Preparation and Characterization of Hormone-sensitive, Resealed Erythrocyte Ghosts*

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In the past 15 years, the investigation of hormone-activated adenyl cyclase has yielded information concerning the mechanism of adenosine 3',5'-monophosphate formation. It is now believed that not only hormones but other ligands such as GTP and Ca²⁺ control the activity of adenyl cyclase. The investigations to date have utilized either intact tissues, suspensions of isolated intact cells, or isolated plasma membranes from disrupted cells. Although of great value, each of these experimental systems has its own limitations. Intact cells or tissues cannot be easily manipulated for, although one may alter the extracellular milieu, the experimentalist has little control over the nature and concentration of intracellular metabolites. Although this limitation is overcome by the use of broken cells in the form of isolated plasma membranes, the experimentalist in this case must always recognize that the processes involved in membrane disruption and isolation may alter the behavior of membrane components and receptor-activated processes. In addition, once the membrane barrier is lost, the investigator can no longer selectively alter either the extracellular or the intracellular environment individually, as these spaces become continuous. Also, with broken cells, one can no longer determine whether specific membrane components are located on the inner or the outer surface of the membrane.

A method for preparing resealed turkey erythrocyte ghosts is described which utilizes hypotonic lysis and resealing following restoration of isotonicity. The resealed ghosts are isolated above 55% sucrose. The resealed ghosts are shown to be capable of maintaining high intracellular K⁺ concentrations in the presence of a low K⁺ extracellular environment. When ATP and an ATP-regenerating system are included during the resealing stage, (R)-(−)-epinephrine- and NaF-stimulated cyclic AMP accumulation, which is linear for 20 min, can be demonstrated. The concentration of (R)-(−)-epinephrine producing a half-maximal response in resealed ghosts is 1.0 ± 0.4 x 10⁻⁶ M. This is the same as that for (R)-(−)-epinephrine in the intact erythrocyte. The resealed ghosts are impermeable to Ca²⁺, but Ca²⁺ inhibition of cyclic AMP accumulation is noted if the divalent cation ionophore, A-23187, is present or if Ca²⁺ is included during the resealing stage.

Many of these difficulties can be overcome by using resealed cell ghosts. Resealed ghosts are cells which have had their cytoplasmic contents removed but which retain an intact plasma membrane and are capable of maintaining transmembrane ionic gradients. By selectively altering the agents which are present during the resealing stage, the investigator is capable of manipulating the composition of the intracellular compartment. Resealed ghosts have been extensively utilized to study human erythrocyte membrane topography and ion transport; unfortunately, however, the human erythrocyte does not possess an active adenyl cyclase. Therefore, resealed human erythrocyte ghosts have not been useful for the study of hormone activated processes such as hormone activation of adenyl cyclase and subsequent cyclic AMP mediated processes.

In this communication, we describe the preparation and characterization of hormone-sensitive resealed ghosts from turkey erythrocytes. Data are presented which show that these resealed ghosts possess many of the permeability characteristics of their parent native erythrocytes. In addition, these resealed ghosts are shown to respond to catecholamine stimulation by forming cyclic AMP. The requirements for cyclic AMP formation by these resealed ghosts will be described. To our knowledge, no previous reports have described the preparation of hormone-sensitive resealed ghosts.

EXPERIMENTAL PROCEDURE

Materials

Dithiothreitol was purchased from Nutritional Biochemicals. Beef heart phosphodiesterase, (R)-(−)-epinephrine bitartrate, creatine

The abbreviations used are: cyclic AMP, adenosine 3',5'-monophosphate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.
phosphate, creatine phosphokinase (160 units/ml), ATPase, and cyclic AMP were purchased from Sigma. Cyclic ["H]AMP, and [2-3H]ATP were purchased from New England Nuclear. A-23187 was a gift from Eli Lily & Co. All other reagents were of the highest purity commercially available and all solutions were prepared using doubly distilled water.

Preparation of Resealed Ghosts

Washed Red Cells—Heparin-treated blood was obtained from donor turkeys and washed three times in 150 mM NaCl. Each wash consisted of suspending the cells in precooled (4°C) saline (0.9% NaCl solution) followed by centrifugation at 2000 x g for 5 min. After each wash, the buffy coat and supernatant fluid were removed. Examination of the sample by phase contrast microscopy after the final wash revealed no cells other than erythrocytes.

