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# MEMBRANE PROTEIN CARBOXYL METHYLATION DOES NOT APPEAR TO BE INVOLVED IN THE RESPONSE OF ERYTHROCYTES TO CYTOSKELETAL STRESS

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**SUMMARY:** We have investigated the effect of changes of human erythrocyte cell shape on the degree of covalent modification by carboxyl methylation of membrane cytoskeletal proteins. The results indicate that the cell probably does not utilize carboxyl methylation to respond to cytoskeletal perturbations caused by such agents as A23187, 2,4-dinitrophenol, and chlorpromazine, all of which are known to cause large changes in cell shape. Protein carboxyl methylation also remained unchanged in the presence of cytochalasin B, which prevents such changes in cell shape. These results are not consistant with a cytoskeletal regulatory role for protein methylation reactions in the intact erythrocyte.

The unique structural properties of the human erythrocyte membrane are thought to be determined by the presence of a "cytoskeleton", an array of proteins which underlies the cell membrane (cf. refs. 1-3 for reviews). The membrane-stabilizing influence of the cytoskeleton is thought to maintain the normal discoid shape of the cell while permitting membrane flexibility which is necessary to allow the cell to squeeze through the narrow capillaries as well as to permit low viscosity blood flow (4,5). Alterations in this cytoskeletal array are thought to be responsible for the various changes in cell shape which can be chemically induced (6-8). Any given shape achieved in this manner (9,10) is apparently a steady-state balance between the tendency to form the spiny echinocytic morphology at one extreme ("crenation" which occurs, for example, in the presence of 2,4-dinitrophenol (DNP)[1] or $Ca^{++}$ and the divalent ionophore, A23187) and a counteracting tendency towards invaginated forms at the other extreme ("cupping" which occurs in the presence of, for example, chlorpromazine). Understanding the processes which cause such shape changes or which regulate the structural properties of the membrane to adapt to such structural stress are clearly important to understand the normal functioning of the membrane.

Since cytoskeletal proteins are major acceptors for carboxyl methylation in the human erythrocyte (11), it was possible that this reversible covalent modif-

[1] The abbreviations used are: DNP, 2,4-dinitrophenol; SDS, sodium dodecyl sulfate; AdoMet, S-adenosyl-L-methionine; EGTA, ethyleneglycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid; EDTA, (ethylenedinitrilo)-tetraacetic acid.

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ication reaction was involved in regulating the structural properties of the cytoskeleton in response to physical or chemical stress. For instance, Alhanaty and Sheetz (12) have shown that erythrocytes undergoing DNP-dependent crenation can become refractory to this cytoskeletal stress. In the continued presence of DNP these cells gradually (within a few hours) revert from echinocytes to their normal discoid shape and alter their subsequent response to other shape-changing compounds, apparently by reversibly modifying the properties of the membrane (12). To determine whether membrane protein carboxyl methylation functions in this sort of cytoskeletal regulatory capacity, we have determined the effect of known cytoskeletal perturbants on the level of membrane protein carboxyl methylation. The results indicate that the extent of this protein covalent modification reaction is largely unaltered as a result of cytoskeletal stress.

## EXPERIMENTAL PROCEDURES

**Chemicals** - A23187 (grade A-mixed $Ca^{++}$ and $Mg^{++}$ salt) was from Calbiochem and cytochalasin B was obtained from Sigma. A stock solution of each was prepared in ethanol at concentrations of 100 μM. The solvent was evaporated before the experiments were begun. DNP was a product of Eastman and chlorpromazine was obtained from Sigma. L-[methyl-$^3$H]methionine (specific activity, 70-80 Ci/mmol) was purchased as an ethanol:water mixture (7:3) from New England Nuclear.
**Determination of Cell Shape** - Cells were fixed at 0°C in solutions of 0.5% glutaraldehyde in a buffer with the same composition as the cell suspension being analyzed. Cells were less than 5% of the total volume of the fixing solution. Shape was then observed by phase contrast microscopy.
**Determination of Protein Carboxyl Methylation** - Human erythrocytes were obtained from healthy volunteers and washed with 118 mM Na phosphate, pH 7.4 as described (13). Cells were incubated at a 20% hematocrit at 37°C with 6 μM L-[methyl-$^3$H]-methionine and 18 mM D-glucose in 118 mM Na phosphate, pH 7.4. To measure protein [$^3$H]methyl esters formed during this incubation, membranes were isolated, their polypeptides separated by SDS-polyacrylamide gel electrophoresis at pH 2.4, and the relative radioactivity determined by autofluorography as described (13). It has been demonstrated that greater than 90% of the radioactivity determined in this manner is in the form of protein carboxyl methyl esters (11). A relative measure of protein methylation was determined by densitometry of the resultant autofluorogram standardized to the amount of protein determined by densitometry of the Coomassie-stained wet gel. Where indicated, [$^3$H]methyl esters were instead determined by organic extraction of [$^3$H]methanol released by base hydrolysis of precipitated membrane protein as described (13).
**Analysis of Cellular [$^3$H]AdoMet** - [$^3$H]AdoMet was quantitated by amino acid analysis as described (13).

