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GUANIDINE HYDROCHLORIDE-INDUCED SHEDDING OF A *DICTYOSTELIUM DISCOIDEUM* PLASMA MEMBRANE FRACTION ENRICHED IN THE CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE RECEPTOR

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The cell surface cyclic AMP receptor of *Dictyostelium discoideum* is under study in a number of laboratories with respect to both its role in development of the organism and the physiology of excitation-response coupling. We report here that when starved amoebae are exposed to the chaotrope guanidine hydrochloride at 1.8 M, they shed a particulate cyclic AMP binding activity into the medium. This activity is due to membrane vesicles which originate from the cell surface. The vesicles are enriched up to 150-fold in cyclic AMP binding activity and up to 14-fold in phospholipid content when compared to the starting amoebae. The cyclic AMP binding activity of the membrane vesicles is identical to that of the cell surface receptor with respect to the following properties; (i) it is lacking in preparations from unstarved, vegetative amoebae; (ii) it is not inhibited by cyclic GMP and is stimulated by calcium ions; (iii) it has very rapid rates of association and dissociation of bound cyclic AMP; (iv) it has two classes of binding sites with dissociation constants similar to those of the surface receptors of whole amoebae. The binding activity of the isolated membranes is stable for several days at 4°C and the lower affinity binding sites are stable up to several months when stored at −80°C. Due to enrichment and stability of the receptor in this preparation, it should be highly suitable for many types of studies. The usefulness is enhanced by the fact that the preparation does not contain detectable cyclic AMP phosphodiesterase activity.

Introduction

When amoebae of the cellular slime mold *Dictyostelium discoideum* enter a period of starvation, they develop the competence to aggregate chemotactically into multicellular masses. These tissue-like aggregates subsequently transform both biochemically and morphogenetically into fruiting bodies composed of a spore mass supported by a cellular stalk. The chemotactic factor which mediates aggregation is cyclic AMP. During aggregation cyclic AMP interacts with specific receptors [1–4] on the cell surface inducing the cells to move in a directed fashion [5–7] and to release a pulse of cyclic AMP [8–10] which relays the signal to neighboring cells. Aggregation-competent amoebae also produce cyclic AMP phosphodiesterases [11–13] which rapidly destroy the signal and thus are thought to aid in restoration of sensitivity to the next stimulus. The potential in this organism

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for a combination of genetic [14–18] and biochemical analyses of the cyclic AMP receptor has drawn many workers to its study.

Biochemical studies to date have lagged behind those at genetic and cellular levels because of lack of progress in purification of an active receptor. We have observed rapid loss of receptor binding activity following cell lysis by a variety of conventional techniques. Also, cyclic AMP induces a number of rapid responses in competent cells [10,19–22] and, of those tested [23–25], none are observed when cyclic AMP is added to broken cell preparations. While it is possible that the responses tested are secondary rather than primary, receptor inactivation could also be the cause. A related possibility is that on cell lysis the receptor assumes an 'off' conformation since its binding of cyclic AMP has been shown to oscillate with time [26–28].

We report here a novel method for preparation of plasma membranes with stable cyclic AMP receptors. This preparation should facilitate many biochemical studies as well as provide a source for purification of the receptor. The discussion considers the possibility that other kinds of receptors might preferentially be enriched by such treatments.

Methods

Growth and starvation conditions. Dictyostelium discoideum strain A3 was grown axenically in HL5 broth [29] on a gyrotary shaker at 22°C to mid- to late-log phase. Harvesting was by centrifugation for 2 min at $500 \times g$ followed by two washes in ice-cold 0.0167 M potassium phosphate (pH 6.1) containing 2 mM $MgSO_4$ (potassium phosphate/magnesium sulfate buffer). This preparation is referred to as vegetative amoebae in the text. To initiate differentiation the amoebae were resuspended at $1 \cdot 10^7$ per ml in potassium phosphate/magnesium sulfate and incubated with shaking at 22°C. The amoebae were harvested and washed after 10–17 h of starvation during which cell surface receptors are expressed. These cells are referred to in the text as aggregation competent.

Cyclic AMP binding assays. Unless otherwise noted, the assay mixtures contained 1 pmol [^{3}H]cyclic AMP (approx. 20000 cpm/pmol), 20 nmol 5′-AMP, 1 μmol dithiothreitol, half-strength potassium phosphate/magnesium sulfate buffer and the preparation to be assayed in a final volume of 100 μl. For each assay, control tubes contained 5 nmol of unlabelled cyclic AMP which completely competes the binding of [^{3}H]cyclic AMP [3]. Separation of bound and unbound cyclic AMP was by two methods depending on the sample to be assayed.

Centrifugation assay: These assays are conducted at room temperature in 1.4 ml microcentrifuge tubes with pointed tips. Centrifugation is begun within 30 s of addition of the cells to the cocktail and is for 30 s (Beckman Microfuge B). The supernatant is removed by aspiration, the pellet surface gently rinsed with 2–3 drops (0.1–0.15 ml) potassium phosphate/magnesium sulfate buffer and aspiration repeated. The samples are dissolved in 1 ml of a scintillation cocktail containing an emulsifier (Scintiverse, Fisher Scientific Co.), and the entire tube is placed in a scintillation vial and counted. Binding is determined by comparing the competed and uncompeted cases. All assays are in duplicate. This assay was used for whole cells unless otherwise noted.