Lysis—The washed red cells were slowly lysed by suspending and centrifuging them a total of five times in hypotonic buffer containing 30 mM sodium phosphate, pH 7.4, and 4 mM MgSO4. Each wash consisted of suspending the cells in 3 volumes of precooled buffer (4°C) followed by centrifugation for 10 min at 12,100 x g at 4°C in a Sorvall RC-2B centrifuge. In preliminary experiments, lysis was attempted using either Tris-HCl or phosphate buffers, pH 7.4, ranging from 5 to 60 mM. Incomplete lysis was observed when buffers of over 30 mM concentration were used, while nucleic acid vacuolization and nuclear disruption and gel formation followed lysis with buffers less concentrated than 30 mM.

Resealing—Lyzed erythrocytes were resealed using a modification of the technique described by Bodeman and Passow (1). The final pellet of lysed cells was suspended in 3 volumes of 30 mM sodium phosphate buffer, pH 7.4, with 4 mM MgSO4. Isotonicity was restored by the dropwise addition of 0.039 volume of a 1:6 mixture of 3 M KCl and 3 M NaCl. This addition was performed with constant stirring on ice. The final concentrations of K+ and Na+ were 109 and 65 mM, respectively, as determined by flame spectrophotometry. The final Mg2+ concentration at the time of resealing was 4 mM. When other agents were to be resealed within the ghosts, they were added just before restoration of isotonicity and, when appropriate, the concentrations of K+ and Na+ added were reduced in order to maintain isotonicity. The final osmolality at the time of resealing was 298-305 mOsm/liter as determined using an Advanced Instruments model 65-31 osmometer. After restoration of isotonicity, the ghosts were incubated on ice for 5 min and, subsequently, at 37°C for 30 min in a shaking water bath.

Isolation of Resealed Ghosts—The resealed ghosts were washed twice in isotonic Tris/NaCl buffer.2 Each wash consisted of suspending the ghosts in 5 to 10 volumes of precooled (4°C) buffer followed by recentrifugation at 12,100 x g at 4°C for 10 min in a Sorvall RC-2B centrifuge. The final pellet of washed, resealed ghosts was suspended in 2 to 3 volumes of isotonic Tris/NaCl buffer (4°C) and layered above 55% (w/v) sucrose in 25 mM Tris-HCl, pH 7.4, with 25 mM NaCl (4°C). This was followed by centrifugation (4°C) at 34,800 x g for 30 min in a Sorvall RC-2B centrifuge. At the completion of this centrifugation, a distinct pellet of washed resealed ghosts was observed. This was followed by centrifugation (4°C) at 12,100 x g at 4°C for 1 min in an Eppendorf table-top centrifuge and 5-11 aliquots of supernatant fluid were taken for determination of K+ concentration. The supernatant was then aspirated and the ghosts were suspended in 1.0 ml of isotonic Tris/NaCl buffer containing 11 mM glucose. The initial concentration of K+ in this mixture was 120 mM. The ghosts were centrifuged (8,000 x g for 1 min at 4°C in an Eppendorf table-top centrifuge) and 5-11 aliquots of supernatant fluid were taken for determination of K+ concentration. The supernatant was then aspirated and the cells were suspended and centrifuged (8,000 x g for 1 min) in isotonic Tris/NaCl buffer containing 11 mM d-glucose. This cell washing in K+-free buffer was continued for a total of five washes, and each time a 5-11 aliquot of supernatant fluid was taken for K+ determination while the spheroplast was replaced with fresh K+-free buffer. After the last wash, the ghosts were suspended in 1.0 ml of isotonic Tris/NaCl buffer with glucose, boiled, and centrifuged. A 5-11 aliquot of this supernatant was then taken for intracellular K+ determination.

Parallel determinations using 2 x 106 intact native erythrocytes were also performed to allow for a comparison between the native cell and resealed ghost.

