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Demonstration of Receptor Heterogeneity and Affinity Modulation by Nonequilibrium Binding Experiments

THE CELL SURFACE cAMP RECEPTOR OF *DICTYOSTELIUM DISCOIDEUM**

Peter J. M. Van Haastert and René J. W. De Wit

From the Cell Biology and Morphogenesis Unit, Zoological Laboratory, University of Leiden, Kaiserstraat 63, NL 2311 GP Leiden, The Netherlands

The binding of [³H]cAMP to *Dictyostelium discoideum* cells was analyzed on a seconds time scale under both equilibrium and nonequilibrium conditions. The binding of [³H]cAMP increases rapidly to a maximum obtained at about 6 s, which is followed by a decrease to an equilibrium value reached at about 45 s. This decrease of [³H]cAMP binding is not the result of ligand degradation or isotope dilution by cAMP secretion but is due to a transition of high-affinity binding to low-affinity binding.

Analysis of the dissociation rate of [³H]cAMP from the binding sites indicates that these high- and low-affinity binding sites are both fast dissociating with a half-life of about 1 s. In addition, these dissociation experiments reveal a third binding type which is slowly dissociating with a half-life of about 15 s. The number and affinity of these slowly dissociating sites does not change during the incubation with [³H]cAMP.

The drugs caffeine and chlorpromazine do not change the total number of binding sites, but they change the ratio of the three binding types. In the presence of 10 mM caffeine almost all binding sites are in the low affinity conformation, while in the presence of 0.1 mM chlorpromazine the ratio is shifted to both the high-affinity type and slowly dissociating type.

The results indicate that the cAMP-binding activity of *D. discoideum* cells is heterogeneous. In the absence of cAMP about 4% of the sites are slowly dissociating with K_d = 12.5 nM, about 40% are fast dissociating with high affinity (K_d = 60 nM), and about 60% are fast dissociating with low affinity (K_d = 450 nM). During the binding reaction the number of slowly dissociating sites does not change. The number of high-affinity sites decreases to a minimum of about 10% with a concomitant increase of low-affinity sites to about 90%. This transition of binding types shows first-order kinetics with a half-life of about 9 s. A half-maximal transition is induced by 12.5 nM cAMP.

The binding of many hormones to specific receptor proteins shows nonlinear Scatchard plots, which may have different molecular mechanisms (1) such as (i) binding site heterogeneity, *i.e.* two or more receptor sites with different affinities,

(ii) a hormone-dependent alteration of the number of binding sites (*cf.* down regulation), (iii) a hormone-dependent alteration of the affinity of the binding sites by site-site interaction (cooperativity) or by increased probability of occupied binding sites to attain another conformation (affinity modulation). Discrimination between these possibilities is necessary to understand the mechanism of action of the hormone. Equilibrium binding experiments are in general not sufficient to reveal the processes underlying the nonlinear nature of the Scatchard plot. In nonequilibrium kinetics the approach to a new equilibrium state is measured after disturbing an old equilibrium. This may often yield the desired information to describe the hormone receptor interaction at a molecular level (2–9). In this report we describe those nonequilibrium binding experiments for the cell surface cAMP receptor of *Dictyostelium discoideum*.

cAMP acts as a first messenger in the cellular slime mold *D. discoideum* (10). Cells of this species feed on bacteria. Exhaustion of their food supply induces cell aggregation which is mediated by chemotaxis (11) to cAMP (12). This nucleotide does not penetrate the cells (13) but is detected by cell surface receptors (14–17).

Equilibrium binding of cAMP to *D. discoideum* cells shows nonlinear Scatchard plots (15, 18), which have been interpreted as one class of binding sites with negative cooperativity or as two classes of binding sites with different affinities. Several observations suggest the involvement of negative cooperativity or at least a very close resemblance of the different binding types. (i) cAMP accelerates the dissociation of the cAMP-receptor complex (18); (ii) the binding of low or high [³H]cAMP concentrations is competed by cAMP derivatives with very similar specificity (19); (iii) photoaffinity labeling with 8-azidoadenosine 3′,5′-[³²P]monophosphate yields only one radioactive labeled protein (20).

The addition of cAMP to a suspension of *D. discoideum* cells induces several responses such as a transient increase of cGMP levels, the excretion of protons, the methylation of proteins and phospholipids, dephosphorylation of myosin, and the entrance of calcium (see Refs. 21–24). The transient increase of cGMP levels is the first response observed; cGMP levels change within 2 s after stimulation and reach a peak after 10 s. Recently we have analyzed the temporal components of the cAMP-mediated cGMP response (24) which revealed that cAMP is detected within 1 s. Transduction of the cAMP signal is terminated within a few seconds by an adaptation process (24). Although we have only scarce information on the biochemical nature of this adaptation process, experiments with different stimuli have revealed that it must be localized in the transduction chain at the cAMP receptor or somewhere between receptor and guanylate cyclase (25).

To obtain more information on the properties of cAMP detection, transduction of the cAMP signal, and adaptation to cAMP, we have developed a receptor binding assay, which can detect cAMP binding within 2 s after the addition of the ligand to the cells. Three binding types with different affinities are recognized. cAMP affects the proportioning of these binding types, thus giving rise to the concave downward Scatchard plot observed at equilibrium.

EXPERIMENTAL PROCEDURES

Materials—[2,8-³H]cAMP (1.5 TBq/mmol) and the cGMP radioimmunoassay kit were purchased from the Radiochemical Centre, Buckinghamshire, U.K.; cAMP was from Boehringer Mannheim, F.R.G.; caffeine was from the British Drug House, Poole, U.K.; and chlorpromazine and dithiothreitol were from Sigma. Siliconoil AR 20 and AR 200 were obtained from Wacker Chemie, München, F.R.G.

Culture Conditions—D. discoideum cells NC-4(H) were grown in association with *Escherichia coli* 281 on a solid medium containing 3.3 g of peptone, 3.3 g of glucose, 4.5 g of KH_2PO_4, 1.5 g of $Na_2HPO_4\cdot2H_2O$ and 15 g of agar per liter. Cells were harvested in the late logarithmic phase with 10 mM sodium/potassium phosphate buffer, pH 6.5 (Pb buffer), and freed from bacteria by repeated centrifugations at $100 \times g$ for 4 min.

cAMP Binding Assay—Cells were starved for 5 h by shaking in Pb buffer at a density of 10^7 cells/ml, washed twice in Pb buffer, and resuspended in Pb buffer at a density of 1.1×10^8 cells/ml. During the experiment the cell suspension was aerated at a flow rate of about 15 ml of air/ml of suspension/min. cAMP binding was measured at 22 °C by mixing 80 μl of the cell suspension with 40 μl of a solution containing Pb buffer, 22.5 mM dithiothreitol, different concentrations [³H]cAMP, and additives such as caffeine and chlorpromazine. After mixing, the solution was added to a test tube containing 10 μl of 10% sucrose and 220 μl of siliconoil (AR 20:AR 200 = 2:1). At the times indicated the cells were separated from the incubation liquid by centrifugation through the oil in a homemade small swing-out rotor at about $10,000 \times g$ for 15 s.

