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Membrane processes associated with the osmotic-pulse incorporation of inositol hexaphosphate

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In previous studies (Biochem. Biophys. Res. Commun. 144, 779–786 (1987); Prog. Clin. Biol. Res. 292, 65–75 (1989)), we showed that inositol hexaphosphate (IHP), when added to erythrocyte membrane ghosts in the range 0.6–2.5 mM, caused a large disruption of skeletal protein–protein interactions as monitored by electron paramagnetic resonance techniques. IHP incorporated into intact cells by an osmotic-pulse method (J. Cell. Physiol. 129, 221–229 (1986)) leads to cells with markedly decreased oxygen affinity. Exposure of the red cells to higher levels of IHP during the osmotic pulse leads to less lysis and more normal cellular indices after healing of the transiently-disrupted membrane (J. Lab. Clin. Med. 113, 58–66 (1989)). In order to determine what effect higher levels of IHP had on skeletal proteins and bilayer lipids of membrane ghosts, spin labeling studies were performed. The main findings were: (a) There was a concentration-dependent alteration in skeletal protein interactions. At concentrations greater than 25 mM IHP, the effectiveness of IHP to disrupt skeletal protein interactions was diminished. (b) No apparent alteration of the motion or order of phospholipids or the lipid water interface of intact cells into which IHP was incorporated occurred, suggesting that higher levels of IHP do not alter the physical state of the lipid bilayer.

Introduction

Inositol hexaphosphate (IHP), a compound which does not cross the red blood cell (RBC) membrane, may be incorporated into intact erythrocytes by a DMSO-induced osmotic pulse technique [1,2]. This procedure yields cells with markedly decreased oxygen affinity, lowered mean corpuscular hemoglobin concentration (MCHC), and acceptable 24-h survival in baboons [3] and humans [4].

IHP binds to the 2,3-DPG site of hemoglobin resulting in decreased oxygen-affinity as evidenced by a right shift of the Hb-oxygen dissociation curve [5]. The advantage of right-shifted low-affinity red cells is the enhancement of oxygen delivery to tissue under adverse conditions, especially decreased blood flow rate. The experimental evidence demonstrating this advantage has been reviewed [6].

A proposed mechanism for the incorporation of IHP by the osmotic pulse been presented previously [1]. The cells are first equilibrated with DMSO, which enters the RBC and causes a high intra- and extracellular osmolality. The cells are then rapidly diluted in a flow system causing a transient DMSO concentration gradient across the membrane. During the time it takes for the DMSO to reequilibrate, water enters the cells, causing them to swell and allowing normally impermeant compounds to cross the membrane. The composition of the 'diluent' has been shown to markedly effect the behavior of the cells during this pulse, with the presence [1] and the concentration [2] of the polyanion such as IHP having an important effect. One of the critical features of this mechanism is that IHP disrupts skeletal protein–protein interactions while leaving skeletal-bilayer interactions unaltered [7]. Previously, we showed that IHP, when added to isolated ghost membranes, caused a concentration-dependent decrease in skeletal protein interactions at concentrations as low as 0.6 mM at pH 8.0 when compared to the corresponding control [8,9]. Such ac-

Abbreviations: IHP, inositol hexaphosphate; MCHC, mean corpuscular hemoglobin concentration; 2,3-DPG, 2,3-diphosphoglycerate.

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tion by IHP would allow the disrupted skeleton, still attached to the membrane, to expand with the bilayer during the transient osmotic pulse and is consistent with the reported effect of IHP on Triton shells [7].

