Control of the Erythrocyte Membrane Shape: Recovery from the Effect of Crenating Agents

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ABSTRACT Intact erythrocytes become immediately crenated upon addition of 2,4-dinitrophenol (DNP) or pyrenebutyric acid (PBA). However, when cells are incubated at 37°C in the presence of the crenating agents with glucose, they gradually (4-8 h) recover the normal biconcave disc form. The recovery process does not reflect a gradual inactivation of DNP or PBA since fresh cells are equally crenated by the supernatant from the recovered cells. Further, after recovery and removal of the crenating agents, cells are found to be desensitized to the readdition of DNP as well as to the addition of PBA, but they are more sensitive to cupping by chlorpromazine. This alteration in the cell membrane responsiveness was reversible upon further incubation in the absence of DNP. Recovery is dependent upon cellular metabolic state since an energy source is needed and incubation with guanosine but not adenosine will accelerate conversion to the disc shape. It is suggested that the conversion of cells from crenated to disc shape in the presence of the crenators, represents an alteration or rearrangement of membrane components rather than a redistribution of the crenators within the membrane. This shape recovery process may be important for erythrocyte shape preservation as well as shape control in other cells.

The morphology of eukaryotic cells provides a visual manifestation of many cell properties and is a valuable indicator of cell state. For example, the complex morphological changes in cell adhesion and spreading are correlated with an alteration of protein synthesis (1). In human erythrocytes, cell shape change is an indication of a major dysfunction and is normally associated with hemolysis (2-4).

Because the erythrocyte lacks organelles or structured cytoplasmic elements, its morphology is determined by the cell volume and the plasma membrane properties. Under isovolumic conditions, erythrocytes will normally become either echinocytes (crenated cells) or stomatocytes (cupped cells) when the shape is perturbed. The crenation of erythrocytes is similar to the formation of many long villi during the rounding of eukaryotic cells since both involve an increase in the relative area of the outside surface of the membrane over that of the inner surface as described by the bilayer couple hypothesis (5). In any closed membrane surface, the relative area of the inner vs. outer membrane surface is an important factor in determining cell morphology. Because of the relative simplicity of the erythrocyte membrane, the mechanisms of membrane shape control can be studied apart from the influence of cytoplasmic structures.

The erythrocyte biconcave disc shape is normally preserved for its 120-d life-span in vivo, despite the fact that its membrane shape is extremely susceptible to morphological changes when exposed to a variety of mild treatment conditions in vitro (6), which may occur in vivo as well. This suggests that the erythrocyte might possess a system for sensing alterations in shape and correcting them. We have indeed found that mild crenation caused by amphipathic agents can be reversed by a process that appears to involve the alteration of membrane properties.

MATERIALS AND METHODS

Materials

BLOOD: Human erythrocytes were drawn from healthy donors into heparinized tubes. Cells were then washed three times in phosphate-buffered saline (PBS) buffer (130 mM NaCl, 9 mM Na2HPO4), pH 7.4, which contained 10 mM glucose. Cells were used for experiments within 2-4 h after washing.

CHEMICALS: 2,4-Dinitrophenol (DNP) was obtained from Sigma Chemical Co. (St. Louis, Mo.) and 1-pyrenebutyric acid (PBA) from Eastman Organic Chemicals. DNP and PBA were recrystallized from ethanol-water three times before use. Chlorpromazine was obtained from Sigma Chemical Co. [3H]DNP was purchased from New England Nuclear (Boston, Mass.). Reagents were prepared in PBS and adjusted to pH 7.4 before use. All other chemicals were the best available grade from commercial sources.

Methods

All incubations of erythrocytes (5% hematocrit) with various agents were conducted at 37°C in PBS containing 10 mM glucose. Cells were preincubated for 1 h at 37°C in PBS-glucose before addition of the reagents. Incubations were stopped by adding four parts of cell suspension to one part of 10% glutaraldehyde.
in PBS. The initial extent of crenation (0h) was determined by mixing the cell suspension with glutaraldehyde within 3–5 s after adding the crenating agent (37°C). Samples were cooled to 0°C for 1 h before counting under a dark-field light microscope. Various degrees of crenation or cupping of shapes could be identified. Therefore, for simplicity, only the percent of disc cells was calculated and presented in the figures. At least 200 cells were counted for each determination in a blind fashion. Counting variations (of the same sample) were found to be small, within 3–5%. However, variations in the recovery rates of 20–30% were occasionally observed between one blood sample and another.

