J. Membrane Biol. 85, 159–168 (1985)

The Journal of
Membrane Biology

Proteolysis of Cardiac Gap Junctions during their Isolation from Rat Hearts

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Summary. Gap junctions (GJ) isolated from rat hearts in presence of the protease inhibitor phenylmethylsulfonylfuoride (PMSF) contain a M_r 44,000 to 47,000 major polypeptide and have a urea-resistant layer of fuzz on their cytoplasmic surfaces, whereas junctions isolated without PMSF are proteolyzed to a M_r 29,500 polypeptide by a serine protease and have smooth cytoplasmic surfaces (C.K. Manjunath, G.E. Goings & E. Page *Am. J. Physiol.* **246**:H865–H875, 1984). Rat liver GJ isolated with or without PMSF contain a M_r 28,000 polypeptide and have smooth cytoplasmic surfaces. Here we examine the origin, type and inhibitor sensitivity of the heart protease; why similar proteolysis is absent during isolation of rat liver gap junctions; and whether the M_r 44,000 to 47,000 cardiac GJ polypeptide is the precursor of the M_r 29,500 subunit. We show that the M_r 44,000 to 47,000 polypeptide corresponds to the unproteolyzed connexon subunit; that proteolysis of this polypeptide occurs predominantly during exposure to high ionic strength solution (0.6 M KI) which releases serine protease from mast cell granules; that this protease is inhibitable with PMSF and (less completely) soybean trypsin inhibitor and chymostatin; and that *in vivo* degranulation of mast cells by injecting rats with compound 48/80 fails to prevent breakdown of cardiac GJ during isolation. The results support the concept that GJ from rat heart and liver differ in protein composition.

Key Words gap junctions · cell-to-cell channels · mast cell protease · cardiac membranes

Introduction

Gap junctions, which contain the cell-to-cell channels responsible for intercellular communication, have been isolated from mammalian liver, heart and lens tissues. Although electron micrographs show that junctions isolated from different mammalian tissues appear structurally similar, a wide range of molecular weights has been reported for the protein components of the isolated junctions. These differences in molecular weight have been shown to result in part from the proteolytic enzymes formerly used in isolating gap junctional membranes. With the introduction of isolation methods that eschew the use of exogenous proteolytic enzymes, the range of molecular weights of the apparent polypeptide subunits in gap junctions from different tissues became much narrower: Polypeptide subunits with M_r 26,000 to 28,000 were obtained for liver gap junctions (Hertzberg et al., 1978; Henderson et al., 1979; Hertzberg, 1980; Nicholson et al., 1981), M_r 28,000 to 29,000 for cardiac gap junctions (Kensler & Goodenough, 1980; Manjunath et al., 1982*a*,*b*; Gros et al., 1983), and $M_r \sim 26{,}000$ for lens gap junctions (Brookhuyse et al., 1976; Alcala et al., 1978; Goodenough, 1979). The inference of apparent similarity based on the molecular weights of junctional subunits from different tissues was not, however, confirmed by subsequent studies. Two-dimensional peptide mapping (Gros et al., 1983; Nicholson et al., 1983) showed few or no homologies between the polypeptides of junctions isolated from liver, heart and lens. Furthermore, the gap junctional origin of the polypeptide obtained from lens now seems uncertain (*see* Gros et al., 1983; Nicholson et al., 1983).

We have recently reported that rat heart gap junctions prepared in the presence of the serine protease inhibitor phenylmethylsulfonylfluoride (PMSF) contain a major polypeptide of M_r 44,000 to 47,000 and conspicuously lack the M_r 29,500 polypeptide characteristic for cardiac gap junctions prepared without PMSF (Manjunath et al., 1984*b*; Manjunath & Page, 1984). Moreover, electron micrographs showed that cardiac gap junctions prepared with PMSF have a fuzzy layer on their cytoplasmic surfaces which could not be removed with 8 M urea, whereas the cytoplasmic surfaces of junctions prepared without PMSF were invariably smooth. By contrast, rat liver gap junctions prepared either with or without PMSF had a M_r of 28,000 and were devoid of cytoplasmic fuzz (Nicholson et al., 1983; Manjunath et al., 1984*a*). We interpreted our results as suggesting that cardiac

(but not liver) gap junctions have a M_r 14,500 to 17,500 cytoplasmic surface component that is covalently bound to the M_r 29,500 component in the lipid bilayer and interstitial gap. We further suggested that, in the absence of PMSF, this cytoplasmic surface component is cleaved during the isolation procedure by a PMSF-inhibitable serine protease, presumably originating in mast cells (Woodbury et al., 1978*a*,*b*).

These new observations, indicating that rat cardiac gap junctions differ from rat liver gap junctions in previously unrecognized major respects, seemed to us to raise three questions. First, why is the PMSF effect that is observable in rat cardiac gap junctions absent in rat liver gap junctions? Secondly, is the 44,000 to 47,000 molecular weight of the polypeptide present in rat heart gap junctions prepared with PMSF the precursor of the proteolyzed M_r 29,500 subunit? And, thirdly, how does the enzymatic proteolysis that cardiac gap junctions undergo during isolation of junctional membranes fit into what is known about enzymatic proteolysis in heart muscle?

