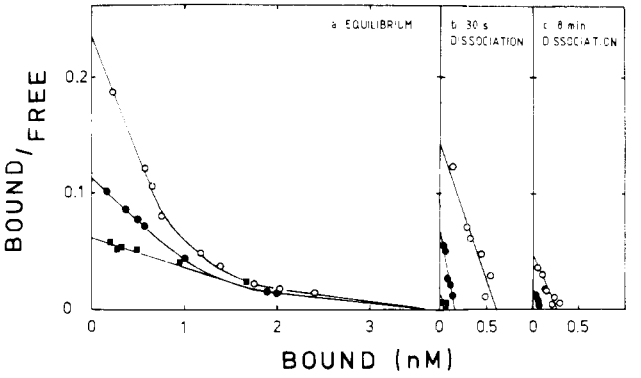


FIGURE 8: Scatchard plots of cAMP binding to isolated membranes at equilibrium and after 30-s and 8-min dissociation. Membranes (10 $\mu$L) were equilibrated with [$^3$H]cAMP concentrations between 1.5 and 160 nM in 130 $\mu$L of binding medium in the presence of $10^{-4}$ M Gpp(NH)p (●) or $10^{-4}$ M GTP (■) or without guanine nucleotides (○). Subsequently, binding was measured directly (a), or dissociation was induced by adding unlabeled cAMP to a concentration of 0.1 mM. The residual binding of [$^3$H]cAMP was measured after 30-s (b) or 8-min (c) dissociation by centrifugation of the membranes through a layer of 5% sucrose, yielding binding curves for S + SS and SS, respectively. Calculation of "free cAMP" for all curves was done by subtracting at each concentration the *total* amount of cAMP bound at *equilibrium* from the cAMP presented. A binding curve of SS after equilibrium in the presence of $10^{-4}$ M GTP could not be obtained, because no significant binding was detectable after 8-min dissociation. Each point is the mean of a determination in duplicate of an experiment reproduced 3 times.

1985). Thus, GTP and GDP are more potent than Gpp(NH)p in reducing the affinity of the receptor for ligand, similar to what has been observed for the vertebrate glucagon receptor (Rojas & Birnbaumer, 1985). In agreement with this, low amounts of SS were found in the presence of $10^{-4}$ M Gpp(NH)p (Figures 5 and 8c), while SS was completely absent in the dissociation process after equilibrium in the presence of $10^{-3}$ M Gpp(NH)p or $10^{-4}$–$10^{-3}$ M GTP and GDP (Figures 6–8).

Analyses of the dissociation process during the first few minutes after initiation of dissociation revealed that guanine nucleotides also reduced the contribution of the S component ($k_{-1} = 1.3 \times 10^{-2}\ s^{-1}$; Figure 6c,f). We were unable to determine the effect of guanine nucleotides on the dissociation process designated F ($k_{-1} > 0.1\ s^{-1}$) because this process was insufficiently resolved by our methods.

*Equilibrium Binding Properties of S and SS.* Assuming that the S and SS components of the dissociation process represent different receptor forms, their affinity and the number of sites were estimated as follows. Membranes were equilibrated with different concentrations of [$^3$H]cAMP in the absence or presence of guanine nucleotides. After initiation of dissociation by dilution or by chase with excess unlabeled cAMP, binding was measured 30 s or 8 min later. After 8-min dissociation, the contribution of F and S ($k_{-1} > 0.1\ s^{-1}$ and $k_{-1} = 1.3 \times 10^{-2}\ s^{-1}$, respectively) to residual binding is less than 10%. Therefore, essentially only SS is observed. Binding at each [$^3$H]cAMP concentration, as observed after 8-min dissociation, is directly proportional to the binding to SS at equilibrium, according to $B_0 = B_t/e^{-k_{-1}t}$. Here $B_0$ represents the binding at equilibrium, while $B_t$ represents the residual binding after dissociation for time $t$. Following the same argument, after 30-s dissociation, F contributes less than 10% to binding, so the binding observed is mainly binding to S and SS.

As reported earlier (Janssens et al., 1985), a Scatchard plot of the equilibrium cAMP binding to isolated membranes is concave, indicating site heterogeneity or negative cooperativity (Figure 8a). The total number of cAMP binding sites was 24.3 ± 14.1 pmol of sites·(mg of protein)$^{-1}$ ($n$ = 4). The binding curve of the SS form of the receptor, deduced from the residual binding after 8-min dissociation, gave a straight line in Scatchard representation, described by $K_D$ = 6.5 (±2.6) nM ($n$ = 6) (Figure 8c). The same binding curve of SS was obtained after dissociation induced by excess unlabeled cAMP or 100-fold dilution. Only in the latter case was the Scatchard plot shifted to the right, indicating a 3-fold higher total number of SS sites than found after dissociation by chase (Table I).