RESULTS

The Recovery of DNP- and PBA-treated Erythrocytes from Crenation to Disc Shape

When cells were treated with DNP, they became immediately crenated (Fig. 1B), and the extent of crenation was a function of DNP concentration (Fig. 2, 0h). When these treated cells were incubated with DNP at 37°C in the presence of glucose, a slow, time-dependent recovery from crenation to normal disc shape was observed at all DNP concentrations (Fig. 2 and Fig. 1C and D). In an attempt to study the mechanism that allowed cells to restore their original shape, we considered first the possibility that after long incubation at 37°C, DNP became gradually inactive (as a crenating agent) and, as a result, recovery of the disc shape occurred. This possible explanation was examined as follows: cells were treated with DNP (1 mM final concentration) and incubated at 37°C in the presence of glucose. At 0 h, just after the cells were mixed with DNP, and after 24 h of incubation, aliquots were removed and centrifuged at 12,000 g for 3 min. The abilities of the resulting supernatants (containing unadsorbed DNP) to produce crenation of freshly prepared erythrocytes

![Figure 1](image1.png) **Figure 1** The recovery process of DNP-treated cells as observed with Nomarski differential interference contrast optics. Cells were incubated in PBS buffer and 10 mM glucose at 37°C in the absence or presence of DNP (0.6 mM). At 0, 2, and 12 h, aliquots were fixed with glutaraldehyde (as described under Materials and Methods). (A) Untreated cells, (B) DNP-treated cells at 0 h, (C) DNP-treated cells after 2 h of incubation, and (D) DNP-treated cells after 12 h of incubation. X 800.

![Figure 2](image2.png) **Figure 2** Time-dependent recovery of cells from crenated to disc shape, in the presence of DNP. Cells were mixed with various concentrations of 2,4-DNP and incubated at 37°C. At various time intervals, aliquots were removed, fixed with glutaraldehyde (as described under Materials and Methods), and counted. DNP concentrations used were 0.4 (C), 0.6 (●), 0.8 (Δ), and 1 mM (▲).
were examined after the appropriate dilutions. As seen in Fig. 3, both supernatants were found to have equal potential to crenate cells, indicating that the prolonged incubation of DNP in the presence of the cells at 37°C did not cause any detectable inactivation of this agent. This suggested that the recovery process could not be explained simply by DNP inactivation under these conditions. It was noticed, however, that both DNP supernatant solutions were slightly less effective as crenators, compared with the DNP solution alone, which was incubated under the same conditions in the absence of cells (Fig. 3). These results were expected since part of the DNP molecules were believed to be associated with the erythrocyte membranes (5) and were probably removed from the DNP solution by centrifugation, causing a minor, but detectable, reduction in the DNP content of the supernatant solutions.

If recovery were caused by an action of DNP or an impurity in the DNP, then recovery from another crenating agent, PBA, would not be expected to proceed at the same rate and to the same extent. (DNP, an uncoupler of oxidative phosphorylation, is known to cross the membrane readily whereas PBA does not [7].) As seen in Fig. 4, recovery from PBA-induced crenation is very similar to recovery from DNP-induced crenation in rate and extent of recovery. Further, with both the crude and the recrystallized reagents, superimposable recovery curves were obtained. It is unlikely that the rates of shape recovery from two different crenating agents are caused by the same impurity of the reagents or a similar redistribution of them within the membrane.

Another possibility is that the recovery is related to membrane oxidation catalyzed by the amphipath, involving dissolved oxygen. To test for this possibility, erythrocytes were incubated with both DNP and PBA in deoxygenated solutions in a glove box under an argon atmosphere. The anaerobic incubation conditions, instead of inhibiting recovery, caused a slight acceleration in the recovery rate. All the above results suggested that a membrane alteration, independent of crenating agent, was responsible for shape recovery.

DNP-recovered Cells Are Desensitized to Crenators but More Readily Become Cupped

The findings presented above indicate that cells become progressively desensitized to DNP, since they restore their biconcave disc shape in the presence of DNP that is still fully active as a crenating agent (Figs. 2 and 3). If this process is caused by the alteration of membrane components rather than chemical modification or rearrangement of the drug itself in the membrane, it is expected that washed erythrocytes (in a DNP-free PBS) will conserve their desensitized state towards DNP. As seen in Fig. 5A, DNP-recovered cells after washing are much less susceptible to DNP-induced crenation as compared with control cells that were incubated in the absence of DNP and then washed simultaneously. DNP washout efficiency was examined in the presence of 0.6 mM [3H]DNP (2.5 Ci/mol). When cells were washed just after the addition of DNP to the cells or after 12 h of incubation with DNP, <0.5% [3H]DNP could be detected in the washed erythrocyte pellet after both incubation periods. Desensitization of the cells after DNP treatment is not restricted to DNP alone, since PBA, another crenating agent, failed to crenate DNP-recovered cells but still was active on control cells (Fig. 5B). Since recovery from a crenated form implies an expansion of the internal half of the membrane bilayer based upon the bilayer couple hypothesis (5), the shape-recovered cells should be more "cupped" than control cells after the DNP is washed away. Although actual cupping was observed only occasionally, DNP-recovered cells became readily cupped in the presence of low concentrations of chlorpromazine (15 µM), which hardly affected control cells (Fig. 5C). This further suggests that recovery involves an alteration of the membrane state.

