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Proteolysis of Erythrocyte-Type and Brain-Type Ankyrins in Rat Heart after Postischemic Reperfusion[1]

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Ankyrin links cytoskeleton and integral membrane proteins and is proteolyzed *in vitro* by calpain, a Ca^{2+}-dependent protease. In the present study, we examined the localization of two ankyrin isoforms, erythrocyte (red blood cell)-type ($ankyrin_R$) and brain-type ($ankyrin_B$), and their proteolysis after ischemia-reperfusion in the subcellular fractions of perfused rat heart by immunoblotting and by immunohistochemistry using specific antibodies. Both isoforms were observed to be distributed chiefly in the myofibril-nucleus ($1,000\times g$ pellet: P1) fraction, while $ankyrin_R$ was located substantially in the membrane ($100,000\times g$ pellet: P2) fraction. Reperfusion after 10 min or more of global ischemia induced preferential proteolysis of $ankyrin_R$ in the P2 fraction and $ankyrin_B$ in the P1 fraction. The proteolysis of $ankyrin_R$, but not $ankyrin_B$, was effectively inhibited by the synthetic calpain inhibitor acethyl-leucyl-leucyl-norleucinal. The immunohistochemical examination showed that anti-$ankyrin_R$ delineated striations, sarcolemma and nuclei, and the staining was decreased after ischemia-reperfusion, while anti-$ankyrin_B$ showed diffuse staining. The proteolysis of $ankyrin_R$ may interfere with force conduction through disruption of the linkage between integral membrane proteins and the myofibril-cytoskeleton.

Key words: ankyrin isoform, calpain, ischemia-reperfusion, membrane-skeleton.

Ankyrins are a family of proteins that link several integral membrane proteins and a spectrin-based membrane cytoskeleton (*1, 2*). The anion exchanger (*3, 4*), Na^+-K^+-ATPase (*5, 6*), a voltage-dependent Na^+ channel (*7*), an Na^+/Ca^{2+}-exchanger (*8*), a ryanodine receptor (*9, 10*), and adhesion molecules (*11*) have been reported to interact with ankyrin. Spectrin is a major constituent of the membrane-skeleton, forming a two-dimensional meshwork under the erythrocyte membranes (*1*). A family of spectrin-related proteins, referred to as non-erythroid spectrin, calspectin or fodrin, has been identified in non-erythroid cells (*1*). In kidney and epithelial cells, ankyrin, calspectin, Na^+-K^+-ATPase, and an adhesion protein, cadherin, form a complex that restricts Na^+-K^+-ATPase to the basolateral surface (*5, 6*), while ischemia induces disruption of the complex, resulting in the redistribution of Na^+-K^+-ATPase to the apical surface (*6*). Degradation of calspectin and ankyrin was reported in the ischemic rat kidney and brain, but not in the ischemic heart (*12*). In the heart, Na^+-K^+-ATPase activity has been shown to decline after ischemia-reperfusion (*13-15*), though the underlying mechanism remains to be determined.

Two major isoforms of ankyrins are known: erythrocyte (red blood cell)-type ($ankyrin_R$) and brain-type ($ankyrin_B$) (*1, 2*). The two isoforms show different spatial and temporal (age) localization in the brain (*16*) and kidney (*6, 17*). We recently reported that reperfusion after brief ischemia induces proteolysis of calspectin at the sarcolemma and intercalated discs through calpain, a Ca^{2+}-dependent protease, and that inhibition of the protease remarkably improved the contractile dysfunction during postischemic reperfusion (*18, 19*).

In the present study, we investigated the localization of $ankyrin_R$ and $ankyrin_B$ in the perfused rat heart and their proteolysis during ischemia-reperfusion.

MATERIALS AND METHODS

Materials—Anti-rat brain ankyrin ($ankyrin_B$) was a generous gift of Dr. Manabu Kunimoto (The National Institute for Environmental Studies, Tsukuba) and was characterized previously (*20*). Anti-chicken erythrocyte ankyrin ($ankyrin_R$) antibody was obtained from Transformation Research, the ECL Western blotting detection kit from Amersham, acetyl-leucyl-leucyl-norleucinal (ALLnaL) from Nacalai Tesque, benzyloxycarbonyl-leucyl-leucyl-leucinal (ZLLLal) from Protein Research Foundation (Osaka), porcine kidney m-calpain from Chemicon International, the ABC kit from Vector Laboratories, the biotin-blocking system from Dako, OCT compounds from Miles, and prestaining markers for electrophoresis from Bio-Rad.

