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Rapid report

Transfer of second messengers through gap junction connexin 43 channels reconstituted in liposomes

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Abstract

Gap junction channels reconstituted in liposomes provide a pathway for the transfer of second messengers. Gap junction channels were formed in the artificial unilamellar liposomes using immunoaffinity-purified connexin 43 gap junction protein from rat brain. Sucrose-permeable and -impermeable liposomes were separated on the basis of sucrose permeability in the iso-osmolar sucrose density gradient. The liposomes permeable to sucrose were also permeable to a communicating dye molecule, Lucifer yellow. In the present study, we examined the transfer of second messengers through the connexin 43 channels reconstituted in liposomes and first report the direct evidence that the gap junction channels are permeable to second messengers including adenosine 3′,5′-cyclic phosphate and inositol 1,4,5-trisphosphate.

Keywords: Connexin 43; Second messenger; Liposome; Cell communication; Gap junction

Gap junctional intercellular communication provides a direct intercellular flow of signal information among cells. Several researchers have presented the hypothesis that extracellular signals acting on one cell are transmitted to neighboring cells by transmitting common second messengers through gap junctions [1–4]. There are reports supporting that second messenger molecules, such as adenosine 3′,5′-cyclic phosphate (cAMP) and inositol 1,4,5-trisphosphate (IP_3), are transmitted through gap junction channels [5–7]. However, the study of messenger transfer through gap junction channels is severely constrained by the fact that the gap junction channels are not exposed to extracellular space and access to the channels is only available via cytoplasm. Gap junctions are membrane channels that link end to end across the space between juxtaposed cells. In the present study, gap junction channels were made accessible by splitting the connexin 43 (Cx43) gap junction channels and reconstituting them in artificial lipid membrane. We isolated intact gap junction protein under non-denaturing condition by means of immunoaffinity purification and the activities of the gap junction channels incorporated in the lipid vesicles were studied.

Cx43 is a major type of gap junction protein in rat brain [8,9]. Cx43 was immunoaffinity-purified from rat brain by using antisera against the synthetic pep-

Abbreviations: cAMP, adenosine 3′,5′-cyclic phosphate; CHAPS, 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate; Cx43, connexin 43; IP_3, inositol 1,4,5-trisphosphate; octylglucoside, *n*-octyl β-D-glucopyranoside; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine

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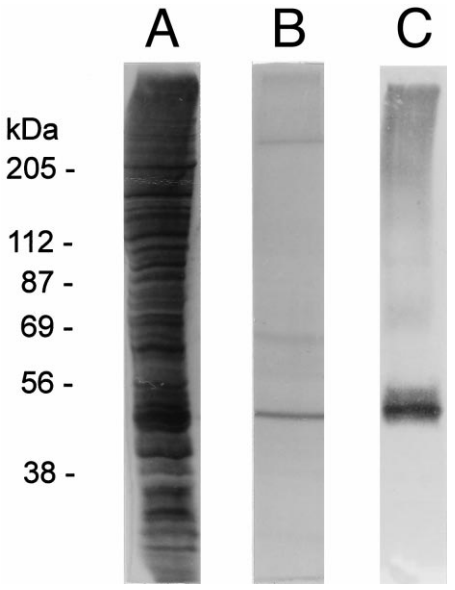

Fig. 1. SDS-PAGE of Cx43 and the corresponding immunoblot. Solubilized membrane proteins of rat brains were electrophoresed on 8% SDS-PAGE and visualized by silver staining (lane A). Cx43 was immunoaffinity-purified by antibodies specific to C-terminal of Cx43 (amino acids 368–382) (lane B). The immunoblot analysis was carried out using the same antibodies specific to C-terminal of Cx43 (lane C).

