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Self-phosphorylation modulates the gating of rat liver gap junction channels: A nonstationary noise analysis

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Abstract

The effect of phosphorylation on the gating of rat liver gap junction hemichannels (Cx 32) has been investigated. It has been shown that self-phosphorylation of rat liver Cx 32 protein reduces the permeability of proteoliposomes as well the current flowing through multichannels in lipid bilayer membrane (BLM). The low frequency power spectral density analyses of nonstationary noise evolved due to the gating of Cx 32 multichannels demonstrated that self-phosphorylation modulated the channel functioning. A clear change in the power spectrum slopes (α) of the nonstationary noise profiles confirmed the modulation of the channel dynamics due to self-regulation.

Keywords: Connexin 32 hemichannels; Proteoliposomes; Bilayer reconstitution; Multichannel current; Phosphorylation; Nonstationary noise

1. Introduction

In an ensemble of many ion channels, the macroscopic ion current fluctuates due to transition in different conduction states. At a fixed membrane voltage, the current level oscillates at different states because of the stochastic opening and closing of the channels and the amplitudes of these oscillations in turn are a reflection of single channel current and the number of functional channels incorporated [1]. The transitions between different states in turn occur due to a large conformational change of the channel protein evolving nonstationary noise. Thus nonstationary noise is the fluctuations in membrane conductance caused by the opening and closing of ion channels. The analysis of these fluctuations through multichannels gives possible estimation of the elemental properties of active channels, which is called as nonstationary noise analysis (NSNA) and was first introduced by Sigworth to study the properties of voltage dependent sodium channels [2]. There are evidences that the nonstationary noise in any multichannel ensemble depends on N, the number of channels present in the membrane [3]. Gap junction proteins or Connexins (Cx) channels electrically and metabolically connect interiors of adjacent cells by forming cell-to-cell communicating hexameric channels [4–7]. The central aqueous pore of the channel permeable to ions and small molecules mediates the communication amongst the neighboring cells. In a multichannel ensemble these passive diffusion channels function in a collective way, which is, very much cooperative and depends on the applied potentials and also the number of channels open at various potentials [8,9]. The functional state of gap junction channels can be modified dynamically (a process known as "gating") resulting in restriction and augmentation of gap junction in intercellular communication (GJIC). Gating mechanisms involve phosphorylation in connexin protein, elevation of cAMP, gAMP levels, acidification, changes in the intracellular calcium concentration [10,11] or modulation by chemicals like Cyclodextrans (CDs, i.e., a series of hollow cyclic glucosaccharides), 7 ketosterol etc [12,13]. It has been reported that phosphorylation can act as an inhibitor or activator of channel functioning [14–17], i.e., it might close down the channels or open up more channels. Moreover, it has also been reported that the functioning of gap junction channels requires both, the presence of ATP and protein kinase activity to counteract the tonic activity of endogenous phosphatases [18].

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But what happens to the dynamics and thereby the nonstationary noise of channel gating in any multichannel ensemble whenever the channels undergo phosphorylation is not yet worked out.

In our earlier communication, we have demonstrated that rat liver Cx 32 can be self phosphorylated in presence of ATP, which decreases the conductance value at a single channel level [19]. In this context, it appeared to be interesting to find out the possible role of self phosphorylation on the gap junction channel kinetics in multichannel ensemble and as a consequence the changes in the nonstationary noise of the channel transitions between different open and closed states to demonstrate the channel modulation.

In the present study, we have investigated the effect of self-phosphorylation on the gating of multichannels ensemble of rat liver gap junction protein. It has been demonstrated that self-phosphorylation of Cx 32 hemichannels decreases the permeability of proteoliposomes and also the total current flow through the multichannels in bilayer membrane due to the modulatation of the channel dynamics as has been analyzed from the changes in patterns of spectral densities of nonstationary noise.

2. Materials and methods

2.1. Chemicals

Lipids were bought from Avanti Polar Lipids, (Alabama, USA). Tris, Hepes, Potassium Chloride Magnesium Chloride and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Isolation of gap junction

Gap junction protein was prepared from rat liver plasma membrane following alkali digestion method of Hertzberg [20]. The purity of the preparation was assayed by 10% SDS PAGE according to Laemmli [21] and also by western blotting with Cx 32 specific antibody as has been mentioned in our earlier publication [8].