Perfusion Procedure—Male Wistar rats weighing about 200 g were anesthetized with sodium pentobarbital, and the hearts were quickly excised. As we previously reported (*18, 19, 21*), the hearts were perfused with Krebs-Hensleit (KH) solution gassed with 95% O_2-5% CO_2 at a constant

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Abbreviations: B, brain-type; R, red blood cell (erythrocyte)-type; KH solution, Krebs-Hensleit solution; ALLnaL, acetyl-leucyl-leucyl-norleucinal; ZLLLal, benzyloxycarbonyl-leucyl-leucyl-leucinal; DMSO, dimethylsulfoxide; PLP, paraformaldehyde-lysine-phosphate.

pressure of 80 cmH_2O for about 10 min, and then subjected to global ischemia at 37°C. For the inhibitor experiments, the perfusion buffer contained either ALLnaL (3-100 μM) or ZLLLal (3-100 μM), both in 0.1% dimethylsulfoxide (DMSO), for 5 min before ischemia. DMSO (0.1%) did not affect the proteolysis of ankyrin (data not shown) as we previously reported for calspectin (*18*). For low Ca^{2+} reperfusion, $CaCl_2$ was removed from the KH solution during reperfusion. After the perfusion, the ventricles were quickly frozen in liquid nitrogen and stored at −70°C until the biochemical analyses.

Subcellular Fractionation—As we have previously reported (*22, 23*), the frozen hearts were homogenized in the STE buffer containing 0.32 M sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 5 mM NaN_3, 10 mM β-mercaptoethanol, 20 μM leupeptin, 0.15 μM pepstatin A, 0.2 mM phenylmethanesulfonyl fluoride, and 50 mM NaF with a Polytron homogenizer (PT1200, Kinematica AG). The homogenate was centrifuged at 1,000×g for 10 min, and then the supernatant was spun at 100,000×g for 60 min. The 1,000×g pellet and 100,000×g pellet were designated as the P1 and P2 fractions, respectively, while the 100,000×g supernatant was referred to as the S fraction. The membrane-cytoskeleton was isolated as the Triton X-100 (1%)-insoluble (10,000×g) pellet of the P2 fraction. A cycle of freezing-thawing of the heart did not affect the distribution of $ankyrin_{R,B}$ (data not shown). Protein concentrations were determined by the method of Lowry (*24*), using bovine serum albumin (BSA) as a standard.

In Vitro Proteolysis of Ankyrin with Calpain—μ-Calpain was purified from human erythrocytes as we previously described (*21*). The P1 or P2 fraction (2.0 mg/ml) was incubated with 0.1 U/ml of μ- or m-calpain in the buffer containing 20 mM Tris-HCl, pH 7.4, 10 mM β-mercaptoethanol, and 1% Triton X-100 in the presence of 2.0 mM $CaCl_2$ at 25°C.

Immunoblotting and Quantification of Ankyrin—The fractions were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) using 6.5% polyacrylamide gels by the method of Laemmli (*25*), followed by immunoblotting according to the method of Towbin *et al.* (*26*) with modifications (*18*). The blots were blocked with 5% skim milk in buffer containing 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 0.05% Tween-20, and incubated with one of the 1,000-2,000-fold diluted antibodies against $ankyrin_B$ and $ankyrin_R$ for 1 h at room temperature. The ankyrin isoforms were then visualized by using an ECL Western blotting detection kit. The amounts of ankyrin isoforms on the immunoblots were measured with an image analyzer (Densitograph AE-6900, Atto) as described in our reports (*22, 23*).

Immunohistochemistry—The ventricles were removed after ischemia-reperfusion, immersed in OCT compounds, and rapidly frozen in liquid nitrogen. After sectioning at 6 μm, the specimens were fixed on glass slides either with paraformaldehyde-lysine-phosphate buffer (PLP) (pH 7.4) or with 70% acetone-30% methanol (−20°C) for 10 min, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS; 0.3 M NaCl, 20 mM Tris-HCl, pH 7.8, 0.05% Tween-20; only after PLP-fixation) for 10 min, treated with cold 0.3% H_2O_2 in methanol for 30 min, biotin-blocked with the kit from Dako, and blocked with 2% normal horse serum in PBS for 1 h. The sections were incubated with anti-$ankyrin_R$ or anti-$ankyrin_B$ antibody (1,000-fold dilution in 1% BSA in PBS) for 1 h at room temperature or overnight at 4°C, and then immunostained by the avidin-biotin-peroxidase complex (ABC) method of Hsu *et al.* (*27*) using an ABC kit. The peroxidase label was visualized by exposing the sections to diaminobenzidine. For preadsorption of the antibodies, the epitopes of $ankyrin_R$ and $ankyrin_B$ were extracted from the Triton X-100 residue of erythrocyte ghost membrane and brain microsomes, respectively, with 1.0 M KCl by the method of Bennett and Stenbuck (*28*).