The experiments described in this paper were designed to investigate these questions. The results strongly support the idea that rat heart and liver gap junctions differ in protein composition; they indicate that the basic channel subunit of cardiac gap junctions is broken down during isolation of the junctions by a serine protease originating in mast cell granules and liberated during the extraction of myofibrillar proteins with high ionic strength solutions; they suggest that the conditions used to isolate liver gap junctions do not release the proteases present in mast cell granules, hence do not result in proteolysis of the junctions; and they lead to the conclusion that the molecular weight of the polypeptide subunit isolated from cardiac gap junctions in the presence of PMSF is probably that of the native subunit.

Materials and Methods

Isolation of Rat Heart Gap Junctions

Control gap junctions were isolated from the hearts of Sprague-Dawley rats of either sex (four animals per preparation) as described in Manjunath et al. (1984*b*) with a single modification, as follows: Myofibrillar proteins were extracted overnight with 0.6 M KI, 6 mM $Na_2S_2O_3$, 1 mM $NaHCO_3$, pH 8.2, at 4°C. The resulting suspension was layered directly over 35% sucrose, 0.6 M KI, 5 mM $Na_2S_2O_3$, 1 mM $NaHCO_3$, pH 8.2, and centrifuged at 22,000 rpm for 60 min at 4°C in a Beckman SW 28 rotor. After completion of this centrifugation, cardiac membranes were collected from the sample/35% sucrose interface and pelleted after dilution with 0.6 M KI, 6 mM $Na_2S_2O_3$ in 5 mM Tris, pH 9.0, by centrifugation at 15,000 rpm for 30 min in a Sorvall SS-34 rotor. The pellets were washed by resuspension and recentrifugation in 5 mM Tris, pH 9.0, and processed by detergent solubilization and density gradient centrifugation exactly as described in Manjunath et al. (1984*b*).

When gap junctions were prepared in the presence of a protease inhibitor, the inhibitor was included in all the isolation media except the 0.3% deoxycholate (DOC) solution and the sucrose/DOC solutions used in density gradient centrifugations.

PMSF (Sigma Chemical Co., St. Louis, Missouri) was used at a final concentration of 1 mM by hourly additions of a stock solution (0.2 M) in ethanol.

Chymostatin (Sigma) was dissolved in dimethylsulfoxide (DMSO) to give a final concentration of 10 μM at all steps except the step during which myofibrillar proteins were extracted with 0.6 M KI; during this step, the inhibitor concentration was increased to 50 μM without increasing the DMSO concentration.

Soybean trypsin inhibitor (STI, Type 1-S, Sigma) was directly dissolved in the buffers to give a final concentration of 100 μg/ml.

In Vivo Degranulation of Mast Cells by Treatment of Rats with Compound 48/80

The drug treatment protocol of Pastan and Almqvist (1966) as modified by McKee et al. (1979) was used. Compound 48/80 (condensation product of N-methyl-*p*-methoxyphenethlamine with formaldehdye, Sigma) was dissolve in 0.9% saline and injected intraperitoneally into male Sprague-Dawley rats (350 to 400 g). Control rats received saline injections. The control and drug-injected rats were anesthetized with ether and sacrificed, and cardiac gap junctions were prepared without adding protease inhibitors at any step of the isolation procedure.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gap junctional pellets were dissolved in the sample buffer (2.5% SDS, 5% β-mercaptoethanol) by heating for 2 min at 100°C. Electrophoresis was performed on 12.5% slab gels according to Laemmli (1970). The reference proteins (Pharmacia Fine Chemicals, Piscataway, New Jersey) were: lactalbumin (M_r 14,400), soybean trypsin inhibitor (20,100), carbonic anhydrase (30,000), ovalbumin (43,000), bovine serum albumin (67,000) and phosphorylase b (94,000). Protein bands were visualized by staining the gels with Coomassie Brilliant Blue R250 (Sigma).

Electron Microscopy

Pellets enriched in gap junctions were fixed with glutaraldehyde, post-fixed with OsO_4, dehydrated, embedded, thin-sectioned, stained with uranium and lead salts, and studied in the electron microscope as previously described (Manjunath et al., 1984*b*).

Results

Figure 1 is an electron micrograph of thin-sectioned and positively stained rat heart gap junctions prepared in the presence of 1 mM PMSF by the modi-