## RESULTS AND DISCUSSION

**$Ca^{++}$-dependent Erythrocyte Crenation and Protein Carboxyl Methylation.** We observed that the introduction of 5 μM to 10 mM $Ca^{++}$ into human erythrocytes by the divalent cation ionophore, A23187, resulted in the transformation of the cells from the normal discoid morphology to the spiny morphology of the echinocyte in a manner analogous to previous reports (14). As shown in Fig. 1, the elevation of intracellular $Ca^{++}$ under these conditions significantly inhibited

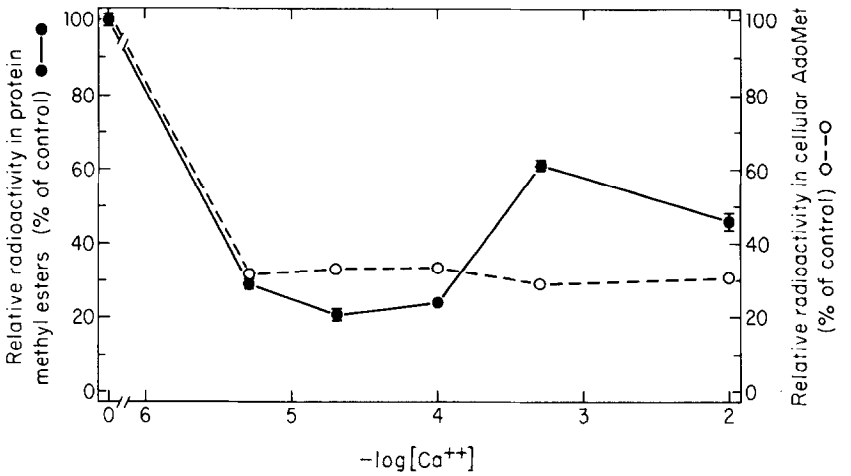


Fig. 1. **Membrane protein carboxyl methylation and the level of [$^3$H]AdoMet during $Ca^{++}$-dependent crenation.** Cells were incubated at 37°C with L-[methyl-$^3$H]methionine, 20 μM A23187, and the indicated concentrations of $CaCl_2$ except for the control which was incubated without A23187 or $CaCl_2$. After 3 h the amount of [$^3$H]AdoMet (○) and the degree of membrane protein carboxyl methylation (●) was determined as described in "Experimental Procedures". Error bars indicate the range of duplicate measurements.

the incorporation of label from L-[methyl-$^3$H]methionine into membrane protein methyl esters. Analysis of individual polypeptides by gel electrophoresis and autofluorography revealed that methylation was inhibited to a similar extent for all protein species. When the amount of [$^3$H]AdoMet, the substrate for the transmethylation reaction, was measured, it was found that the $Ca^{++}$-ionophore-induced inhibition of methylation is paralleled by a quantitatively similar decrease in the intracellular [$^3$H]AdoMet concentration (Fig. 1).

These changes in [$^3$H]AdoMet levels do not, however, appear to be a direct response to the cytoskeletal stress. For example, the ionophore alone, in the presence of either of the divalent cation chelators[2], EDTA or EGTA, was sufficient to significantly decrease the amount of cellular [$^3$H]AdoMet even though these conditions do not result in crenation (Table I). The level of [$^3$H]AdoMet was found to be lower in the presence of EDTA than it was in the presence of EGTA. Since both chelators bind $Ca^{++}$ similarly but EDTA binds $Mg^{++}$ more tightly than does EGTA, these data suggest the possibility that it is the dilution of $Mg^{++}$ across the cell membrane, facilitated by the ionophore, which caused this ionophore-dependent decrease in [$^3$H]AdoMet. In fact, the addition of excess $Mg^{++}$ to these cells in the presence of the ionophore and either EDTA or EGTA restores the [$^3$H]AdoMet to levels similar to that observed in the control (no ionophore, see Table I). This $Mg^{++}$ effect on [$^3$H]AdoMet levels may

[2]Since A23187 was obtained as the $Ca^{++}$, $Mg^{++}$ salt, chelators were necessary to remove these divalent cations added in association with the ionophore.