The results are shown in Fig. 1. With progressive washing, the extracellular K+ concentration fell to 0.2 mM and, thereafter, K+ concentrations remained relatively constant. The

*Isotonic Tris/NaCl buffer, in this communication, refers to 130 mM NaCl in 25 mM Tris-HCl, pH 7.4. Isotonic Tris/KCl buffer refers to 130 mM KCl in 25 mM Tris-HCl, pH 7.4. The osmolality of each is approximately 300 mOsm/kg.

formation (2). When necessary, the sample cyclic AMP was concentrated by extraction with trichloroacetic acid followed by ether and, in these cases, cyclic AMP recovery always exceeded 93% when followed using cyclic ["H]AMP as an internal marker. Cyclic AMP content was determined by the method of Gilman (3) using the cyclic AMP-binding protein prepared according to the method of Miyamoto (4) and the phosphodiesterase inhibitor prepared by the method of Appleman (5). The bound cyclic ["H]AMP was recovered on Millipore filters (0.45-μm pore size, 25-mm diameter) and counted in a liquid scintillation counter in a mixture of Cellusolve and dioxane/POP/POP-POP. Portions of samples to be assayed for cyclic AMP were preincubated with beef heart phosphodiesterase (0.26 unit/ml) in 55 mM Tris-HCl, pH 7.4, containing 38 mM MgSO4 for 30 min at 37°C, placed in a boiling water bath for 3 min, and then assayed for cyclic AMP as described above. These phosphodiesterase-treated portions did not inhibit cyclic ["H]AMP binding, whereas those not treated with phosphodiesterase did inhibit cyclic ["H]AMP binding. This confirmed our impression that all of the material assayed as cyclic AMP was cyclic AMP. In addition, in all experiments appropriate samples of buffers containing the added agents were processed as controls and their ability to inhibit cyclic ["H]AMP binding was determined and subtracted from the experimental values when indicated. All results are expressed as pmol of cyclic AMP/106 ghosts.

RESULTS

Integrity of Resealing Process

Our initial attention was directed towards establishing whether or not true resealing had occurred after restoration of isotonicity. Examination of the resealed ghosts by phase contrast microscopy revealed only cells which appeared to consist of a central nucleus surrounded by a plasma membrane envelope. The persistence of a central nucleus established these to be "rightside-out" ghosts (6) while the absence of an opaque cytoplasm clearly distinguished these ghosts from the unlysed, hemoglobin-filled, turkey erythrocyte. The above findings were confirmed by examining the resealed ghosts by electron microscopy at 60,000 x magnification. No cyttoplasmic organelles or hemoglobin were noted in the resealed ghosts.

These morphological findings show that these ghosts might retain membrane integrity but do not establish them as resealed ghosts. A series of experiments were performed to determine if these ghosts were capable of maintaining high intracellular K+ concentrations while suspended in K+-free buffer since such an observation would suggest that the ghosts were functionally "resealed." For these studies, 2 x 106 resealed ghosts suspended in 1 ml of isotonic Tris/KCl buffer containing 11 mM d-glucose. The initial concentration of K+ in this mixture was 120 mM. The ghosts were centrifuged (8,000 x g for 1 min at 4°C in an Eppendorf table-top centrifuge) and 5-11 aliquots of supernatant fluid were taken for determination of K+ concentration. The supernatant was then aspirated and the cells were suspended and centrifuged (8,000 x g for 1 min) in isotonic Tris/NaCl buffer containing 11 mM d-glucose. This cell washing in K+-free buffer was continued for a total of five washes, and each time a 5-11 aliquot of supernatant fluid was taken for K+ determination while the spheroplast was replaced with fresh K+-free buffer. After the last wash, the ghosts were suspended in 1.0 ml of isotonic Tris/NaCl buffer with glucose, boiled, and centrifuged. A 5-11 aliquot of this supernatant was then taken for intracellular K+ determination.

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erythrocytes were washed in K+-free buffer. This suggests that extracellular K+ concentration with resealed ghosts did not fall each wash was determined. After the final wash, the suspension was boiled and centrifuged, and the supernatant K+ was determined. Further details are given in the text. Results represent mean of duplicate determinations from one representative experiment. ○• resealed ghosts; ○ — ○, intact erythrocytes.

extracellular K+ concentration with resealed ghosts did not fall as low with washing in K+-free buffer as it did when intact erythrocytes were washed in K+-free buffer. This suggests that the resealed ghosts slowly leak K+. However, this K+ leakage must be very small relative to the total K+ content of the ghosts, since boiling the ghosts resulted in a 10-fold increase in K+ concentration in the supernatant fluid. In these experiments, the packed ghost volume was 2.5 to 3.0% of the total sample volume. Thus, a 10-fold increase in K+ concentration would suggest that, before boiling, the K+ concentration inside the ghosts is 200 to 300-fold greater than the extracellular K+ concentration. This K+ gradient is, however, considerably less than that found when intact erythrocytes are studied (Fig. 1).