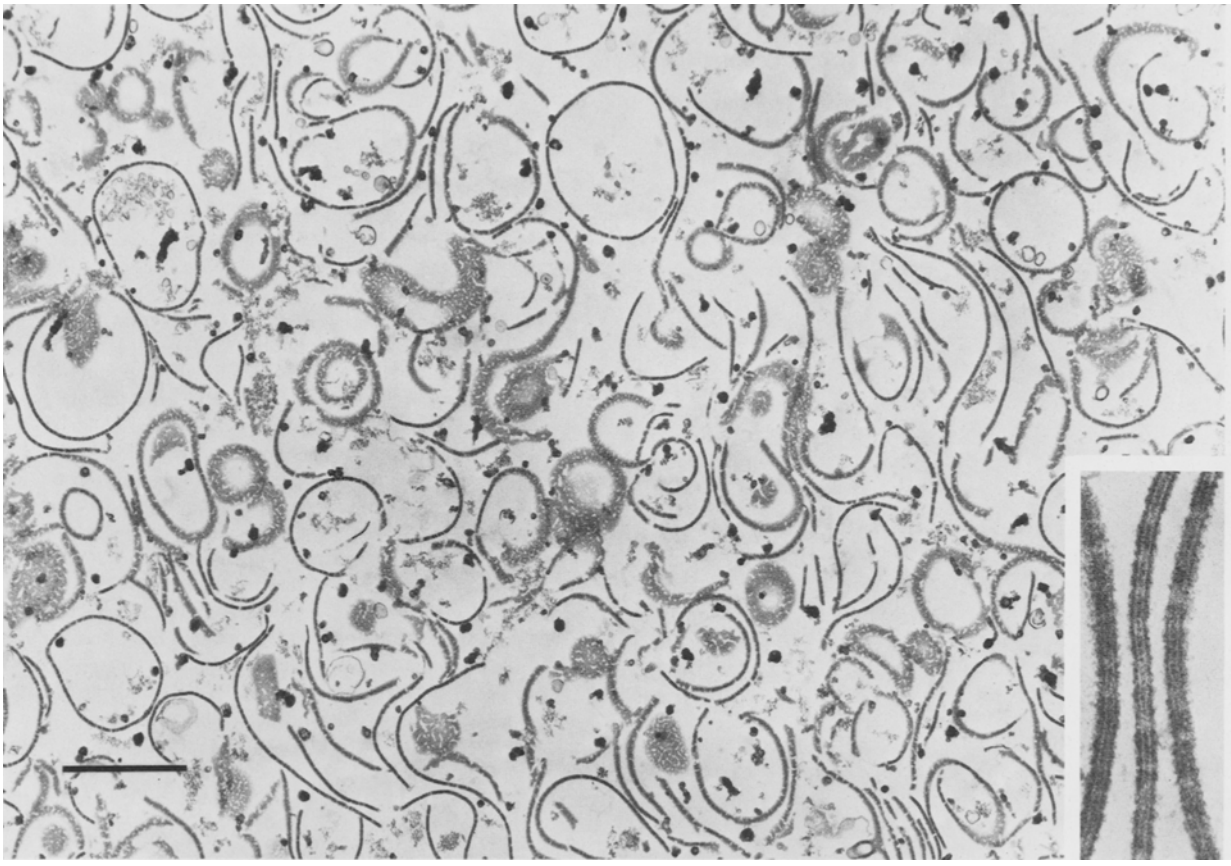

Fig. 1. Electron micrograph of thin-sectioned pellet of rat heart gap junctions isolated in the presence of 1 mM PMSF. The pellet shows gap junctional membranes intermixed with amorphous material. Calibration bar: 1 μm. Inset ($\times$ 119 $\times$ 10^3) shows the fuzzy cytoplasmic surfaces of the junctions

fied isolation procedure. The preparation is highly enriched in gap junctions but is contaminated with a significant amount of amorphous material. The cytoplasmic surfaces of the gap junctions appear fuzzy (insert).

SDS-PAGE of the preparation (Fig. 2*B*) reveals the presence of multiple protein bands. Virtually identical SDS-PAGE patterns were obtained when gap junctional pellets were dissolved in the sample buffer (2.5% SDS, 5% β-mercaptoethanol), either at room temperature for 30 min or at 100°C for 2 min. The two most prominent bands are the diffuse band at M_r 44,000 to 47,000 and the band at M_r > 100,000. We have shown previously (Manjunath et al., 1984*b*) that the bands at M_r 44,000 and > 100,000 and also most of the minor bands above M_r 47,000 are nonjunctional contaminants.

Figure 2 also demonstrates the effect of added PMSF during the early stages of the isolation procedure on the SDS-PAGE pattern of purified junctions. In this experiments (Fig. 2, lanes C–F), rat hearts were homogenized in 1 mM $NaHCO_3$, pH 8.2, and the membrane-rich pellets were prepared in the absence of PMSF. The pellets were then suspended in 0.6 M KI, 6 mM $Na_2S_2O_3$, 1 mM $NaHCO_3$, pH 8.2, and stirred at 4°C. PMSF was added to the different suspensions at intervals of 0, 3, 9 and 16 hr after the start of stirring. All samples were extracted with 0.6 M KI for a minimum of 16 hr to eliminate adsorption artifacts. During the subsequent preparation of gap junctions from these samples, 1 mM PMSF was included in each step of the isolation procedure.

