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Studies on collective behaviour of gap junction channels

Paramita Ghosh [a], Subhendu Ghosh [b,*]

[a] Department of Biophysics, University of Delhi South Campus, Benito Juarez Road, New Delhi-110021, India
[b] Department of Animal Sciences, School of Life Sciences, University of Hyderabad, Hyderabad 500046, India

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Abstract

Gap junction provides low resistance pathways for cell-to-cell passive diffusion of ions, metabolites, second messengers etc. and thus, controls development, differentiation in embryonic tissues, and communication in adult tissues. It has been pointed out in our previous work that these passive diffusion channels behave cooperatively which in turn depends on the structural parameters and also membrane potentials. In the present paper, we have analyzed the multichannel bilayer electrophysiological data of rat liver gap junction Connexin 32 (Cx32) hemichannels. Through the measurements of relaxation time it has been demonstrated that one of the relaxation time constants follows a decay pattern with the number of channels open at various potentials applied across the bilayer membrane. This leads to the conclusion that the collective behaviour of rat liver gap junction hemichannels is cooperative in multichannel ensembles.

Keywords: Gap junction; Bilayer electrophysiology; Relaxation studies; Collective behaviour; Channel-to-channel cooperativity

1. Introduction

Gap junctions are hexameric connexon hemichannel units from two adjacent cells, which are joined in the extra cellular space to form a channel providing direct intercellular communication. The channel facilitates passive diffusion of ions, metabolites, second messengers, and also serves as electrical synapse [1–9]. The synchronization achieved from the diffusional and electrical coupling by means of gap junction plays pivotal role in controlling the development, differentiation, growth and impulse propagation [10–16], and thereby enables cells to function as a coherent unit. The regulation of the gap junction mediated intercellular coupling (GJIC) can be done by several ways, e.g., by altering channel gating characteristics pharmacologically or by generalized blockage of transcription or translation, and by directly controlling connexin gene expression [17]. Also, phosphorylation of connexin by endogenous mechanisms [18,19] or by exogenous kinases [20,21] has been proved to be a protective response of GJIC to stress.

It has been shown that generally ion channels do function in clusters in a cooperative way [22,23] to control the intercellular ion-flux [24–27]. Clustering is also reported to be required for the operation of gap junction channels [15]. However, only a few reports are available to show the gap junction mediated cooperativity for the cellular growth and cell coupling etc [28–30]. We have earlier suggested that the mutual interactions among the gap junction protein channels on a cell membrane matrix is a viable mechanism of controlling ion channel conductance and this collective behaviour depends on the structural parameters and also membrane potentials [31,32]. We feel, there is a need to highlight the biological importance of cooperative functioning of gap junction channels (or hemichannels) as a result of their mutual interactions.

In the present work, we undertake analyses of multichannel electrophysiological data of rat liver gap junction hemichannels of Cx32 with a view to understand the channel cooperativity under different applied potentials. Based on the experiments on bilayer electrophysiology we have carried out relaxation studies of multichannel ensembles of gap junctions, which reveal some interesting features on the cooperative behaviour of the channels and demonstrate the physiological consequences behind it.

* Corresponding author. Tel.: +91 40 23134579; fax: +91 40 23010120/45.
E-mail address: sgsl@uohyd.ernet.in (S. Ghosh).

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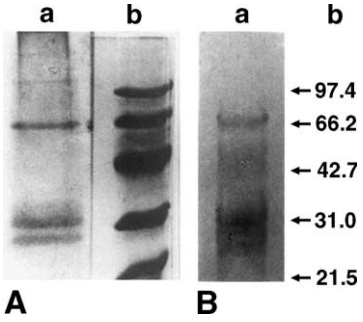

Fig. 1. Characterization of gap junction protein isolated from rat liver by immunoblotting using affinity purified anti-connexin 32 antiserum. A. Electrophoretic profile of gap junction protein on 10% polyacrylamide gel stained with Commassie blue. Lane a: 5 μg of isolated protein was loaded. The major protein of gap junction migrates at 32 kDa along with its higher aggregated form of 67 kDa. A 27-kDa band is also prevalent in this lane. Lane b: shows the low molecular weight marker proteins (Phosphorylase B, 97.4 kDa; BSA, 66.2 kDa; Ovalbumin, 42.7 kDa, Carbonic Anhydrase, 31 kDa; Soybean Trypsin Inhibitor, 21.5 kDa) B. Western blot of a gel identical to that in A. Lane a: The 32 kDa, its higher aggregate of 67 kDa along with the low molecular weight band of 27 kDa are labeled with affinity purified anti-connexin 32 antiserum. In control experiments, with either no primary antibody or preimmune mice IgG fraction instead of primary antibody, no labeling was detected (data not given). Lane b: The positions of the molecular mass standard proteins are shown by arrows.