Table I

**Erythrocyte [$^3$H]AdoMet levels in the presence of ionophore.** Cells were incubated for 3 h with L-[methyl-$^3$H]methionine with the following additions and the amount of cellular [$^3$H]AdoMet was measured as described in "Experimental Procedures". Where indicated, A23187 was added to a concentration of 20 μM, EDTA and EGTA were 1.0 mM, and $Mg^{++}$ and $Ca^{++}$ as the $Cl^-$ salts were 2.0 mM.

| sample | [$^3$H]AdoMet (% of control) |
|---|---|
| control - no additions | (100) |
| A23187 + EDTA | 9 |
| A23187 + EGTA | 66 |
| A23187 + EDTA + $Mg^{++}$ | 116 |
| A23187 + EGTA + $Mg^{++}$ | 122 |
| A23187 + EDTA + $Ca^{++}$ | 25 |
| A23187 + EGTA + $Ca^{++}$ | 34 |

be explained by the requirement of free $Mg^{++}$ for the enzymatic synthesis of AdoMet in the erythrocyte (15). $Mg^{++}$ is normally impermeable to the cell membrane (cf. ref. 16).

The addition of excess $Ca^{++}$ to ionophore-treated cells in the presence of EGTA, however, also resulted in lowered levels of [$^3$H]AdoMet (Table I). These conditions of high intracellular $Ca^{++}$ are known to deplete ATP levels due to stimulation of the $Ca^{++}$-dependent ATPase (17). Since ATP is a substrate for AdoMet synthesis, conditions which lower ATP levels may lower AdoMet levels and consequently result in lower levels of protein carboxyl methylation (J. Barber and S. Clarke, unpublished observations).

From these considerations, it appears that the decrease in the relative number of protein esters observed in Fig. 1 for cells exposed to $Ca^{++}$ and the ionophore, A23187, is probably not a direct effect of crenation but is a secondary result of lowered AdoMet levels apparently due to the loss of cellular $Mg^{++}$ and ATP. This is substantiated by the fact that exposure of cells to the ionophore in the presence of EDTA or EGTA results in a decrease in the level of protein carboxyl methylation even though this does not result in appreciable crenation (see Table II). Furthermore, when cytochalasin B is added along with $Ca^{++}$ and the ionophore, crenation is prevented but the inhibition of protein carboxyl methylation still occurs (Table II).

This $Ca^{++}$-ionophore-dependent decrease in the already substoichiometric levels of protein carboxyl methylation has no effect itself on membrane structural properties since cells whose transmethylation reactions are inhibited by intracellular S-adenosyl-L-homocysteine remain discoid (18). Thus, crenation is not caused by the lowered levels of protein methyl esters resulting from decreased AdoMet concentrations and the lowered levels of methyl esters is not caused by crenation.

Table II

**The lack of correlation between $Ca^{++}$-dependent crenation and inhibition of membrane protein methylation reactions.** Cells were incubated for 3 h with L-[methyl-$^3$H]methionine under the conditions described below and the cell shape and level of protein carboxyl methylation determined as described in "Experimental Procedures". A23187 and $CaCl_2$ were used at a concentration of 20 μM and cytochalasin B was used at a concentration of 30 μM.

| sample | cell shape (% discoid) | $^3$H-protein methyl esters (% of control) |
|---|---|---|
| control - no additions | 95 | (100) |
| A23187 + $Ca^{++}$ | <5 | 27 |
| A23187 + EGTA | 95 | 52 |
| A23187 + EDTA | 90 | 3 |
| A23187 + $Ca^{++}$ + cytochalasin B | >99 | 29 |
| cytochalasin B | >99 | 99 |

**DNP-dependent Crenation and Protein Carboxyl Methylation** - The effect of DNP-dependent crenation on membrane protein carboxyl methylation was also tested. Phase contrast microscopy confirmed that crenation occurred (<5% discoid) at the concentrations of DNP used. After 6 h of incubation with 3 mM DNP cells adapted to this cytoskeletal stress to the extent that 20% of the cells were discoid, similar to previous observations (12). As shown in Table III, DNP also lowers the amount of cellular [$^3$H]AdoMet but the level of total membrane protein carboxyl methylation is largely unaltered during this adaptation process in the presence of DNP.

