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RECONSTITUTION OF CARDIAC GAP JUNCTION CHANNELING ACTIVITY INTO LIPOSOMES: A FUNCTIONAL ASSAY FOR GAP JUNCTIONS

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Cardiac gap junctions were reconstituted into liposomes. To determine if reconstitution resulted in membrane channel formation, we developed an assay for channel function that used a liposome-entrapped peroxidase to detect entry of a substrate into the liposome. The data demonstrate, for the first time, that reconstituted gap junctions from heart are capable of channel-forming activity in artificial membranes.

Reconstitution of functional gap junction channels or connexons into liposomes and planar bilayers has been accomplished with MIP26K, the lens junctional protein (1-6). Furthermore, a 27,000 dalton junctional protein from liver has been shown to functionally assemble into planar bilayers (7). However, at this time there are no reports of functionally reconstituted cardiac gap junctions. Although certain heart proteins cross-react with antibodies and cDNA probes to liver gap junctions (8-11), the absence of reconstitution studies leaves open the question of whether these putative HGJ proteins are actually channel-forming molecules.

In the present study, chicken HGJs were tested for their ability to reconstitute into liposomes. To detect functional channels in these artificial membranes, an assay was developed which monitored accessibility of added substrate to liposome-entrapped enzyme.

MATERIALS AND METHODS

Preparation of Gap Junctions. Gap junctions were prepared from frozen chicken hearts (Pel-Freeze) according to published methods (12). Briefly, homogenized hearts were washed with low salt buffers, treated with 0.6 M KI overnight, subjected to a series of salt-removal washes, and the resulting cardiac membranes were suspended in 5 mM Tris, pH 10. At this point, the membranes were solubilized with an equal volume of 0.4% N-lauroylsarcosine, instead of the slightly higher concentration used in the published methods (12). Then gap junctions were isolated by discontinuous sucrose gradient centrifugation as previously described (12). Five adult chicken ventricles yielded HGJs containing approximately 200 μg of protein.

Abbreviations: HGJ, cardiac gap junction; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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FITC-Labelled Microperoxidase. Microperoxidase (MP-11; Sigma) was labelled with FITC using the ultra-rapid FITC-celite labeling method (13). To ensure labeling of each enzyme molecule, excess FITC was used. Thus, 64 mg of FITC-celite was dissolved in 4 ml of 25 mM sodium carbonate, pH 11, and added to 10 mg of microperoxidase in 0.5 ml of sodium bicarbonate, pH 7.5. After shaking the mixture for 1 hr at room temperature, 12.5 mg of glycine was added to bind unconjugated FITC. Celite was removed by centrifugation and FITC-microperoxidase was purified on a P-2 column (BRL) and concentrated by speed vac.

Gap Junction Reconstitution in Liposomes. HGJs (~200 μg) and FITC-microperoxidase (270 μg) were dissolved in 2% octyl glucoside (n-octyl-β-D-glucopyranoside, Sigma) and dialyzed in the presence of a sphingomyelin-brain phospholipid mixture, according to Gooden et al. (2). Control liposomes were formed identically, but without HGJs. Following liposome formation, FITC-microperoxidase not entrapped in liposomes was removed by gel filtration on Sephacryl S-200 (Pharmacia) as previously described (14).

Reconstituted Channel Function Assay. Channeling activity was monitored by movement of substrate into liposomes and oxidation by entrapped enzyme. For a determination, 500 μl of substrate solution (60 μM Azure A, 3.6 mM hydrogen peroxide) was added to a cuvette containing column-purified liposomes (50 μl) and pH 7.5, 50 mM Tris buffer (50 μl). In control experiments, Blue Dextran was substituted for Azure. Changes in percent transmittance were followed spectrophotometrically (Beckman Model 25) at 612 nm and recorded over time. Substrate oxidation, expressed as the change in %T, was recorded over the 20 seconds following substrate addition.

In some experiments, channeling activity was assessed in HGJ-reconstituted liposomes (50 μl) that had been preincubated for 10 min with a rabbit antiserum (50 μl) against a 34,000 dalton HGJ polypeptide (anti-34kD) or with preimmune serum (50 μl) from that same rabbit (PI).

