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Effects of the Calcium-Mediated Enzymatic Cross-Linking of Membrane Proteins on Cellular Deformability

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Summary. Excess calcium binding affects the shape and dynamics of cellular deformation of human erythrocytes. It may be hypothesized that incorporation of calcium may modify cellular deformability by processes which include specific cross-linking of membrane proteins with resultant changes in cell shape and deformability. Since previous studies indicate that accumulation of calcium ions causes development of γ-glutamyl-ε-lysine bridges in membrane proteins, under control of a membrane transamidating enzyme which specifically requires calcium ions for activation, experiments were devised to examine the relationship between cross-linking and deformability and to determine the effects of specific inhibitor of membrane protein cross-linking on the calcium-dependent modification of erythrocyte to the echinocytic shape. The elastic shear modulus of the membrane was not significantly affected by calcium-induced cross-linking, indicating that induced shape change, not altered elasticity, causes the observed reduction in cellular deformability. These findings support the interpretation that Ca^{++}-induced and transamidase-catalyzed cross-linking of membrane proteins contributes to fixation of altered cellular shape and decreased cellular deformability.

Key words. Ca^{++}-mediated cross-linking, erythrocyte proteins, transglutaminase, erythrocyte deformability.

It is known from the work of several investigators that the increase of intracellular concentration of calcium ions has an effect on the shape and on the membrane viscoelastic properties of human erythrocytes [5, 6, 21–23]. The cellular deformability of the erythrocyte, defined as the capacity for passive change in cellular configuration in response to shear forces, is thought to depend on the viscoelastic properties of the membrane, the viscosity of the cytoplasm, and the shape in the normal erythrocyte. The biconcave disc shape has excess area compared to the minimum area to enclose the erythrocytes' volume and, given its membrane's elastic properties and the relatively low viscosity of its intercellular contents, passive change of shape occurs at relatively small forces. Factors that reduce the surface area, i.e., make the cell more spherical, have a pronounced effect on cellular deformability, particularly if deformation occurs at a rapid rate, and both increase in cytoplasmic viscosity or reduction of membrane elastic properties would also be expected to reduce cellular deformability. It may be postulated that incorporation of calcium in erythrocyte membranes may modify cellular deformability by the specific cross-linking of membrane proteins with consequent changes in cell shape and membrane elastic properties.

Calcium binding occurs primarily on the inner membrane surface [7]. It was previously shown that accumulation of calcium ions in human red cells causes the fusion of membrane proteins by γ-glutamyl-ε-lysine bridges [11, 12, 19]. This cross-linking process is under the control of an intrinsic transamidating enzyme of the endo-γ-glutamine-ε-lysine transferase type (often referred to by the trivial name, transglutaminase) which specifically requires calcium ions ($>10^{-5}$M) for its conversion from latent to an active form. Thus the enzymatic polymerization reaction may have an effect on the calcium-induced transformation of discocytes to echinocytes as well as increased membrane rigidity, both of which are poten-

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tially irreversible. This paper examines the effect of specific inhibitors of membrane protein cross-linking on the calcium-dependent fixation of echinocyte shape and loss of cellular deformability in human erythrocytes.

Materials and Methods

Venous blood was obtained from normal adult donors in vacutainer tubes containing EDTA. The red cells were harvested by repeated centrifugation and washing at 4 °C (3 × 1,000 *g* × 10 min) using either 5 mM Tris-HCl, 0.16 M NaCl, 10 mM glucose, pH 7.4 (Buffer A) or 5 mM Tris-HCl, 0.1 M KCl, 0.06 M NaCl, 10 mM glucose, pH 7.4 (Buffer K), and the cells were used for experiments on the day of collection. A 5-mM stock solution of ionophore A23187 (a gift from Eli Lilly Laboratories, Indianapolis, Ind.) was prepared in dimethylsulfoxide and was diluted fivefold with either Buffer A or K just prior to use.

Bovine serum albumin was obtained from Sigma Chemical Co. Histamine, cystamine, aminoacetonitrile (Aldrich), and glycine methylester (Mann Research Laboratories), purchased as the hydrochloride salts, were dissolved in water and adjusted to pH 7.4. N,N-dimethylhistamine was prepared by Dr. K.N. Parameswaran at Northwestern University. ATP[1] assays were performed by the ATP-utilizing coupled reaction system of hexokinase and glucose-6-phosphate dehydrogenase, as described by Beutler [2]. Hemoglobin was measured by the method given in Sigma Technical Bulletin No. 525 (1976).