Millipore filtration assay: This modified Gilman [30] assay can be used for unbroken cells as well as for subcellular fractions. The assay mixtures are prepared in small glass tubes and kept on ice. Immediately prior to filtration the mixture is diluted with 1.5 ml ice-cold potassium phosphate/magnesium sulfate containing 10 mM dithiothreitol and rapidly transferred with a disposable pipette to a 24 mm HAWP Millipore filter (0.45 μm pore size) under gentle suction. The filter is not washed further. Binding is determined by comparison of competed and uncompeted cases. All assays are in duplicate. Dilution and filtration are achieved within a few seconds. Washing of the filter caused a significant loss of specifically-bound counts. Rapid dilution allowed much faster filtration rates with concentrated samples. The dilution volume was chosen for optimal reduction of background and retention of specific binding. This assay detects at least 90% of the binding activity for intact cells measured by the centrifugation assay. Since the pore size of the filter is larger than some of the structures to be assayed, as is true when it is used for soluble binding proteins, we

initially compared results on subcellular fractions by the Millipore assay with the method of Franklin and Potter [31] which uses ammonium sulfate with carrier proteins to precipitate bound ligand which is collected by centrifugation. This assay detected the same amount of binding as the Millipore method but is more cumbersome to use.

These assays do not detect the total number of sites present since 10 nM [^{3}H]cyclic AMP is subsaturating [2–4]. However, the assays are proportional to the number of input binding sites when less than one-third of the total [^{3}H]cyclic AMP is bound.

Protein determination. Measurement of protein was by the Lowry method as described by Layne [32] using bovine serum albumin as standard.

Treatment of amoebae with guanidine hydrochloride. Amoebae were suspended at $2 \cdot 10^8$/ml in potassium phosphate/magnesium sulfate buffer and chilled in an ice bath. A 6 M solution of guanidine · HCl was added dropwise with gentle magnetic stirring to a final concentration of 1.8 M unless otherwise indicated. Stirring on ice was continued for 30 min and the amoebae harvested by centrifugation at $5000 \times g$ for 5 min at 4°C. The cell pellet (P5) was resuspended in potassium phosphate/magnesium sulfate.

For membrane isolation, the supernatant (S5) was centrifuged for 30 min at $40000 \times g$ to yield a supernatant (S40) and a pellet (P40) which was resuspended in potassium phosphate/magnesium sulfate buffer. When optimal resolution of membranes from other P40 components was desired, P40 was overlayed on a sucrose step gradient of 0.9, 1.2, 1.5 and 1.8 M in potassium phosphate/magnesium sulfate buffer and centrifuged for 16 h at $50000 \times g$, 4°C, in an SW27 rotor. The membranes banded at the 0.9/1.2 M and 1.2/1.5 M interfaces whether or not the gradient contained 1.8 M guanidine · HCl.

Cell surface labelling. Tritiated and nonradioactive isethionyl acetimidate were synthesized according to Whiteley and Berg [22]. For labelling of cells, an ice-cold solution of 35 mM unlabelled isethionyl acetimidate was prepared and adjusted to pH 8.0 with NaOH. Solid [^{3}H]isethionyl acetimidate (20 Ci/mmol) was added to yield about $1.3 \cdot 10^8$ dpm per ml. This solution was mixed with an equal volume of cells at $2 \cdot 10^8$/ml in 17 mM potassium phosphate (pH 8.0). The pH was readjusted to 8.0 with potassium hydroxide at 5–10-min intervals during 30 min of shaking at 22°C. The cells were then harvested at 4°C by centrifugation at $500 \times g$ and washed four times with potassium phosphate/magnesium sulfate buffer.

Incorporation is specific for the cell surface by the following criteria. First, lysis of the cells in the presence of the reagent leads to increases of 300–350-fold in incorporation of trichloroacetic acid-precipitable counts. Second, when intact cells are labelled, washed, lysed and fractionated by sodium dodecylsulfate polyacrylamide gels, the patterns of proteins detected by Coomassie blue staining and fluorographic analysis of tritium [34] are completely different (not shown). A dramatic example is actin which is a major cytoplasmic protein but is not detectably labelled. By these criteria, the vast majority of reactive sites are protected from the reagent when the plasma membrane is intact (Das, O.P., Barclay, S.L. and Henderson, E.J. submitted).

Lysis of amoebae by forced filtration. Amoebae were suspended at $4 \cdot 10^7$/ml in cold potassium phosphate/magnesium sulfate buffer, warmed to room temperature (2–4 min), and in a single pass were forced by a syringe through a 5 μm pore size Nuclepore polycarbonate filter. The pores are smaller than the cell diameter, and lysis by this treatment is always over 95%, usually complete, as monitored by phase contrast microscopy.

All the input protein was recovered in the filtrate, and, when cells were surface labelled with [^{3}H]isethionyl acetimidate, no radioactivity remains adherent to the filter. All protein-bound radioactivity in the lysate is sedimentable at $30000 \times g$ (Das, O.P., Barclay, S.L. and Henderson, E.J., submitted).