The technique for very short incubation times ($t \le 5$ s) was as follows. The piston of a 500-μl automatic pipette (Gilson P1000 set at 500 μl) was pressed completely. The cell suspension (80 μl), an air bubble (~50 μl), and the [³H]cAMP solution (80 μl) were carefully taken into the pipette. The tip of the pipette was placed close to the siliconoil-containing tube placed into a centrifuge, which was slightly modified so that it could operate without a lid. A $t = 0$ s the piston of the pipette was released quickly, by which about 300 μl of air enter the pipette. This results in the removal of the barrier caused by the air bubble, and the cell suspension is rapidly and vigorously mixed with [³H]cAMP. The piston was then pressed again, and the cell suspension was layered on top of the siliconoil. Then the pipette was withdrawn from the centrifuge, and centrifugation was started. The actual incubation time (between the release of the piston and the start of the centrifuge) was recorded with a stopwatch. After some experience an incubation period of 1.5 s was routinely obtainable.

After centrifugation, the tubes were frozen in liquid nitrogen, the bottoms of the tubes containing the sucrose and the cell pellet were cut, and the radioactivity was determined by liquid scintillation counting. Nonspecific binding was measured by including about 0.1 mM cAMP in the incubation mixture and was subtracted from all data shown.

cGMP Stimulation—Cells were starved on non-nutrient agar at a density of 1.5×10^6 cells/cm² for 4–5 h, harvested, washed twice, and suspended in Pb buffer at a density of 10^8 cells/ml. Aliquots (100 μl) were preincubated with 20 μl of caffeine or chlorpromazine for 30 s. Then 20 μl of cAMP (50 mM final concentration) were added, followed by the addition of 100 μl of 3.5% perchloric acid (v/v). Lysates were neutralized with 50 μl of $KHCO_3$ (50% saturated at 20 °C) and centrifuged at $8000 \times g$ for 2 min. The cGMP content in 100 μl of the supernatant was measured radioimmunologically (24).

RESULTS

Binding of 30 nM [³H]cAMP to *D. discoideum* cells increases rapidly after adding the ligand (Fig. 1A). Half-maximal binding is reached at about 1.5 s. A maximum of [³H]cAMP binding is obtained at about 6 s, which is followed by a decrease to an apparent equilibrium value approached at

about 45–60 s. Fig. 1B demonstrates that the decrease of [³H]cAMP binding between 10 and 30 s follows first-order kinetics with a rate constant of 0.08 s^{-1} ($t_{1/2} = 9$ s). [³H]cAMP association experiments were also performed at other [³H] cAMP concentrations. The decay of [³H]cAMP binding, observed at 10 and 100 nM, also showed first-order kinetics with about the same rate constant. However, at 2 nM [³H]cAMP this time-dependent decrease is absent, while at concentrations above 200 nM it becomes relatively small (data not shown).
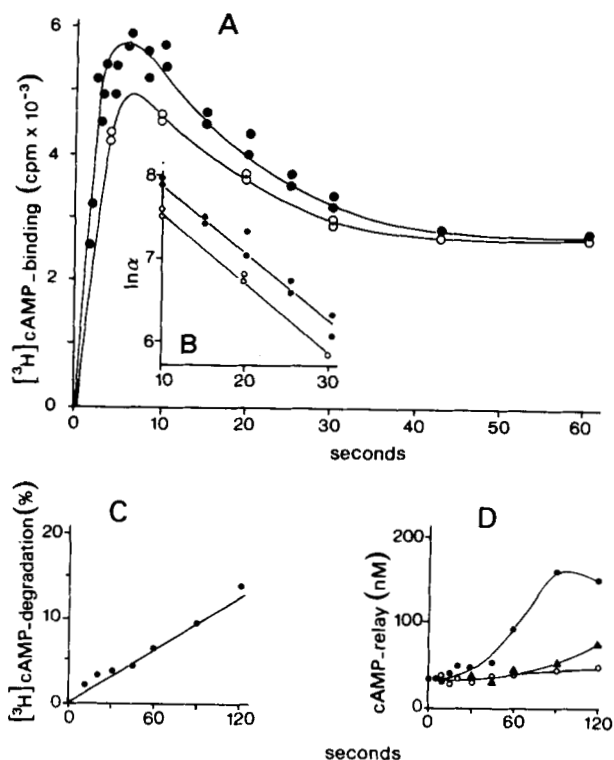
FIG. 1. **The kinetics of association of [³H]cAMP to *D. discoideum* cells.** *A*, in a pipette were sucked up 80 μl of a cell suspension (8.8×10^6 cells), about 50 μl of air, and 40 μl of a solution which yields after mixing 10 mM phosphate buffer, pH 6.5, 7.5 mM dithiothreitol, 30 nM [³H]cAMP (about 135,000 cpm) and 0 or 2 mM caffeine. At $t = 0$ s, 300 μl of air were sucked into the pipette, which caused mixing of cells with [³H]cAMP. The suspension was immediately layered on top of 220 μl of siliconoil. At the times indicated the cells were rapidly separated from the liquid by centrifugation in a swing-out rotor. The cell-associated radioactivity was determined. Nonspecific binding was measured by including 0.1 mM cAMP in the incubation mixture. The value (900 cpm) was constant between 6 and 60 s and was subtracted from all data. Each *point* is a single determination, except the points at 45 and 60 s which are the means of triplicate determinations. ●, control; ○, with 2 mM caffeine. *B*, semilogarithmic plot of the data of *A*. α = cpm bound at t − cpm bound at equilibrium (60 s). The *straight line* indicates a decay of [³H]cAMP binding between 10 and 30 s which follows first-order kinetics with a rate constant $k = 0.08 \text{ s}^{-1}$. *C*, experiment as described in *A*, except that at the indicated times the suspensions were pipetted into a tube containing 100 μl of perchloric acid 3.5% (v/v). Degradation of [³H]cAMP was measured as described (24). *D*, eighty μl of a cell suspension (8.8×10^6 cells) were mixed with 40 μl of cAMP solution (final concentrations 30 nM cAMP, 7.5 nM dithiothreitol, with or without 2 mM caffeine). At the times indicated 100 μl of perchloric acid were added for the detection of total cAMP, or samples were centrifuged briefly (5 s at $10,000 \times g$), and the supernatant was added to 100 μl of perchloric acid for the determination of extracellular cAMP. After neutralization of the lysates, cAMP was determined by the Gilman assay (27). ●, total cAMP; ○, total cAMP in the presence of 2 mM caffeine; ▲, extracellular cAMP. The results shown are from a typical experiment repeated two times.