Shape Change Protocol

Washed, packed erythrocytes were first diluted with equal volumes of Buffer K, then 2 ml cell suspensions were mixed with 50 µl of either aminoacetonitrile, histamine, or cystamine solutions (giving final concentrations for these compounds of 20 mM) and were incubated for 2 hr at 37 °C. Then, 40 µl each of ionophore and $CaCl_2$ solutions were added to yield 20 µM and 1.5 mM concentrations for ionophore and Ca^{2+}, respectively. The mixtures were then allowed to incubate for another 2 hr at 37 °C. Appropriate controls, containing no added amines, were prepared with and without Ca^{2+} present. In the latter case 1.5 mM $MgCl_2$ was substituted for $CaCl_2$.

Following the treatment of cells as above, 5 µl aliquots were pipetted into 0.4 ml of 0.5% glutaraldehyde (Sigma; high purity) made up with Buffer K but without glucose (Buffer K-G) and were fixed at room temperature overnight.

Other aliquots of 1 ml each were mixed with 40 ml of cold Buffer K to which 0.4% serum albumin and 1 mM EDTA were added. Cells were collected by centrifugation (1,000 *g* × 10 min) and were resuspended at 37 °C in 40 ml of the same solution of Buffer K-0.4% BSA-1 mM EDTA for a period of 2 hr, following which they were again harvested by centrifugation. The cells were washed twice more for 1 hr each, in 40 ml Buffer K containing 0.05% BSA and 1 mM EDTA, in each instance followed by centrifugation. Finally, 5 µl of cell suspensions were taken for fixation with 0.4 ml of glutaraldehyde, as described for the unwashed cells above.

Glutaraldehyde-fixed cells were placed on Nucleopore filters of 13 mm diameter (Nuclepore Corp., 3 µm holes) by aspirating 20 µl of cell suspensions followed by ample washing with Buffer K-G. The cells were then exposed for a period of 45 min to osmium vapor, which was removed by washing with distilled water. Dehydration from water to ethanol and transfer to freon 113 was carried out by the serial application of 5, 10, 30, 45, 80 and 100% water/ethanol for 5 min each, followed by 100% ethanol for 10 min, and ethanol/freon mixtures in the same serial fashion. Critical point drying with freon 13 (at 44 °C and 900 psi) was followed by mounting the samples on aluminum stubs and gold-palladium coating of about 150 Å thick. The cells were examined in a Joule 50 A scanning electronmicroscope fitted with a Polaroid camera at magnifications ranging from 1,000 to 5,000-fold for the assignment of echinocytic shape ; approximately 2,000 cells (representing about 20–50% of all cells) were counted between the edges of the specimen at randomly selected areas.

Deformability Experiments

Packed red cells (0.25 ml) were first diluted into Buffer A (1.8 ml) followed by a preincubation period of 1 hr at 37 °C. Then 0.04 ml of $CaCl_2$ (or $MgCl_2$) were added to yield the desired concentration (0–0.6 mM) followed by 0.04 ml of A23187 ionophore (10 µM final concentration). Erythrocytes were also preincubated with amines as above, except that the 1.8 ml contained the desired concentration of amine in H_2O plus Buffer A such that the final osmotic strength remained essentially constant, i.e., 320 mosm. Final hematocrits were approximately 10% in all samples. Following incubation at 37 °C for the desired times, cells were washed (2 × 15 ml) at 4 °C in 0.25% BSA dissolved in Buffer K. Aliquots of cells were taken for deformability measurements and the remaining cells lysed in 5 mM sodium phosphate of pH 8.0 for isolation of ghosts and subsequent SDS gel electrophoresis [3, 20].