Do DNP-recovered Cells Regain Their Original Sensitivity to DNP?

In an attempt to examine whether the observed DNP desensitization of DNP-recovered cells is a reversible process, cells were incubated at 37°C in the presence of DNP (0.6 mM) for 12 h, and their return from crenation to disc shape was followed. As seen in Fig. 6, after 12 h most of the cells regained the biconcave disc shape. Cells were then washed twice and incubated in DNP-free PBS for an additional period of 12 h.
At various time intervals, aliquots were removed and treated with 0.6 mM DNP. A gradual resensitization process of the cells to DNP was observed (Fig. 6). While at 0 h (just after the cells were washed), DNP did not induce detectable crenation, 2 h later ~50% of the cells became crenated. After an additional 10 h of incubation, almost all cells were as susceptible to DNP as control cells that were incubated simultaneously under the same conditions but without pretreatment with DNP. At that time (12 h after washing), DNP-pretreated cells were found to be sensitive not only to DNP but to PBA as well and had lost their hypersensitivity towards chlorpromazine-induced cupping (data not shown).

Adenosine and Guanosine Effects on the Recovery of DNP-treated Cells

It was previously demonstrated that ATP is involved in the maintenance of the normal shape of the erythrocyte. Since erythrocytes require adenosine base to synthesize ATP, adenosine was added to enable the cell to raise its ATP concentration. Adenosine (up to 2 mM) was found to have only a minor effect on the recovery of cell shape (Fig. 7). Unexpectedly, when guanosine (2 mM) was used instead of adenosine, a dramatic increase in the recovery rate was observed. Moreover, when erythrocytes were preincubated with 2 mM guanosine (2 h 37°C) and then exposed to DNP in the absence of guanosine (which was washed away before DNP treatment), a similar acceleration of their recovery back to normal discs was observed. Guanosine treatment itself did not affect cell sensitivity to the crenator agents at 0 h. Since guanosine raises erythrocyte GTP levels (8), it is possible that guanosine or its metabolites are involved in this shape recovery process.

DISCUSSION

The mechanism by which PBA- or DNP-treated cells recover from echinocyte to normal biconcave disc is not clear yet. In principle, this recovery process may occur as a result of a
redistribution of the drugs in the membrane during incubation. Indeed, it was initially suggested by Hoffman (9) that spontaneous recovery of cells in the presence of a sphering agent occurred when the uptake of the sphering agent by the cells was complete. Moreover, it was previously demonstrated (10) that when erythrocytes were treated with methochlorpromazine, they were first crenated, but upon incubation at 37°C they progressively assumed a normal biconcave disc shape. At that point, when cells were washed with a drug-free buffer, all of them became cupped. On the basis of the bilayer couple hypothesis (5), it was argued that methochlorpromazine interacted first with the outer half of the lipid bilayer causing it to expand relative to the inner lipid bilayer and producing the observed crenated shape. With time, methochlorpromazine flipped from the outer to the inner half of the membrane where it interacted preferentially at equilibrium. A similar kinetic phenomenon was observed by Matayoshi (7) with pyrenebutyrylcholine (PBC). Matayoshi was able to establish with fluorescence quenchers that the reversal of crenation was correlated with a movement of PBC away from external quenchers (presumably into the cell).

The recovery process described here can hardly be explained on the basis of the hypothetical mechanism of drug redistribution in the erythrocyte membrane at equilibrium since:

(a) The time-scale of recovery is different. While the recovery of rose bengal- (9), methochlorpromazine- (10), and PBC-treated (7) cells occurs within 20-60 min, the recovery of DNP- and PBA-treated cells requires 4-8 h in the presence of glucose.

(b) DNP- and PBA-recovered cells do not become cupped upon further incubation after recovery (up to 12 h) and only few (<1%) become occasionally cupped after the agents are washed away.

(c) The shape recovery by drug redistribution does not require the presence of a metabolic energy source whereas the recovery from DNP or PBA crenation will not occur in PBS alone. It is not known, however, whether energy is required to restructure membrane components or simply to maintain (during long incubation periods) the lability in the skeleton needed for slow passive relaxation to disc shape. Further studies are required to clarify this point.

(d) No slow, time-dependent interaction of DNP with the membrane was detected. [3H]DNP could be readily washed out, and to the same extent (>99.5%), from cells at 0 h and after 12 h of incubation.