RESULTS AND DISCUSSION

An Assay for Functional Reconstitution in Liposomes. Demonstration that gap junction reconstitution into liposomes is a functional assembly requires evidence of channeling activity. In earlier studies, channel-forming ability of reconstituted lens MIP26K was demonstrated both by osmotic swelling of vesicles (3,4) and by ascorbate reduction of liposome-encapsulated cytochrome C (2). These assays of the lens protein channeling activity both use a low molecular weight molecule that transits membrane channels and produces a signal. We have now developed an assay which uses enzymatic oxidation of a low molecular weight substrate to detect channeling. The signal is generated when the substrate is able to pass through membrane channels into liposomes containing trapped enzyme. Such a system, using substrate conversion as a signal, is advantageous in simplicity, the ability to produce strong signals, and, potentially, in production of signals of relatively long duration.

The enzymatic oxidation/reduction reaction shown in Fig. 1 was chosen as the signaling system in the present study. A low molecular weight substrate, Azure A (292 MW), and a high molecular weight substrate, Blue Dextran (2×10^6 MW), both colored when reduced, are rapidly oxidized to non-colored compounds in the presence of peroxidase and hydrogen peroxide. Neither dye is sensitive to oxidation by hydrogen peroxide in the absence of enzyme.

In selecting an enzyme with peroxidase activity, microperoxidase was found to be preferable to HRP for use in the liposome assay. The problem with HRP is a technical difficulty in separating unincorporated enzyme from trapped enzyme following liposome

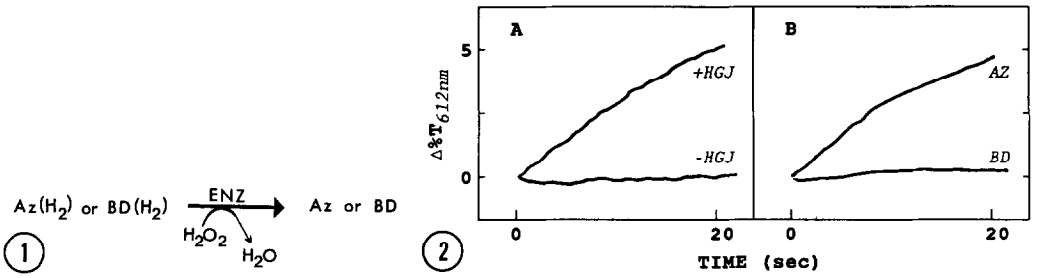

Figure 1. Signaling system for detection of channeling. In their reduced form, Azure A ($Az(H_2)$) and Blue Dextran ($BD(H_2)$) are colored and absorb maximally at 612 nm. The dyes are oxidized to colorless compounds by peroxidase (ENZ) in a coupled reaction that reduces hydrogen peroxide (H_2O_2) to water (H_2O).

Figure 2. HGJs form channels in liposomes, with molecular size limitations. (A) Liposomes formed with entrapped FITC-microperoxidase, but no HGJ (lower curve), fail to oxidize Azure A. Liposomes formed with HGJ (upper curve), as well as enzyme, rapidly oxidize the Azure substrate. (B) Oxidation of Azure substrate (upper curve), but not Blue Dextran (lower curve), shows that HGJ channels allow the 292 MW Azure (AZ) molecule to enter liposomes, but do not allow the 2268 MW entrapped enzyme to exit liposomes and oxidize Blue Dextran (BD).

formation. The small size of microperoxidase (1879 MW), on the other hand, permits easy separation by column filtration, but presents the potential for enzyme leakage out gap junction channels (15). To increase the size of microperoxidase we covalently attached FITC to microperoxidase, producing FITC-microperoxidase with a molecular weight of 2268 daltons. FITC-microperoxidase has been reported to be incapable of channel passage (15), but is still readily separated from enzyme trapped in liposomes by column filtration.