Two trivial explanations for the decrease of [³H]cAMP binding after 10 s are the degradation of the ligand or the dilution of [³H]cAMP with cAMP secreted by the cells. Degradation is less than 5% during the first 30 s of the binding assay (Fig. 1C). [³H]cAMP stimulation induces a strong cAMP response in the cells; however, this cAMP is secreted only after 1 min (Fig. 1D). Furthermore, caffeine has been shown to be a strong inhibitor of the cAMP-mediated cAMP response (26). Although this drug alters cAMP binding, relatively low concentrations completely inhibit the cAMP response, while the decrease of [³H]cAMP binding is still present (Fig. 1, A and D).

These observations indicate that changes of the binding site rather than of the ligand concentration are responsible for the peculiar binding kinetics of [³H]cAMP.

The number of binding sites as well as the binding affinity may decrease during the incubation with [³H]cAMP. Therefore, Scatchard analysis was made at the time point of maximal [³H]cAMP binding (6 s) and at the moment of apparent equilibrium binding (45 s). This reveals (Fig. 2) that the number of binding sites does not change during the incubation. Furthermore, both curves intersect the ordinate at approximately the same point, which represents the association of the first cAMP molecule to a binding site. These results suggest that at first cAMP binds to high-affinity sites which convert to low-affinity sites in a time-dependent manner. This conversion is also cAMP dose dependent with almost no conversion at 2 nM cAMP and maximal conversion at concentrations above 100 nM.

The dissociation of the [³H]cAMP-receptor complex was measured to obtain more information on these high- and low-affinity binding types. Cells were incubated with 2 nM [³H]cAMP (high-affinity binding) or 100 nM [³H]cAMP (low-affinity binding) till equilibrium (45 s). Then excess cAMP was added and bound radioactivity was measured (Fig. 3).
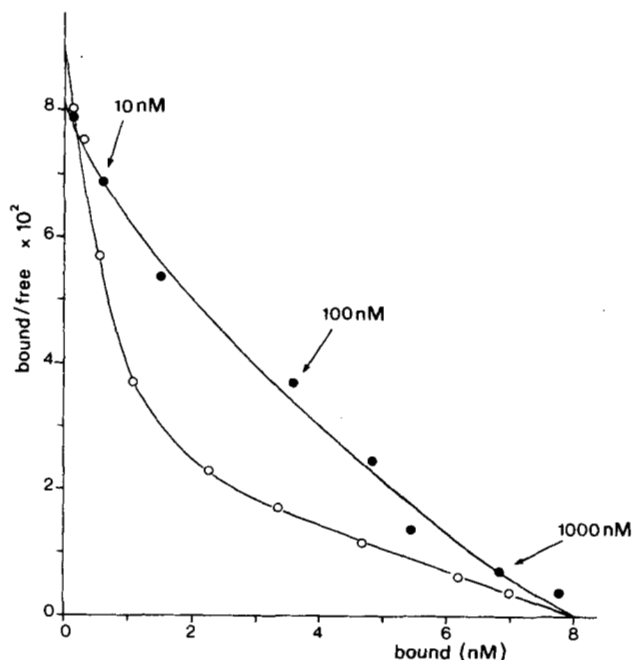
FIG. 3. **Dissociation of the [³H]cAMP-receptor complex.** A suspension (120 μl) containing 8.8×10^6 cells, 2 or 100 nM [³H]cAMP, 7.5 mM dithiothreitol, and Pb buffer was incubated for 45 s and then rapidly mixed with 6 μl of 2 mM cAMP with a technique as described in the legend to Fig. 1A. At the times indicated, cells were centrifuged through siliconoil, and the cell-associated radioactivity was determined. Nonspecific binding was measured by including 0.1 mM cAMP during the entire incubation mixture. $b(t)$ is the specific binding at t seconds after the cAMP chase. ●, dissociation after binding with 2 nM [³H]cAMP; ○, with 100 nM [³H]cAMP.

The release of cell-associated [³H]cAMP is very fast. Most of the radioactivity dissociates after binding at 2 or 100 nM [³H]cAMP with a half-life of about 1.5 or 0.7 s, respectively. However, a small but significant portion of the radioactivity dissociates more slowly with a half-life of about 15 s at both ligand concentrations. About 27% of the specific binding of 2 nM [³H]cAMP is bound to this slowly dissociating binding type; this figure is 13% at 100 nM [³H]cAMP.

The slowly dissociating binding type (which is further referred to as "slow," abbreviated as S) was studied in more detail by Scatchard analysis (Fig. 4). Cells were incubated with different [³H]cAMP concentrations for 45 s followed by the release of [³H]cAMP from the fast-dissociating complexes during a 8-s incubation with excess cAMP. Residual binding after 8 s of dissociation shows a linear Scatchard plot with a dissociation constant of 12.5 nM. During the 8-s period at least 96–99% of the radioactivity is chased from the fast-dissociating types; however, also 33% of the radioactivity bound to S dissociates during this period. The *dashed line* in Fig. 4B represents the Scatchard plot of S at the onset of dissociation. The number of S-binding sites is approximately 4% of the total number of cAMP-binding sites.

S has high binding affinity. Is this binding type identical to the high-affinity binding which converts during the incubation period into low-affinity binding? If this were the case we would expect that the binding of [³H]cAMP to S is high at 6 s and decreases as the conversion from high-affinity to low-affinity binding proceeds. Therefore, the association of [³H]cAMP to S was measured (Fig. 5). [³H]cAMP was allowed to bind to the total population of binding sites during different time periods. Then excess cAMP was added, and S-specific binding was measured 8 s later. The results of Fig. 5A demonstrate that the hypothesis that S is identical to the high-affinity site which transfers to low-affinity binding is not valid.