Figure 2 shows that, when PMSF is omitted from the initial homogenization medium but present in the media used for all subsequent isolation steps (including the 0.6 M KI solution used to extract myofibrils), a small but significant breakdown of the gap junctional protein occurs (*compare* lane C with lane B). This leads to a decrease in the intensity of the M_r 44,000 to 47,000 band and the intensification of the bands at M_r 29,500, 31,000 and 34,000. After 3

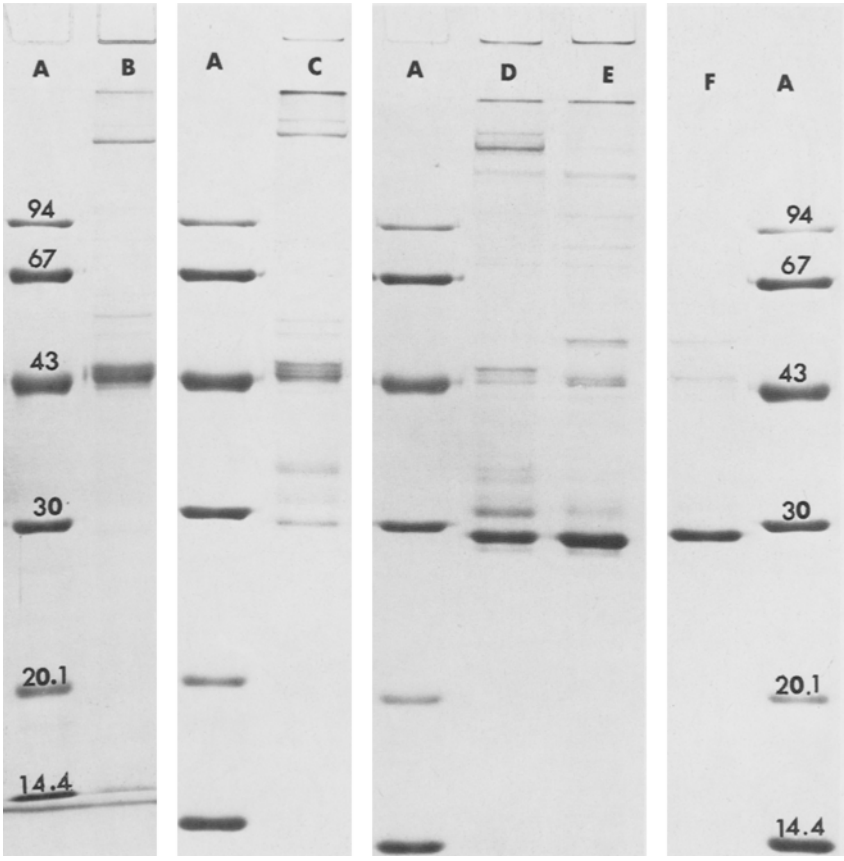

Fig. 2. SDS-polyacrylamide gel electrophoretic analysis of the proteolysis of rat heart gap junctions during isolation. Rat heart gap junctions were isolated as described under Materials and Methods. PMSF was added at different stages in the isolation procedure to terminate proteolysis. Lane *A*, reference proteins and molecular weights in kilodaltons. Lane *B*, PMSF present throughout the isolation procedure. Lane *C*, PMSF absent during initial homogenization but present during 0.6 M KI extraction and all subsequent steps. Lane *D*, PMSF added after 3 hr of extraction of cardiac membranes with 0.6 M KI. Lane *E*, PMSF added after 9 hr of extraction of cardiac membranes with 0.6 M KI. Lane *F*, PMSF added after 16 hr of extraction of cardiac membranes with 0.6 M KI

hr of incubation in 0.6 M KI (lane D), the M_r 29,500 begins to appear as a major band, associated with the somewhat less intense M_r 31,000 band. Decreases in the intensities of the M_r 44,000 to 47,000 band and (to a lesser extent) of the M_r 34,000 band are evident at this stage. After 9 hr of incubation in the 0.6 M KI (lane E) the breakdown of gap junctional proteins to produce the M_r 29,500 band is almost complete, with the consequence that the M_r 31,000, 34,000 and 44,000 to 47,000 bands are reduced to minor bands on the gel. In addition to the gap junctional bands, the M_r > 100,000 nonjunctional band is also proteolyzed after 6 hr of incubation. Further incubation of the suspension for up to 16 hr does not generate any product smaller than M_r 29,500 (lane F). The M_r 29,500 band produced during the 0.6 M KI-extraction is not degraded further even when PMSF is absent during the rest of the isolation procedure (Fig. 5, lane B).

The proteolysis of gap junctions when they are extracted with 0.6 M KI at slightly alkaline pH and the inhibition of this proteolysis by PMSF strongly suggest the involvement of a serine protease originating in mast cells. In addition to PMSF (Murakami & Uchida, 1978; Bird & Carter, 1980) this protease is also known to be inhibited by chymostatin (Libby & Goldberg, 1980*a*,*b*) and soybean trypsin inhibitor (Griffin & Wildenthal, 1978; Murakami & Uchida, 1978). We therefore tested the effects of these two inhibitors on cardiac gap junctions. Figure 3 shows the SDS-PAGE patterns of rat heart gap junctions prepared in the presence of 10 μM chymostatin (lane A) and 100 μg/ml soybean trypsin inhibitor (lane B). The SDS-PAGE patterns of the two preparations are similar: both preparations contain several bands in the range between M_r 29,500 to 47,000. Of these, the most prominent bands, those at M_r 29,500, 31,000 and 47,000, are of roughly equal intensity. We consider it particularly noteworthy that the SDS-PAGE patterns of the preparations made with soybean trypsin inhibitor and chymostatin resemble the patterns of those gap junctional preparations made with PMSF in which PMSF was omitted during the initial homogenization but was present during the 0.6 M KI extraction and all subsequent steps (lane D and Fig. 2C, shown here for comparison). The soybean trypsin inhibitor and chymostatin preparations also show a trace amount of the M_r > 100,000 band, but the band at M_r 44,000 is conspicuously absent (arrow).