2. Materials and methods

2.1. Chemicals

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

2.2. Isolation and characterization of gap junction

We have isolated and purified gap junction (Cx32) from Wister rat liver plasma membrane following alkali digestion method [33]. The characterization of purified protein was performed by 10% SDS PAGE as well as by western blotting with Cx32 specific antibody as has been detailed in our previous publication [34]. Polyclonal antibody was raised against electroeluted Cx32 protein in balb/c mice by intraperitoneal injection. After subsequently given booster doses, anti-Cx32 IgG was purified from those mice by affinity purification [35].

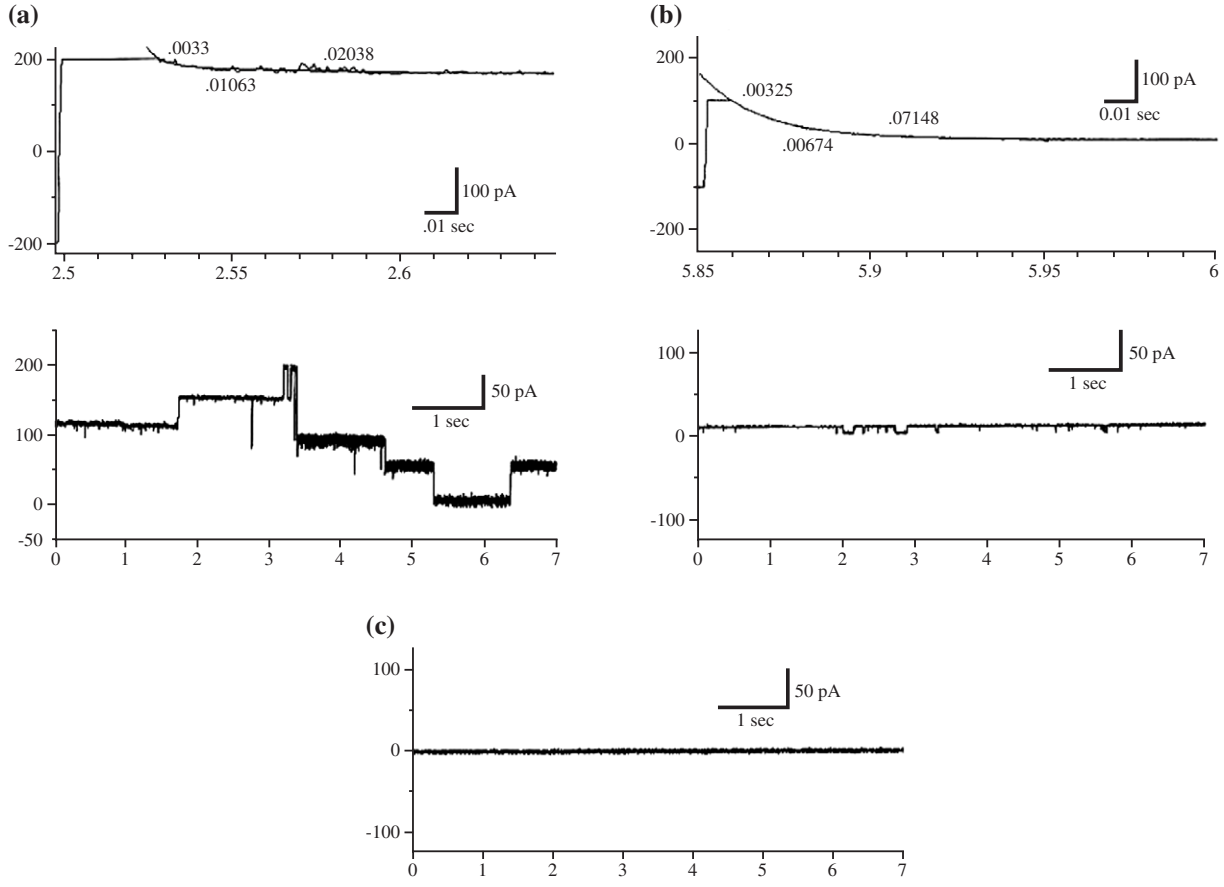

Fig. 2. Multichannel current trace of gap junction channel in BLM. (a) represents a current trace at +60 mV having a total number of 110 functional channels (N is 110) and the decay with time (relaxation) is given at the top of the trace. (b) represents a current trace having less number of functional channel (N is 7) at the same voltage (+60 mV) and its relaxation is given at the top. (c) represents current trace at 0 mV after the incorporation of functional channels. Channel numbers are similar as those of (a). 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.