After equilibration in the presence of Gpp(NH)p ($10^{-4}$ M), the Scatchard curve of the SS form, observed after 8-min

dissociation, was shifted to the left (Figure 8c). This indicated that the total number of SS sites was reduced about 3-fold, apparently without a significant change in $K_D$. The same result was obtained after dissociation induced by 100-fold dilution (Table I).

The Scatchard plot of the residual binding after 30-s dissociation by chase with unlabeled cAMP is shown in Figure 8b. This binding curve could very well be described by a single binding component, with $K_D = 6.5 \pm 3.6$ nM ($n = 3$). Because both S and SS contribute to the residual binding after 30-s dissociation, this result indicates that S and SS have a similar affinity for cAMP, i.e., a $K_D$ of about 6 nM. After equilibration with [$^3$H]cAMP in the presence of $10^{-4}$ M Gpp(NH)p and subsequent dissociation by chase for 30 s, a linear Scatchard plot was obtained, indicating a $K_D$ of $6.9 \pm 3.0$ nM ($n = 3$). However, the number of sites was 3-fold lower compared to the situation in the absence of Gpp(NH)p (Figure 8b). With $10^{-4}$ M GTP, the reduction in S + SS site numbers, as determined after 30-s dissociation, was even more dramatic (Figure 8b).

To investigate whether guanine nucleotides, besides the SS sites, also reduced the number of S sites, we calculated the number of SS sites contributing to the residual binding after 30-s dissociation from the number determined after 8-min dissociation (Table I). It turned out that the observed reduction in the number of SS sites, caused by guanine nucleotides, was insufficient to explain the observed reduction in the number of S + SS sites (Figure 8b). Therefore, we conclude that guanine nucleotides also cause a reduction in the number of S sites.

## Discussion

We intend to study the molecular mechanism of signal transduction in the lower eukaryote *Dictyostelium discoideum*. Therefore, we have isolated membranes enriched in cell-surface cAMP receptors. Here, we present a detailed analysis of the kinetic properties of cAMP receptors in isolated membranes and of the effects of guanine nucleotides.

As with intact cells (Green & Newell, 1975; Coukell, 1981; van Haastert & De Wit, 1984), binding of cAMP to isolated membranes yields equilibrium binding curves that cannot be described by a single dissociation constant (Figure 8a). In general, such binding curves can be explained by site heterogeneity and/or cooperativity of binding (Boeynaems & Dumont, 1980). Both explanations have been shown to be applicable to cAMP binding to intact *Dictyostelium* cells (van Haastert & De Wit, 1984). The observations described in this paper indicate that similar processes also occur in isolated membranes. First, site heterogeneity was suggested by multiphasic association and dissociation kinetics. Particularly, dissociation kinetics could be resolved in several components, as we have shown earlier (Janssens et al., 1985). The binding curve from two components, observed in dissociation kinetics, could be described by a single dissociation constant, which suggests that these represent separate receptor forms. Second, cooperative effects in cAMP binding to isolated membranes were suggested by the difference that was observed in the dissociation of the [$^3$H]cAMP–receptor complex in the absence and presence of excess unlabeled cAMP (Figure 5). This method to establish cooperativity in binding systems was first described by De Meyts et al. (1973).

A summary of the kinetic parameters of the different receptor forms, present in isolated membranes, is given in Table I. The S and SS forms have a high affinity ($K_D$ about 6 nM). These forms probably contribute to the high-affinity component in the equilibrium cAMP binding curve (Figure 8a). It is likely then that the fast dissociating F form(s) has (have) a low affinity and represent(s) the low-affinity component in the equilibrium binding curve, for which dissociation constants between 100 and 200 nM have been reported (Green & Newell, 1975; Coukell, 1981; Devreotes, 1982). It is probable that our F form is composed of either the H or the L receptor form, or both, which before were observed on intact cells and in a crude membrane preparation (van Haastert & De Wit, 1984; van Haastert, 1984). The H and L forms both have a dissociation rate constant ($k_{-1}$) greater than 0.1 $s^{-1}$ (measured at 22 °C), and their $K_D$ values are 60 and 450 nM, respectively (van Haastert & De Wit, 1984).