RESULTS

We previously reported the P1 and P2 fractions as myofibril-nucleus and membrane fractions, respectively (*22, 23*). As shown in the immunoblots presented in Fig. 1, large portions of $ankyrin_B$ and $ankyrin_R$ were distributed to the P1 fraction, while 4.9% of $ankyrin_R$ was associated with the P2 fraction, as shown in Table I. The molecular mass of myocardial $ankyrin_B$ was 222 kDa, being larger than that of the brain counterpart (212 kDa), while both myocardial and brain $ankyrin_R$ migrated at 239 kDa, being slightly larger than erythrocyte $ankyrin_R$.

We then examined the effect of ischemia duration (5-40 min) on the amounts of the two isoforms following subsequent reperfusion for 30 min. Figure 2 shows that as ischemia duration increased, $ankyrin_B$ was preferentially reduced in the P1 fraction, while $ankyrin_R$ was selectively decreased in the P2 fraction.

The proteolysis progressed rapidly during reperfusion after 20 min of ischemia, as shown in Fig. 3. The preferential proteolysis of $ankyrin_B$ in the P1 fraction and that of

TABLE I. **Distribution of $ankyrin_R$ and $ankyrin_B$ in the control heart.** Each expressed as % of the total. For details, see the text.

Fraction	$Ankyrin_R$	$Ankyrin_B$	Protein
P1	90.6%	84.2%	61.7%
P2	4.9%	2.5%	17.9%
S	4.5%	13.3%	22.0%

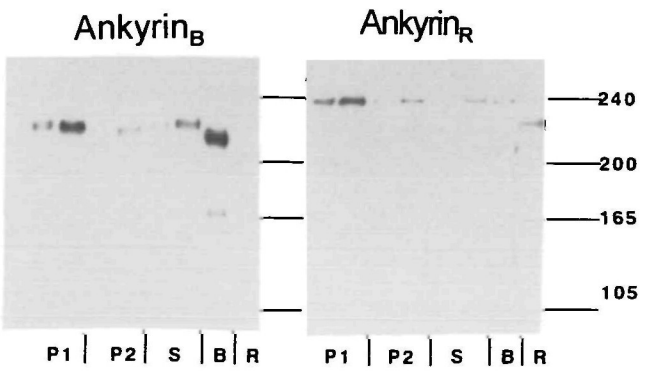


Fig. 1. **Distribution of $ankyrin_R$ and $ankyrin_B$ in the subcellular fractions of the rat myocardium.** The P1 (myofibril-nucleus), P2 (membrane), and S (cytosolic) fractions (5 and 20 μg), brain microsomes (B) and red blood cell ghosts (R) were immunoblotted with anti-$ankyrin_R$ antibody or anti-$ankyrin_B$ antibody. The molecular weight of the standard proteins is shown on the right. For details, see the text.