2.3. Electrophysiology

The electrophysiological experiments were carried out following our earlier protocol [19]. In brief, bilayer membranes were formed across an aperture (150 μm diameter) in a Delrin® cup (Warner Instruments, USA) with diphytanoyl phosphatidyl choline and cholesterol (6 : 1) solution in n-decane (21 mg/ml). 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 (IVM, USA).

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 rat liver gap junction protein and 1% (final concentration) triton X100, which was subjected to SM2 biobeads (80 mg wet beads/ml) mediated detergent removal method [19]. For single channel incorporation, 2 μl of proteoliposomes were added to the *trans* chamber and allowed to fuse to the BLM by slow stirring (as per our earlier standardization, [19]). After 5–10 min of stirring, the single channel was achieved, and the solution of the trans chamber was dialysed out to avoid further fusion of more channels. Then 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).

At the second step of our studies to find out the cooperativity of the channels, we recorded the functioning of multichannels in the same way. For these experiments, 10 μl of proteoliposomes (from the similar proteoliposomal preparations as mentioned above) were allowed to fuse to BLM. After incorporation of multichannels, the trans chamber solution was dialyzed out and the recordings were performed. The electrophysiological data were analyzed using the software Axograph (Axon Instruments, USA) in an Apple Macintosh computer after filtering

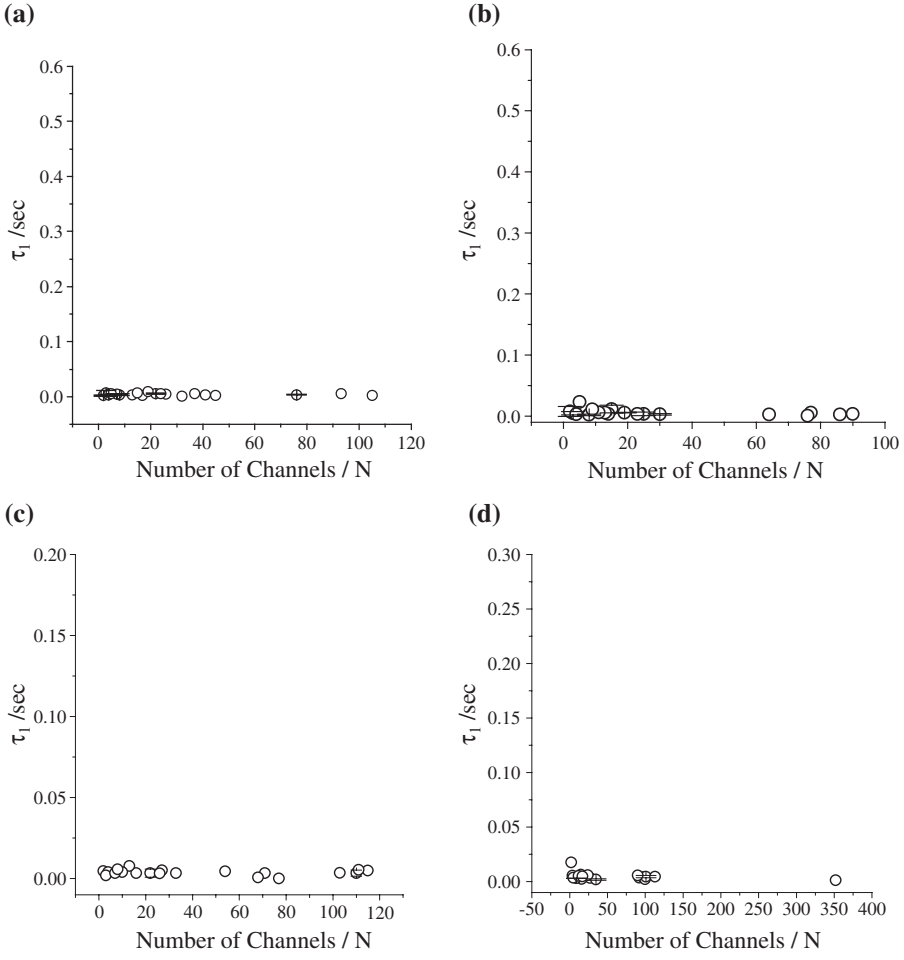

Fig. 3. Variation of relaxation time constant (τ_1) as a function of number of channels (N) incorporated in lipid bilayer at different voltages, (a) +80 mV, (b) −80 mV, (c) +60 mV and (d) −60 mV. The data points are average of five different experiments (±standard deviations). The experimental conditions remained the same as in Fig. 2.