2.3. Effect of phosphorylation on the permeability of proteoliposomes

To monitor the effect of phosphorylation on the permeability of proteoliposomes, the kinetic assay for liposomally entrapped Glucose 6 Phosphatase Dehydrogenase enzyme in the absence or presence of 100 μM (final Concentration) of ATP (Mg^{2+}) was studied. For these studies, proteoliposomes with reconstituted gap junction channels and entrapped Glucose 6 Phosphate dehydrogenase enzyme were prepared from Soya PC and Cholesterol (7:3) following the method by Tokunage et al, [22]. In brief, a lipid film (5.42 mg SPC:1.15 mg Cholesterol) was made under nitrogen and kept in evacuated desiccator for 30 min at room temperature. It was then dispersed in 250 μl-distilled water by ultrasonication for 5 min (Branson 1510) at 20 °C. Then 10 μg of gap junction protein was added to the solution and the mixture was dried under nitrogen and kept desiccated as above. The dried film was then resuspended in 250 μl of buffer (100 mM Tris–Cl, pH 7.4) containing 5 unit of Glucose 6 Phosphate dehydrogenase by ultrasonication for 10 min. The control liposomes were also prepared in the same way except no gap junction was added to that. The liposomal preparations were passed through minicolumn (2 ml) of Sephadex G50, which was pre-equilibrated with the above-mentioned buffer by centrifugation as has been detailed in the earlier publication [23]. Small fractions (100 μl) from the recovered control and proteoliposomes were assayed for the entrapped Glucose 6 phosphate dehydrogenase enzyme according to Anderson and Nordlie [24]. The kinetic assay was started with the addition of NAD, Glucose 6 Phosphate and ± ATP to the liposome solution. The increase in the absorbance of produced NADH was measured at 340 nm by spectrophotometer up to 15 min. Then the liposomes were disrupted by 1% (final concentration) triton X100 to get absorbance of total NADH produced. The final equilibrium value for each experiment was calculated as follows: $F=(F_T/F_{Tot})\times 100$. F_T is the absorbance of NADH after 15 min of starting the experiment. F_{Tot} is the absorbance observed for total NADH formed after breaking the liposomes with 1% (final concentration) triton X100. The relative final equilibrium value was calculated as:$(F_{Gj}-F_0)/F_0$ X100. Where, F_{Gj} corresponds to the final equilibrium value of proteoliposomes and F_0 to that of the control liposomes.

2.4. Effect of self-phosphorylation on the current flow and open channel noise of Cx 32 multichannels in BLM

Phosphorylation of reconstituted gap junction channels was carried out following our earlier publication [19]. In brief, bilayer membranes were formed across a 150 μM of diameter aperture in a Delrin® cup (Warner Instruments, USA) with diphytanoyl phosphatidyl choline and cholesterol (6:1) solution in *n*-decane (21 mg/ml). Channels were incorporated into the membrane by fusing the proteoliposomes with it in a symmetric buffer solution (10 mM Hepes, 500 mM KCl, 5 mM $MgCl_2$, pH 7.4). Proteoliposomes were prepared by dispersing a lipid film (5.42 mg SPC: 1.15 mg cholesterol) in 250 μl of the above-mentioned buffer, containing 10 μg of gap junction protein and 1% (final concentration) triton X100. Detergent was removed from the solution by adding SM2 biobeads (80 mg wet beads/ml) following the method of Levy et al. [25], as has been detailed in our earlier publication [19]. 5 μl of proteoliposomes were added to the trans chamber and allowed to fuse to the BLM by slow stirring. The cis chamber was connected to the 201-head stage input and the trans chamber was held at virtual ground via a pair of matched Ag–AgCl electrodes (Warner Instruments, USA). After 5–10 min of stirring, the channels were inserted (as designated by the opening and closing) and the solution of the trans chamber was dialyzed out to avoid further fusion of more channels. Multichannel current recordings were carried at different clamping potentials with an Axopatch 200A integrating patch clamp amplifier (Axon Instruments, USA) and stored in unfiltered form (5 kHz bandwidth) on videocassettes after digitization through an analog to digital converter (VR

10B, Instrutech, USA). After it, 100 μM ATP (Mg^{+2}) was added to the trans chamber and allowed to react for 30 min. Then the solution of the trans chamber was again dialyzed out, and channel currents were recorded at different holding potentials. For noise analysis, Fast Fourier Transformation (FFT) analyses of time series experimental data were carried using the software Axograph (Axon Instruments, USA) in an Apple Macintosh computer [26] after the signal was filtered through an 8-pole bassel filter (902 LPF, Frequency Devices, Haverhill, MA). Sampling was done using ITC-16 interface (Instrutech Corporation, USA) at a rate of twice the corner frequency. The power spectrums S(f) i.e., square of the FFT vs. frequency (*f*) for native as well as phosphorylated Cx 32 were analyzed at different applied potentials.