Cellular deformability was determined by the micropipette-aspiration technique [15, 16]. Randomly selected cells were subjected to negative pressure, ΔP, applied slowly (> 3 sec) to the cell by glass micropipettes of 1.2×10^{-6}m internal diameter, and their deformation was observed as the extension of cell membrane into the pipette as a function of pressure. The pipette dimension was confirmed by electron microscopy. The dynamics of deformation were recorded on videotape with simultaneous recording of pressures such that frame-by-frame replay of tapes permitted analysis of membrane extension *vs.* pressure over the range 1.0 to 5.0×10^2 dynes/cm^2. The membrane elastic shear modulus was calculated from the extensions *vs.* force. The maximal extension of $\Delta P = 7.5 \times 10^3$ dynes/cm^{2}served as an estimate of the effective excess surface area of the cell to volume enclosed. Very small extensions indicated relative sphericity and are predictive of reduced cellular deformability [5].

Results

As presented in Fig. 1 and Table 1, the results of control experiments with Mg^{2+} showed that most of the erythrocytes retained discocytic forms during the entire period of incubation with ionophore A23187. However, following the addition of Ca^{2+}-ions, a high proportion of the cells assumed echinocytic or, rather preponderantly, spheroechinocytic shapes, and even washing with EDTA and serum albumin [17] did not significantly alter the number of echinocytically transformed cells. The exposure of erythrocytes to inhibitors of transglutaminase catalyzed cross-linking, prior to and during loading with Ca^{2+}, did not prevent the enchinocytic transition as such, but greatly reduced the number of *irreversibly*

[1] *Abbreviations:* EDTA – ethylenediaminetetraacetic acid; ATP – adenosine triphospate; BSA – bovine serum albumin; SDS – sodium dodecyl sulfate; Hb – hemoglobin; DMSO – dimethyl sulfoxide; DTT – dithiothreitol.

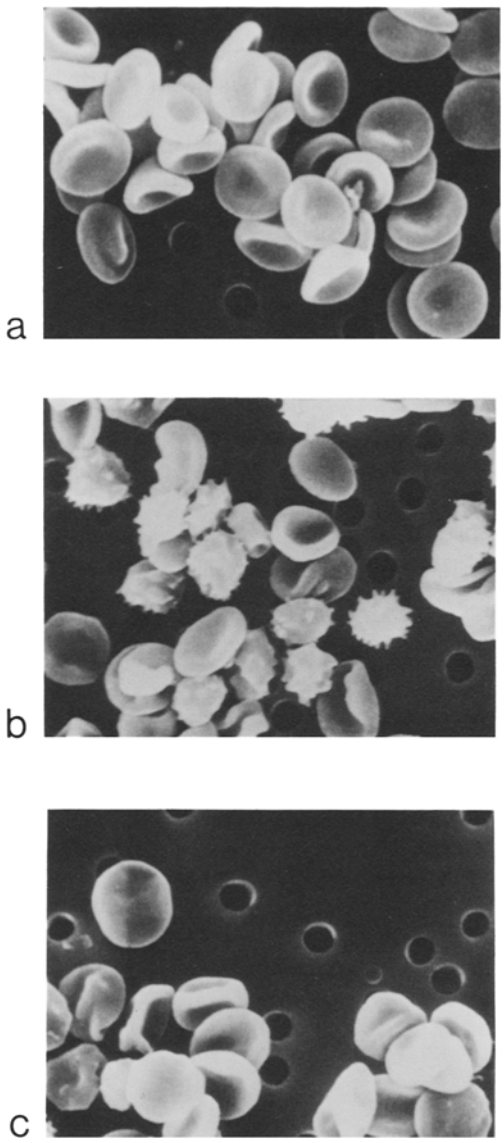

Fig. 1. Inhibition of shape change fixation. Packed human erythrocytes were mixed with equal volumes of Buffer K (0.1 M KCl, 0.06 M NaCl, 5 mM Tris-HCl, 10 mM glucose, pH 7.4). Two ml of this mixture was used for each sample. Samples *a* and *b* were incubated for 1 hr at 37 °C without any additions; sample *c* contained 50 mM histamine. At this time, A23187 dissolved in DMSO was added to a final concentration of 20 μM to each sample . After 5 min, Mg^{2+} was added to sample *a* to a final concentration of 1.5 mM and Ca^{2+} was added to samples *b* and *c* to a final concentration of 1.5 mM. The samples were incubated for 30 min at 37 °C. After incubation, all samples were suspended in 40 vol of cold Buffer K with 0.4% BSA and 1 mM EDTA. They were centrifuged at 2,000 rpm in a Beckmann JA-20 rotor and resuspended in the same buffer for 2 hr at 37 °C. The cells were centrifuged and resuspended in Buffer K with 0.05% BSA and 1 mM EDTA twice for 1 hr at 37 °C and centrifuged to pack the cells. Five μl of each packed cell sample was added to 400 μl of 2.5% glutaraldehyde and was allowed to sit overnight at 4 °C. The glutaraldehyde-treated cells were placed on nuclepore filters and processed as described in Materials and Methods for examination with a scanning electron microscope