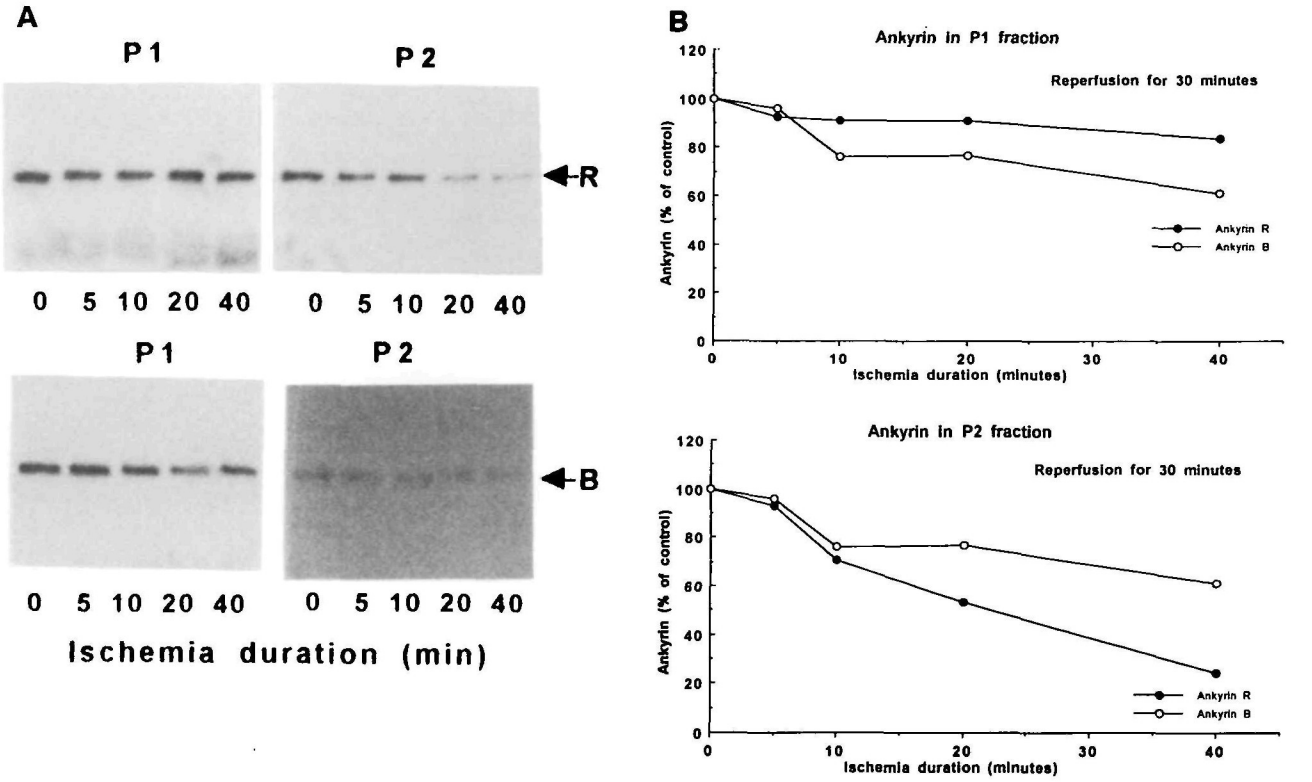


Fig. 2. **Effect of ischemia duration on the proteolysis of ankyrin isoforms.** Proteolysis of ankyrin$_R$ (R, closed circles) and ankyrin$_B$ (B, open circles) of the P1 and P2 fractions in the heart after various durations of ischemia followed by a fixed time (30 min) of reperfusion is shown in representative immunoblots (panel A) and in the graph showing the quantification of the blots (panel B, mean of two experiments for each point, shown as % of no-ischemia value). For details, see the text.