3. Results and discussion

3.1. Effect of phosphorylation on the permeability of Cx 32 channels in reconstituted proteoliposomes

Fig. 1 shows the relative final equilibrium value of NADH produced in Glucose 6 Phosphate dehydrogenase assay. It depicts that the final equilibrium rate for proteoliposomes is 22.30±2.57%, whereas, that of the ATP treated proteoliposomes comes down to 7.95±1.83%. The fact demonstrates that self-phosphorylation regulates the permeability of proteoliposomes indicating the closing down of many channels.

3.2. Phosphorylation inhibits the current flow through the multichannels in BLM and modulates the channel dynamics; a nonstationary noise analysis

Fig. 2 represents the current traces of rat liver connexin multichannels at two different voltages and their corresponding

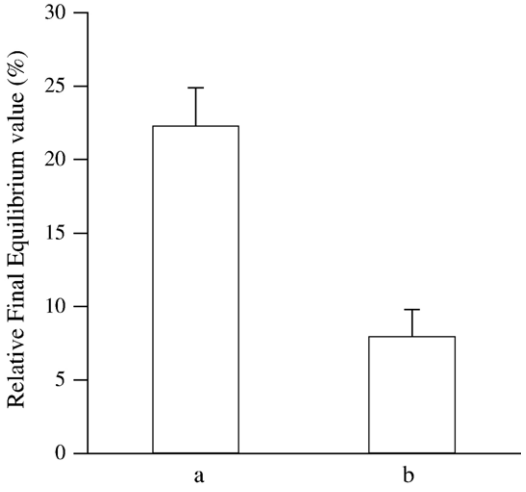

Fig. 1. Effect of phosphorylation on the permeability of Cx 32 channels reconstituted proteoliposomes: The relative final equilibrium rate of NADH production for proteoliposomes containing native gap junction protein channels was found to be 22.30±2.57% (a), whereas, that under phosphorylation decreases to 7.95±1.83% (b). The experimental details are given in the Materials and methods section.