Table 1. Prevention of irreversible echinocytic shape change in erythrocytes by inhibiting transglutaminase catalyzing cross-linking of membrane proteins during loading with Ca^{2+}

Incubation with buffer A (0.1 M KCl, 0.06 M NaCl, 10 mM glucose, 5 mM Tris-HCl, pH 7.4; hematocrit 50%) 2 hr, 37 °C, plus 20 mM of	Incubation (with Buffer B 20 μM A23187, 0.4% DMSO), 2 hr, 37 °C, plus 1.5 mM of	Percentage of crenated cells[a]	
		Before wash	After wash in BSA-EDTA[b]
	$MgCl_2$	15	5
	$CaCl_2$	90	77
H_2NCH_2CN	$CaCl_2$	90	40
$H_2NCH_2CH_2$-(imidazole)	$CaCl_2$	85	38
$H_2N(CH_2)_2SS(CH_2)_2NH_2$	$CaCl_2$	88	30

[a] Crenation monitored in SEM, counting about 2,000 cells (echinocytes II to spheroechinocyte).

[b] Wash: 40 vol 0.4% BSA-1 mM EDTA in buffer A for 5 min at 4 °C, then 2 hr at 37°C, followed by 40 vol 0.05% BSA-1 mM EDTA in buffer A for 2 × 1 hr at 37 °C.

transformed cells. As shown in Table 1, regardless of the primary amine used, the irreversible formation of the echinocytic shape is inhibited, as judged by the reversion of shape to either flattened discs or cups devoid of surface spikes. This inhibition is clearly dependent on the primary amine function, since an analogue of histamine, N,N-dimethylhistamine, which is not a primary amine and is thus not a substrate for the enzyme, did not inhibit irreversible shape changes. The concentration of ATP, which dropped sharply as a result of Ca^{2+} loading of the cells remained low in all instances (less than 600 nmol/g hemoglobin).

The elastic shear modulus, estimated from small extensions of membrane into the micropipette without induction of tension throughout the membrane did not differ from the value observed in normal cells: 10^{-2} dyn/cm. This finding does not exclude significant change in elasticity, as would be expected to result from the demonstrated cross-linking, since the transducer sensitivity and optical resolution are such that small pressure differences (< 20 dyn/cm^2) and extensions lengths (< 0.1 μm) cannot be detected. These data suggest that the contribution of altered elasticity to reduced cellular deformability in these static equilibrium measurements is small. The bending stiffness of the membrane contributes to the behavior of the membrane during the deformation caused by micropipette aspiration and certainly is an important determinant in the shear response observed in the *in vivo* circulation. The effect of bending stiffness,

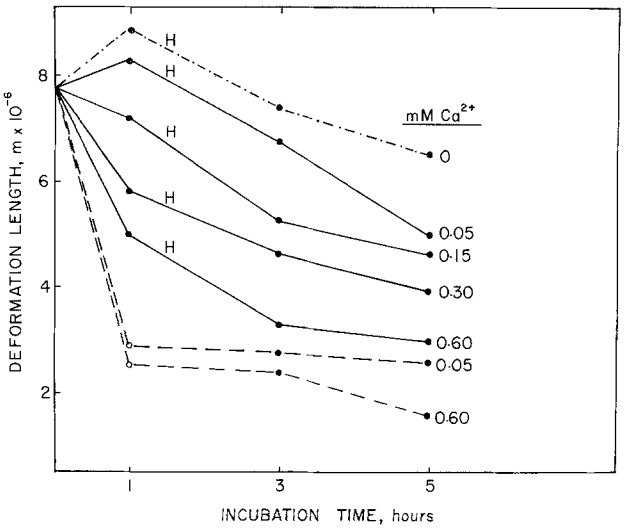

Fig. 2. The effect of a transamidase inhibitor, histamine, on the Ca^{2+}-induced loss of membrane deformability was measured by the micropipette technique. Washed erythrocytes were preincubated for 1 hr at 37 °C in the presence (*H*) or absence of 80 mM histamine, ionophore A23187 (10 μM) and increasing levels of Ca^{2+} (or Mg^{2+}). At the indicated times, samples were removed, washed twice in Buffer K containing 0.25% BSA to remove Ca^{2+} and ionophore, and then subjected to deformation analysis as given in Methods