Another possibility is that an impurity in DNP or PBS could cause recovery, but the data are likewise inconsistent with this possibility. If the impurity is cationic, it would not explain the data since, after recovery and washing, cells would be expected to become crenated. If the impurity reacts with the membrane, the reaction would have to be reversible (see Fig. 6) and would have to occur on the same time-scale for both DNP and PBA impurities. This explanation is even more unlikely because with either crude or recrystallized reagents the same time-scale for recovery is observed.

It is conceivable that alternative processes may be proposed to explain this phenomenon. However, the mechanism that is consistent with our observations and that we favor is that the recovery from DNP- and PBA-induced crenation involves a redistribution or alteration of membrane components rather than an action of the creasing agents within the membrane. Indeed, after recovery the washed cells are resistant to creasing agents and sensitive to cupping agents without detectable incorporation of the drug into the membrane. In addition, strong support for this suggestion comes from the observation that preincubation with guanosine but not adenosine is effective in increasing the recovery rate. This indicates that a nucleotide or other guanosine metabolite may be involved in producing a relative expansion of the inner surface of the membrane, i.e., shape recovery.

Since the recovery from echinocyte to biconcave disc involves only a small, relative change in the membrane area of <1% (11), it is possible that protein or lipid alterations could produce such a change in shape. For example, it was suggested that energy-dependent shape changes in ghosts can be affected by antispectrin antibodies (10) or that lipid rearrangements could produce shape changes, based on the correlation between 1,2-diacylglycerol phosphorylation and the conversion from echinocyte to disc shape (12, 13). Although preliminary studies have demonstrated a change in lipid phosphorylation with recovery (our unpublished results) the exact identity of the protein or lipid components that are involved in the recovery process remains to be established.

From these studies it is clear that the crenation of erythrocytes by DNP and PBA is reversible. The results presented here further suggest that this process involves a restructuring of membrane components. This implies that erythrocytes can detect alterations in their shape and can set in motion processes to restore the normal shape. As mentioned earlier, the relative area of the inner and outer membrane surfaces is an important parameter in determining membrane shape in any cell. Thus, we believe that these phenomena may play an important role not only in maintaining the disc shape of erythrocytes in vivo but also in the control of shape in other cell types as well.

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REFERENCES

1. Ben-Ze'ev, A., S. R. Farmer, and S. Peuman. 1980. Protein synthesis requires cell-surface contact while nuclear events respond to cell shape in anchorage-dependent fibroblasts. Cell. 21:365-372.

2. Deuticke, B. 1968. Transformation of and restoration of biconcave shape of human erythrocytes induced by amphiphilic agents and changes of ionic environment. Biochim. Biophys. Acta. 163:494-500.

3. Matayoshi, E. D. 1980. Distribution of shape-change compounds across the red cell membrane. Biochemistry. 19:3414-3422.

4. Brown, P. R., and R. E. Parks, Jr. 1973. Use of high pressure liquid chromatography to investigate the "in vitro" reactions of human erythrocytes with guanosine. Anal. Chem. 45:948-951.

5. Hoffman, J. F. 1972. Quantitative study of factors which control shape transitions of human red blood cells of constant volume. News. Rev. Fr. Hématol. 12:771-774.

6. Shertz, M. P., and S. J. Singer. 1974. Biological membranes as bilayer couplets. A molecular mechanism of drug-erythrocyte interactions. Proc. Natl. Acad. Sci. U. S. A. 71:4457-4461.

7. Deuticke, B. 1968. Transformation of and restoration of biconcave shape of human erythrocytes induced by amphiphilic agents and changes of ionic environment. Biochim. Biophys. Acta. 163:494-500.

8. Brown, P. R., and R. E. Parks, Jr. 1973. Use of high pressure liquid chromatography to investigate the "in vitro" reactions of human erythrocytes with guanosine. Anal. Chem. 45: 948-951.

9. Hoffman, J. F. 1972. Quantitative study of factors which control shape transitions of human red blood cells of constant volume. News. Rev. Fr. Hématol. 12:771-774.

10. Shertz, M. P., and S. J. Singer. 1977. Equilibrium and kinetic effects of drugs on the shapes of human erythrocytes. J. Cell Biol. 70:247-251.

11. Beck, J. S. 1978. Relations between membrane monolayers in some red cell shape transformations. J. Theor. Biol. 75:487-501.

12. Quist, E. F., and K. L. Reece. 1980. The role of lipids in the process of erythrocyte shape regulation. Biochim. Biophys. Acta. 945:97-103.

13. Allas, D. A., and Michell. 1978. Calcium activated polyphosphoinositide and phosphodiesterase in the plasma membrane of human and rabbit erythrocytes. Biochim. Biophys. Acta. 508:277-286.