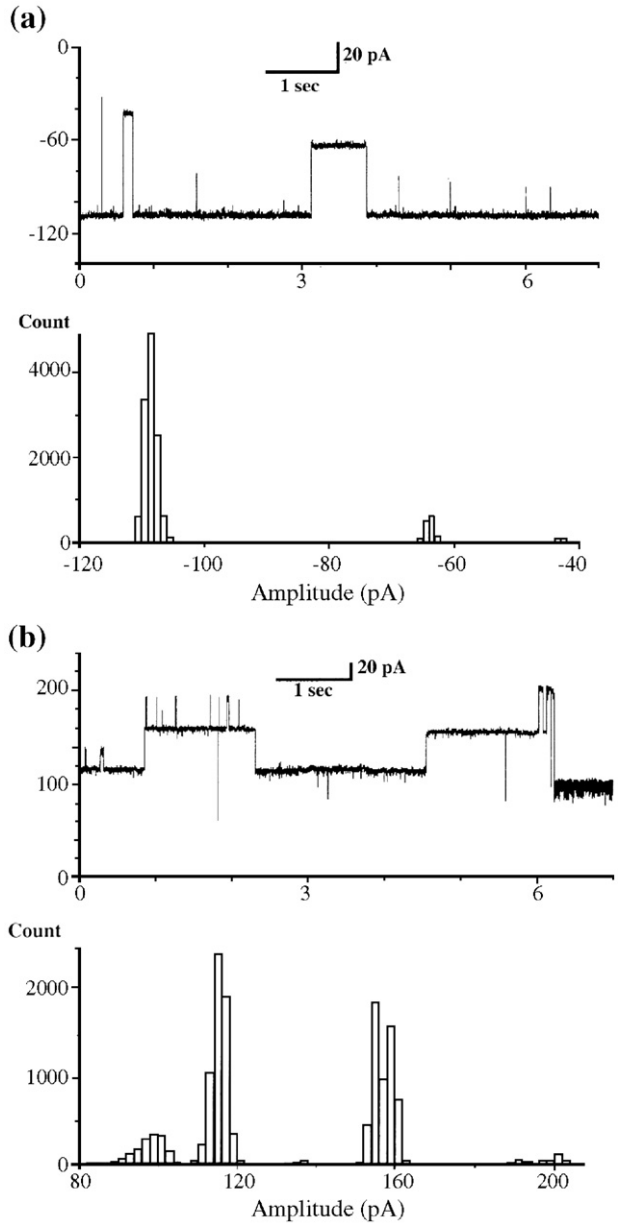

Fig. 2. Multichannel current traces of native rat liver Cx 32 gap junction channels and the corresponding amplitude histograms: The figure represents the current traces of native rat liver connexin multichannels at two different voltages and their corresponding histograms. (a) −40 mV and (b)+60 mV. The traces and the histograms show that the channels are opening and closing stochastically at different conducting states. The bilayers were formed between symmetric solutions of 500 mM KCl, 5 mM $MgCl_2$ and 10 mM Tris–HEPES at pH 7.4. The signal was filtered at 1 kHz and sampled at 2 kHz.

histograms. The traces show that the channels are opening and closing stochastically at different conducting states. The amplitude histogram showed a number of peaks for different open states. But when incubated with 100 μM ATP (final concentration) in trans chamber for a period of 30 min, the channel current decreases due to the closure of many channels as has been shown by the current traces as well as the corresponding amplitude histograms of Fig. 3. The experiments were repetitively performed with various total numbers of reconstituted channels in BLM (as the multichannel insertion in

membrane bilayer is always random insertion in case of any ion channels), and every time a decrease in the total current level was monitored. This suggests that self-phosphorylation of channels by addition of ATP closes down most of the multichannels to a minimal conducting state.

A very interesting result was observed when we looked into the nonstationary noise profiles of native and phosphorylated gap junction multichannels in bilayer. Fig. 4 illustrates the representative current spectral density of native Cx 32 multichannels at two different holding potentials over a frequency of

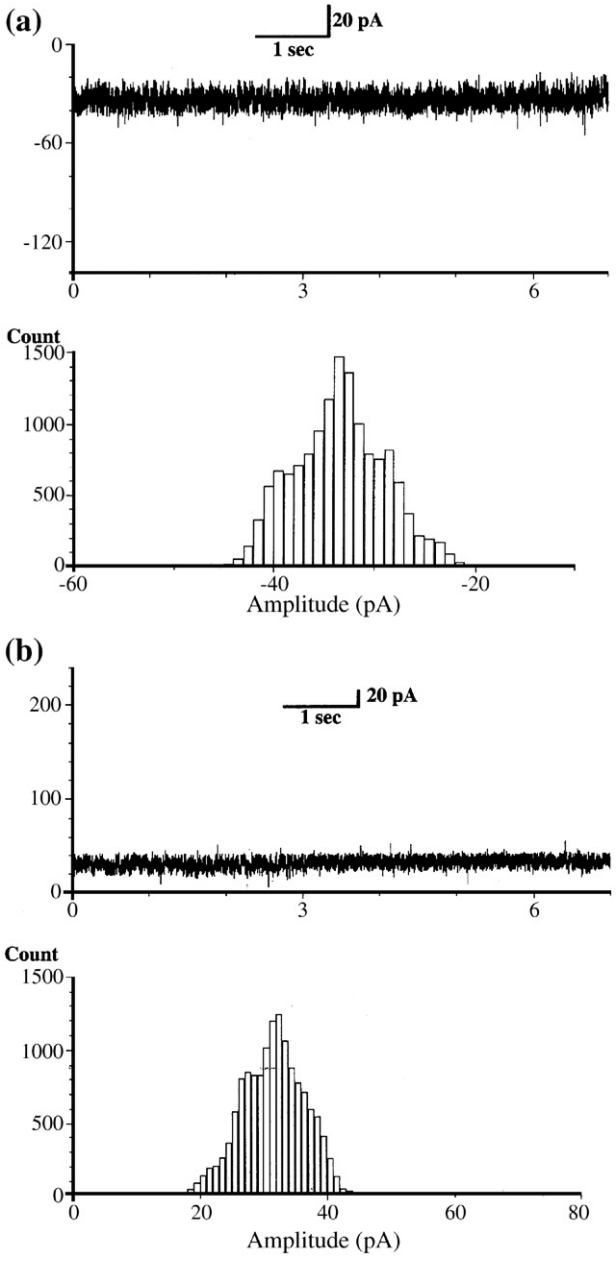

Fig. 3. Multichannel current traces of phosphorylated rat liver Cx 32 gap junction channels and the corresponding amplitude histograms: After incubation with 100 μM ATP (final concentration) in trans chamber for a period of 30 min, the channel current decreases due to self phosphorylation followed by the closure of many channels as has been shown by the current traces as well as the corresponding amplitude histograms. (a) −40 mV and (b)+60 mV. The experimental details remain the same as of Fig. 2.