Table 2. Prevention of reduction in erythrocyte deformability by inhibition of transglutaminase cross-linking of membrane proteins

Sample	Percent of control (mean deformation length)	
	1 hr	3 hr
0.3 mM Mg^{2+}	100	92
0.3 mM Ca^{2+}	48	31
0.3 mM Ca^{2+} + 25 mM glycine methylester	71	–
0.3 mM Ca^{2+} + 55 mM glycine methylester	79	–
0.3 mM Ca^{2+} + 75 mM glycine methylester	89	59

A typical experiment with one donor's cells: 37 °C, 10 μM A23187 ionophore $100 = 9.00 \pm 0.38 \times 10^{-6}$ m (mean from measurement of 7 cells in 0.3 mM Mg^{2+} at 1 hr).

however, cannot be distinguished by the micropipette method. The effect of curvature of the surface and its complex relationship to spicule formation and resolution likewise cannot be estimated by present methods.

The combined effects of Ca^{2+} and the cross-linking inhibitor, histamine, on erythrocyte cellular deformability are shown in Fig. 2. After treatment with Ca^{2+}-ion and ionophore, the red cells show a reduction in length of membrane extension when compared to controls incubated with Mg^{2+}-ion and ionophore.

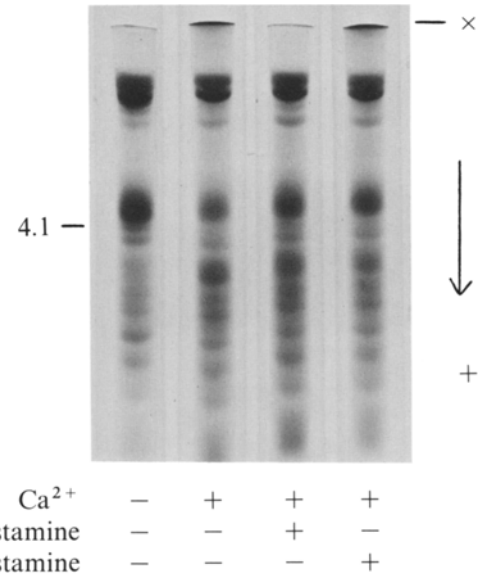

Fig. 3. Sodium dodecyl sulfate-polyacrylamide (4.5%) gel electrophoretic analysis of membrane fractions obtained from the cell populations of Table 3. Erythrocytes were preincubated for 1 hr in the presence (gel 3-histamine; gel 4-N,N-dimethylhistamine) or absence (gels 1 and 2) of amines. Ionophore A23187 (10 μM) was added, followed by 0.3 mM $MgCl_2$ (gel 1) or 0.3 mM $CaCl_2$ (gels 2–4). After incubation at 37 °C for 4 hr, membranes were isolated, solubilized in 1% SDS-40 mM DTT, and subjected to electrophoresis. Polymer is denoted by *X*

Table 3. Effects of transglutaminase inhibitor[a] histamine and the noninhibiting analogue N^{α}-dimethylhistamine in prevention of reduced deformability.

Sample	Percent of control (mean deformation length)	
	1 hr	3 hr
0.3 mM Mg^{2+}	100	92
0.3 mM Ca^{2+}	56	38
0.3 mM Ca^{2+} + 25 mM Histamine	62	–
0.3 mM Ca^{2+} + 55 mM Histamine	69	–
0.3 mM Ca^{2+} + 75 mM Histamine	77	54
0.3 mM Ca^{2+} + 25 mM N^{α}-dimethylhistamine	60	–
0.3 mM Ca^{2+} + 75 mM N^{α}-dimethylhistamine	37	37

[a] A typical individual experiment with erythrocytes from one donor: 37 °C, 10 μM A23187 ionophore.