Phospholipid determination. Cell or membrane samples were washed twice with 20 volumes of 20 mM potassium chloride. Lipids were extracted according to Folch et al. [35]. Dried lipid extracts were digested for 1 h at 150°C in 70% perchloric acid [36], cleared with one-third volume of 30% hydrogen peroxide followed by a further incubation for 30 min at 150°C. The samples were cooled to room temperature and used directly for phosphate determination as described by Bartlett [37] but omitting the sulfuric acid treatment. This pro-

tocol, when applied to aqueous dispersions of egg phosphatidylcholine, resulted in quantitative yields of inorganic phosphate.

Precipitations with trichloroacetic acid. Samples were loaded onto Whatman cellulose filter discs and dried under a heat lamp. The dried discs were shaken for 10 min in 10% (w/v) trichloroacetic acid, washed twice with 10% acetic acid, once with 95% ethanol, all at room temperature, and then dried prior to radioactivity determination in 10 ml Scintiverse (Fisher).

Materials. Tritiated cyclic AMP was from Amersham and tritiated acetonitrile from New England Nuclear. Dithiothreitol and guanidine · HCl were from Sigma. Other chemicals were reagent grade and used without further purification.

Results

Extraction of cyclic AMP binding activity by guanidine · HCl

When cell lysis was accomplished by a number of conventional techniques, membrane-associated cyclic AMP binding was highly unstable. For example, when cells were ruptured by Dounce homogenization or by −20°C freeze/thaw, binding of cyclic AMP (10 nM) to particulate material was only rarely observed and then decayed with a half-life of a few minutes to low or undetectable levels. Addition of serine-protease inhibitors was without effect (data not shown). We therefore attempted to extract and solubilize the binding activity from unbroken cells with the chaotropic agent guanidine · HCl which has been used to extract D-lactate dehydrogenase from *Escherichia coli* membranes [38]. Fig. 1 shows that binding activity is progressively lost from cells by incubation with increasing concentrations of guanidine · HCl. Phase contrast microscopy showed that the amoebae shrink in the presence of guanidine · HCl but do not usually lyse at concentrations less than 2.2 M. A concentration of 1.8 M guanidine · HCl was used in all subsequent experiments.

The guanidine · HCl-containing supernatant (S5) obtained following removal of cellular debris by centrifugation at 5000 × *g* was assayed for cyclic AMP binding by the Millipore technique. The total activity recovered in the extracted cell sediment (P5) and the supernatant (S5) usually varied

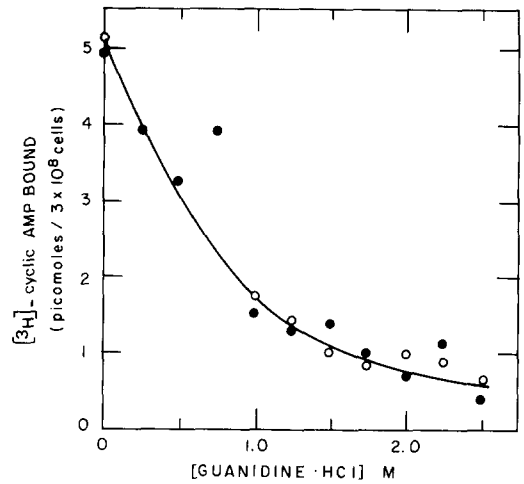

Fig. 1. Guanidine·HCl-induced loss of cell-associated cyclic AMP binding. Guanidine·HCl treatment was as described in Methods, except that the final concentrations varied as indicated on the abscissa. The cell pellet (P5) recovered at the end of the treatment was assayed for cyclic AMP binding activity by the Millipore filtration method. The open and closed symbols represent two separate experiments.

between 100 and 150% of activity in the untreated starting cells (data not shown). However, in contrast to the case of D-lactate dehydrogenase noted above, the activity in the S5 supernatant is not truly soluble and sediments at 40000 × *g* (P40) as shown in Table I. As seen in the table, while the amoebae did not appear to lyse, they lost 25–30% of their total protein into the medium during this treatment. However, only 3.4% of the extracted protein sedimented at 40000 × *g* whereas 80% of the binding activity sedimented.

There is variability in the P40 preparation. For example, in seven experiments the fold purification of binding activity varied from 5 to 58 (average 21) and the yield of binding activity varied from 12 to 77% (average 39%). These variables did not correlate with hours of starvation of the amoebae prior to treatment nor with cell density in the culture prior to starvation. In some experiments, the amoebae release as much as 60 to 70% of their protein into the medium during guanidine treatment, and in these cases the fold purification of binding by P40 is low (around 10-fold). However, in nearly all cases, the binding activity can be purified to the same final specific activity by an

TABLE I

DISTRIBUTION OF CYCLIC AMP BINDING FOLLOWING TREATMENT WITH GUANIDINE·HCl

A total of $3.4 \cdot 10^8$ amoebae (starved 17 h in suspension) were treated with 1.8 M guanidine·HCl followed by centrifugation at $5000 \times g$. The supernatant fraction (S5) was then centrifuged for 30 min at $40000 \times g$, and the supernatant (S40) and pellet (P40, resuspended in potassium phosphate/magnesium sulfate buffer) were assayed for cyclic AMP binding activity and protein concentration.