Studies on the effect of varying the IHP concentrations of the isotonic diluents from approximately 27 mM (low-) to 65 mM (high-)IHP have shown that for cells pulsed in the presence of higher concentrations less lysis occurs for the same DMSO concentration, and P_{50} shift and cellular indices undergo much less change relative to control cells. Even for the same degree of IHP incorporation and P_{50} shift, there is less cell lysis and smaller changes in cellular indices for cells exposed to higher IHP concentrations during the pulse [2]. The in vivo survival of small volumes of chromium-labeled, IHP-incorporated, autologous human red blood cells, prepared by the osmotic pulse method, has been measured [4]. Treatment with 46 mM IHP (50/50 IHP) resulted in 24-h posttransfusion RBC survivals which were slightly (but not significantly) better than those for cells treated with 27 mM IHP (low-IHP). Cells from both treatment groups had normal survival after the first 24 h. Use of the higher IHP concentration also gave a higher in vitro hemoglobin recovery, more normal RBC indices, and a more consistent correlation between 24-h RBC survival and P_{50} shift.

The mechanism responsible for these observations is unknown. The present study investigates the possible role of a ionic strength-dependent decrease in the effectiveness of IHP-induced disruption of skeletal-protein interactions at higher IHP concentrations.

Materials and Methods

Inositol hexaphosphate and polyethylene glycol were obtained from Sigma. The spin labels 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl (MAL-6), 4-(*N*,*N*-dimethyl-1-*n*-hexadecyl)-ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl (CAT-16), and 5-doxylstearic acid (5-NS) were obtained from Aldrich.

Effect of IHP on erythrocyte membrane proteins

Blood was obtained from healthy human volunteers by venipuncture into heparinized tubes, immediately placed on ice, and processed within 30 min of collection. Intact cells were isolated by centrifugation at 4°C at $600 \times g$ and three subsequent resuspensions and washings in PBS buffer (150 mM NaCl, 5 mM sodium phosphate (pH 8.0)). The buffy coat was carefully removed. Erythrocyte ghost membranes were obtained by hypotonic lysis with 5P8 (5 mM sodium phosphate buffer (pH 8.0)) employing one volume of cells to 20 volumes of 5P8, and subsequent centrifugation at 4°C and $27\,000 \times g$. The ghosts were resuspended in ice-cold 5P8 and this process was continued until the membranes were free of residual hemoglobin. Protein content was estimated by the method of Lowry et al. [10].

Membrane proteins can be selectively labeled with MAL-6, a spin label that covalently binds primarily to cysteine SH groups of membrane proteins (reviewed in Refs. 11–13). Under our labeling conditions 70–90% of MAL-6 is covalently bound to the major skeletal protein (spectrin) and the cytoplasmic pole of Band 3 [11–13]. Others have confirmed this location of MAL-6 in erythrocyte membrane ghosts by protein extraction and immunological techniques [14,15].

The effect of high IHP concentrations (4–75 mM) on the physical state of skeletal proteins was examined by the addition of one volume of 20–375 mM IHP solution (pH 7.2) or one volume of a pH and ionic strength matched control solution (NaCl added to 5P8 to match the ionic strength) to four volumes of MAL-6 spin-labeled ghosts membranes, previously warmed to room temperature for 15 min, and incubated 30 min at room temperature. EPR spectra were recorded on a Varian E-109 spectrometer with computerized data acquisition under conditions of constant temperature (20 ± 0.5°C); spectrometer settings are given in the appropriate figure legends.

Effect of osmotic pulse on erythrocyte membrane lipids

Three IHP diluents were employed to prepare low-affinity cells: low-IHP (27 mM), high-IHP (65 mM) and 50/50-IHP. The latter was an equal mixture of the former two solutions. All three diluents contained inositol hexaphosphate (IHP), 1% polyethylene glycol (PEG), and NaCl and had a pH of 7.2 and an osmolality of 305–315 mosmol/kg. The distinction between the diluents was due to different IHP concentrations and molecular forms of IHP used. The low-IHP diluent (27 mM IHP) was prepared from the sodium salt of IHP, while the high-IHP (65 mM IHP) diluent was prepared from both the acid and salt, and 50/50 was prepared as an equal mixture of high and low-IHP diluents [2].

Low-affinity IHP-incorporated red cells were prepared by the DMSO-induced osmotic pulse method as previously described [2]. Treated and control intact cells were spin labeled with the lipid-specific spin labels 5-NS or CAT-16 as previously described [11–13,16].