Figure 4 compares the electron microscopic appearance, in thin sections, of rat heart junctions prepared in the presence of soybean trypsin inhibitor (STI, 100 μg/ml) (4*B*) with those prepared in the absence of protease inhibitors (4*A*) and in the presence of 1 mM PMSF (4*C*). The STI-treated pellet contains both gap junctions with fuzzy cytoplasmic surfaces and gap junctions with smooth cytoplasmic surfaces (arrow). The appearance of this preparation is thus intermediate between that of PMSF-treated junctions (all cytoplasmic surfaces fuzzy) and proteolyzed control junctions (all cytoplasmic surfaces smooth).

We next studied the effect of inhibiting the mast cell protease *in vivo* on the electron-microscopic appearance and protein composition of isolated cardiac gap junctions. Injection of rats with compound 48/80 degranulates mast cells and thereby abolishes up to 90% of the alkaline serine proteolytic activity of rat heart tissue (McKee et al., 1979). Figure 5 compares the SDS-PAGE patterns of cardiac gap junctions from controls and 48/80-injected rats. The junction-enriched pellets were prepared without adding any protease inhibitors; the controls were saline-injected rats whose mast cells were not degranulated (lane B) and the experimental animals were rats injected with compound 48/80 whose mast cells were degranulated (lane C). The yield of gap junctions from the drug-injected rats was found to be much higher than that from the control rats as judged by the intensity of the protein bands on the gel and the number of gap junctions seen in unit area of thin sections. The control preparation shows a single major protein band at M_r 29,500, a pattern typical for proteolyzed rat heart gap junctional preparations. Even in the preparation made from rats treated with compound 48/80, the M_r 29,500 band is the major band, but the gel also shows two other prominent bands at M_r 31,000 and ~34,000, respectively.

Figure 6 shows the effect of treatment with compound 48/80 on the electron-microscopic appearance of isolated rat heart gap junctions. Gap junctions prepared from both control (Fig. 6*A*) and drug-treated hearts (Fig. 6*B*) have smooth cytoplasmic surfaces (inserts) and do not discernibly differ in appearance.

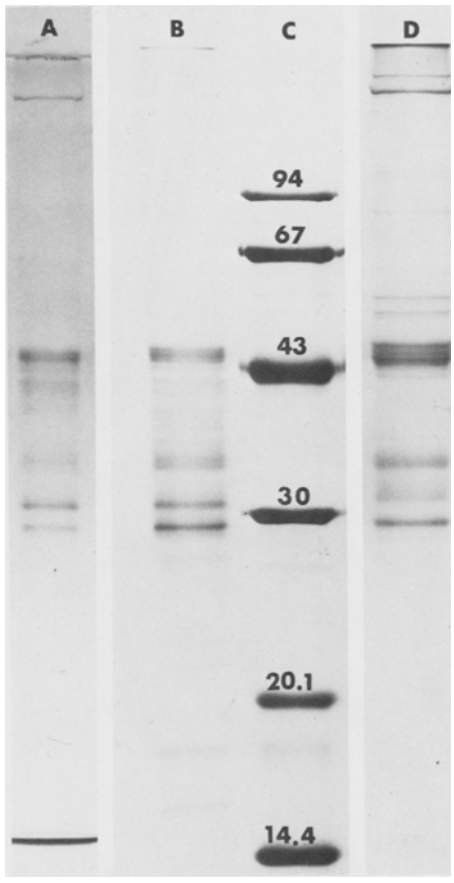

Fig. 3. SDS-polyacrylamide gel electrophoresis of rat heart gap junctions prepared in presence of chymostatin and soybean trypsin inhibitor. Lane *A*, gap junctions prepared in presence of chymostatin (10 μM). Lane *B*, gap junctions prepared in presence of soybean trypsin inhibitor (100 μg/ml). Lane *C*, reference proteins. Lane *D*, rat heart gap junctional preparation where PMSF was absent during the initial homogenization but present during the 0.6 M KI extraction and all subsequent steps. Arrow indicates the position of the M_r 44,000 band

Discussion

Proteolysis of Cardiac Gap Junctions During Membrane Isolation

To understand proteolytic breakdown of cardiac gap junctions during isolation of the junctional membranes, as well as to prevent or minimize such proteolysis, it is necessary to consider the properties of the proteolytic enzymes present in mammalian hearts. Several studies have demonstrated that homogenates of hearts contain both soluble cytoplasmic and particulate proteases that are active in the range of neutral and alkaline pH's used by us and others to isolate cardiac gap junctions (reviewed in Morgan et al., 1980). The soluble mammalian muscle neutral and alkaline proteases include the Ca^{2+}-activated neutral protease (Busch et al., 1972; Reddy et al., 1975; Dayton et al., 1976; Waxman & Krebs, 1978; Croall & DeMartino, 1983), the sulfhydryl-dependent neutral protease which is inhibitable by high Ca^{2+} concentrations (Koszalka & Miller, 1960) and the insulin-degrading