FIG. 2. **Scatchard plot of equilibrium and pre-equilibrium binding.** The binding of different [³H]cAMP concentrations (2, 4, 10, 30, 100, 200, 400, 1000, 2000 nM) was measured at equilibrium (45 s, ○) and at the maximum of cAMP binding (6 s, ●). The results shown are the means of 15 (○) or 3 (●) independent experiments each in triplicate. A maximum binding of 8 nM equals about 8×10^4 binding sites/cell.
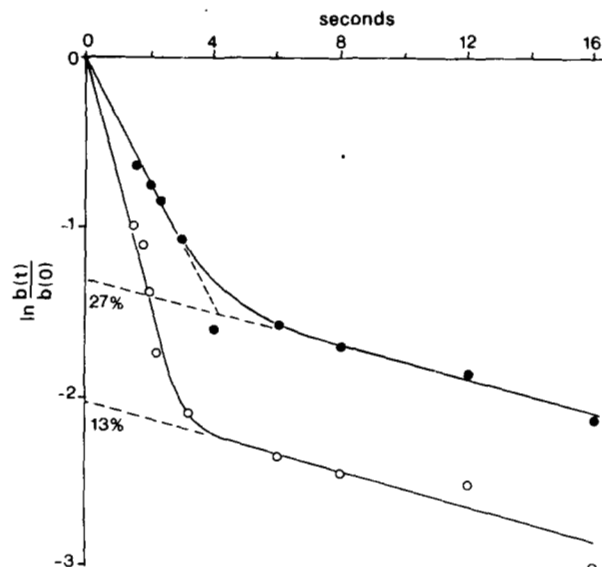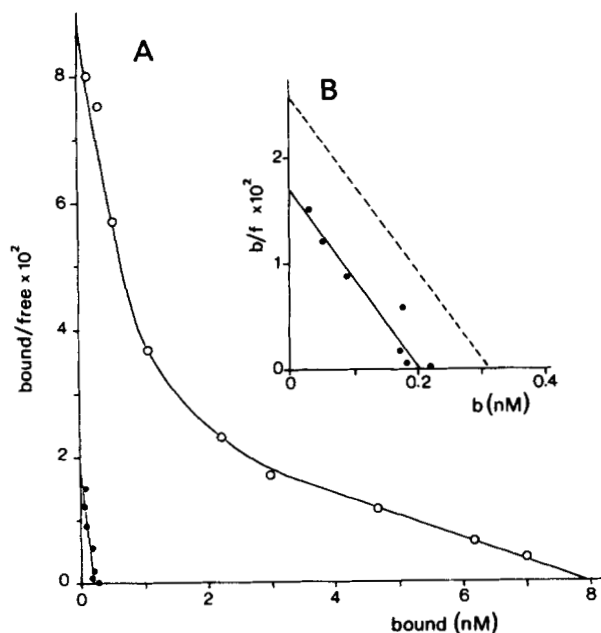
FIG. 4. **Scatchard plot of S.** *A*, cells were allowed to bind different [³H]cAMP concentrations till equilibrium (45 s). Then excess cAMP (2 μl of 10^{-2} M) was added, and [³H]cAMP binding was measured 8 s later. O, binding before, and ●, binding after the 8-s chase with excess cAMP. *B*, enlargement of a part of *A*. The *dashed line* represents the Scatchard plot of S at the start of the cAMP chase (the rate constant of dissociation (0.05 s^{-1}) predicts that about 33% of the occupied S sites dissociate during 8 s. The number of S sites (0.31 nM = 3100 sites/cell) is about 4% of the total binding sites. The slope represents the apparent dissociation constant, K_d = 12.5 nM. The results shown are the mean of 15 (O) or 3 (●) independent experiments each in triplicate.

This may suggest that S is a stable nonconvertible binding type for which the law of mass action is applicable,

$$cAMP + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} cAMP \sim S. \qquad (1)$$
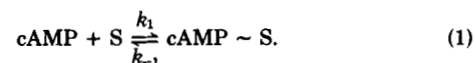
Then, association of [³H]cAMP to S follows the equation,

$$b(t) = b(\infty)\{1 - e^{-(k_1[cAMP]+k_{-1})t}\} \qquad (2a)$$

or

$$-\ln\{1 - b(t)/b(\infty)\} = (k_1[cAMP] + k_{-1})t \qquad (2b)$$

where $b(t)$ is the binding at t, and $b(\infty)$ is the binding at equilibrium (45 s).

A replot of the data of Fig. 5*A* as the left-hand portion of Equation 2b *versus* time yields straight lines for all concentrations (Fig. 5*B*). The slopes (β) of these lines equal $k_1[cAMP] + k_{-1}$. A replot of β *versus* [cAMP] also yields a straight line (Fig. 5*C*). The intersection with the abscissa yields the dissociation constant (K_d = 17 nM), the intersection with the ordinate yields the rate constant of dissociation (k_{-1} = 0.054 s^{-1}), and the slope yields the rate constant of association (k_1 = 3.3 × 10^6 M^{-1} s^{-1}). This experiment shows that S has normal kinetic properties without changes of the number of binding sites or their affinity during the incubation with [³H]cAMP. This suggests that still two other binding types must exist to account for the observed negative cooperative interactions. These binding types are both fast dissociating (Fig. 3) and have high- and low-binding affinity (H and L, respectively).

In Fig. 4*B* the binding of [³H]cAMP to S at equilibrium (*dashed line*) was calculated. Subtraction of these data from the total equilibrium binding (*open symbols* in Fig. 4*A*) yields
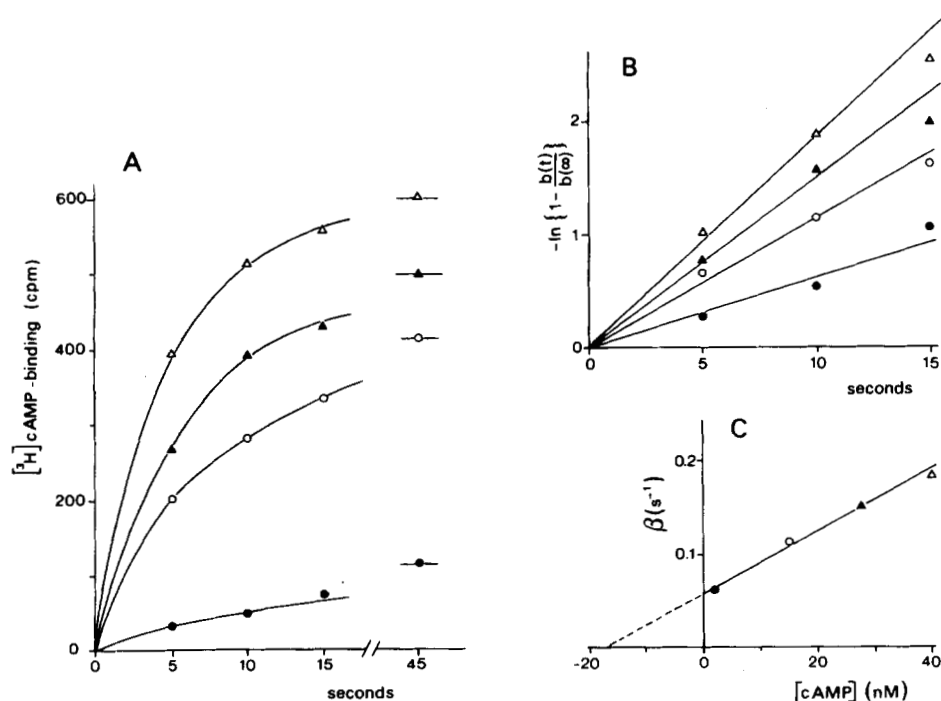

FIG. 5. **Association of [³H]cAMP to S.** *A*, a 80-μl cell suspension was mixed with 40 μl of [³H]cAMP. At the times indicated further binding of [³H]cAMP was prevented by the addition of excess cAMP (2 μl of 10^{-2} M), and S-specific binding was measured by centrifugation of the samples after 8 s. ●, 2 nM; O, 15 nM; ▲, 27.5 nM; △, 40 nM [³H]cAMP. The binding at 45 and at 60 s is approximately equal, indicating the attainment of equilibrium at 45 s. Nonspecific binding was subtracted from all data. The results shown are the means of triplicate determinations from a single experiment. *B*, replot of the data of *A* (same symbols). $b(t)$ equals the specific binding at t, and $b(\infty)$ at equilibrium (45 s). *C*, replot of the data of *B* (same symbols). β equals the slopes in *B*; $\beta = k_1[cAMP] + k_{-1}$. The intercept with the abscissa equals $-K_d$, yielding K_d = 16.5 nM, and the intercept with the ordinate equals k_{-1} = 0.056 s^{-1}.

the binding to H + L (Fig. 6*A*). At low cAMP concentrations the binding is mainly to H, while at high concentrations (>100 nM), binding is mainly to L. However, the proportioning of H and L at these extremes of the cAMP concentration is not known. The fraction of H + L sites in H (α^H) in the absence of cAMP is called ψ, and α^H in the presence of large cAMP concentrations is called ϕ ($\psi > \phi$). The cAMP dose dependence of the conversion of H to L is shown in Fig. 6*B*; a half-maximal conversion is induced by 12.5 nM.