Results

A typical EPR spectrum of the $M_I = +1$ low field resonance lines of MAL-6 attached to membrane proteins is shown in Fig. 1. The spectrum of MAL-6 demonstrates the presence of at least two classes of membrane protein SH binding sites for this spin label, discernable by their motion: those that are strongly immobilized (S sites) and those that are weakly immobilized (W sites).

The ratio of the spectral amplitude of the low-field weakly immobilized component to that of the strongly

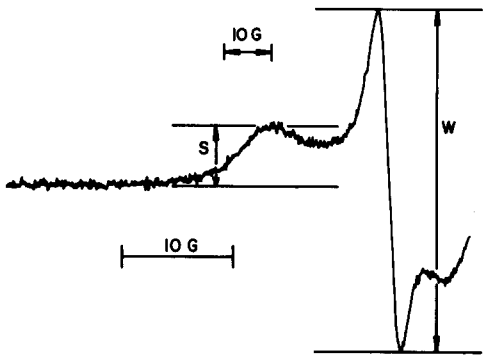


Fig. 1. Typical ESR spectrum of the $M_I = +1$ low-field lines of MAL-6 attached to skeletal proteins in human erythrocyte ghost membranes. S and W are the spectral amplitudes of MAL-6 covalently bound to strongly- and weakly-immobilized binding sites, respectively [11–13,17–19]. Spectrometer conditions: 40 G scan width, 12 mW power incident on the resonance cavity, and 0.32 G modulation amplitude at 100 kHz.

immobilized component (W/S ratio) has been repeatedly shown by us and others (reviewed in Refs. 11–13) to be a convenient and highly sensitive monitor of the conformation and organization of membrane skeletal proteins and is reflective of the segmental motion of protein binding sites. In addition, our laboratory has demonstrated that the W/S ratio is sensitive to the state of aggregation of spectrin in MAL-6 labeled ghost membranes [17]. Decreased (increased) skeletal protein–protein interactions are associated with increased (decreased) values of W/S of spin-labeled ghosts relative to controls [17–19]. Others have shown that our MAL-6-labeling procedures yield highly reproducible spectra that can be used with confidence [20].

As discussed above, red cells incorporated with IHP by the osmotic pulse possess differing morphology depending on the isotonic IHP diluent used. Initial studies of the effects of these diluents added to MAL-6-labeled ghosts suggested that the effectiveness of IHP to disrupt skeletal protein–protein interactions decreased with increasing IHP concentration in the diluent (data not shown). Given this observation we employed electron paramagnetic resonance to examine the effect of concentrated IHP solutions on erythrocyte membrane skeletal proteins labeled with MAL-6.

Addition of IHP solutions (final IHP concentration was 2.5–75 mM) to isolated MAL-6 spin-labeled ghost membranes resulted in a diminution of the disruptive effect of IHP on skeletal protein–protein interactions, as judged by the W/S ratio, at concentrations greater than 12.5 mM compared to ionic strength- and pH-matched controls (Fig. 2). This finding suggests that a less disruptive (i.e., skeletal-stabilizing) effect is observed at high IHP concentrations. This suggestion could be due to a IHP-specific effect or to the influence of ionic strength of higher concentrations of IHP. In order to distinguish between these alternatives, the following experiment was performed. 12.5 mM IHP was added to spin-labeled ghosts to give the maximum difference between subject and control W/S values. Then additional increments of NaCl were added to both the IHP-treated and control samples until an ionic strength equivalent to 75 mM IHP was obtained. EPR spectra showed that the W/S ratio of the IHP-treated sample decreased to a value indistinguishable from NaCl-treated control samples, suggesting that at higher concentrations of IHP, the stabilizing effect seen in Fig. 2 may be associated with the increased ionic strength present.