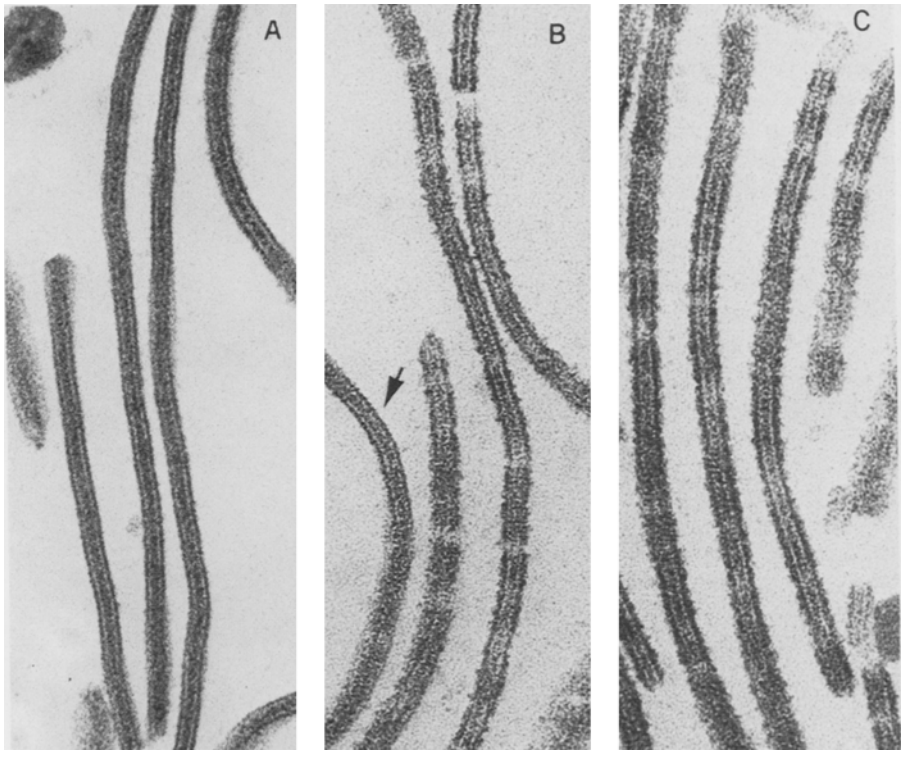

Fig. 4. Electron-microscopic appearance, in thin sections, of rat heart gap junctions isolated in the absence of protease inhibitors (*A*, proteolyzed), in the presence of soybean trypsin inhibitor (100 μg/ml) (*B*), and in the presence of 1 mM PMSF (*C*). The proteolyzed junctions have smooth cytoplasmic surfaces and the PMSF-treated junctions have fuzzy cytoplasmic surfaces. The soybean trypsin inhibitor-treated junctions show both smooth (arrowhead) and variably fuzzy regions. Magnification 156,250×

protease (Duckworth et al., 1972). These soluble cytoplasmic proteases are probably not involved in the *in vitro* degradation of gap junctions described in this paper. The vigorous homogenization during the first step of our isolation procedure would solubilize them, and would also release most of the lysosomal proteases. The proteolysis of cardiac gap junctions occurred predominantly during the second step of our isolation procedure (extraction of myofibrillar proteins at high ionic strength), at which stage the other proteases solubilized or released from lysosomes during the first step would have been removed by dilution and washing. Additional evidence against gap junctional proteolysis by soluble and lysosomal proteases released during homogenization was the failure of EDTA and leupeptin to prevent proteolysis during isolation of gap junctions by our procedure (Manjunath et al., 1984*b*). EDTA inhibits the Ca^{2+}-activated neutral protease (Croall & De Martino, 1983) (a soluble protease), and leupeptin inhibits lysosomal cathepsins B and L (Huisman et al., 1974; Kirshke et al., 1977; Libby & Goldberg, 1978).

Strong as is the argument against the involvement of soluble cytoplasmic and lysosomal proteases in the *in vitro* proteolysis of cardiac gap junctions described in this paper, the evidence implicating the particulate alkaline protease activity is even stronger. This activity, most of which is presumably localized within the granules of the mast cell (Woodbury et al., 1978*a*,*b*), is described in the literature as solubilizable only in solutions of high ionic strength, e.g., 0.5 to 0.8 M phosphate (Sanada et al., 1978; Everitt & Neurath, 1979), 1.1 M KI (Noguchi & Kandatsu, 1976) and 1 M KCl (Drabikowski et al., 1977; Griffin & Wildenthal, 1978). The initial homogenization step of our procedure for isolating cardiac gap junctions, during which the tissue is homogenized at low ionic strength in 1 mM $NaHCO_3$, pH 8.2, would therefore fail to solubilize the protease of mast cell granules. Instead, this protease would be present in the membrane pellet produced by the initial homogenization, either in a membrane-bound form or as a free precipitate. It would be released from the granules and solubilized during the exposure to 0.6 M KI solution in the second (myofibrillar extraction) step of our procedure. Moreover, the properties of the purified serine protease from mast cell granules are ideally suited for degrading cardiac gap junctions under the conditions used to isolate these junctions. These properties include its alkaline pH optimum; its inhibitability by PMSF, chymostatin, and soybean trypsin inhibitor, all three of which reagents protect cardiac gap junctions in our system against proteolysis to varying degrees; and the failure of the purified enzyme to be inhibited by EDTA and leupeptin, two reagents which also fail to protect

cardiac gap junctions against proteolysis in our isolation method. That soybean trypsin inhibitor and chymostatin gave only incomplete protection against proteolysis to cardiac gap junctions in our experiments may be explained by the large size and consequent slow diffusion of these molecules to the sites of proteolysis, particularly during the initial homogenization.