tide SSRASSRPRPDDLEI, corresponding to residues 368–382 of the amino acid sequence of Cx43. The brain of Sprague–Dawley rat (approximately 1.3 g) was minced in 25 ml of 5 mM $NaHCO_3$, 5 mM EDTA, 3 mM NaN_3 and 1 mM PMSF (pH 8.2) and centrifuged at 48 000 × *g* for 10 min. The crude membrane fraction in the pellets was solubilized in the buffer A containing 50 mM Na-phosphate, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM PMSF, 1 mM leupeptin and 0.75% 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate (CHAPS) (pH 7.4). After centrifugation at 100 000 × *g* for 1 h, the supernatant was loaded onto immunoaffinity column in which antibodies against the Cx43 were cross-linked to CNBr activated Sepharose 4B. Following successive washing with the buffer A and the buffer A plus 0.5 M NaCl, the column was equilibrated with the buffer A in which CHAPS was replaced with 80 mM *n*-octyl β-D-glucopyranoside (octylglucoside). Bound Cx43 was eluted from the column by the brief exposure to the elution buffer containing 50 mM Na-acetate, 10 mM KCl, 459 mM urea, 1 mM EDTA, 80 mM octylglucoside, 1 mM PMSF and 1 mM leupeptin (pH 2.4). The eluent was rapidly neutralized by dropping directly into 1 M HEPES (pH 7.4). All the procedures were carried out at 4°C. The preparation was resolved on 8% SDS-PAGE. A single band of 41 kDa protein was detected by silver staining and immunoblotting (Fig. 1).

The purified Cx43 was then reconstituted in unilamellar phospholipid vesicles composed of phosphatidylcholine (PC), phosphatidylserine (PS) and lissamine rhodamine B-labeled phosphatidylethanolamine (PE). The fluorescently labeled PE was used as a marker for liposomes in the experiment. The procedure used for the incorporation of gap junction proteins in unilamellar liposomes has been described by Rhee et al. [10]. The lipids of PC, PS and lissamine rhodamine B-labeled PE were dissolved in chloroform at a molar ratio of 10:5:0.03. The lipid mixture was dried to a thin film under a stream of nitrogen gas and the lipid film was suspended in the urea buffer containing 10 mM HEPES, 459 mM urea, 10 mM KCl, 0.1 mM EDTA, 1 mM PMSF, 1 mM leupeptin and 80 mM octylglucoside (pH 7.4). The immunoaffinity-purified Cx43 was then added to the urea buffer and the protein/lipid ratio was adjusted to 1:50 (w/w). The protein–lipid mixture was applied onto a Sephadex G-50 column (2 × 100 cm). The column was eluted with the urea buffer at the flow rate of 0.1 ml/min. The liposomes were collected in the void volume while octylglucoside was retained in the column. The liposomes were concentrated by centrifugation at 240 000 × *g* for 14 h.

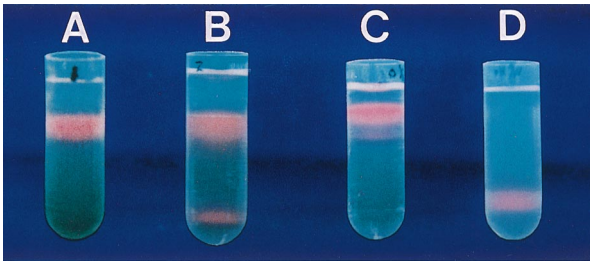

Fig. 2. Transport-specific fractionation of liposomes containing open and closed channels. Unilamellar liposomes composed of PC, PS and lissamine rhodamine B-labeled PE at a molar ratio of 10:5:0.03 were formed with purified Cx43 at the protein/lipid ratio of 1:50. The Cx43-reconstituted liposomes were fractionated in iso-osmolar sucrose density gradient on the basis of sucrose permeability. Liposomes marked with the fluorescently labeled lipid were illuminated with UV light in the dark. Liposomes formed without Cx43 (A). Liposomes formed with Cx43 (B). Liposomes containing closed Cx43 channels (C) and open Cx43 channels (D).