Fraction	Total binding		Total protein		Specific binding	
	pmol	%	mg	%	pmol/mg	x-fold
Untreated cells	0.95	100	36.6	100.0	0.026	1.0
S5	0.53	54	9.6	26.4	0.055	2.1
P40	0.48	50	0.4	0.9	1.520	58.0
S40	0.12	13	9.4	25.4	0.013	0.5

additional step of sucrose gradient centrifugation (see below).

The composition and origin of P40

Since the binding activity of P40 was not soluble, a number of possible sources of this activity had to be tested. The first possibility considered was that somewhat hydrophobic proteins, including the receptor, were extracted but then self-associated as is common for integral membrane proteins. By electron microscopy of P40 two types of structures were apparent (Fig. 2a): particles of approx. 0.05 μm diameter and collapsed vesicles of 0.2–0.5 μm diameter. While the vesicles shown in this electron micrograph were single and isolated, aggregated vesicles were also present. The following experiments were performed to determine whether the vesicles originated from the cell surface and whether cyclic AMP binding was to the vesicle population or the particles.

If the vesicles in P40 are derived from the cell surface, they should be labelled by reagents which react with proteins and aminophospholipids but which do not pentrate membranes. Accordingly, aggregation-competent amoebae were reacted with tritiated isethionyl acetimidate which reacts with primary amines and is membrane-impermeable in erythrocytes [33] and *D. discoideum* (see Methods). The labelled amoebae were treated with guanidine · HCl and fractionated to obtain P40. The distribution of trichloroacetic acid-precipitable radioactivity is shown in Table II.

Of the radioactivity in the labelled cells, 8% appeared in S40 and 17% in P40 in this experiment. The specific activity of P40 was 31-fold greater than that of S40 and 4.6-fold greater than the starting cells indicating that at least some component of P40 originates from the cell surface. Since control experiments (see Methods) indicated little, if any, pentration of the reagent into the cytoplasm during labelling period, the protein-bound radioactivity of S40 may be due to elution of some cell surface proteins by the chaotrope.

Since P40 contains both particles and vesicles, we further fractionated P40 to determine whether the cyclic AMP binding and cell surface material

TABLE II

DISTRIBUTION OF RADIOACTIVITY AFTER GUANIDINE·HCl TREATMENT OF CELLS LABELLED WITH [^{3}H]ISETHIONYL ACETIMIDATE

Amoebae were starved for 11 h, washed and reacted with the imido ester as described in Methods. After washing free of unreacted label, the amoebae were treated with guanidine·HCl and aliquots of the fractions were precipitated with 10% trichloroacetic acid. Total precipitable counts are shown.

Preparation	Total radioactivity (cpm)	Total protein (mg)	Specific activity (cpm/mg)
Washed cells	98100	189	519
S40	8224	105	78
P40	16835	7	2405

were due to the same structural component. A preparation of starved amoebae was divided into two equal portions and one was treated with [^{3}H]isethionyl acetimidate. P40 was prepared from both populations and subfractionated on parallel sucrose step gradients. The distribution of tritium