Since all the proteases present in the heart (lysosomal and nonlysosomal, particulate and soluble) would be released during the initial homogenization, we cannot be certain which serine proteases are responsible for the degradation of gap junctional proteins which occurs at this step (Fig. 2). The prevention of proteolysis during this step by PMSF does, however, rule out involvement of proteases other than serine proteases.

Unlike proteolyzed gap junctions prepared without PMSF, gap junctional pellets prepared in the presence of PMSF are contaminated with substantial amounts of amorphous material (Fig. 1). We have been unable to purify junctions made with PMSF to the same degree as proteolyzed gap junctions. By using sucrose-KI gradients, we succeeded in separating most but not all of the proteins that apparently precipitate in the presence of PMSF, but dissolve in the absence of the inhibitor, possibly due to proteolysis. The high degree of purity of cardiac gap junctions prepared without PMSF may be due to proteolysis of the contaminant bands such as that at $M_r > 100{,}000$ (Fig. 2*E*) during extraction with 0.6 M KI.

Injection of compound 48/80 into rats has been reported to inhibit up to 90% of the mast cell protease in the heart (McKee et al., 1979). However, most of the gap junctions isolated in the absence of PMSF from the hearts of compound 48/80-injected rats were proteolyzed. This finding could mean that the serine protease involved in the proteolysis of cardiac gap junctions was of nonmast cell origin and hence unaffected by compound 48/80. Alternatively, the 10% residual mast cell protease activity present in compound 48/80-treated hearts sufficed to proteolyze most of the gap junctions. We prefer the latter explanation in view of the prolonged incubation in 0.6 M KI and the high efficacy of the protease. Our interpretation is supported by the observation that, in the absence of PMSF, substantial proteolysis of gap junctions occurred within 3 hr of incubation at 4°C (Fig. 2). A similar proteolysis by a PMSF-inhibitable mast cell alkaline protease has also been observed during the preparation of rat cardiac myosin (Uchida et al., 1977). The tissues of rats contain especially high levels of the mast cell protease as compared to those of other mammals such as rabbits (Drabikowski et al., 1977). This difference between rats and rabbits in tissue mast cell protease content correlates well with our finding that gap junctions prepared in the absence of PMSF from rabbit hearts contain polypeptides larger than M_r 28,500 (Manjunath et al., 1982*a*), whereas rat heart junctions isolated without PMSF are completely degraded to the M_r 29,500 polypeptide.

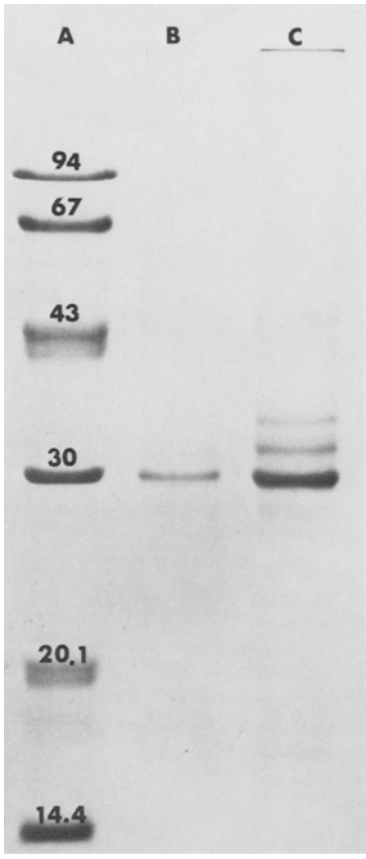

Fig. 5. Effect of injection of compound 48/80 on the SDS-PAGE pattern of rat heart gap junctions isolated in the absence of protease inhibitors. Rats were injected with compound 48/80 in saline or saline alone (controls) as described under Materials and Methods. Lane *A*, reference proteins. Lane *B*, gap junctions prepared from control rats. Lane *C*, gap junctions prepared from rats injected with compound 48/80

Differences in Proteolysis and Structure Between Liver and Cardiac Junctions

Purification of mammalian hepatic gap junctions is done without exposure to salt solutions of high ionic strength. Even the extraction of gap junctional membranes with 0.1 M Na_2CO_3, pH 11.0, to remove urate oxidase (Hertzberg & Gilula, 1979) is too brief and too alkaline to permit significant proteolysis by the serine protease of mast cell granules. It is there-