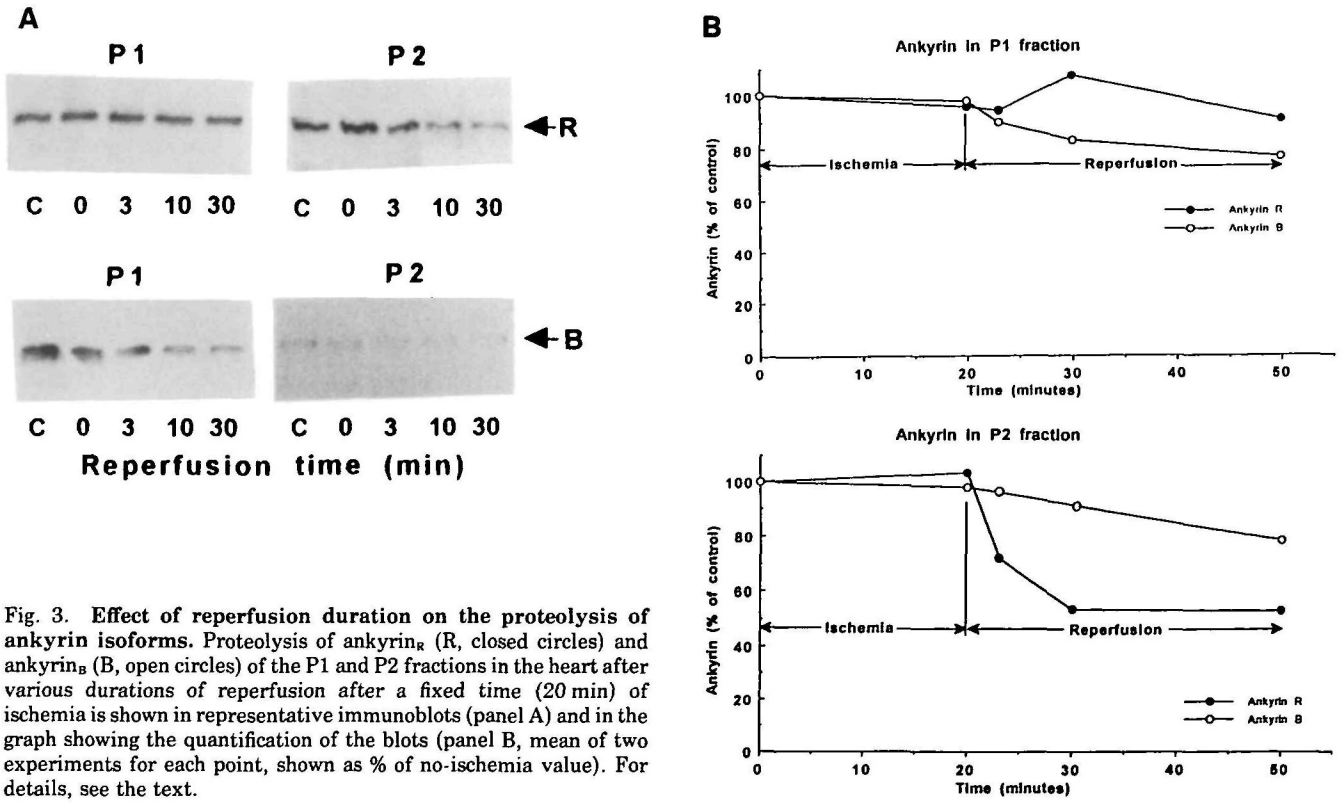


Fig. 3. **Effect of reperfusion duration on the proteolysis of ankyrin isoforms.** Proteolysis of ankyrin$_R$ (R, closed circles) and ankyrin$_B$ (B, open circles) of the P1 and P2 fractions in the heart after various durations of reperfusion after a fixed time (20 min) of ischemia is shown in representative immunoblots (panel A) and in the graph showing the quantification of the blots (panel B, mean of two experiments for each point, shown as % of no-ischemia value). For details, see the text.

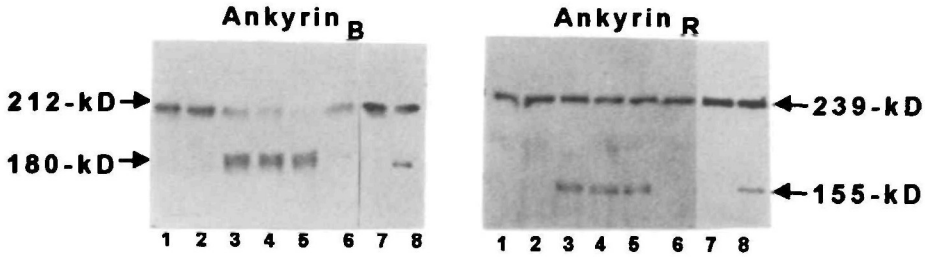
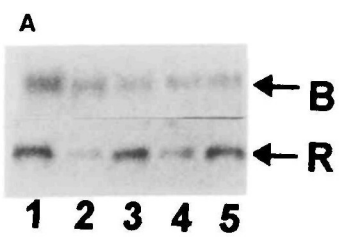


Fig. 4. **Comparison of ankyrin proteolysis after ischemia-reperfusion and *in vitro* proteolysis.** The sizes of the fragments after *in vitro* proteolysis of ankyrin$_R$ and ankyrin$_B$ in the P1 fraction by purified μ-calpain (Lanes 1-6) and those after ischemia-reperfusion (Lanes 7 and 8) are shown (arrows) with their molecular masses. In the *in vitro* proteolysis experiment, incubation times (lane numbers are shown in parenthesis) were 0 (1), 3 (2), 10 (3), 30 (4), and 60 (5) min and 60 min in the presence of ALLnaL (6). ALLnaL (100 μM) inhibited the proteolysis. The molecular weights of the proteolytic fragments in the *in vitro* experiments were the same as those of ankyrin$_R$ in the P2 fraction and ankyrin$_B$ in the P1 fractions generated after ischemia (20 min)-reperfusion (30 min) (Lane 8) but not seen in the control heart (Lane 7). Ankyrin$_B$ shows a faster rate of proteolysis than does ankyrin$_R$.