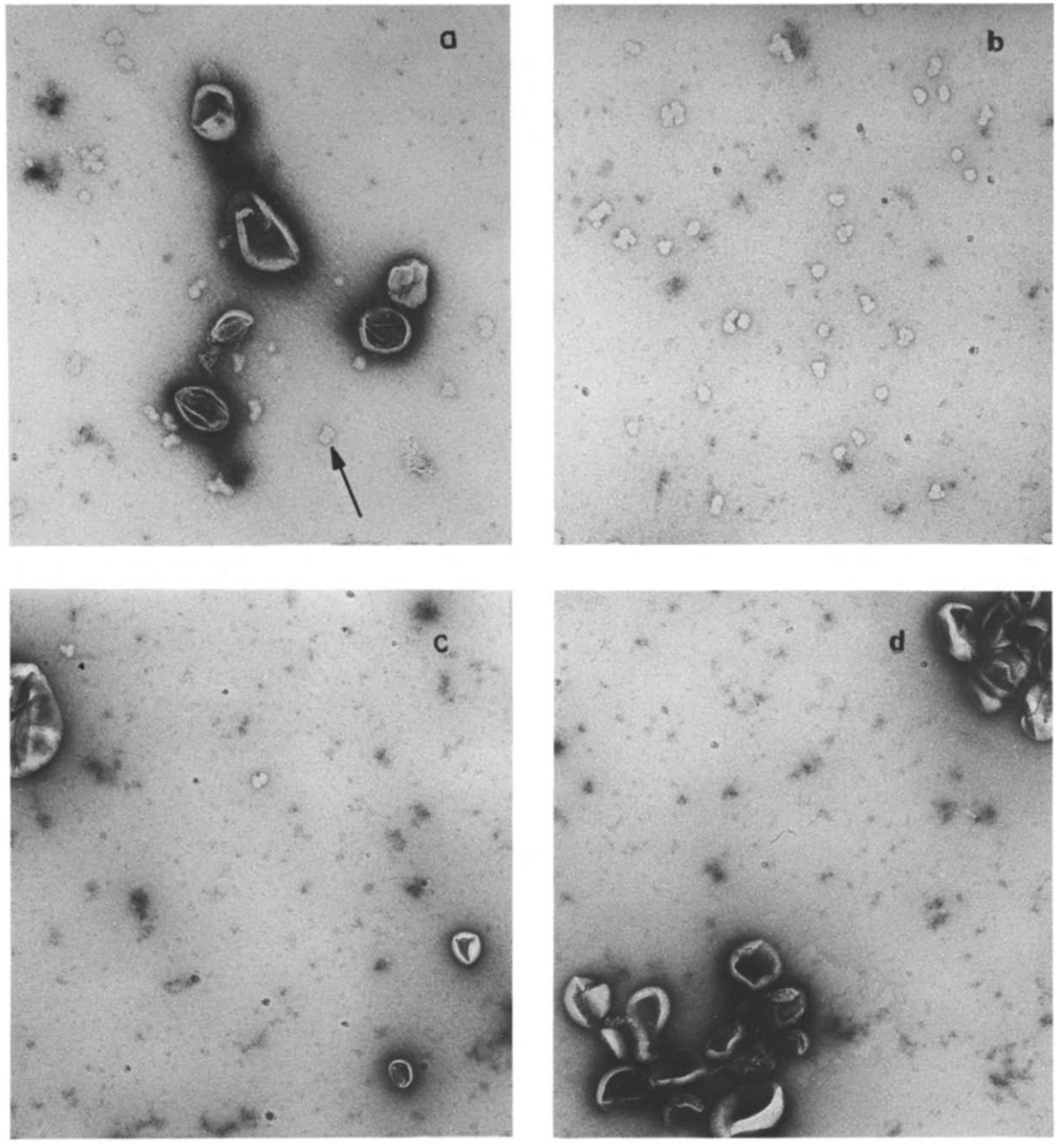

Fig. 2. Electron micrographs of fractionated P40. Samples were from the experiment described inTable III and were prepared using wet mounts fixed on carbon grids with uranylacetate. (a) P40 sample applied to the sucrose gradient. Vesicles and particles (see arrow) are apparent. Magnification: 52500×. (b) Fraction 2 from the sucrose gradient. Magnification: 52500×. (c) Fraction 3 from the gradient. Magnification: 37500×. (d) Fraction 4 from the gradient. Magnification: 45000×.

TABLE III

SUCROSE GRADIENT FRACTIONATION OF P40

Amoebae were starved for 11 h, harvested, washed and resuspended to $2 \cdot 10^8$/ml. Half of the suspension was subjected to labelling with [^{3}H]isethionyl acetimidate as described in Methods. Both the labelled and unlabelled cells were then used to prepare P40. The isolated P40 samples were overlayed on parallel step gradients composed of equal volumes of 1.0, 1.5, 2.0 and 2.5 M sucrose all containing 1.8 M guanidine·HCl. Centrifugation was at 50000× *g* in an SW 27 rotor for 18 h at 4°C. The total gradient was collected from the bottom into five fractions. Binding of cyclic AMP was measured in fractions from the unlabelled P40. The protein distribution was essentially the same in both gradients, and the average of the two is shown. The [^{3}H]isethionyl acetimidate ([^{3}H]IAI) incorporation data are total counts rather than trichloroacetic acid precipitated. Fraction 1 is the bottom of the gradient. Fraction 2 is the region containing the 1.5/2.0 M sucrose interface. Fractions 3 and 4 banded just below and just above the 1.0/1.5 M sucrose interface, respectively. Fraction 5 contains the region of sample overlay and half the 1.0 M sucrose. Visually, the bulk of the material was in fractions 2, 3, and 4. n.d., not determined.

Fraction	Total protein (mg)	[^{3}H]cAMP binding			Incorporation from [^{3}H]IAI	
		Total cpm ($\times 10^{-3}$)	pmol/mg	%	Total cpm ($\times 10^{-3}$)	cpm/mg ($\times 10^{-3}$)
P40	3.55	66.0	0.90	100	23.0	6.5
5	n.d.	9.8	–	15	7.0	–
4	0.60	24.2	2.06	37	9.2	15.3
3	0.17	6.1	1.86	9	8.7	51.0
2	1.26	14.7	0.58	22	3.8	3.0
1	n.d.	1.7	–	3	0.0	–

and of cyclic AMP binding in these gradients is shown in Table III, and electron micrographs of the fractions are shown in Fig. 2(b–d). While all fractions had cyclic AMP binding activity and four of the five contained label from amidination, only fractions 2 to 4 have been studied in more detail.

Fraction 2 contained the small particles observed in P40 (Fig. 2b) with few contaminating vesicles. It also contained label from amidination and bound cyclic AMP but in both cases at a specific activity lower than P40. Fractions 3 and 4 had higher specific activities of both cyclic AMP binding and amidination than did the input P40.

TABLE IV

PHOSPHOLIPID AND PROTEIN COMPOSITION OF cAMP BINDING FRACTIONS

The data represent the average of two experiments. Amoebae were starved 12 and 14.5 h in shaken suspension, were harvested, washed and resuspended to $2 \cdot 10^8$/ml. The guanidine·HCl treatment was performed on $4 \cdot 10^9$ total cells. An aliquot of the resultant P40 preparation was applied to a sucrose step gradient of 0.9, 1.2, 1.5 and 1.8 M sucrose in potassium phosphate/magnesium sulfate buffer. Centrifugation was for 16 h at 50000× *g*, 4°C, in an SW 27 rotor. Fractions banding at the 1.2/1.5 and 0.9/1.2 M sucrose interfaces are labelled 1 and 2 and correspond to fractions 3 and 4 of Table III. All fractions were washed, subjected to chloroform/methanol extraction and analyzed for phosphate release as described in Methods.

