Short Sequence-Paper

Self-regulation of rat liver GAP junction by phosphorylation

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Abstract

We have studied the functioning of rat liver Connexin 32 (Cx32) at the single channel level in presence of ATP. It was observed that ATP regulates the functioning of the channel by running down the junctional conductance. A non-specific exogenous protein phosphatase (alkaline phosphatase) reversed the rundown of junctional activity to its normal functioning state. Autoradiograhic studies demonstrate autophosphorylation of rat liver Cx32. These findings indicate a self-regulatory mechanism of the channel.
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Cell-to-cell communication is known to take place via low-resistance passages created by gap junctions. These are plasma membrane specialized proteins called 'connexins' forming transmembrane channels in both the excitable and nonexcitable cells. The communication is carried out by passive diffusion of metabolites, ions, and morphogens etc. through the gap junction channels [1]. Phosphorylation has been found to play an important role in regulation of biological activities of these channels such as gating, trafficking, assembly or metabolic stability, disassembly, and degradation etc. [2–6]. Reports show that phosphorylation of gap junction can be initiated either directly by extrinsic protein kinases A, C [7–9], v-Src tyrosine protein kinase [4] or by the effectors of protein kinases such as dbcAMP, 8 Br-cAMP, 8 Br-cGMP, TPA, OAG, nafenopin etc. [10–12]. Phosphorylation mainly takes place at the cytoplasmic C terminal domain of the protein, which is thought to be important for the regulation of junctional permeability [13]. The consequences of the phosphorylation vary from system to system; in some cases, phosphorylation reduces junctional permeability and conductance, whereas, in some other cases, it does just the opposite [14–18]. In case of rat liver gap junction protein, only Connexin 32 (Cx32) has been demonstrated to undergo phosphorylation. So far, reports are available on the phosphorylation of rat liver Cx32 either in vitro or in hepatocytes by cAMP-dependent protein kinase, protein kinase C, Ca^{2+}/Calmodulin-dependent protein kinase II, TPA or PMA [7,8,19] etc; or by the activators like epidermal growth factor (EGF) receptor [20], the non-genotoxic hepatocyte carcinogen nafenopin [12] etc. On the contrary, rat liver Cx26 does not undergo phosphorylation in the presence of protein kinases, due to the lack of sites for phosphorylation of rat liver Cx32 [21–23]. However, little is known about the consequences of channel functioning due to phosphorylation of rat liver Cx32. In this report, we have shown that the channel current of rat liver Cx32 hemichannels in lipid bilayer membrane declined due to addition of ATP. Autoradiographic experiments demonstrated that Cx32 protein undergoes self-phosphorylation in the presence of ATP.

Gap junction was prepared from rat liver plasma membrane by alkali digestion method [24] and the purity of the preparation was assessed by 10% SDS-PAGE [25] as well as by Western blotting with Cx32-specific antibody as detailed in our earlier publication [26].

Bilayer membranes were formed across a 150-μM-diameter aperture in a Delrin (R) cup (Warner Instruments, USA) with diphytanoyl phosphatidyl choline and cholesterol (Avanti Polar Lipids, Birmingham, AL, USA), (6:1:: w:w) solution in *n*-decane (21 mg/ml). Channels were incorporated into the membrane by fusing the proteoliposomes with it in a symmetric buffer (10 mM HEPES, 500 mM KCl, 5 mM $MgCl_2$, pH 7.4) solution. Proteoliposomes were prepared by dispersing a lipid film (5.42 mg SPC: 1.15 mg cholesterol) in 250 μl of the abovementioned buffer con-