To make a further comparison of the receptor forms observed in isolated membranes with receptor forms, described before, on intact cells and in a crude membrane preparation, it must be noted that several of the earlier binding studies (van Haastert & De Wit, 1984; van Haastert, 1984) were performed at 22 °C, while our measurements were done at 0 °C. The dissociation rate constants of S and SS in isolated membranes and of the forms on intact cells are 1.4–4-fold higher if measured at 22 °C instead of 0 °C (data not shown). Therefore, we think that the S form in isolated membranes ($k_{-1} = 1.3 \times 10^{-2}$ $s^{-1}$ at 0 °C) is identical with the S form observed on intact cells and in a crude membrane preparation ($k_{-1} = 5 \times 10^{-2}$ $s^{-1}$ at 22 °C; $K_D$ = 12–15 nM) (van Haastert & De Wit, 1984; van Haastert, 1984) and is also identical with the form with $k_{-1} = 1.6 \times 10^{-2}$ $s^{-1}$ at 0 °C described for intact cells (van Haastert, 1985). Our SS form ($k_{-1} = 0.9 \times 10^{-3}$ $s^{-1}$) is probably similar to the form with $k_{-1} = 1.6 \times 10^{-3}$ $s^{-1}$ at 0 °C described for intact cells (van Haastert, 1985). Thus, it seems that at least several of the receptor forms that have been distinguished on intact *Dictyostelium* cells can also be observed in isolated membranes.

It is remarkable that we obtained binding curves for SS that could be described by a single dissociation constant (Janssens et al., 1985; Figure 8), because the contribution of SS in the dissociation process was strongly influenced by the presence of millimolar concentrations of unlabeled cAMP during dissociation (Figure 5). In fact, the latter observation would suggest that the binding curves of SS should manifest cooperativity at higher ligand concentrations, which was not observed. Similar, apparently paradoxical, observations have also been made in other systems [see Levitzki (1984)]. As an explanation, it might be suggested that the allosteric effect of cAMP on SS only becomes apparent at much higher cAMP concentrations, when the SS sites are already almost saturated. In such a case, a deviation from a linear Scatchard plot will easily be missed. For this explanation to be feasible, the allosteric effect has to be mediated by cAMP binding sites which have a low affinity than SS, for instance, F sites.

Guanine nucleotides decrease the affinity of membranes for cAMP (Figure 8; Janssens et al., 1985; van Haastert, 1984). This effect proves to be the result of a decreased contribution of S and SS sites to total cAMP binding, rather than of a decrease in affinity of the S and SS receptor forms. GTP, Gpp(NH)p, and GDP all have very similar effects on dissociation kinetics (Figure 7) as well as on equilibrium binding (van Haastert, 1984; Janssens et al., 1985). Because guanine nucleotides do not change the total number of cAMP binding sites (Figure 8; van Haastert, 1984; Janssens et al., 1985), it is probable that S and SS forms are converted to F sites.

The effects of guanine nucleotides on the binding properties of the *Dictyostelium* cAMP receptor are remarkably similar to those on receptors for hormones and neurotransmitters in vertebrate cells (Stadel et al., 1982). In the latter case, it has

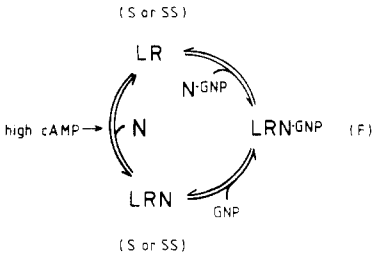


FIGURE 9: Model of the interaction of cAMP receptors with guanine nucleotide binding protein to explain the occurrence of F, S, and SS receptor forms and their interconversions. Abbreviations: L, ligand; R, receptor; N, G protein; GNP, guanosine di- or triphosphate. The possible identity of the different forms with F, S, and SS is indicated.

been shown that the effects of guanine nucleotides are mediated by guanine nucleotide binding proteins (G or N proteins). For vertebrates, it has been proposed that the different receptor forms represent different complexes between receptor and G protein (De Lean et al., 1980; Stadel et al., 1982). By analogy, the presence and the interconversions of the F, S, and SS receptor forms in *Dictyostelium* membranes might be explained by interaction of the cAMP receptor with a putative G protein (Figure 9). Evidence for the presence of a guanine nucleotide binding protein in *D discoideum* membranes was obtained by Leichtling et al. (1981), who showed the existence of a $M_r$ 42000 protein that could be ADP-ribosylated. In the model we propose, receptor (R) interacts with G protein (N), which can be occupied with guanine nucleotides or can be empty. These different complexes with the receptor are assumed to have different affinities for the ligand cAMP (L).

Addition of guanosine di- or triphosphates to the system will shift the equilibria toward the $LRN^{GNP}$ form, concomitantly reducing the fraction of LR and LRN forms. In this model, it is likely that LR and LRN are identical with the S and SS receptor forms, which are converted to the F form(s) (identical with $LRN^{GNP}$) in the presence of guanine nucleotides. At present, we have no information whether S or SS represents LR or LRN. The F form is present in isolated membranes, not exposed to guanine nucleotides (Figure 6). This might indicate that additional receptor forms with properties of F exist which are not complexed with guanine nucleotide occupied G protein. Alternatively, guanine nucleotides might remain bound to G protein throughout the membrane isolation procedure, similar to what has been described for vertebrates (Cassel & Selinger, 1978; Lad et al., 1980). In our model, the reduced contribution of SS after dissociation by chase, i.e., at high ligand concentration, compared to dissociation by dilution (low ligand concentration) (Figure 5), can be explained by a conversion of SS to S at high cAMP concentrations.