Sample	Total protein		Total phospholipid		Ratio μmol phosphate/ mg protein
	mg	%	μmol phosphate	%	
Untreated cells	286.0	100	34.8	100	0.12
P40	6.0	2.1	5.1	15	0.85
Gradient fraction 1	0.4	0.1	0.6	2	1.72
Gradient fraction 2	1.8	0.6	2.5	7	1.36

These fractions contained the vesicle population with fraction 3 composed of single and double vesicles (Fig. 2c) and fraction 4 of aggregated vesicles (Fig. 2d).

The vesicular structures appeared membranous, and this was confirmed by their high content of phospholipid in a Folch extraction. Table IV shows the protein and phospholipid content of the vesicle fractions compared to starting amoebae and P40. The sucrose concentrations in this experiment were altered from those of Table III to afford maximum resolution of the two vesicle populations. The more dense material (the 0.05 μm particles) pellets through this gradient and was not analyzed. In the vesicle fractions the phospholipid-to-protein ratios were 11- and 14-fold increased over the starting cells and are in the range of expected values for biological membranes. For example, rat liver plasma membranes have a phosphate/protein ratio of 0.825 [39]. It should be noted that the values for the vesicles may be different from native plasma membranes of *D. discoideum*.

Our interpretation of the above observations is that in the presence of guanidine · HCl the cell surface vesiculates and sheds receptor-containing plasma membrane into the medium. However, since the cells release a significant amount of their total protein on exposure to guanidine · HCl, it is possible that the released binding activity could be due to cytoplasmic binding proteins which adhere to membranes or become insoluble in the presence of the chaotrope. The experiments below argue against this possibility. Cells were lysed gently by passage through polycarbonate filters (see Methods) and the sedimentation behavior of binding sites in an S5 supernatant in the absence and presence of guanidine · HCl was investigated (Table V). The data are compared with the standard guanidine · HCl extraction. The first point apparent is that binding activity in the filter lysate which does not sediment at 5000 × *g* also does not sediment at 40000 × *g* in opposition to the behavior of the guanidine · HCl-extracted material (compare lines 2 and 3 of the table). When S40 from the filter lysate was adjusted to 1.8 M guanidine · HCl and centrifuged at 40000 × *g*, no detectable pellet was obtained (data not shown). However, the binding activity of this S40 was 7-fold inhibited by guanidine · HCl (compare lines 4 and 5). Storage at 4°C in guanidine resulted in further losses of activity (line 6). In contrast, the binding activity of P40 prepared by guanidine extraction is completely stable in the presence or absence of guanidine · HCl for up to four days at 4°C (not shown).

TABLE V

SEDIMENTATION BEHAVIOR AND EFFECT OF GUANIDINE·HCl ON CYTOPLASMIC cAMP BINDING SITES. COMPARISON WITH GUANIDINE·HCl EXTRACTION

Amoebae were starved for 12 h in shaken suspension, harvested, washed in potassium phosphate/magnesium sulfate buffer and divided into two aliquots. One sample was subjected to guanidine·HCl extraction. The other sample was lysed by filtration as described in Methods. The samples were centrifuged 10 min at 5000 × *g* (4°C) yielding supernatants (S5) which were then subjected to centrifugation for 30 min at 40000 × *g* further yielding supernatants (S40) and pellets (P40) which were resuspended in potassium phosphate/magnesium sulfate buffer. All binding assays were by the Millipore method. The starting cells bound 0.156 pmol cAMP per 10^7 amoebae. n.d., not determined.

Fraction	% of cAMP binding to untreated cells	
	Filtration lysed	Guanidine·HCl extracted
Crude lysate	14.2	n.d.
S5	18.4	33.2
P40	0.3	48.0
S40	18.9	2.2
S40 + 1.8 M guanidine·HCl	2.7	n.d.
S40 + 1.8 M guanidine·HCl, 48 h	0.7	n.d.

In a separate experiment addition of guanidine · HCl to the crude filtration lysate did not cause the binding activity in the S5 fraction to become sedimentable at 40000 × *g* (data not shown). These experiments indicate that soluble binding sites do not become insoluble or adsorb to membranes in the presence of guanidine · HCl.

We conclude from these experiments that the binding activity of P40 is due to plasma membrane vesicles containing the cell surface receptor. Both as a confirmation of this conclusion and to assure that the treatment does not alter the receptor, we

have compared a number of properties of the binding activity with the receptor on intact cells.

Cyclic AMP binding properties

The cell surface receptor has extremely rapid rates of association and dissociation. This is also true of P40 and the membrane vesicles. First, maximal binding is achieved at the earliest time points we can achieve, i.e., within a few seconds. Second, the off rate has been reported to produce 50% dissociation in less than 4 s following a dilution 5.3-fold greater than used in our Millipore assay [40]. To determine whether the off rates for P40 and the cell surface receptor were equally high, we examined the rate at which bound [^{3}H]cyclic AMP dissociates following addition of an unlabelled cAMP chase. As shown in Fig. 3, 50% dissociation of bound radioactivity occurred within a few seconds for both aggregation-competent amoebae and P40 prepared from them.