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taining 10 μg of gap junction protein and 1% (final concentration) Triton X-100 which was subjected to SM2 biobeads (80 mg biobeads/ml) mediated detergent removal method [27]. Two microliters of proteoliposomes were added into the *trans* chamber and allowed to fuse to bilayer by slow stirring. The *cis* chamber (voltage command side) was connected to the CV 201A head stage input and the *trans* chamber was held at virtual ground via a pair of matched Ag–AgCl electrodes (IVM, USA). With the achievement of single channel insertion, the solution in
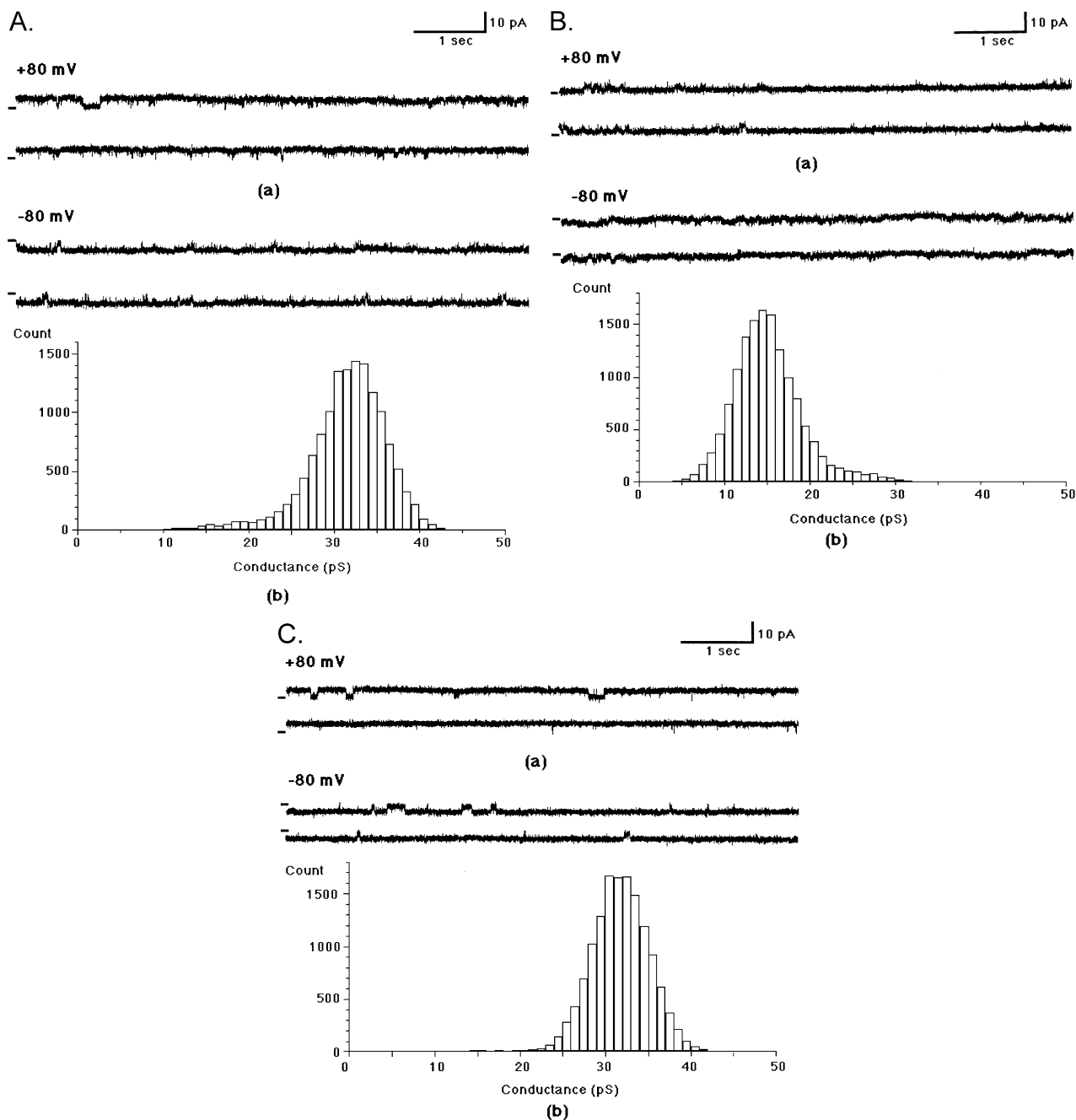

Fig. 1. Effect of phosphorylation on the functioning of gap junction channel at the single channel level. The bar at the side of each trace indicates the position of base line. Data were filtered at 1 kHz and sampled at 2 kHz. A. (a) Continuous single channel current traces of rat liver gap junction functioning in planar BLM at +80 and −80 mV. (b) Conductance histogram of averaged single channel conductances showing a peak at 33 pS. B. (a) The continuous current traces of the same samples as in A at +80 and −80 mV are shown after 40 min treatment with 100 μM (final concentration) ATP. (b) The single channel conductance histogram averaged from the current traces of (a) shows a decrease in conductance value after treatment with 100 μM ATP. C. Dephoshorylation of gap junction protein demonstrated at the single channel level after 5 min of addition of 1 unit of alkaline phosphatase to the phosphorylated channel of B. (a) Represents the continuous current traces at +80 and −80 mV. (b) Shows averaged all points conductance histogram obtained from current traces of (a).