In order to determine the effect of the osmotic pulse procedure on the lipid bilayer and at the extracellular lipid/water interface of the erythrocyte membrane, the lipid specific spin labels 5-NS and CAT-16, respectively, were employed. Previously, we showed that no irreversible alterations in membrane skeletal protein interactions occurred in ghosts isolated from low-affinity intact cells into which low-IHP was incorporated compared to control cells [8]. Employing two lipid specific spin labels, IHP-incorporated cells have been examined to determine if the motional environment in the lipid bilayer or at the extracellular lipid/water interface is altered following the osmotic pulse with high-IHP. In contrast to the studies with MAL-6 reported above, these investigations employed intact cells. As discussed in previous publications from our laboratory [11–13], the order and motion of the membrane lipids can be assessed by measuring the order parameter derived from the parameters of the spectrum of 5-NS (Fig. 3). The quaternary amine spin label, CAT-16, is a lipid-specific, cationic, paramagnetic molecule whose nitroxide moiety reports on the lipid/water interface of the erythrocyte membrane [16]. The motional environment of the extracellular lipid/water interface of erythrocyte membranes is

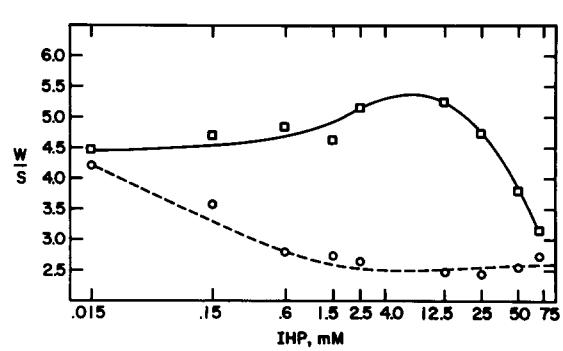


Fig. 2. Plot of the W/S ratio of MAL-6 covalently bound to erythrocyte membrane proteins in ghosts versus the logarithm of the final IHP concentration (in millimolar units) (□) and of pH- and ionic strength-matched controls (○). The latter solutions were prepared by NaCl addition to 5P8 solutions. For example, for 2.5 mM IHP, the control was prepared by addition of NaCl to 5P8 to make a 195 mM NaCl solution, since the ionic strength for the sodium salt of IHP is 195 mM. Likewise, for 75 mM IHP, the control contained 4.68 M NaCl. Other concentrations of IHP were similarly controlled.

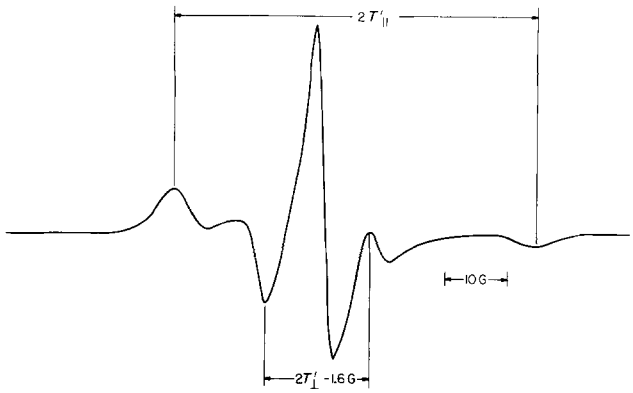


Fig. 3. Typical ESR spectrum of 5-NS incorporated into the lipid phase of intact erythrocytes. Spectral parameters from which the order parameter is calculated [11–13] are indicated. Spectrometer conditions: 100 G scan range, 16 mW power incident on the resonance cavity, and 0.32 G modulation amplitude.

TABLE I

The effect of IHP incorporation into intact erythrocytes by the osmotic-pulse method on the physical state of membrane lipid components

	Diluent [a]	Spectral value [b,c]	*N*
Bilayer lipids	Control	0.682 ± 0.008	5
5-NS	low-IHP	0.682 ± 0.008	5
Order parameter	50/50-IHP	0.685 ± 0.006	3
	high-IHP	0.685 ± 0.008	3
Lipid/water Interface	Control	1.35 ± 0.04	4
CAT-16	low-IHP	1.38 ± 0.03	4
Apparent rotational	50/50-IHP	1.34 ± 0.05	2
Correlation time (ns)	high-IHP	1.36 ± 0.04	2

[a] Defined in text.