Green and Newell first reported [4] Scatchard plots of binding of cAMP to aggregation competent amoebae which indicated either two types of binding sites (high and low affinity) or possibly negative cooperative interactions. Fig. 4 shows Scatchard plots of binding to aggregation competent amoebae and the highest binding capacity membrane fraction from a sucrose gradient (equivalent to fraction 4 of Table III). High and low

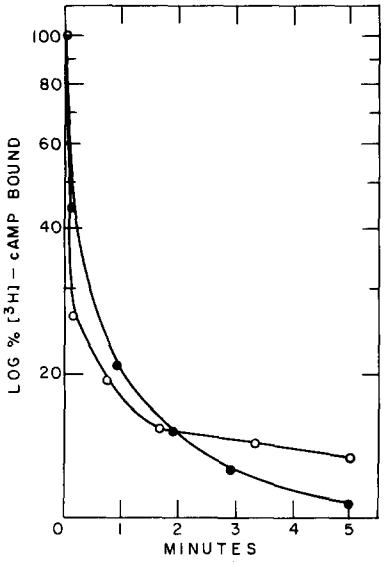

Fig. 3. Rate of dissociation of bound [^{3}H]cyclic AMP following addition of a cold cyclic AMP chase. Amoebae were starved in shaken suspension for 12 h and harvested. One aliquot was stored on ice. Another aliquot was used to prepare P40. Cells were adjusted to $1.5 \cdot 10^8$ per ml and bound 0.105 pmol cyclic AMP per 10^7 cells in the Millipore assay. The P40 preparation was adjusted to the same binding capacity per volume. Both preparations were mixed with the standard binding cocktail containing 10 nM [^{3}H] cyclic AMP and an aliquot taken for determination of bound radioactivity. Concentrated, nonradioactive cyclic AMP was added to yield 10 μM final, and aliquots were removed at differing times for determination of remaining bound radioactivity by the Millipore assay. The open symbols represent binding to cells, the closed symbols to P40.

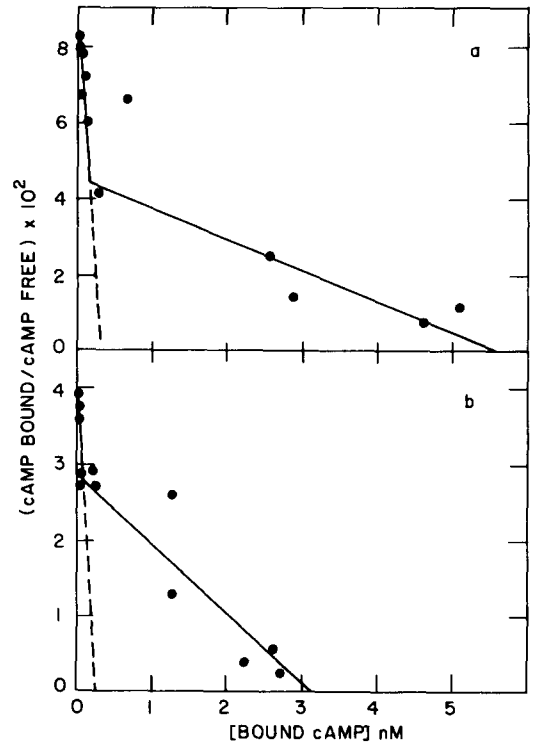

Fig. 4. Scatchard plots of cyclic AMP binding to aggregation-competent amoebae and isolated membranes. (a) Amoebae were starved 10 h and binding was assayed by the Millipore method using $1.25 \cdot 10^7$ amoebae per assay tube. (b) Amoebae starved 12.5 h were treated with guanidine·HCl and the resulting P40 fraction subjected to sucrose step gradient centrifugation as described in the legend to Table IV. Binding studies were performed on the fraction which banded at the 0.9/1.2 M sucrose interface using 19 μg of protein per assay tube. In both graphs, the lines are drawn to indicate the extrapolations used to calculate dissociation constants and number of sites. The cyclic AMP concentrations varied from 0.4 to 420 pmol and the specific activity from $5 \cdot 10^3$ to 690 cpm/pmol.

affinity sites are present in both preparations. The dissociation constants calculated from the data for cells (Fig. 4a) were 6.3 and 119 nM. The data for isolated membranes (Fig. 4b) yielded dissociation constants of 4 and 107 nM and a maximum binding capacity of 0.83 and 16.4 pmol per mg protein for the high and low affinity sites, respectively. In seven different experiments, the data always indicated that the dissociation constants in P40, vesicles and cells were the same. The data of Fig. 4 also represent a typical final preparation in that while the fold purification of cyclic AMP binding sites during isolation of P40 is variable, further subfractionation by sucrose gradient nearly always yields material of this specific binding activity (approx. 2 pmol cyclic AMP bound per mg protein in the standard assay at 10 nM cyclic AMP).