The Effect of Drugs on cAMP Binding—Since the proportioning constants ψ and ϕ are unknown for the present, it is not possible to use the data of Fig. 6*A* or Fig. 2 to calculate the binding affinities of H and L. Therefore, we have tested several drugs for an effect on the distribution of binding types with the aim to find a drug which fixes the binding sites in one of the H, L, or S binding types. Additionally, such a drug may reveal the binding type which transduces the cAMP signal.

The screening procedure consists of three steps. First the effect of different concentrations of drugs on the cGMP response and on the equilibrium binding of 2 nM [³H]cAMP was tested. At this low [³H]cAMP concentration all three binding types will be detected. In a second step the positively responding drugs were tested for an effect on (*a*) the binding of 2 nM [³H]cAMP at equilibrium and at 8 s after a chase with excess cAMP (which yields information on S) and (*b*) binding of 100 nM [³H]cAMP after 6 s of association and at
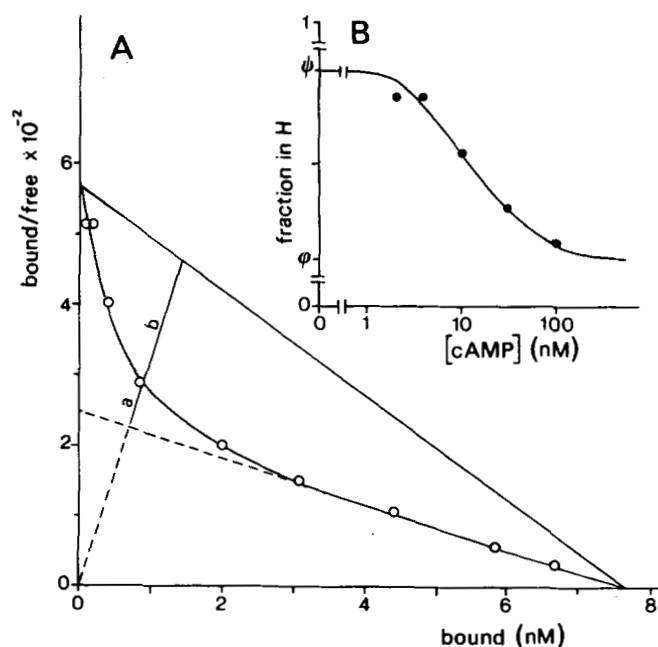

FIG. 7. **Effect of caffeine and chlorpromazine on [³H]cAMP binding and on the cAMP-mediated cGMP response.** *A,* the binding of 2 nM [³H]cAMP was measured after 45 s in the presence of different concentrations of caffeine or chlorpromazine. Nonspecific binding in the presence of the drugs was also measured and subtracted from total binding. *B,* cells were preincubated with the drugs for 30 s, followed by the addition of 50 nM cAMP (final concentration). The cells were lysed at 40 s by perchloric acid, and cGMP was measured in the neutralized lysates. The results shown are the means and standard deviations of three to six experiments each in triplicate. *, significantly above control; **, significantly below control. ●, chlorpromazine; ○, caffeine.

FIG. 6. **Scatchard plot of H + L.** Replot of the data of Fig. 4. Binding to S at equilibrium (*dashed line* in Fig. 4*B*) was subtracted from total binding at equilibrium (*open symbols* in Fig. 4*A*). The result is shown in Fig. 6*A*. The fraction of H + L sites in the H conformation is called α^H. Assume that $\alpha^H = \psi$ if [cAMP] = 0 and $\alpha^H = \phi$ if [cAMP] is large; then α^H at any concentration of cAMP is given by $\alpha^H = \psi - (\psi - \phi)\dfrac{b}{a+b}$, where *a* and *b* are segments on the line which connects the measured point with the origin. In *B* α^H is presented *versus* [cAMP]. The *curve* represents the equation

$$\alpha^H = \psi - (\psi - \phi)\frac{[cAMP]}{[cAMP] + 12.5}.$$

See "Calculation of Binding Parameters" for an estimation of ψ and ϕ.

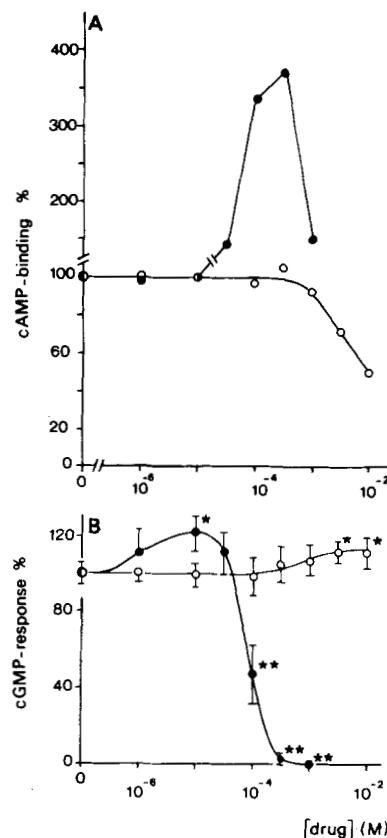equilibrium (which yields information on the transfer of H to L). In the third step the drugs were tested for their effect on all binding parameters by Scatchard analysis at equilibrium conditions and at nonequilibrium conditions (6-s association and 8-s chase). The two most interesting drugs, caffeine and chlorpromazine, will be treated below.[1]

Caffeine inhibits the binding of 2 nM [³H]cAMP to about 40% while it simultaneously enhances the cGMP response (Fig. 7). Chorpromazine has a different effect; cAMP binding is stimulated by this drug, the cGMP response is slightly but significantly stimulated at low drug concentrations, and completely inhibited at high drug concentrations. The effects of these drugs on nonequilibrium binding data are shown in Table I. Caffeine has no effect on nonspecific binding. Chlorpromazine increases nonspecific binding to 220% at 0.1 mM. This effect increases at higher chlorpromazine concentrations up to 618% at 1 mM (data not shown). Caffeine inhibits 2 nM equilibrium binding about 47%. After a 8-s chase with excess

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[1] Caffeine and chlorpromazine were investigated because these drugs have been reported to interfere with cAMP relay and the cGMP response (23, 26, 28), and, in the case of chlorpromazine, with cAMP binding (report of P. C. Newell at the *British Cellular Slime Mould Meeting*, Stirling, Sept. 15, 1983).