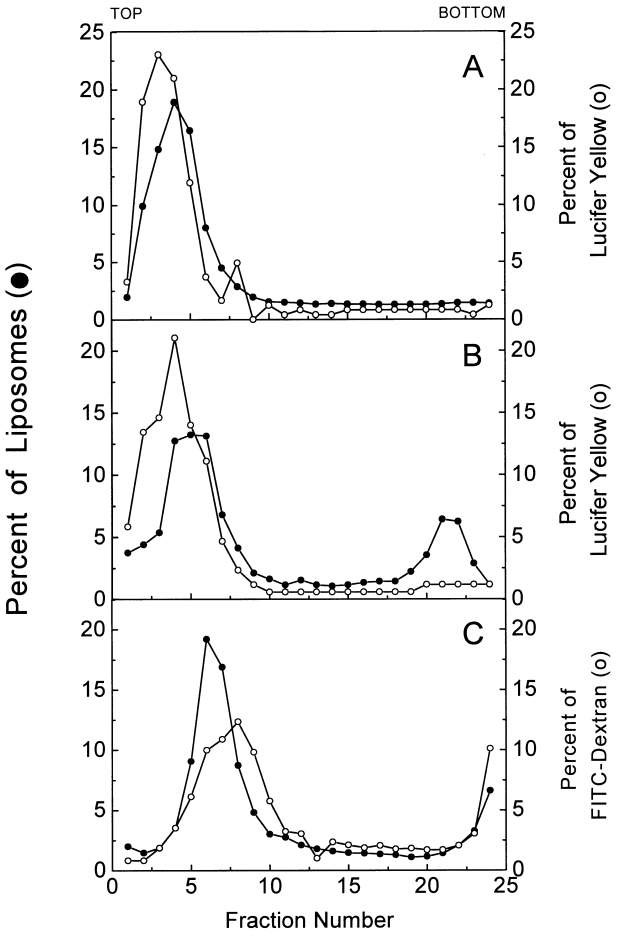

Fig. 3. Permeability of reconstituted Cx43 channels to Lucifer yellow and FITC-dextran. Liposomes were formed in the presence of 3 mM Lucifer yellow or 3 mM FITC-dextran. The distribution of liposomes without Cx43 in iso-osmolar density gradient was determined after sedimentation (A). The distribution of liposomes formed with Cx43 was examined (B,C). The fractions were eluted from top to bottom and the amount of liposomes in each fraction was quantified by measuring the fluorescent intensity of lissamine rhodamine B-labeled PE in the liposome membrane (●). The distribution of Lucifer yellow and FITC-dextran within the gradient was measured (○).

The size of the liposomes is strongly influenced by the lipid/detergent ratio and by the chain length of the detergent molecules. Rhee et al. [10] constructed phospholipid unilamellar vesicles under the same experimental condition as ours and reported an average vesicle diameter of 45 nm. Based on a bilayer thickness of 4.0 nm and an average area of 0.7 nm^2/phospholipid molecules [11], the vesicles were designed to contain an average of one gap junction channel per vesicle. The Poisson distribution predicts that 63% of the vesicles contain at least one gap junction channel. The permeability of the Cx43 channels reconstituted in liposomes was analyzed by using the transport-specific density shift technique described [10]. A linear gradient of sucrose was formed from 0 to 459 mM in 10 mM HEPES, 10 mM KCl and 1 mM EDTA (pH 7.4). The liposomes were layered onto the iso-osmolar density gradient in which a reverse linear gradient of 459 to 0 mM urea in the same buffer compensated the sucrose gradient osmotically. The preparation was centrifuged at $250\,000 \times g$ for 8 h. It was found that liposomes formed without Cx43 were sucrose-impermeable and moved into the gradient a short distance banding near the top of the gradient (Fig. 2A). However, the liposomes formed with Cx43 were fractionated into two populations within the gradient; sucrose permeable fraction which migrates near the bottom of the gradient and sucrose-impermeable fraction