the *trans* chamber was dialyzed out with fresh buffer to avoid fusion of more channels. Single channel currents for different holding potentials were recorded with an Axopatch 200A integrating patch clamp amplifier (Axon instruments, USA) and stored in unfiltered form (5 kHz band width) on video cassettes after digitization through an analog to digital converter (VR 10B, Instrutech, USA). Channel recordings were analyzed using Axodata and Axograph software (Axon Instrument, Foster City, CA) in a Macintosh computer (Macintosh IICi, Apple Computer, USA) after filtering through 8 pole Bessel filter (902 LPF, Frequency Devices, Haverhill, MA) as has been mentioned in our earlier reports [28,29]. Sampling was done using ITC-16 interface (Instrutech Corp. USA) at a rate of twice the corner frequency. Fig. 1A [(a) and (b)] shows some representative current traces and the conductance value of a single hemichannel functioning in bilayer membrane. After recording the single channel current of gap junction, Mg^{2+} ATP was added to the *trans* solution at a final concentration of 100 μM, incubated for appropriate times, followed by dialyzing out the *trans* solution with fresh buffer. When the channel was treated with Mg^{2+} ATP (100 μM, final concentration), the junctional conductance progressively declined and after a time of 40 min, the channel conductance achieves a residual state (14 pS) as has been demonstrated by the continuous current traces as well as the representative histograms in Fig. 1B [(a) and (b)]. Fig. 2 summarizes the results of five independent experiments under control condition and after addition of ATP to the bath solution. On the average, the

Fig. 3. Phosphorylation of 32 kDa rat liver gap junction protein. Lane a: 10% SDS polyacrylamide characterized and coomassie blue-stained protein; lane b: the corresponding autoradiogram; lane c: molecular weight markers (in kilodaltons). Phosphorylation was performed by incubating 10 μg gap junction with γ [^{32}P]-ATP along with buffer solution at 30°C as mentioned in the text, for a time period of 40 min.

relative conductance of gap junction single-channel decreases significantly due to treatment of ATP. As discussed in our previous paper, relative conductance is linearly related to opening probability [30]. Accordingly, the abovementioned result also signifies that opening probability decreases due to ATP treatment. These studies in the bilayer system demonstrate that ATP regulated the function-
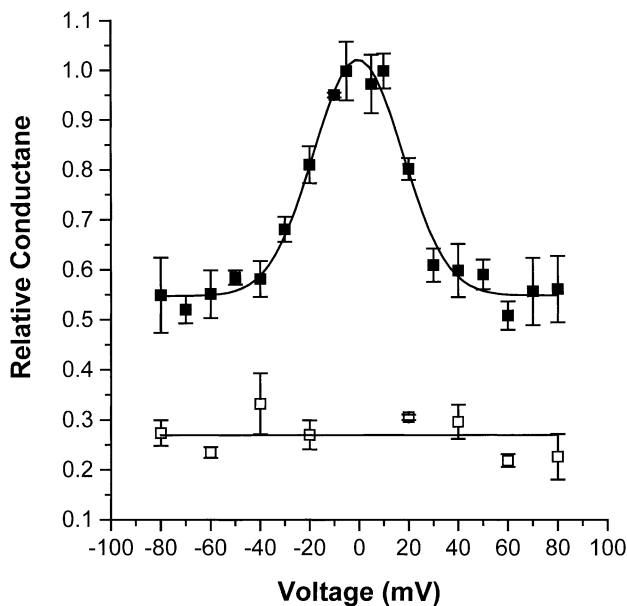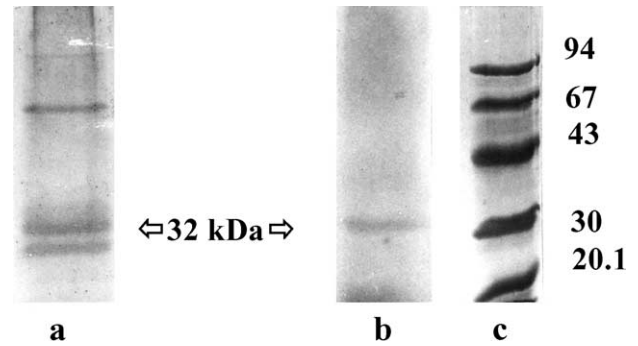