The liberation of K+ when resealed ghosts were lysed by boiling was also used to study the requirements for resealing. Washed erythrocytes were lysed and resealed as described previously, but the duration of the 37° incubation step after restoration of isotonicity with KCl/NaCl was varied. After isolation, the resealed ghosts were extensively washed in isotonic Tris/NaCl buffer containing 11 mM d-glucose. Aliquots were removed for cell count, and the remainder was centrifuged. After removing an aliquot of the supernatant fluid for K+ measurement, the pellet was resuspended in this same supernatant fluid, boiled for 3 min, and centrifuged again. Another aliquot of supernatant fluid was taken for determination to measure intraghost K+. The change in supernatant fluid K+ concentration upon boiling the ghosts was determined by subtracting the K+ concentration before boiling from that found after boiling the ghosts. Because there was a slight variation in the number of ghosts present in different samples, the K+ liberated upon boiling was normalized by expressing it as K+ liberated/107 ghosts.

The results relating the change in intracellular K+ concentration per 107 ghosts as a function of the time of incubation at 37° during resealing are shown in Table I. When the 37° incubation period was less than 15 min, very few ghosts were recovered after centrifugation above the sucrose cushion, making an accurate measurement of K+ release after boiling impossible. Even with 15-min incubation at 37°, the recovery was poor and results were quite variable. This suggests that little or no resealing occurs unless the ghosts are incubated in isotonic media for more than 15 min at 37°. Maximum K+ liberation is seen to occur from ghosts resealed at 37° for 25 to 30 min, while longer incubation at 37° appears to decrease the efficiency of resealing.

Catecholamine-stimulated Cyclic AMP Accumulation by Resealed Ghosts

Requirements for Cyclic AMP Formation—When ATP was not included during the resealing phase, cyclic AMP accumulation could not be detected when the resealed ghosts were later incubated in the presence of (R)-(−)-epinephrine (Table II). Inclusion of theophylline during the assay period did not alter this finding. Similarly, inclusion of an ATP-regenerating system at the time of resealing or assay did not result in demonstrable cyclic AMP accumulation when the ghosts were later incubated in the presence of epinephrine (data not shown). However, when ATP and the ATP-regenerating system were present at the time of resealing, accumulation of cyclic AMP after (R)-(−)-epinephrine stimulation was easily seen (Table II). Addition of ATP and the regenerating system after resealing (ie, at the time of assay) did not increase the amount of cyclic AMP accumulated during the assay period (basal or epinephrine-stimulated) if the ghosts had previously been resealed in the presence of ATP and the regenerating system (data not shown).

Time Dependence of Cyclic AMP Accumulation—Cyclic AMP was found to accumulate in a linear fashion for at least 20 min in the presence of (R)-(−)-epinephrine (Fig. 2). Basal cyclic AMP accumulation was negligible (Table II).

Epinephrine Concentration Dependence—Washed, intact erythrocytes or washed, resealed ghosts containing 2 mM ATP and the ATP-regenerating system were suspended in isotonic Tris-NaCl buffer containing varying (R)-(−)-epinephrine concentrations and incubated at 37°. After a 10 min incubation period, the ghost and intact cell samples were placed in a boiling water bath for 3 min and cyclic AMP content was determined.

The results of these experiments are shown in Fig. 3. The epinephrine concentration which gave half-maximal activity was 1.0 ± 0.4 x 10−4 M for the resealed ghosts and 0.8 ± 0.2 x 10−4 M for the intact erythrocytes. Although these values were similar, resealed ghosts and intact cells did not accumulate cyclic AMP at the same maximal rate after epinephrine stimulation. For resealed ghosts, the maximal rate of epinephrine-stimulated cyclic AMP accumulation was 0.7 ± 0.1 pmol/min/107 ghosts, while that for intact erythrocytes was 2.5 ± 0.1 pmol/min/107 cells. The value obtained with resealed ghosts, however, was noted to vary somewhat between different experiments, ranging from 0.7 to 1.8 pmol/min/107 ghosts (Figs. 2 to 4 and Tables II and III).