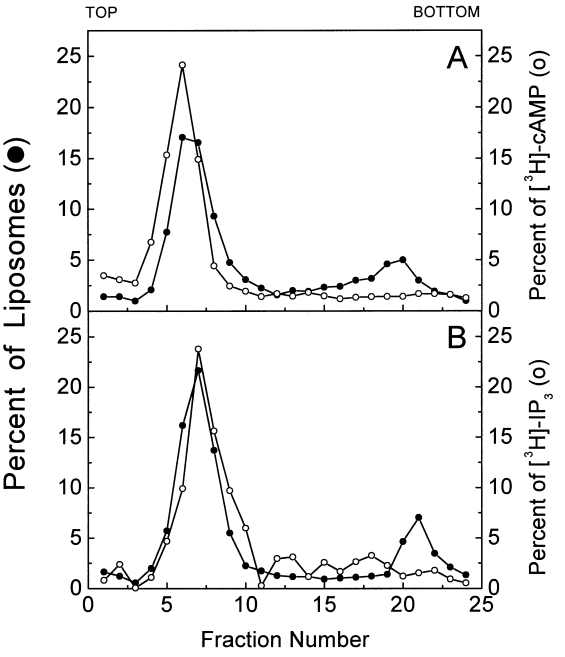

Fig. 4. Permeability of reconstituted Cx43 channels to second messengers. Liposomes were formed with Cx43 in the presence of 10 μCi/ml of [^{3}H]cAMP (A) or 1 μCi/ml of [^{3}H]IP_3 (B). After transport-specific fractionation, the distribution of radiolabeled cAMP or IP_3 within the iso-osmolar density gradient was examined. The amount of second messengers in each fraction was radiometrically measured (○). The distribution of fluorescent lissamine rhodamine B-labeled PE in the liposome membrane also assessed (●).

near the top of the gradient (Fig. 2B). Sucrose-permeable liposomes containing open Cx43 channels seemed to continuously equilibrate their internal solution with external solution and moved to a lower position in the gradient. However, sucrose-impermeable liposomes containing closed form of Cx43 channels were buoyed by the internal urea buffer (the urea buffer is lighter than the external sucrose buffer). It shows that the permeation through Cx43 channels was maintained during and after sedimentation. Liposome fractions were eluted from top to bottom of the gradient, and aliquots of permeable and impermeable liposomes were separately reapplied onto the iso-osmolar density gradient. It shows that the liposomes migrated to their original position in the gradient (Fig. 2C,D).

Lucifer yellow is a well-known communicating dye molecule and its transfer through reconstituted channels was examined. Liposomes were formed with or without Cx43 in the presence of 3 mM Lucifer yellow and the loss of Lucifer yellow from the liposomes was monitored. The loss of FITC-dextran (average MW = 12 000 Da), a non-communicating dye entrapped into liposomes was also monitored as a negative control. Following transport-specific fractionation, the liposomes in the iso-osmolar density gradient were eluted from top to bottom of the gradient. The distribution of the lipid vesicles was examined by monitoring the fluorescence of lissamine rhodamine B-labeled PE in the liposome membrane at 590 nm with an excitation at 560 nm. The amount of Lucifer yellow retained in liposomes were quantified by measuring its fluorescence intensity at 530 nm excited at 428 nm, while the amount of FITC-dextran was monitored by the fluorescence at 520 nm with an excitation at 490 nm. Liposomes formed without Cx43 moved into the gradient a short distance banding near the top of the gradient and Lucifer yellow was retained in the liposomes (Fig. 3A). However, liposomes formed with Cx43 were fractionated into two populations in the gradient and Lucifer yellow was only detectable in sucrose-impermeable fraction (Fig. 3B). Sucrose-permeable liposomes which contain open Cx43 channels specifically lost Lucifer yellow whereas sucrose-impermeable liposomes retained the dye. The data suggested that sucrose-permeable channels formed by Cx43 are also permeable to Lucifer yellow. In contrast, the sucrose-permeable liposomes were not permeable to a non-communicating dye, FITC-dextran (Fig. 3C). The permeable liposomes migrated even to the lower position in the gradient with the large FITC-dextran.

The same experimental procedure was adapted to verify the transfer of second messengers through gap junction channels. The liposomes were formed with Cx43 in the presence of 10 μCi/ml of [^{3}H]cAMP or 1 μCi/ml of [^{3}H]IP_3. The distribution of radioactivity in the gradient was assessed after transport-specific fractionation. Sucrose-permeable liposomes lost second messengers, whereas sucrose-impermeable liposomes retained the radioactivity (Fig. 4). The specific loss of labeled second messengers from the sucrose-permeable liposomes demonstrated that the sucrose-permeable channels formed by Cx43 are also permeable to second messengers.

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