Table III

**The effect of DNP and chlorpromazine on membrane protein carboxyl methylation.** Cells were suspended with L-[methyl-$^3$H]methionine as described in "Experimental Procedures" and then incubated at 37°C with the following additions. At the times indicated samples were removed, the cytoplasm was analyzed for [$^3$H]AdoMet, membranes were isolated and the total level of $^3$H-protein methyl esters determined by organic extraction as described in "Experimental Procedures". Values are reported as % of controls for each time point.

| time (h) | additions | [$^3$H]AdoMet (% of control) | $^3$H-methyl esters (% of control)[a] |
|---|---|---|---|
| 2 | none | (100) | (100 ± 2) |
| | 3 mM DNP | 71 | 70 ± 2 |
| | 10 mM DNP | 36 | 90 ± 3 |
| | 50 μM chlorpromazine | 138 | 108 ± 2 |
| 6 | none | (100)[b] | (100 ± 2)[c] |
| | 3 mM DNP | 58 | 66 ± 2 |
| | 10 mM DNP | 24 | 90 ± 2 |
| | 50 μM chlorpromazine | 123 | 108 ± 4 |

[a] reported as the mean ± S.E.M. for triplicate measurements
[b] this value is actually 45% of the 2 h time point due to the fall in AdoMet pool levels during incubation (18)
[c] this value is actually 153 % of the 2 h time point due to the approach of steady-state labelling of the [$^3$H]-methyl esters (13)

**Chlorpromazine-dependent Cupping and Protein Carboxyl Methylation** - Invaginated shapes ("cups") are thought to represent the opposite extreme from echinocytes (9,10). Chlorpromazine at the concentration used here caused >95% cup formation. However, as seen by the data in Table III, the invaginated form assumed by the erythrocyte under these conditions does not result in detectable changes in protein carboxyl methylation.

**Manipulation of Cytoskeletal Actin and Protein Carboxyl Methylation** - Filamentous actin (polymers composed of approximately 10-15 monomeric subunits) appears to exist normally as a complex with band 4.1 and spectrin of the cytoskeleton (cf. refs. 19-21). Both band 4.1 and the spectrin anchoring protein, band 2.1, are major sites of carboxyl methylation in intact cells (11). Since it is known that cytochalasin B binds to these complexes (22) and, in the case of purified actin, results in depolymerization of actin filaments, we measured the effect of this compound on erythrocyte membrane protein carboxyl methylation. Control experiments confirmed the known effects of cytochalsain B on D-glucose transport into intact cells (23) as well as the inhibition of polymerization of purified actin *in vitro* (data not shown).

Although the consequent depolymerization of filamentous actin in the erythrocyte cytoskeleton might be expected to result in observable changes in erythrocyte shape, no such changes were observed (Table II). However, cytochalasin B does affect the cytoskeleton as observed by the inhibition of $Ca^{++}$-dependent crenation reported in Table II. Nevertheless, the degree of carboxyl methylation of cytoskeletal proteins is unchanged in the presence of 1-30 μM cytochalasin B (Table II and data not shown). Analysis of individual proteins by gel electrophoresis and autofluorography demonstrated that there were also no detectable changes in any of the minor methylated proteins which might have only small effects on the overall degree of methylation. From this we infer that the cell apparently does not utilize protein carboxyl methylation to adapt to changes in the state of the cytoskeletal spectrin-actin-band 4.1 complex.

**Functional Implications for Erythrocyte Protein Carboxyl Methylation** - It was conceivable that alterations in the level of protein carboxyl methylation of cytoskeletal membrane proteins might regulate the properties of the membrane in response to experimental alterations of the cytoskeleton. Even though this modification reaction is substoichiometric (average degree of modification <5% cf. ref. 13), modification of even such a small subpopulation of proteins might have profound effects on the whole cytoskeletal array of proteins much as the strength of a chain is determined by the strength of its weakest link. Alternatively, this substoichiometric degree of modification might represent only a basal level which could be elevated to stoichiometric levels in response to the appropriate stimulus.

However, we find no large changes in carboxyl methylation of individual cytoskeletal proteins under conditions known to alter the structural properties of the cytoskeleton. Since the inhibition of membrane protein carboxyl methylation does not detectably alter cellular anion transport (L.L. Lou and S. Clarke, manuscript in preparation) or membrane structural properties such as cell shape (18), these data suggest a non-regulatory function for this modification reaction. This indirectly gives further evidence for the hypothesis that this reaction is involved in the "housekeeping" metabolism of proteins which have suffered spontaneous chemical damage (24,25). According to this model, only proteins containing "damaged" residues such as D-aspartate (24) or L-aspartyl isopeptide linkages (25) would be sites for carboxyl methylation and thus manipulation of the cytoskeleton would not alter the degree of covalent modification unless it also altered the rate of formation of these "damaged" residues. In this regard, it is observed that although both crenating agents used here ($Ca^{++}$ and DNP) lower the levels of [$^3$H]AdoMet and consequently the number of protein methyl esters, at very high concentrations of crenators, protein methylation begins to return to control levels even though [$^3$H]AdoMet levels do not (Fig. 1 and Table III). This may indicate that under these extreme conditions of cytoskeletal duress, the formation of such damaged residues in proteins is facilitated.

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