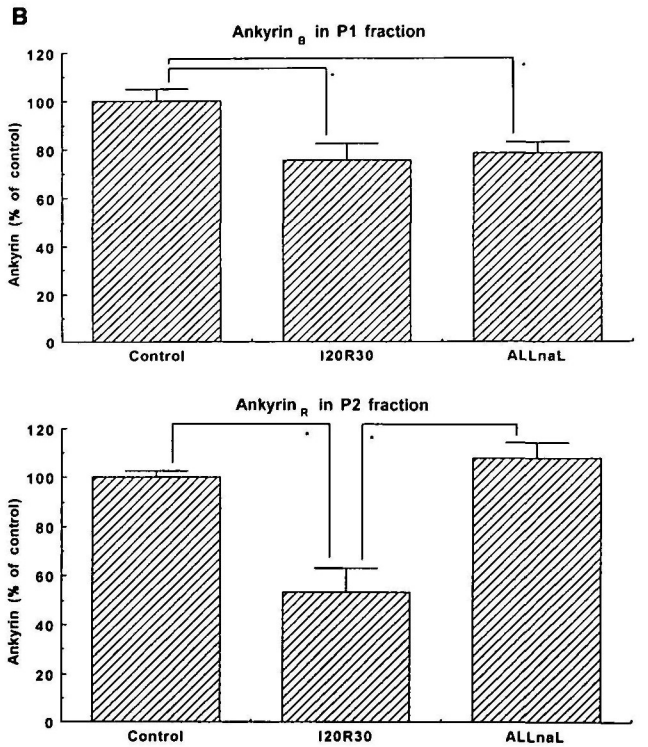


Fig. 5. **Inhibitory effect of protease inhibitors on the proteolysis of ankyrin isoforms after ischemia-reperfusion.** Panel A contains the representative immunoblots showing the bands of ankyrin$_R$ of the P2 fraction (R) and ankyrin$_B$ of the P1 fraction (B) in the control heart (Lane 1), the heart after ischemia (20 min)-reperfusion (30 min) alone in 0.1% DMSO (Lane 2, solvent for inhibitors), after ischemia-reperfusion in the presence of ALLnaL (Lane 3) or ZLLLal (Lane 4) at 30 μM and after ischemia with low Ca^{2+} reperfusion (Lane 5). Panel B shows the quantification of the blots (number of animals for control=4, ischemia-reperfusion=6, 100 μM ALLnaL=6). * indicates significant difference ($p<0.05$).

ankyrin$_R$ in the P2 fraction was confirmed. Ischemia for 60 min did not induce ankyrin proteolysis (data not shown), as reported previously (*12*).

μ-Calpain treatment of the P1 fraction generated a 155-kDa fragment of ankyrin$_R$ and a 180-kDa fragment of ankyrin$_B$, as shown in Fig. 4. Fragments with the same sizes were detected after ischemia-reperfusion, although the amount of the fragments was small and they were not consistently detectable under the conditions used to measure native ankyrin$_B$ and ankyrin$_R$.

To examine the involvement of calpain in the ankyrin proteolysis during ischemia-reperfusion, we examined the effect of inhibitors of calpain, ALLnaL and ZLLLal, that also inhibit proteasome (*29*). ALLnaL and ZLLLal suppressed the *in vitro* proteolysis of ankyrin$_R$ and calspectin (α-subunit) in the P2 fraction by m-calpain with attaining maximum inhibition at 3-10 μM (data not shown). Figure 5 shows the effect of 30 μM ALLnaL or 30 μM ZLLLal on the proteolysis after ischemia (20 min)-reperfusion (30 min). ALLnaL inhibited the proteolysis of ankyrin$_R$ in the P2 fraction (maximally at 30-100 μM) but not that of ankyrin$_B$ in the P1 fraction, while ZLLLal did not inhibit the proteolysis of ankyrin$_R$ or ankyrin$_B$. Low Ca^{2+} reperfusion with KH buffer without $CaCl_2$ also reduced ankyrin$_R$ proteolysis. Careful examination of ZLLLal at 30 μM or higher in the KH solution revealed fine turbidity, indicating insolubility of ZLLLal in the solution.

The immunohistochemical localization of the ankyrin isoforms is shown in Fig. 6. In the acetone-methanol-fixed sections, ankyrin$_R$ was localized in the striations, sarcolemma and nuclei (panel A), and the staining for striations and sarcolemma was reduced after ischemia-reperfusion (panel C). Preadsorption of anti-ankyrin$_R$ antibody with ankyrin$_R$ epitope derived from erythrocyte ghosts reduced the labeling (panel B). In the PLP-fixed section, the myocardium was diffusely stained, with the striations and sarcolemma being less visibly delineated (panel D). On the other hand, there was barely visible staining for ankyrin$_B$ in the sections after acetone-methanol fixation (data not shown), while the staining for ankyrin$_B$ was diffuse and did not delineate specific organelles except for the nucleus after PLP-fixation (panel E). The ankyrin$_B$ staining was reduced after ischemia-reperfusion (panel G). Preadsorption of anti-ankyrin$_B$ antibody with ankyrin$_B$ epitope derived from brain microsome (panel F) or incubation without the primary antibodies (panel H) diminished the labeling.