[b] The order parameter was used for 5-NS [11–13], while the apparent rotational correlation time was utilized for CAT-16 [16,21].

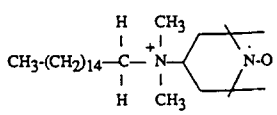
[c] None of the parameters calculated were significantly different than control values.

assessed by the apparent rotational correlation time (τ) calculated from the spectrum illustrated in Fig. 4 [16,21].

Cells incorporated with IHP by the osmotic pulse employing either low-, 50/50-, or high-IHP diluents compared to control intact cells displayed no alteration in the order or motion of 5-NS in the lipid bilayer or τ of CAT-16 at the lipid/water interface, suggesting that IHP incorporation into intact erythrocytes by the osmotic pulse procedure does not irreversibly alter lipid motion (Table I).

Discussion

The applications of low-affinity red blood cells include treatment of pathological states such as ischemia and sickle cell disease [5]. Several methods for preparing low affinity cells are available including the lysis/resealing [22] and the osmotic pulse techniques [1,2]. The purpose of the present studies has been to gain a further understanding of membrane processes associated with the osmotic pulse incorporation of IHP into intact cells.



CAT-16

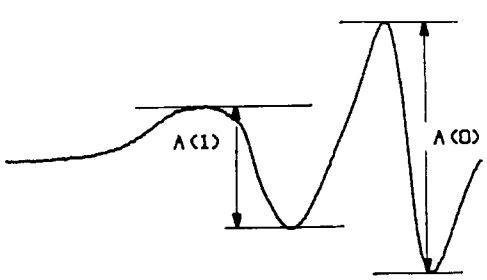


Fig.4. Structure of CAT-16 and the ESR spectrum of the $M_I = +1$ and $M_I = 0$ lines. Parameters from which the apparent rotational correlation time is calculated are given. Spectrometer conditions are the same as in Fig. 3 except the scan range was 40 G.

In the development and optimization of the osmotic pulse incorporation of IHP it has been observed that the IHP concentration of the isotonic diluent affected the Hb recovery of the resulting low-affinity red cells [2]. These data, along with preliminary kinetic measurements, suggest that faster resealing occurs at higher IHP concentrations, limiting hemoglobin loss during the transient osmotic pulse.

Previously, we showed that an IHP concentrations as low as 0.6 mM added to isolated membranes could significantly disrupt skeletal protein–protein interactions [8,9]. In order to determine the effect of higher concentrations of IHP on skeletal protein–protein interactions, IHP was added to MAL-6-labeled ghosts (final [IHP] = 2.5–75 mM). The results presented in Fig. 2 indicate that at IHP concentrations greater than 12.5 mM, the W/S ratio decreases to a value that at 75 mM is indistinguishable from ionic strength and pH matched control, suggesting a stabilizing effect of IHP at these concentrations.

If one assumes that a finite concentration of IHP can associate with and cause a maximal disruption of the skeletal protein network (note in these studies no Hb was present to bind free IHP), excess free IHP may be responsible for the above results. Given this assumption, a possible explanation for this effect is a stabilizing effect of increased ionic strength which counteracts the IHP disruptive effect at lower concentrations. This possibility is supported by the experiment in which increasing levels of NaCl added to spin labeled ghosts in the presence of 12.5 mM IHP lead to ratios indistinguishable from controls.