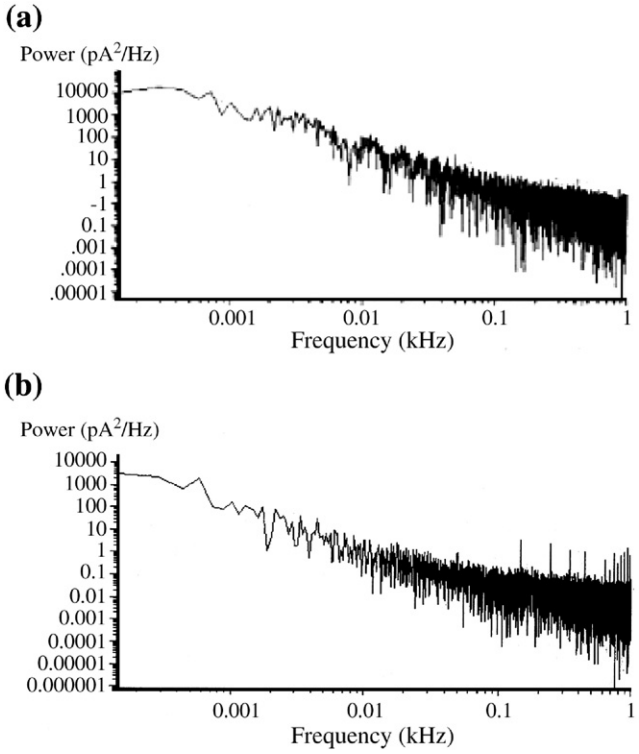

Fig. 4. Current spectral density of native Cx 32 multichannels: Illustrates the plots of power spectrum e.g., spectral density $S(f)$ vs. frequency (f) for the current time traces of native Cx 32 multichannels at two representative holding potentials, (a):+40 mV and (b):−60 mV over a frequency of 1–140 Hz. It is evident from the figure that the slope (α) of the power spectrum is near about 2 indicating the noise of the native channel is highly correlated Brownian noise ($1/f^2$).

1–140 Hz. It is evident from the figure that the slope (α) of the power spectrum is near about 2 indicating the nonstationary noise of the native channels is Brownian (random walk process, [27]). At all applied potentials (from ±40 mV to ±100 mV) the values measured for α were found to be near about 2 on an average. The point to be noted here is that at low potentials (±5 mV to ±20 mV) gap junction channel remained always open at a particular conducting state and do not show any switching in between different conducting states. Due to this we haven't considered the voltages below ±20 mV, as it will not provide the nonstationary noise of the channels, which is essential for this particular study. When the channels are phosphorylated by addition of 100 μÌ ATP in the trans chamber, a significant change in the pattern of the power spectrums was observed to give white noise having $\alpha < 0.5$. As has been shown by Fig. 5, there was a sharp deviation of exponent α from $\alpha = 2$ of native gap junction multichannels to $\alpha < 0.5$ after phosphorylation and thereby, changing the nonstationary noise from Brownian noise to white noise. This phenomenon was independent of all applied electrical fields. Fig. 6 depicts the plot of the slopes (α) of the spectral densities for native as well as phosphorylated Cx 32 hemichannels currents as a function of applied voltages (mV). It clearly demonstrates that phosphorylation changes the nonstationary noise of gap junction channel from Brownian noise or so called from a state of correlated randomness with much smoother landscape ($\alpha \sim 2$) to very