Little can be said about the position of adenylate cyclase in the model of Figure 9 and about its interaction with G protein. Until recently, all attempts have failed to show an interaction of *Dictyostelium* adenylate cyclase with G protein, e.g., by affecting the enzyme activity in vitro with guanine nucleotides, fluoride, or cAMP or by treatment of cells with cholera toxin (Klein, 1976; Renart et al., 1981; Devreotes, 1982; P. M. W. Janssens, unpublished experiments). Only very recently Khachatrian et al. (1985) have presented data that suggest the linkage of an inhibitory G protein to *Dictyostelium* adenylate cyclase. Obviously, further investigations are required to establish the way in which adenylate cyclase in this organism is regulated.

The function of each of the putative receptor forms in the signal transduction process remains to be established. Isolation of receptors, G protein, and other components of the signal transduction system and their reconstitution are now required to demonstrate the interactions we propose at a molecular level.

**Registry No.** cAMP, 60-92-4; Gpp(NH)p, 34273-04-6; GTP, 86-01-1.

## REFERENCES

Boeynaems, J. M., & Dumont, J. E. (1980) *Outlines of Receptor Theory*, Elsevier/North-Holland Biomedical Press, Amsterdam.

Cassel, D., & Selinger, Z. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4155–4159.

Coukell, M. B. (1981) *Differentiation (Berlin) 20*, 19–35.

De Lean, A., Stadel, J. M., & Lefkowitz, R. J. (1980) *J. Biol. Chem. 255*, 7108–7117.

De Meyts, P., Roth, J., Neville, D. M., Gavin, J. R., & Lesniak, M. A. (1973) *Biochem. Biophys. Res. Commun. 55*, 154–161.

Devreotes, P. N. (1982) in *The Development of Dictyostelium discoideum* (Loomis, W. F., Ed.) pp 117–158, Academic Press, New York.

Green, A. A., & Newell, P. C. (1975) *Cell (Cambridge, Mass.) 6*, 129–136.

Janssens, P. M. W., & van Driel, R. (1984) *FEBS Lett. 176*, 245–249.

Janssens, P. M. W., Van der Geer, P. L. J., Arents, J. C., & van Driel, R. (1985) *Mol. Cell. Biochem. 67*, 119–124.

Khachatrian, L., Howlett, A., & Klein, C. (1985) *J. Cyclic Nucleotide Protein Phosphorylation Res. 10*, 179–188.

Klein, C. (1976) *FEBS Lett. 68*, 125–128.

Klein, C., & Juliani, M. H. (1977) *Cell (Cambridge, Mass.) 10*, 329–335.

Lad, P. M., Nielsen, T. B., Preston, M. S., & Rodbell, M. (1980) *J. Biol. Chem. 255*, 988–995.

Leichtling, B. H., Coffman, D. S., Yaeger, E. S., & Rickenberg, H. V. (1981) *Biochem. Biophys. Res. Commun. 102*, 1187–1195.

Levitzki, A. (1984) *Receptors, a Quantative Approach*, pp 31–36, Benjamin/Cummings Publishing Co., Menlo Park, CA.

Peterson, G. L. (1977) *Anal. Biochem. 83*, 346–356.

Rojas, F. J., & Birnbaumer, L. (1985) *J. Biol. Chem. 260*, 7829–7835.

Schaap, P., & Van Driel, R. (1985) *Exp. Cell Res. 159*, 388–398.

Stadel, J. M., De Lean, A., & Lefkowitz, R. J. (1982) *Adv. Enzymol. Relat. Areas Mol. Biol. 53*, 1–43.

Sussman, M. (1982) in *The Development of Dictyostelium discoideum* (Loomis, W. F., Ed.) pp 353–385, Academic Press, New York.

van Driel, R. (1981) *Eur. J. Biochem. 115*, 391–395.

van Haastert, P. J. M. (1984) *Biochem. Biophys. Res. Commun. 124*, 597–604.

van Haastert, P. J. M. (1985) *Biochim. Biophys. Acta 845*, 254–260.

van Haastert, P. J. M., & Kien, E. (1983) *J. Biol. Chem. 258*, 9636–9642.

van Haastert, P. J. M., & De Wit, R. J. W. (1984) *J. Biol. Chem. 259*, 13321–13328.