[b] $100 = 6.60 \pm 0.49 \times 10^{-6}$ m (mean from measurement of 7 cells in buffer containing 0.3 mM Mg^{2+}).

This indicates a functional but not absolute decrease in surface area; at this pressure only the large spicules visible by light microscopy are smoothed out. The direct measurements of surface area and volume have not been achieved in ionophore-treated cells. The presence of histamine prior to and during loading

of the cells with Ca^{2+} results in an inhibition of the loss of extension; the inhibition is more effective at lower Ca^{2+} concentrations and decreases with time. The data presented in Tables 2 and 3 from special individual experiments show that inhibition is dependent on amine concentration at time: the higher the amine level the greater the inhibition of loss deformability. In addition, Table 3 shows that the histamine analogue, N,N-dimethylhistamine, does not affect the loss of membrane extension that results when cellular Ca^{2+} levels are increased. These results can be correlated with the SDS gel electrophoretic analysis of the membranes from cells treated with Ca^{2+} and inhibitor, as shown in Fig. 3. Cross-linked polymers appear in samples containing Ca^{2+} and the inhibitor analogue, dimethylhistamine, whereas polymer formation is inhibited by histamine.

Discussion

The elasticity of the cell membrane [15, 16] and absence of intracellular organelles permit human erythrocytes to respond to factors such as the state of aggregation of hemoglobin (e.g., sickle cells [4]), the movement of water and ions in and out of the cytoplasm [13], the selective interactions of drugs [18] and *in situ* generated compounds (e.g., diacylglycerol [1]) with the membrane by a great variety of changes in shape and surface topology, and such changes are often reversible. As demonstrated above, the Ca^{2+}-induced shape changes, however, can become irreversible in a relatively short period of time. Without addressing the point as to how the echinocytic transition with Ca^{2+} *per se* came about, the purpose of the present paper was to analyze the Ca^{2+}-dependent irreversible component responsible for the fixation of this abnormal shape and the accompanying change in deformability as a result of the reduced membrane area and putative change in elasticity. In specific terms, attention was focused on the role of transamidase-mediated anchoring of the membrane proteins to each other by γ-glutamyl-ε-lysine bridges [11, 19] and the issue was approached by using specific inhibitors of this cross-linking process.

The data presented are in agreement with the idea that the retention of echinocytic shape and change in cellular deformability after removal of Ca^{2+} and ionophore is, indeed, related to the transamidase catalyzed fusion of the membrane proteins. For the majority of cells, the cross-linking inhibitors prevented the Ca^{2+}-induced change of shape from becoming irreversible. Following a preliminary report [8], our findings were quickly confirmed by Palek et al. [14] employing 40 mM histamine for inhibiting cross-linking.

The inhibitors also interfered with the Ca^{2+}-dependent promotion of the membrane rigidity. Inhibition by aminoacetonitrile, glycine methylester, and histamine might be considered to be purely competitive, directed against the ε-lysine functionalities of membrane protein substrates involved in cross-linking [9, 10]. The action of cystamine, however, is probably more complex because this compound can lead to the direct inactivation of transglutaminase by modifying its active center thiol group (E.-M. Svahn, R.B Credo, and L. Lorand, *in preparation*).

It should also be noted that the concentration of ATP dropped appreciably during the loading of erythrocytes with Ca^{2+}, regardless of whether inhibitory amines were present or not, and the ATP concentration remained low in every case even after washing of the cells with EDTA and serum albumin in the glucose-containing buffer. Thus, the observed effects of cross-linking inhibitors in preventing the fixation of echinocytic shape and loss of membrane elasticity cannot be directly related to the bulk concentration of cellular ATP.

In conclusion, it can be stated that the Ca^{2+}-induced and transamidase-catalyzed cross-linking of membrane proteins into high molecular weight polymers contributes to the fixing of altered cellular shape and to the decrease of cellular deformability. The degree and extent of cross-linking when Ca^{2+} levels rise in the erythrocyte *in vivo*, be it as a result of aging, sickling, or some disease state, may modulate cellular shape and deformability and may thus be significant with respect to the mechanisms of cell destruction.

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