The binding activities of aggregation-competent cells and P40 derived from them share a number of additional features. Cyclic GMP at 0.2 mM (a 20000-fold excess) caused reductions of binding to cells and P40 of only 11 and 13%, respectively. Calcium ions (5 mM $CaCl_2$) which have been reported to increase the number of accessible surface receptors [41] caused enhancements of 34 and 22%, respectively. Trypsin (0.1–1.0 mg/ml) as expected [28] did not inactivate either preparation though both were inactivated by heat (80°C, 5 min). Finally, vegetative amoebae have low numbers of surface receptors [2–4] and yield a P40 with specific binding activity 15–30-fold lower than that of aggregation-competent cells.

Stability of P40

The binding activity of the isolated membranes is unstable to freezing and storage at −20°C. However, after rapid freezing in acetone and dry ice and storage at −80°C, the low affinity sites are unchanged in number and dissociation constant but the high affinity sites are undetectable.

Discussion

The mechanism by which the membrane vesicles arise during guanidine · HCl treatment is not clear. The concentration of guanidine · HCl used in our experiments would be expected to have a relatively gentle chaotropic effect but a strong hypertonicity, and we do observe cell shrinkage. Yahara and Kakimoto-Sameshima [42] have shown that under certain conditions of hypertonicity lymphocytes and thymocytes shrink. Under these conditions, even in the absence of ligands normally requirued to induce capping of surface antigens, some of these antigens capped tightly in regions of the cell surface associated with an extraordinary density of microvilli. We speculate that in *D. discoideum* guanidine · HCl may affect the contractile elements or the osmotic balance of the amoebae such that cell shrinkage and membrane extrusion occur. The shear forces generated by stirring could release the extruded membrane into the medium. We do not anticipate that the released vesicles will be representative, random samples of native plasma membrane since the chaotrope could elute some classes of polypeptides and/or cause preferential exclusion/inclusion in the putative vesiculation sites. The cell surface phosphodiesterase is a candidate for an eluted or excluded species since it is not detectable in the vesicles (unpublished data). The receptor is a candidate for preferential inclusion since it may be enriched as much as 150-fold.

A number of observations support the cell surface origin of the P40 vesicles. They are highly enriched in the cell-surface label from [^{3}H] isethionyl acetimidate. The vesicles look membranous in the electron microscope and are greatly enriched in phospholipid. The cyclic AMP binding properties of the vesicles are identical to those of the cell surface receptor and different from those of the known cytoplasmic binding proteins. This is a critical point since it simultaneously reinforces the cell surface origin of the vesicles and assures that binding is not due to adsorption of proteins released from cytoplasmic pools (see below).

From the data based on filtration lysis of the amoeba, in our binding assay cytoplasmic binding proteins are not particulate and do not become so in the presence of guanidine · HCl but are inactivated by this agent. Also, while a number of cytoplasmic binding proteins have been reported [43–48], in each case a significant feature of the protein differs from the identical behavior of the receptor in cells and vesicles.

There are a number of reported methods for isolation of plasma membrane-enriched fractions from *D. discoideum* [49–55]. The lysis techniques used, such as freeze-thaw [49,50], shear techniques

[51] detergent lysis [52,53], all lead to inactivation of the receptor in our hands, though in some cases the treatment may only interfere with the assay.

There have been two reports of cyclic AMP binding to particulate subcellular fractions. The first case [54] was to a crude fraction obtained following sonication of the amoebae. However, this fraction was only 2-fold enriched for a plasma membrane marker enzyme and had a lower specific radioactivity from a cell surface label than the crude homogenate. Binding was not reported in more purified membrane fractions.

The second report was more promising [28]. A particulate fraction was obtained after freezing in liquid nitrogen, thawing at room temperature and homogenization of the pellet obtained from high speed centrifugation. A sucrose step gradient yielded material 4–5-fold enriched in a cell surface radiolabel and phosphodiesterase and 8–10-fold in alkaline phosphatase and cyclic AMP binding. Scatchard analyses were not reported although the binding behavior paralleled several features of the receptor. Although we have not been successful with homogenization, subtle differences in conditions could account for this. This method may well complement the one reported here as the two may be differentially desirable for cerain types of studies.

The vesicles obtained by sucrose density fractionation of P40 are an ideal source for attempts to isolate the receptor as they are already enriched up to 150-fold over starting cells. The material is quite stable; we have observed full retention of activity for four days at 4°C and others (Coukell, M.B., personal communication) have observed full retention for several weeks at 4°C. Thus, large amounts of material could be accumulated as a starting point for further purification. The vesicles also exhibit both the high and low affinity binding sites so that the basis for the curvilinear Scatchard behavior (distinct classes or cooperative interactions) has been retained. For analysis of functions, it could be particularly useful to exploit the preferential loss of the high affinity sites on freezing at −80°C. Also, it has recently been shown that at very low cyclic AMP concentrations positive cooperativity is observed and is present in the vesicles as well as the cells [55].

Another useful feature of the vesicles is the absence of cyclic AMP phosphodiesterase activity. Binding studies have been complicated by the presence of this enzyme since it is sufficient to destroy the ligand before completion of a binding assay. The enzyme is inhibited by thiols, but complete inhibition requirues 5–10 mM of dithiothreitol [3]. The alternative is to assay cyclic AMP binding in the presence of a large excess of cyclic GMP which is a better substrate for the enzyme than a ligand for the receptor [56]. The lack of the enzyme in the guanidine · HCl preparations negates the need for these agents.

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