To explore the physiological implications of ankyrin proteolysis, glycoproteins and Na^+-K^+-ATPase in the membrane-skeleton were examined by Con A-binding and by Na^+-K^+-ATPase immunoreactivity on nitrocellulose membranes. There was no significant dissociation of

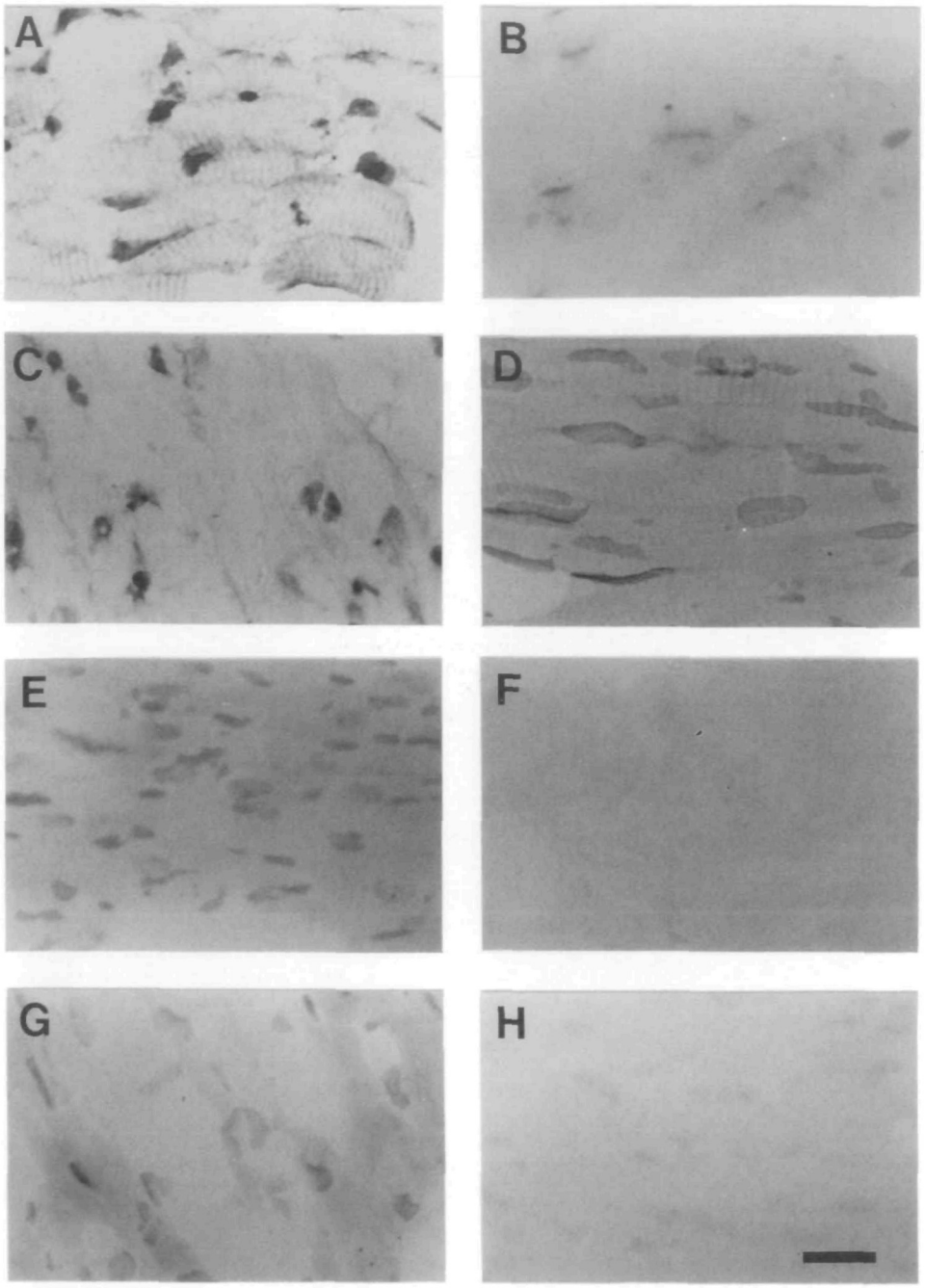


Fig. 6. **Immunohistochemical localization of ankyrin isoforms.** The results of immunostaining for ankyrin$_R$ (panels A-D) and ankyrin$_B$ (panels E-H) in cryosections of control (panels A, B, D, E, F, and H) and ischemia (20 min)-reperfused (30 min) hearts (panels C and G) after acetone-methanol fixation (panels A-C) or after PLP-fixation (panels D-H) are shown. Preadsorption of antibody to ankyrin$_R$ with its epitope (panel B), preadsorption of antibody to ankyrin$_B$ with its epitope (panel F), or incubation without primary antibody (panel H) diminished the staining. Bar = 10 μm.

Na^+-K^+-ATPase or Con A-binding proteins from the membrane-cytoskeleton after ischemia-reperfusion (data not shown).