Fluoride Stimulation of Cyclic AMP Accumulation—The
TABLE I

<table>
<thead>
<tr>
<th>Incubation period at 37°C</th>
<th>K⁺ released (μeq/10¹⁰ ghosts)</th>
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<tbody>
<tr>
<td>0</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>5</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>15</td>
<td>0.08 ± 0.08</td>
</tr>
<tr>
<td>25</td>
<td>0.21 ± 0.06</td>
</tr>
<tr>
<td>30</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td>45</td>
<td>0.14 ± 0.03</td>
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</tbody>
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*Under these conditions, only small quantities of ghosts could be recovered, making these determinations of K⁺ release relatively inaccurate.*

TABLE II

<table>
<thead>
<tr>
<th>Resealing conditions</th>
<th>Cyclic AMP accumulation (pmol cyclic AMP/10¹⁰ ghosts/20 min)</th>
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<tbody>
<tr>
<td></td>
<td>Basal</td>
</tr>
<tr>
<td>Buffer alone</td>
<td>0.12 ± 0.19</td>
</tr>
<tr>
<td>Buffer + ATP + RS*</td>
<td>0.26 ± 0.29</td>
</tr>
</tbody>
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*RS refers to an ATP regenerating system consisting of 2.94 mg/ml creatine phosphate and 17 units/ml creatine phosphokinase.*

concentration dependence for fluoride-stimulated cyclic AMP formation in resealed ghosts is shown in Fig. 4. Half-maximal cyclic AMP accumulation occurred at a NaF concentration of 0.3 ± 0.10 μM, and 0.5 ± 0.10 μM dithiothreitol was included. Cyclic AMP was measured as described in the text. When present during resealing, the ATP concentration was 2 mM. Results represent the mean ± S.E. of duplicate determinations from two or more experiments.

**Effect of Ca²⁺ and (Ca²⁺ + Mg²⁺) Ionophore—Cyclic AMP accumulation by resealed ghosts in the presence or absence of epinephrine was not altered by addition of Ca²⁺ at concentrations up to 10 mM if the Ca²⁺ was added at the time of assay and after resealing had occurred (data not shown). Inclusion of EDTA in the extracellular fluid at the time of assay did not alter the ability of epinephrine to stimulate cyclic AMP accumulation by resealed ghosts. Although 10 mM Ca²⁺ by itself did not inhibit cyclic AMP accumulation, Ca²⁺ concentrations as low as 1 mM in the presence of the (Ca²⁺ + Mg²⁺) ionophore A-23187 resulted in complete inhibition of epinephrine-stimulated cyclic AMP accumulation (Table III). Inclusion of A-23187 alone reduced epinephrine-stimulated cyclic AMP accumulation by 70%.

When ghosts were resealed in the presence of 2.5 mM Ca²⁺, epinephrine did not stimulate cyclic AMP accumulation. This lack of cyclic AMP accumulation could not be overcome by addition of EDTA and/or the ionophore A-23187 (data not shown).

**DISCUSSION**

Resealed ghosts and vesicles from human erythrocytes have been widely employed in studies of membrane topography and membrane transport phenomenon. In general, these ghosts and...
vesicles are prepared from hypotonically lysed cells. The gross characteristics of these resealed membranes are now known. For example, Steck et al. (6, 7) have shown that vesicles may seal in an inside-out or rightside-out manner and that, under certain conditions, one or the other form may predominate. Hoffman (8) has noted that several species of resealed ghosts may result. These include ghosts which reseal spontaneously in an inside-out or rightside-out manner and that, under certain conditions, one or the other form may predominate. Under these conditions, as reported for the human erythrocyte by Dodge et al. (9), some adsorption of hemoglobin to the membrane persists and the resealed ghosts remain slightly pink.

Even under optimal conditions, preparations of rightside-out human erythrocyte ghosts may be contaminated with some inside-out forms. We have found that the avian erythrocyte ghost reseals with the nucleus inside the plasma membrane. This has advantages as it is highly unlikely that these nucleus-containing ghosts have inside-out plasma membranes. Thus, interpretation of topographical data using this type of resealed ghost will be facilitated.