TABLE I

The effect of caffeine and chlorpromazine on nonequilibrium [³H]cAMP binding

Experiment no.	[³H]cAMP	Time	Counts/min bound		
			Control	10 mM caffeine	0.1 mM chlorpromazine
	nM	*s*			
Nonspecific binding					
1	2	6–60[a]	58.7 ± 1.3	61.4 ± 2.0	130 ± 4
2	100	6–60[a]	2531 ± 67	2491 ± 90	6004 ± 192
Specific binding					
3	2	45	428 ± 23	226 ± 15	1462 ± 60
4	2	45 + 8 off[b]	81 ± 3.0	54 ± 3.4	595 ± 29
5	100	6	9873 ± 632	5431 ± 186	16587 ± 371
6	100	45	5902 ± 254	4800 ± 151	16129 ± 447
Affinity modulation (6/5)			1.67 ± 0.13	1.13 ± 0.05	1.03 ± 0.04

[a] Nonspecific binding does not change between 6 and 60 s.
[b] Binding with 2 nM [³H]cAMP for 45 s followed by a chase with 0.1 mM cAMP for 8 s. The results shown are the means and standard deviation of triplicate determinations of a typical experiment.

cAMP it appears that binding to S is inhibited about 33%. Caffeine inhibits the 100 nM equilibrium binding slightly (19%), and binding at 6 s after association is only slightly larger than at equilibrium, which may indicate that the transition from H to L does not take place. These results suggest that caffeine partially inhibits the binding of [³H]cAMP to S and to H, but that binding to L is not strongly inhibited.

Chlorpromazine has a different effect. Equilibrium binding at 2 nM cAMP is increased to 342%, and binding to S is increased even to 735%. The effect on 100 nM binding is less pronounced. As with caffeine, binding at 6 s and at equilibrium (45 s) are almost identical, which suggests that also in the presence of chlorpromazine the transition of H into L is absent. These results suggest that chlorpromazine stimulates the binding to S and to H, while binding to L may be suppressed.

Scatchard analyses of [³H]cAMP binding in the presence of caffeine and chlorpromazine are shown in Fig. 8. Neither of the drugs affects the total number of binding sites. The Scatchard plots at 8 s after a chase with excess cAMP yield approximately identical dissociation constants for S in the absence or presence of caffeine or chlorpromazine (Fig. 8, *B* and *D*). The dissociation rate constant (k_{-1}) of S is also not affected by these drugs (Fig. 9). The drugs change the number of S binding sites; the fraction of binding sites with the S configuration is reduced by caffeine from about 4 to 2.5%, while the fraction is increased to 16% in the presence of chlorpromazine (Fig. 8).

Scatchard plots of H + L are calculated by subtraction of the binding to S before the chase (*dashed lines* in Fig. 8, *A* and *C*) from the total binding at 45 s. The results (Fig. 8*E*) show that [³H]cAMP binding in the presence of caffeine is mainly low-affinity binding with K_d = 450 nM and that binding in the presence of chlorpromazine is almost exclusively high-affinity binding with K_d = 60 nM.

Calculation of Binding Parameters—In this section the kinetic constants (k_{-1} and K_d) of H, L, and S will be calculated by curve fitting of a mathematical equation with the experimental data which have been obtained in the previous sections. The standard deviation of the cAMP binding assay is about 3%. The standard deviation in Scatchard plots is about 4% at low cAMP concentrations (2 nM) and increases at higher cAMP concentrations. The standard deviation becomes above 10% at cAMP concentrations above 1 µM. Therefore, all values of the kinetic parameters are regarded valid if

the calculated curve is within a 10% deviation of the observed data. The kinetic constants of S are (Figs. 3–5, 8, and 9) K_d = 12.5 ± 3 nM and k_{-1} = 0.05 ± 0.015 s⁻¹.

By using the drugs caffeine and chlorpromazine it was possible to alterate cAMP binding. Nevertheless, the total number of binding sites was not affected and neither were the binding constants of S (K_d and k_{-1}). We make the assumption that these drugs also do not affect the binding constants (K_d and k_{-1}) of H and L. Thus, we assume that the drugs only change the proportioning of the three binding types. After having made this assumption it is possible to establish the K_d values of H and L as 60 and 450 nM, respectively.

In Fig. 6*A* the Scatchard plot of H + L is shown, and in Fig. 6*B* the fraction in H (α^H) as a function of the cAMP concentration is shown. The Scatchard plot should fit the equation,

$$b/b_{max} = \alpha^H \frac{[cAMP]}{[cAMP] + 60} + (1 - \alpha^H) \frac{[cAMP]}{[cAMP] + 450} \quad (3a)$$

where

$$\alpha^H = \psi - (\psi - \phi) \frac{[cAMP]}{[cAMP] + 12.5} \quad (3b)$$

where [cAMP] is the nanomolar concentration of cAMP. The first term in the right-hand portion of Equation 3a represents the binding to H and the second term the binding to L. α^H is the fraction of sites in H; ψ is the fraction in H in the absence of cAMP and ϕ is the fraction in H at high cAMP concentrations (Fig. 6*B*). ψ and ϕ were varied to find an optimal fit of Equations 3 for the observed data of Fig. 6*A*, which yields ψ = 0.4 ± 0.05 and ϕ = 0.1 ± 0.04.

The value of ψ can also be estimated from the binding data at 6 s after association (Fig. 2). However, subtraction of the binding to S from the total binding is more complicated, since binding to S has not yet reached equilibrium at 6 s. The association rate of cAMP binding to S is known (Fig. 5); thus it is possible to calculate the binding to S at 6 s (by using Equation 2a). These data are subtracted from the total binding at 6 s, and subsequently the resulting data are fitted to Equation 3a where $\alpha^H = \psi$ (the transition of H to L has not yet taken place). This yields ψ = 0.45 ± 0.1.