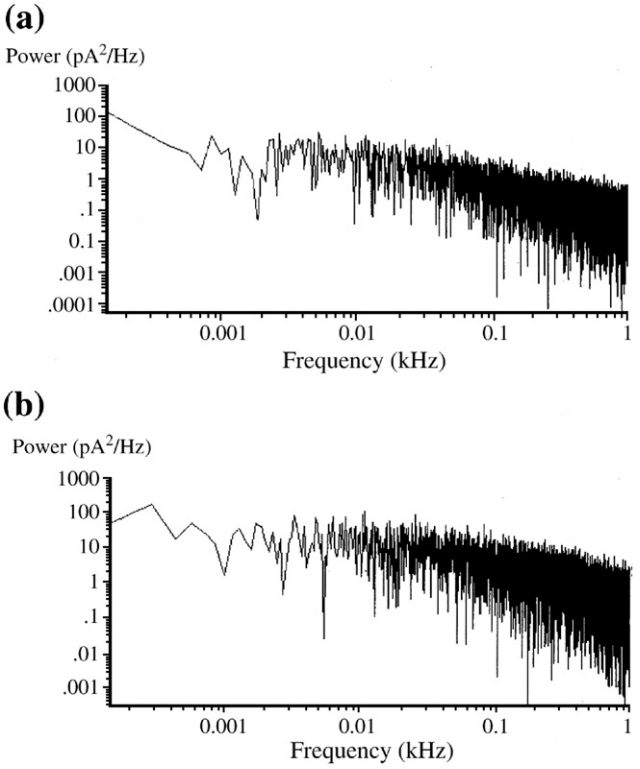

Fig. 5. Current spectral density of phosphorylated Cx 32 multichannels: The spectral patterns of the phosphorylated Cx 32 multichannels are shown at the same two voltages (a):+40 mV and (b):−60 mV. A sharp change of the slope having $\alpha < 0.5$ demonstrates the nonstationary noise to be white noise ($1/f^0$).

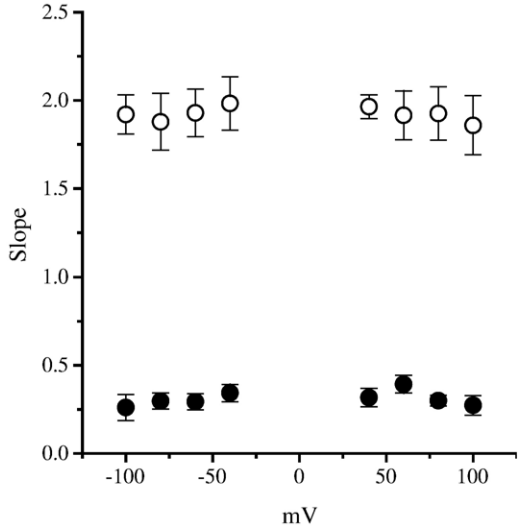

Fig. 6. Slopes (α) of the spectral densities for native as well as phosphorylated Cx 32 hemichannels currents as a function of applied voltages (mV). The slopes of native (o) Cx 32 hemichannels were found to be ~2, whereas after phosphorylation the slopes (•) show values <0.5. Each point represents an average of 5–7 experimental data along with the standard deviations.

rough landscape of white noise having uncorrelated randomness ($\alpha < 0.5$). Thus, it has been clear from our experimental analysis that, self-phosphorylation closes down the channel in BLM and also modulates the channel dynamics to change the nonstationary noise pattern.

Within the lipid atmosphere, the voltage sensitive gap junction channels switch randomly between different conductive and nonconductive states. But this switching is dependent on many external variables, i.e., external electrical fields, ionic strength of the bathing solution etc. These different parameters become the determinant factors for the nonstationary noise of the channels. Since the native gap junction channels in BLM function very smoothly and have greater dynamical activities, hence gating events at different times are correlated, showing the nonstationary noise to be Brownian with a slope reaching to 2. But as soon as the channels start interacting with another external parameter, like channel blocking or activating molecules, immediately a change of state of the system occurs which is reflected by the nonstationary noise. This is what is happening after phosphorylation of the reconstituted rat liver gap junction hemichannels in our system of studies. Addition of phosphates groups to Cx 32 proteins modulates the channels by inducing them to go to a lower conductive state with less dynamical activities in the membrane, which in turn leads to the breakdown of the correlation in gating and hence transformation of nonstationary noise from highly correlated Brownian ($1/f^2$) to uncorrelated white ($1/f^0$) noise.

In conclusion, we demonstrate that self-phosphorylation reduces the channel current of Cx 32 by closing down some of the channels in multichannel ensemble as has been experimentally shown in proteoliposomes as well as in bilayer studies. This phenomenon has a consequence on the nonstationary noise evolved due to opening and closing of gap junction channels. The noise pattern changes from correlated random Brownian noise to uncorrelated random white noise. Thereby self-phosphorylation of rat liver Cx 32 multichannels breaks the correlation in the dynamics of the ion flow through gap junction multichannels, which might be a self-regulatory pathway of the protein under adverse physiological conditions at the cellular level.

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