It is quite likely that both the avian and human erythrocyte plasma membranes reseal by similar mechanisms. Thus, one would expect that preparations of turkey erythrocyte resealed ghosts would consist of a mixture of types I, II, and III ghosts (8). We have modified the method of Bodemann and Passow (1) to isolate the resealed ghosts. This technique involves centrifugation over a sucrose cushion. The leaky (type III) ghosts would be expected to pass through the sucrose leaving behind a preparation enriched in types I and II ghosts. However, the experimentalist need not rely solely on the sucrose cushion to allow for studies which specifically characterize type II ghosts. If certain compounds such as K⁺ or ATP are added after lysis is complete but before restoration of isotonicity, they will not be incorporated in the type I ghosts which reseal before restoration of isotonicity has occurred. Similarly, if, after resealing, the ghosts are extensively washed, only the type II ghosts will retain the added compounds since these agents will be eluted from type III ghosts which never completely reseal. This approach has been utilized in the current investigation. Thus, type I ghosts are not contributing to the observed behavior (K⁺ retention, cyclic AMP accumulation) since this form of ghost would have resealed prior to the addition of critical agents (K⁺ or ATP plus the ATP-regenerating system). Similarly, type III ghosts do not contribute to the observed response because the critical agents (K⁺ or ATP plus the ATP-regenerating system) have been eluted from these ghosts by the extensive washing procedure used.

In this communication, we have shown that resealed turkey erythrocyte ghosts possess several characteristics which closely resemble those of their parent intact erythrocytes. Rsealed ghosts maintain a high intracellular K⁺ concentration in the presence of a very low extracellular K⁺ solution (Fig. 1). In addition, these resealed ghosts retain ATP and the ATP-regenerating system as shown by experiments which reveal that epinephrine-stimulated cyclic AMP accumulation occurs only in ghosts which were resealed in the presence of these agents (Table II). Our observation that resealed ghosts retain less K⁺ after extensive washing in K⁺-free buffer (Fig. 1) and accumulate less cyclic AMP after stimulation with epinephrine (Fig. 2) would suggest that only a fraction of the resealed ghosts being studied are type II ghosts (see below).

In a previous communication (10), we reported that epinephrine-stimulated cyclic AMP accumulation by intact turkey erythrocytes was not affected by high extracellular concentrations of Ca²⁺ but was completely abolished by relatively low extracellular Ca²⁺ concentrations if the cation ionophore A-23187 was present. Presumably, these findings indicate that the intact turkey erythrocyte plasma membrane is relatively impermeable to Ca²⁺ added to the extracellular fluid. In this regard, the resealed ghost appears to closely resemble the intact erythrocyte. Calcium, added after resealing has been completed, does not alter the rate of epinephrine-stimulated cyclic AMP accumulation by resealed ghosts. In the presence
of the ionophore A-23187, however, low concentrations of external Ca\(^{2+}\) completely abolish epinephrine-stimulated cyclic AMP accumulation (Table III). Thus, the resealed ghost also appears to be relatively impermeable to extracellular Ca\(^{2+}\).

Epinephrine-stimulated Cyclic AMP Accumulation—Cyclic AMP accumulation by ghosts resealed in the presence of ATP plus the ATP regenerating system and stimulated by (R)-(−)-epinephrine proceeds linearly for 20 min (Fig. 2) at a rate which varies between different ghost preparations ranging from 0.7 to 1.8 pmol/min/10\(^8\) ghosts. This is 28 to 72% of the rate at which intact erythrocytes stimulated by (R)-(−)-epinephrine accumulate cyclic AMP. This might indicate that, during ghost preparation and/or assay, there is a partial inactivation of the receptor-cyclase complex. However, we have noted that the concentration of (R)-(−)-epinephrine which gives the half-maximal rate of cyclic AMP accumulation by resealed ghosts (1.0 ± 0.4 × 10\(^{-6}\)M) is very similar to that which gives a half-maximal response with intact cells (0.8 ± 0.2 × 10\(^{-6}\)M). Thus, at least from a functional view, the receptor component has not been substantially altered during ghost preparation. We would suggest that the decreased rate of cyclic AMP accumulation by resealed ghosts indicates that only a fraction (28 to 72%) of the ghosts being studied are capable of responding to (R)-(−)-epinephrine stimulation, since only this fraction are type II as opposed to types I and III ghosts and only this fraction has retained ATP plus the ATP regenerating system. According to this hypothesis, the ghosts which respond to hormonal stimulation accumulate cyclic AMP at a rate which is the same as that noted for the intact cells.