DISCUSSION

This study demonstrates the preferential proteolysis of ankyrin$_R$ in the membrane (P2) fraction and ankyrin$_B$ in the myofibril-nucleus (P1) fraction during postischemic reperfusion of the rat heart, although both ankyrin isoforms are located predominantly in the myofibril-nucleus fraction (Figs. 2 and 3). The immunohistochemical analysis showed the distribution of ankyrin$_R$ to the striations, sarcolemma and intercalated discs (Fig. 6). These observations are consistent with the report of Li *et al.* (*8*), showing that the anti-ankyrin$_R$ antibody labels the striation pattern that corresponds to the T tubules strongly and the peripheral sarcolemma weakly. Alternatively, the striation pattern may represent the "constamere" which Nelson and Lazarides found in the skeletal muscle as the labeling of ankyrin$_R$ and $\alpha\beta$-spectrin on the sarcolemma in the form of rings, transverse to the long axis of the muscle fiber, which surround and bind with the myofibrils at the level of the Z-lines (*30*). Ischemia-reperfusion strikingly reduced the ankyrin$_R$ labeling of the striations (Fig. 6), consistent with the substantial decrease in ankyrin$_R$ in the membrane fraction in the immunoblotting analysis (Figs. 2 and 3). In contrast, the anti-ankyrin$_B$ antibody stained myocardium

diffusely, with no particular organelles except nuclei being delineated (Fig. 6). Different expressions of ankyrin$_R$ (restricted) and ankyrin$_B$ (diffuse) were also reported in other tissues. In the brain, ankyrin$_R$ is localized at the nodes of Ranvier (*16*), while in the kidney, ankyrin$_R$ is restricted to the basolateral domains of distal tubular cells and intercalated discs of collecting ducts (*17*).

The preferential proteolysis of ankyrin$_R$ (this study) and calspectin (*18*) in the membrane fraction and the inhibition of the proteolysis either by calpain inhibitors ALLnaL, ZLLLal or by low Ca^{2+} reperfusion (Fig. 5) suggest that the two proteins lie in the vicinity of the membrane and are proteolyzed by calpain. The involvement of calpain was also supported by the identical-sized fragments of ankyrin$_R$ and ankyrin$_B$ produced *in vitro* with calpain and those generated after ischemia-reperfusion (Fig. 4). In addition, we previously showed calpain activation in the membrane fraction after ischemia-reperfusion (*19, 21*). It was reported that 100 μM but not 10 μM ZLLLal inhibited the autolysis of μ-calpain in erythrocytes after Ca^{2+} ionophore treatment, although 10 μM ZLLLal almost completely inhibited m-calpain activity *in vitro* (*29*). The inability of ZLLLal to inhibit the proteolysis (even at 100 μM) of ankyrin and calspectin after ischemia-reperfusion despite its effectiveness in the *in vitro* experiment suggest lower permeability (solubility) of ZLLLal than ALLnaL, rather than differential selectivity of the inhibitors against calpain and proteasome. We previously reported that ALLnaL greatly improved the contractile dysfunction during postischemic reperfusion through inhibiting calspectin proteolysis in the sarcolemma (*18*). Since ankyrin$_R$ is located closer to the sarcolemma than is calspectin (*1, 2*), the proteolysis of ankyrin$_R$ may affect the force conduction from the myofibril to the sarcolemma more profoundly than does calspectin proteolysis. On the other hand, the ineffectiveness of ALLnaL in inhibiting ankyrin$_B$ proteolysis may be because the inhibitor does not readily diffuse to the ankyrin$_B$ location or because calpain is not involved in the proteolysis.

As for the implications of ankyrin proteolysis, Hall and Bennett reported that calpain proteolysis results in a major conformational change in ankyrin$_B$ (*31*), whereas Srinivasan *et al.* did not find a change in the binding of ankyrin$_B$ fragment (195 kDa) with voltage-dependent Na^+ channel after limited proteolysis by calpain (*7*). We did not find evidence for the involvement of ankyrin proteolysis in the dissociation of a membrane protein, Na^+-K^+-ATPase, from the membrane-cytoskeleton. However, the activity of Na^+-K^+-ATPase may still be affected by the ankyrin proteolysis, because the ankyrin binding site of the enzyme was shown to lie in close proximity to the catalytic center of the enzyme (*32, 33*). Besides Na^+-K^+-ATPase, ankyrin interacts with the Na^+/Ca^{2+} exchanger (*8*), the ryanodine receptor (*9, 10*), adhesion molecules (L1, uvomorulin, cadherin), and some glycoproteins (*5, 6, 34, 35*). In addition, Na^+/Ca^{2+} exchanger colocalizes with ankyrin$_R$ at T-tubules (*36*), while the ryanodine receptor lies at the junction of the T-tubules and the sarcoplasmic reticulum, and is very sensitive to endogenous calpain during its purification (*37*). Proteolysis of ankyrin may modulate the functions of these ankyrin-binding proteins during ischemia-reperfusion or under other pathological conditions.

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