By using the calculated values for ψ and ϕ and the observed K_d values for H, L, and S, it is possible to estimate the distribution of radioligand bound to the three binding types
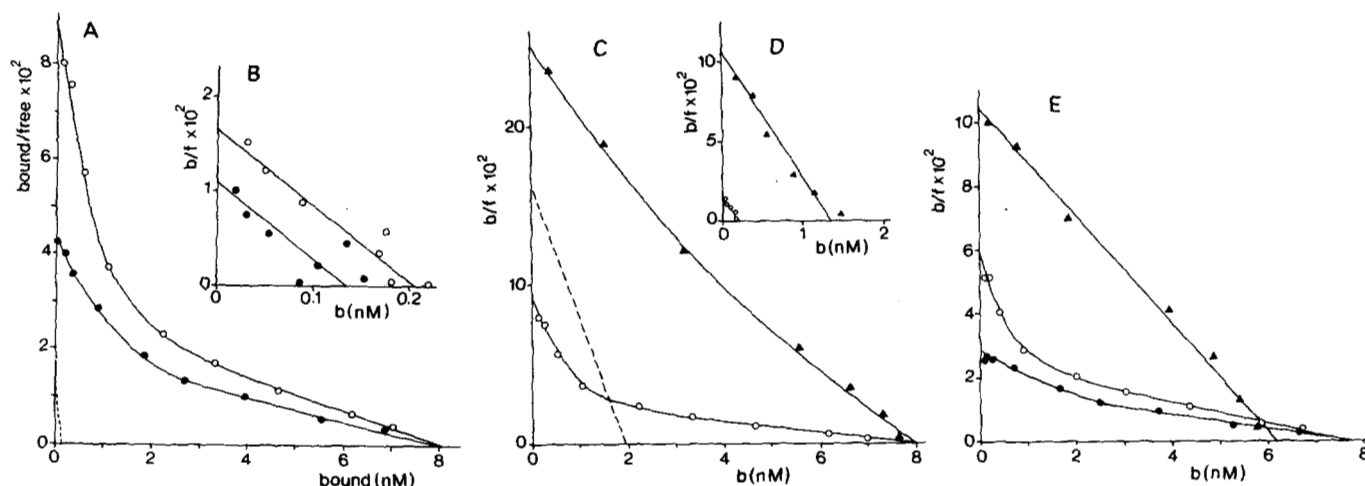
FIG. 8. Effect of caffeine and chlorpromazine on Scatchard plots of [³H]cAMP binding. *A*, the total binding of different [³H]cAMP concentrations was measured in the absence (O) or presence (●) of 10 mM caffeine at equilibrium (45 s). *B*, the binding of different [³H]cAMP concentrations to S was measured by incubation of cells with [³H]cAMP till equilibrium (45 s), followed by the addition of excess cAMP (2 μl, 10^{-2} M). [³H]cAMP binding was detected 8 s later. Same symbols as in *A*. *C* and *D*, same experiment as in *A* and *B*, except that caffeine is replaced by 0.1 mM chlorpromazine. O, control; ▲, chlorpromazine. *E*, Scatchard plot of H + L. The *dashed lines* in *A* and *C* represent the binding to S at equilibrium (45 s) in the presence of the drugs. These data are subtracted from the total binding in the presence of the drugs. The binding of cAMP to H + L in the absence of drugs is shown for comparison (redrawn from Fig. 6*A*). O, control; ●, 10 mM caffeine; ▲, 0.1 mM chlorpromazine. The results shown are the means of triplicate or duplicate determinations from three independent experiments.
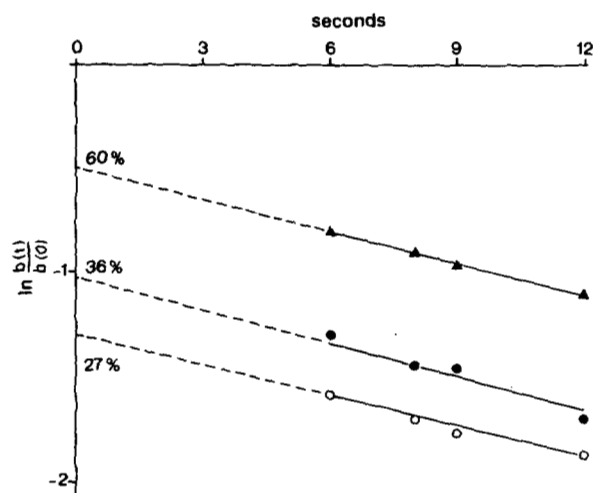

FIG. 9. Dissociation rate of S in the presence of caffeine and chlorpromazine. Cells were preincubated with 2 nM [³H]cAMP and 10 mM caffeine or 0.1 mM chlorpromazine for 45 s. Then (at $t = 0$ s) excess cAMP (2 μl of 10^{-2} M) was added, and [³H]cAMP binding was detected at 6, 8, 9, and 12 s. The intercept with the ordinate represents the fraction of the specifically bound radioactivity which is bound to S. O, control; ●, caffeine; ▲, chlorpromazine. The results shown are the mean of triplicate determinations from a single experiment.

TABLE II

Summary of the cAMP receptor binding parameters

Transition rate constant (H $\overset{k}{\rightarrow}$ L), $k = 0.08 \pm 0.014$ s^{-1}. Half maximal transition at 12.6 ± 2.8 nM.

Parameter	Unit	H	L	S
K_d	nM	60 ± 13	450 ± 140	12.5 ± 3
k_{-1}	s^{-1}	0.45 ± 0.1	1.0 ± 0.2	0.05 ± 0.015
k_1	μM^{-1} s^{-1}	7.5 ± 2.3	2.2 ± 0.8	4 ± 1.5
Site distribution		H	L	S
			%	
Before transition		38.5	57.5	4
After maximal transition		9.6	86.4	4
With caffeine		≤7.5	≥90	2.5
With chlorpromazine		84	0	16

of H and L were changed to find an optimal fit for the release of bound [³H]cAMP observed in Fig. 3. This yields for H, k_{-1} = 0.45 ± 0.1 s^{-1} and for L, k_{-1} = 1.0 ± 0.2 s^{-1}.

All parameters for the complex of cAMP binding sites on the cell surface of *D. discoideum* are summarized in Table II.
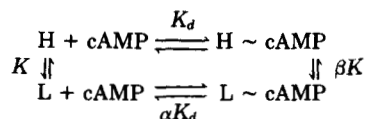
DISCUSSION

Three binding types, H, L, and S, are recognized by kinetic analysis of [³H]cAMP binding to *D. discoideum* cells. The ratio of the number of the three binding types may change during the incubation period and by pharmacological agents. Nevertheless, the total number of binding sites does not change. This suggests that the three binding types belong to the same population of binding sites and that they can be converted into each other. The three binding types are characterized by their kinetic properties. S has high affinity for cAMP (K_d = 12.5 nM) and is slowly dissociating ($t_{1/2}$ = 15 s); H has high affinity (K_d = 60 nM) and is fast dissociating ($t_{1/2}$ = 1.5 s); and L has low affinity (K_d = 450 nM) and is fast dissociating ($t_{1/2}$ = 0.7 s). S is a stable nonconvertible site, while H and L are interconvertible. This indicates that the

at any cAMP concentration. At 2 nM [³H]cAMP the label distribution is 30% to H, 43% to L, and 27% to S; at 100 nM [³H]cAMP this is 12% to H, 75% to L, and 13% to S. The dissociation of [³H]cAMP form all three binding types after a cAMP chase is given by

$$\frac{b(t)}{b(o)} = \sum_{n}^{i} J_i\, e^{-k_{-i}t} \quad (4)$$

where *i* denotes H, L, or S and *J* the fraction of [³H]cAMP bound to these types. The k_{-1} of S has been established as 0.05 s^{-1} (Figs. 3, 5*C*, and 9). The dissociation rate constants

curvilinear Scatchard plot at equilibrium is the result of both binding site heterogeneity (S *versus* H + L) as well as affinity modulation (H → L). The change of binding affinity could be detected because the binding to H and L is much faster ($t_{1/2}$ < 1.5 s) than the transition of H to L which has a half-life of about 9 s.