Substrate Fails to Enter Control Liposomes. Liposomes containing trapped FITC-microperoxidase were prepared in the presence or absence of isolated HGJs (Fig. 2). Liposomes formed in the absence of gap junction material served as controls. When reduced Azure substrate was added to control liposomes and monitored for enzymatic oxidation, no change in transmittance at 612 nm was observed (Fig. 2A, lower trace), demonstrating that substrate is not accessible to enzyme. Thus, in the absence of gap junction material, exogenous substrate cannot enter liposomes. In addition, the absence of an oxidation signal also shows that enzyme not entrapped during liposome formation was, in fact, removed by column filtration. Free (unentrapped) enzyme would have been detected, since addition of exogenous microperoxidase to these controls results in rapid substrate oxidation.

HGJs Reconstitute Functional Channels in Liposomes. The data are dramatically different when experimental liposomes formed in the presence of HGJ are used in place of control liposomes. If gap junction connexons are reconstituted in forming liposomes, the prediction is that the channels will provide a passageway for Azure substrate to reach entrapped enzyme. This prediction is realized. Addition of Azure substrate to experimental liposomes resulted in rapid substrate oxidation as reflected by continuous %T recordings (Fig. 2A, upper trace). Oxidation occurred at a nearly constant rate for

20 sec and continued for at least 40 sec. The results demonstrate, for the first time, liposome reconstitution of HGJ channeling activity.

Reconstituted Channels are Size Selective. To determine if the reconstituted membrane channels exhibited a molecular size restriction, Blue Dextran was compared with Azure A in the assay (Fig. 2B). The comparison was made to determine if FITC-microperoxidase could exit through the channels to oxidize Blue Dextran ($>10^6$ MW). The data showed no oxidation of Blue Dextran (Fig. 2B, lower trace) indicating that these channels are size selective. That is, the 292 MW Azure dye can transit the channel but the 2268 MW FITC-microperoxidase can not. If FITC-microperoxidase had escaped liposomes, Blue Dextran would have been oxidized, as shown by addition of exogenous enzyme to these experiments.

HGJ Antiserum Inhibits Reconstituted Channel Function. If the channeling activity described above is a specific result of HGJ connexon presence in the liposome bilayer, the activity should be sensitive to HGJ antibodies. To test such sensitivity, we used a polyclonal antiserum against a prominent polypeptide of HGJ. Briefly, the antiserum was generated in rabbits against a 34 kD polypeptide species (data not shown) electroeluted from SDS-PAGE-resolved HGJ. Treatment of experimental liposomes with anti-34 kD sharply blocked channeling activity (Fig. 3, lower trace). Specificity of the inhibition is shown by continued channel function following identical preincubation with preimmune serum (Fig. 3, upper trace). Although these data do not provide insight into the organization of the connexon, they do show that the channel is HGJ polypeptide specific and that the 34 kD polypeptide is crucial to structure and/or function of the channel.

Conclusions. The significant new information from this study is that HGJ can be functionally reconstituted into artificial membranes. Reconstitution into artificial membranes is a potentially powerful tool for independently analyzing the channel-forming activity of the various polypeptides present in purified heart (16-18) and liver (19-22) gap junctions. For example, a 27,000 dalton liver gap junction molecule has recently been functionally reconstituted into planar bilayers (7). Also, preliminary data from our

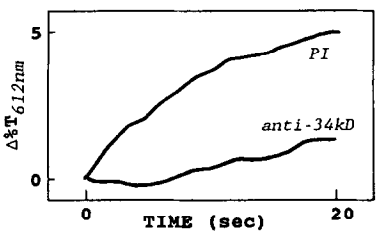

Figure 3. Antibodies to HGJ inhibit channeling activity in HGJ-reconstituted liposomes. An antiserum against a 34,000 dalton polypeptide of HGJ (anti-34 kD) sharply inhibits reconstituted channel function (i.e., oxidation of Azure substrate by entrapped enzyme is sharply retarded). Specificity of the inhibition is shown by the lack of an effect of preimmune serum (PI). While PI treatment has no effect on the rate of substrate oxidation, anti-34kD reduces the PI rate by approximately 60%.

laboratory has shown functional reconstitution of some, but not all, HGJ polypeptide species electroeluted from SDS gels. Clearly, identification of gap junction channel-forming proteins will be best accomplished by channel-forming activity. Absolute relationships between reconstituted channeling activity and cellular gap junctions can then be documented by inhibition of channeling activity with antibodies that also localize to cellular gap junctions and/or inhibit dye transfer between cells.

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