Fluoride Stimulation of Cyclic AMP Accumulation—Resealed ghosts, unlike their parent intact erythrocytes, accumulate cyclic AMP in response to fluoride stimulation. This occurs at 30 to 30% of the rate of epinephrine-stimulated cyclic AMP accumulation. In contrast, purified plasma membranes from turkey erythrocytes also respond to fluoride and epinephrine stimulation by forming cyclic AMP but, in this case, the fluoride response is 150 to 200% of the epinephrine response. These observations might suggest that the observed but decreased fluoride response noted with resealed ghosts results from only partial ghost permeability to fluoride. However, the fluoride concentration dependence for cyclic AMP accumulation by resealed ghosts (Fig. 4) and plasma membrane preparations is similar and, even at very high fluoride concentrations (Fig. 4), no further cyclic AMP accumulation by resealed ghosts is noted. More likely, therefore, is the possibility that resealed ghosts are fluoride-permeable but only partially fluoride-sensitive. Perhaps it is the process of membrane disruption itself which conveys fluoride sensitivity upon the previously fluoride-insensitive intact cells and, in this regard, resealed ghosts represent a species which is intermediate between intact and completely disrupted cells.

Ca\(^{2+}\) Inhibition—In a previous report, Ca\(^{2+}\) was shown to inhibit epinephrine stimulation of turkey erythrocyte adenyl cyclase in plasma membrane preparations (11). This inhibition was shown to result from Ca\(^{2+}\) binding to a cluster of cooperative, high affinity Ca\(^{2+}\) sites. Occupancy of these Ca\(^{2+}\) sites was found to decrease V\(_{\text{max}}\) without altering the K\(_{\text{m}}\) for ATP, Mg\(^{2+}\), or hormone. In this communication, we report that erythrocyte ghosts resealed in the presence of 2.5 mM Ca\(^{2+}\) do not accumulate cyclic AMP when later exposed to epinephrine. Presumably this Ca\(^{2+}\) inhibition of cyclic AMP accumulation also results from Ca\(^{2+}\) binding to the previously described high affinity Ca\(^{2+}\) sites although further studies will be needed to confirm this assumption. When ghosts are resealed in the presence of 2.5 mM Ca\(^{2+}\) and later assayed in the presence of EDTA and/or the cation ionophore A-23187, no epinephrine-stimulated cyclic AMP accumulation can be demonstrated. This suggests that, under these conditions, Ca\(^{2+}\) inhibition cannot be reversed.

The rate of epinephrine-stimulated cyclic AMP accumulation by ghosts resealed in the absence of added Ca\(^{2+}\) is markedly reduced by addition of the ionophore A-23187 even in the absence of added Ca\(^{2+}\) (Table III). A similar observation has been previously reported for intact erythrocytes (10). One explanation for these findings would be that the ionophore itself inhibits cyclic AMP formation by a direct effect on adenyl cyclase but this would seem unlikely in view of our previous observation that concentrations of A-23187 as high as 10 \(\mu\)g/ml do not inhibit adenyl cyclase in plasma membrane preparations (10). An alternative explanation would therefore seem more likely. We suggest that the ionophore induces a change in the distribution of Ca\(^{2+}\) even in the absence of added Ca\(^{2+}\). Presumably, the ionophore releases Ca\(^{2+}\) from membrane Ca\(^{2+}\)-binding sites making more Ca\(^{2+}\) available to adenyl cyclase and resulting in inhibition of the enzyme.

A 8-adrenergic influence on Ca\(^{2+}\) fluxes across the turkey erythrocyte plasma membrane has been previously noted (10), but studies of this phenomenon are made difficult by the presence of multiple Ca\(^{2+}\) pools and the inability to selectively alter intracellular Ca\(^{2+}\) concentrations. The resealed turkey erythrocyte ghost may prove very useful in further studying the relationships between 8-adrenergic stimulation and transmembrane Ca\(^{2+}\) fluxes.

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M L Steer, C Baldwin and A Levitzki


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