The conversion of H to L is time and cAMP dose dependent. We can, therefore, describe the binding to H and L as

$$
\begin{array}{ccc}
& K_d & \\
\text{H + cAMP} & \rightleftharpoons & \text{H} \sim \text{cAMP} \\
K \Updownarrow & & \Updownarrow \beta K \\
\text{L + cAMP} & \rightleftharpoons & \text{L} \sim \text{cAMP} \\
& \alpha K_d &
\end{array}
$$

SCHEME 1

where K = H/L and βK = H ~ cAMP/L ~ cAMP. If the system is closed and receives no input of materials of energy, the principle of detailed balance or microscopic reversibility implies that $\alpha = \beta$. We know that in the absence of cAMP, K = H/L = 0.4/0.6 = 2/3, and that in the presence of high cAMP concentrations, βK = H ~ cAMP/L ~ cAMP = 0.1/ 0.9 = 1/9, thus β = 1/6. Furthermore, we know that K_d = 60 nM and that αK_d = 450 nM; thus α = 7.5 and $\alpha \neq \beta$. This indicates that Scheme 1 is not a closed system and that there must be a driving force beyond H and L to account for the time- and concentration-dependent transition from H to L. What is the nature of this driving force? We have observed that the transition (defined .as a decrease of 100 nM [³H] cAMP binding between 6 and 45 s) takes place at 0 °C and in cells preincubated at 22 °C with 1 mM NaN₃ for 10 min which depleted the ATP content. This suggests that the driving force for the transition of H to L is not solely an input of energy, but that it probably proceeds via the input of materials; this implies that the occupied high-affinity site H interacts with another structure (*e.g.* protein or phospholipid) by which the binding affinity for cAMP is reduced. We may hypothesize two identities for this structure. First, we have observed that half-maximal transition occurs at 12.5 nM cAMP, which is the K_d for S; thus the occupancy of the third binding type S may determine the proportioning of the other two binding types. Second, a similar affinity modulation has been observed in other hormone-signal transduction pathways (7–9). The reduction of binding affinity of the receptor for the hormone appears to be due to the coupling of occupied receptor via a GTP-binding protein to adenylate cyclase activity. The same process may take place in *D. discoideum.*

The drugs caffeine and chlorpromazine were used to characterize the binding constants of H and L. These drugs may also shed light on the function of the binding sites. Binding to H is almost absent in the presence of caffeine, and the cGMP response is not inhibited, which suggests that H does not transduce the cAMP signal to guanylate cyclase. The results with chlorpromazine cannot give a clear answer on the function of the binding sites, because this drug induces a strong increase of nonspecific binding. Therefore, the inhibition of the cGMP response is not necessarily due to the shift of binding types but could be the result of other membrane perturbations, which apparently are induced by this drug.

Receptor heterogeneity and affinity modulation are not unique for the cAMP receptor. By using essentially identical nonequilibrium binding experiments the binding of the chemoattractant folic acid to *D. discoideum* cells appears to take place at five partially interconvertible binding types.² Binding site heterogeneity and interconversion of binding sites has also been observed among the acetylcholine receptor (6) and the β-adrenergic receptor (8). It is tempting to suggest that interconversion of heterogeneous binding sites is a general feature of signal transduction via cell surface receptors.

REFERENCES

1. Boeynaems, J. M., and Dumont, J. E. (1980) *Outlines of Receptor Theory*, p. 221, Elsevier/North-Holland Biomedical Press, Amsterdam
2. Maelicke, A., Fulpius, B. W., Klett, R. P., and Reich, E. (1977) *J. Biol. Chem.* **252**, 4811–4830
3. Quast, U., Schimerlik, M., Lee, T., Witzeman, V., Blanchard, S., and Rafterry, M. A. (1978) *Biochemistry* **17**, 2405–2414
4. Heidemann, T., and Changeux, J.-P. (1979) *Eur. J. Biochem.* **94**, 255–279
5. Boyd, N. D., and Cohen, J. B. (1980) *Biochemistry* **19**, 5344–5353
6. Boyd, N. D., and Cohen, J. B. (1980) *Biochemistry* **19**, 5353–5358
7. Tolkowsky, A. M., Braun, S., and Levitzki, A. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79**, 213–217
8. Toews, M. L., Harden, T. K., and Perkins, J. P. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80**, 3553–3557
9. Neufeld, G., Steiner, S., Korner, M., and Schramm, M. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80**, 6441–6445
10. Konijn, T. M. (1972) *Adv. Cyclic Nucleotide Res.* **1**, 17–31
11. Bonner, J. T. (1947) *J. Exp. Zool.* **106**, 1–26
12. Konijn, T. M., Van de Meene, J. G. C., Bonner, J. T., and Barkley, D. S. (1967) *Proc. Natl. Acad. Sci. U. S. A.* **58**, 1152–1154
13. Moens, P. B., and Konijn, T. M. (1974) *FEBS Lett.* **45**, 44–46
14. Malchow, D., and Gerisch, G. (1974) *Proc. Natl. Acad. Sci. U. S. A.* **71**, 2423–2427
15. Green, A. A., and Newell, P. C. (1975) *Cell* **6**, 129–136
16. Henderson, E. J. (1975) *J. Biol. Chem.* **250**, 4730–4736
17. Mato, J. M., and Konijn, T. M. (1975) *Biochim. Biophys. Acta* **385**, 173–179
18. Mullens, I. A., and Newell, P. C. (1978) *Differentiation* **10**, 171–176
19. Van Haastert, P. J. M., and Kien, E. (1983) *J. Biol. Chem.* **258**, 9636–9642
20. Juliani, M. H., and Klein, C. (1981) *J. Biol. Chem.* **256**, 613–619
21. Gerisch, G. (1982) *Annu. Rev. Physiol.* **44**, 535–552
22. Van Haastert, P. J. M., and Konijn, T. M. (1982) *Mol. Cell. Endocr.* **26**, 1–17
23. Devreotes, P. N. (1982) in *The Development of Dictyostelium discoideum* (Loomis, W. F., ed) pp. 117–168, Academic Press, New York
24. Van Haastert, P. J. M., and Van Der Heijden, P. R. (1983) *J. Cell Biol.* **96**, 347–353
25. Van Haastert, P. J. M. (1983) *Biochem. Biophys. Res. Commun.* **115**, 130–136
26. Teibert, A., and Devreotes, P. N. (1983) *J. Cell Biol.* **97**, 173–177
27. Gilman, A. G. (1970) *Proc. Natl. Acad. Sci. U. S. A.* **67**, 305–312
28. Brenner, M., and Thoms, S. D. (1984) *Dev. Biol.* **101**, 136–146

² R. J. W. De Wit and P. J. M. Van Haastert, manuscript in preparation.