Fig. 2. Relative conductance versus voltage plot of unphosphorylated (■) and phosphorylated (□) gap junction. Relative conductance was calculated as conductance at particular voltage divided by the maximum conductance of unphosphorylated hemichannel at 5 mV. Each data point is the mean of five independent experiments and the standard deviations.
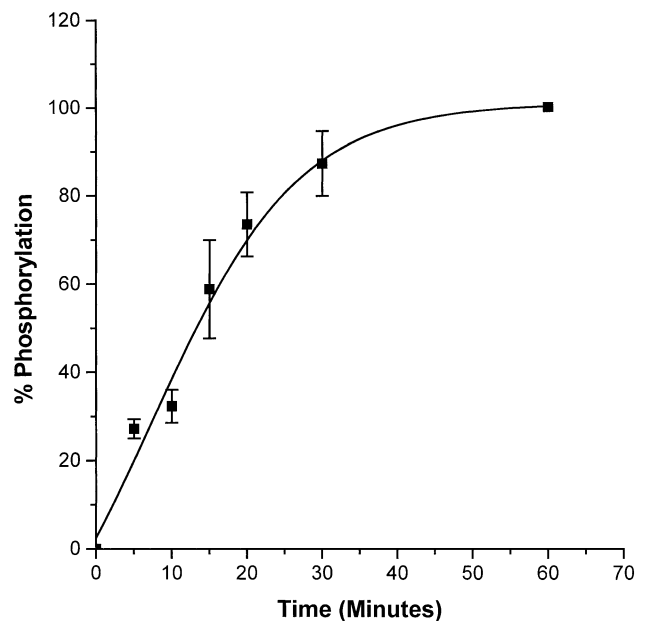

Fig. 4. The time course of gap junction phosphorylation. The phosphorylation reaction was incubated for different times and then subjected to SDS-PAGE as detailed in the text. The bands corresponding to 32 kDa protein were cut out from the gel, dissolved in scintillation fluid and counted. Total count got after 60 min of starting the phosphorylation reaction was taken as 100%. Each point indicates the mean of five individual experiments and the standard deviations.

ing of rat liver Cx32 by closing it down to a residual state of the single channel conductance.

To find if the protein is involved in self-phosphorylation in the presence of ATP, 1 unit of a non-specific exogenous protein phosphates (alkaline phosphatase) was added to the *trans* solution of the same bilayer and allowed to react for 5 min. Then the solution was dialyzed out with fresh buffer and recording was carried out at different holding potentials. The addition of protein phosphatase is expected to dephosphorylate the channel leading to its reversal to the initial conducting state. Fig. 1C [(a) and (b)] shows that the channel is going back to its normal open state. This indicates that at a single channel level, the gating of rat liver Cx32 hemichannel, is regulated by autophosphorylation in the presence of ATP and then dephosphorylation by alkaline phosphatase.

To rule out the possibility of Cx32 autophosphorylation, radioactive experiments were performed by addition of 2 μl of γ [^{32}P]-ATP (specific activity 3500 Ci/mM) to a reaction mixture containing 50 mM HEPES, 2 mM MgCl$_2$, 5 mM EGTA, 100 μM Mg^{2+} ATP and 10 μg gap junction. The reaction mixture was incubated at 30°C for different time periods. The reaction was stopped by adding 3–5 volumes of ice-cold 20% TCA, and then was kept on ice for 20 min. The pellet was washed repeatedly by 20% TCA, solubilized with Laemmli sample buffer, and run on 10% SDS-PAGE [25]. Gels were stained with coomassie blue, dried and exposed to IPC KODAK X-ray film at −70 °C for 12 h and then autoradiographed. For time kinetics studies of phosphorylation, the bands of 32 kDa were cut out from the coomassie blue-stained gels, dissolved in scintillation fluid and counted in a β counter (LKB, USA). The autoradiograph shows the incorporation of γ [^{32}P] into the 32-kDa band of rat liver gap junction (Fig. 3). From this evidence, it is suggested that, the major component of rat liver gap junction protein gets phosphorylated in vitro in the presence of ATP. However, the proteolytic form (28 kDa) and the dimeric form (67 kDa) of Cx32 do not undergo any phosphorylation as evident from the autoradiograph. The time course of the Cx32 autophosphorylation (Fig. 4) indicates that the process reaches equilibrium at 40 min.

As mentioned previously, phosphorylation either deactivates or enhances gap junction channel functioning [31,32], our results for the first time show that phosphorylation of rat liver gap junction can take place without addition of any extrinsic kinase, and also this leads to closure of the channel at the single channel level. As no contamination except the proteolytic and dimeric product of Cx32 has been found in our preparation, it is likely that gap junction itself has a kinase activity. This process of self-phosphorylation–dephosphorylation might have significant consequences in cellular physiology. For example, control of ion flow through gap junction channels is extremely important in maintaining homeostatic balance in cell assemblies. To achieve this, self-control over opening and closing of the channels is necessary. Self-phosphorylation of rat liver Cx32 offers a viable mechanism of the process,—a mechanism by which cell-to-cell communication is self-regulated to maintain the homeostatic balance in liver cells.

Acknowledgements

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