We have previously observed that as the ionic strength of MAL-6-labeled ghost solutions increases, the W/S ratio rapidly decreases to a plateau or baseline value which in our hands typically is 2.75 ± 0.25 [11], as shown for ionic-strength matched controls in Fig. 2. The reason that this value of the W/S ratio results is not fully understood. One interpretation of this finding is that the high ionic strength environment alters ionic interactions, both attractive and repulsive, between skeletal network proteins, resulting in a more stabilized skeleton. For example, since spectrin is highly negatively charged, one can speculate that at high IHP levels, an increase in ionic strength allows spectrin domains to come closer to each other, thereby decreasing the motion of MAL-6 bound to the protein resulting in a decrease in the W/S ratio. Similar reasoning could be applied to the negatively charged spectrin and the negatively charged domain of the cytoplasmic pole of the major transmembrane protein Band 3. This speculation of the IHP results may be conceptualized as a phase shift to a higher ionic strength concentration range at which free-IHP causes a stabilizing effect due to its ionic strength, resulting in a decrease in the W/S ratio of MAL-6-labeled skeletal proteins to the plateau or control value.

The finding, that high IHP levels seem to diminish the destabilizing effect of IHP seen at low concentrations [8,9], may explain the morphological differences between IHP-incorporated low-affinity cells prepared with low- versus high- and 50/50-IHP diluents. According to the proposed mechanism for the osmotic pulse procedure [1], the red cell skeleton is disrupted by IHP in the diluent, but remains attached to the lipid bilayer. It is known that the percentage of DMSO in the cell suspension partially determines the stress of the transient osmotic pulse on the red cells [1]. The morphology experiments also suggest a second determinant of the osmotic-pulse stress, namely, the higher the IHP concentration in the diluent the lower the accompanying stress [2]. Our EPR results appear to be consistent with these observations, in that, the higher the IHP concentration exposed to MAL-6-labeled ghosts, the smaller the resulting disruption of protein–protein interactions in the skeletal protein network. Applied to the pulse mechanism, this finding may suggest that with higher-IHP diluents, the stress of the osmotic pulse, due to disassociation of skeletal protein–protein interactions, would be less, resulting in faster and restored mechanical stability.

While IHP-incorporated red cells have displayed acceptable survivability in baboons [3] and humans [4], we have employed lipid specific spin-labeling techniques to determine if these low-affinity intact cells possessed significant membrane lipid alterations relative to non-treated controls. Based on in vivo survival data [4], the P_{50} shift of treated blood should be 15 mmHg. However, in order to determine the maximal effect on erythrocytes exposed to the osmotic pulse, P_{50} shifts of 25–30 mmHg, compared to untreated controls, were employed in these EPR studies. Our results show that low-affinity intact cells, prepared by the osmotic pulse, employing low-, high- and 50/50-IHP diluents, do not exhibit alterations in the motion of 5-NS in the lipid bilayer or in the rotational motion of CAT-16 at the extracellular phospholipid/water interface relative to untreated intact cells (Table I). These findings suggest that the stress to the lipid bilayer during the osmotic pulse does not cause irreversible changes in the organization and motion of lipid bilayer components. Schlegel et. al. [23] have demonstrated that using the lysis/resealing method of incorporation of molecules into red cells can, under certain conditions, lead to a phospholipid asymmetry. No conclusion about possible lipid-asymmetry alterations can be drawn from our 5-NS-labeling experiment because the label is localized in both bilayer leaflets [11–13]. Since CAT-16 is localized only in the outer leaflet of the bilayer adjacent to bilayer proteins [16], it is conceivable that alterations in the phospholipid asymmetry could be detected. However, no change in the rotational motion of CAT-16 was observed. The apparent lack of an irreversible alteration of erythrocyte membrane lipids in ghosts isolated from intact cells treated with IHP is consistent with our earlier finding of no irreversible membrane protein alterations [8,9].

A summary of the findings of the present study suggests that: (1) following osmotic-pulse incorporation of IHP into red cells there was no apparent alteration in the motion and order of the lipid bilayer or in the motional environment at the phospholipid/water interface of intact cells; and (2) at high (> 25 mM) IHP concentrations the effectiveness of IHP to disrupt skeletal protein interactions is diminished, which may imply that the higher IHP concentrations present in the 50/50- and high-IHP diluents lead to the higher hemoglobin recovery associated with these treated cells.

Acknowledgement

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