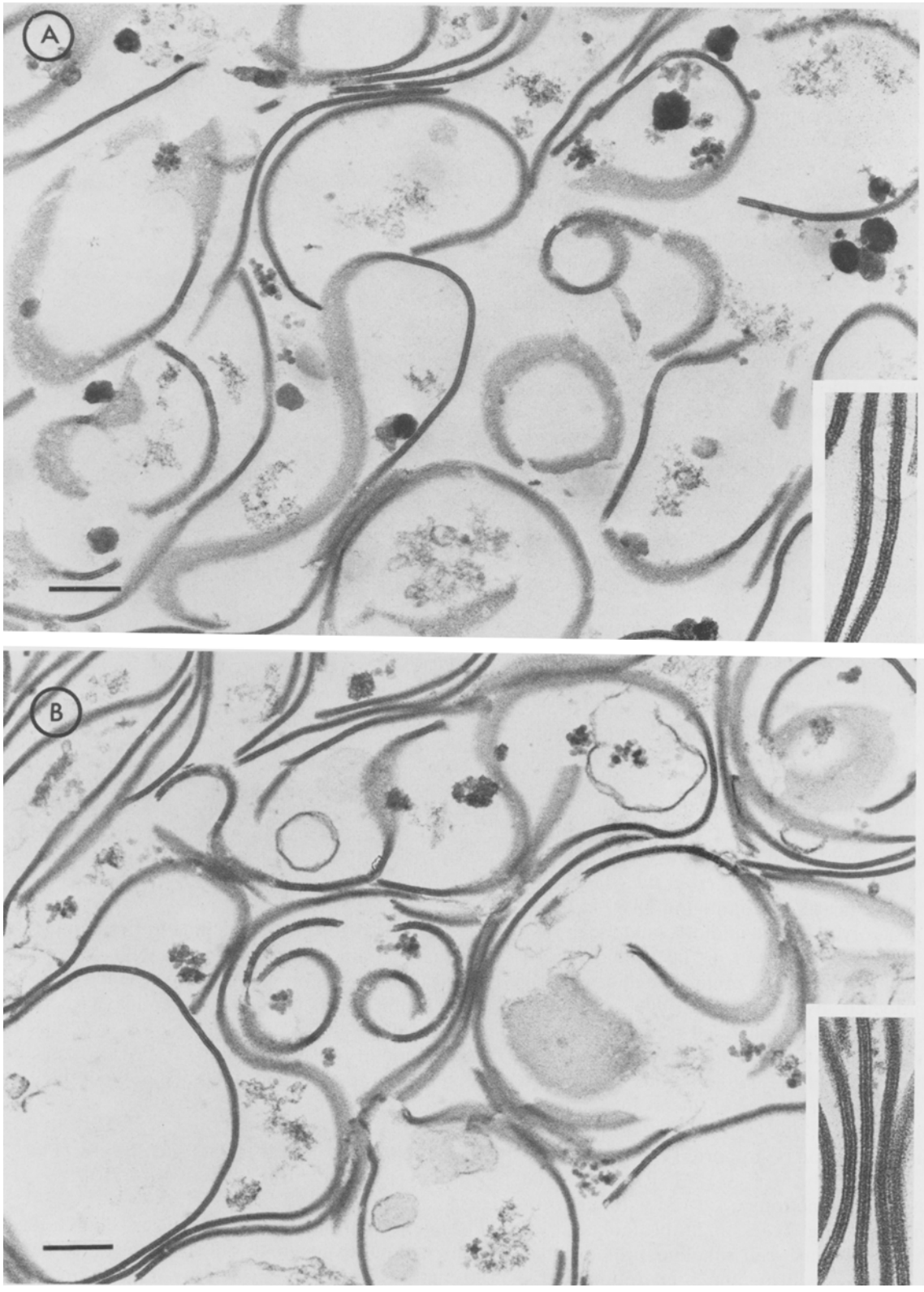

Fig. 6. Electron micrographs of thin-sectioned pellets of rat heart gap junctions isolated in the absence of protease inhibitors from saline-injected controls (*A*) and rats injected with compound 48/80 (*B*). Both pellets show similar enrichment in gap junctions. Calibration bar represents 0.2 μm. Insets ($\times$ 119 $\times$ 10^3) show that gap junctions from both preparations have smooth cytoplasmic surfaces

fore highly improbable that liver gap junctional proteins are degraded during their isolation by serine proteases as are cardiac gap junctional proteins. However, the action of nonserine proteases present in liver homogenates on liver gap junctions cannot be ruled out on the basis of published data. In the absence of detailed experimental evidence on this point, the M_r 28,500 polypeptide subunit obtained by SDS-PAGE of isolated liver junctions still constitutes the best available estimate of the molecular weight of the native channel protein subunit, rather than that of a proteolytic breakdown product. If the M_r 28,500 liver polypeptide is indeed unproteolyzed, it follows that the molecular weights of the polypeptide subunits of cardiac and liver gap junctions are remarkably different: 44,000 to 46,000 for cardiac junctions *vs.* 28,000 for liver junctions. Taken in conjunction with the finding that the cytoplasmic surfaces of unproteolyzed cardiac gap junctions are "fuzzy" (Manjunath et al., 1984*b*) whereas those of liver junctions are smooth (Manjunath et al., 1984*a*), these observations suggest major structural differences. We speculate that these differences may be related to differences in function between cardiac gap junctions, which must continually pass large electric currents, and hepatic junctions, in which the passage of electric current is presumably a less prominent or minor feature.

This work was supported by National Heart, Lung, and Blood Institute Grants HL 10503 and HL 20592.

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Received 24 September 1984; revised 22 January 1985