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.

2.4. Estimation of number of channels open

The maximum number of channel (N) opening at an applied voltage was determined from all point amplitude histograms. At a particular voltage, N is determined from the maximum channel current I_{max} (corresponding to the furthest peak in the amplitude histogram) divided by the corresponding single-channel current (I_{single}). The single channel current, I_{single} for different voltages has been obtained from the single-channel data.

2.5. Relaxation studies

With the sudden change in transmembrane potential the channel current immediately jumps to a high value and then gradually relaxes to a stable value [27]. The time taken in the latter process is called the relaxation time (τ). Determination of relaxation time (τ) was done by the software Axograph (Axon Instruments, USA) in Apple Macintosh Computer using the following relation involving triple exponential function [27,36].

$$I(t - t_p) = I_{t=\infty} + \sum_{i=1,2,3} I_p \exp\left\{-(t - t_p)/\tau_i\right\} \quad (1)$$

Here, I_p denotes the peak value of the channel current (positive or negative) at a particular voltage at a time $t = t_p$ (when the voltage is switched on). The current (I) decays exponentially to a stable value $I_{t=\infty}$. In our experiment we considered $i = 1$, 2 and 3.

3. Results and discussions

Fig. 1 depicts the denaturing electrophoresis profile of gap junction protein from rat liver and its corresponding western blot pattern. The preparation shows one major band of 32 kDa, along with a lower band of 26 kDa and a higher aggregate of approximately 67 kDa (Fig. 1A). The affinity purified antibody raised against electroeluted 32 kDa monomer can recognize all the three bands suggesting

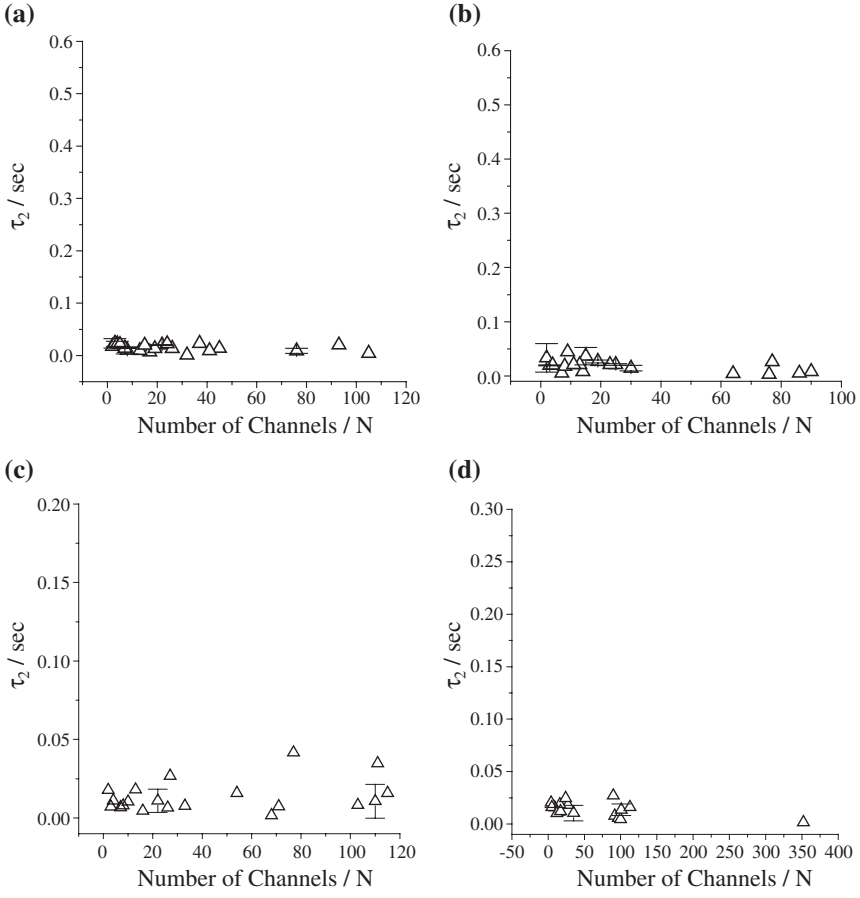

Fig. 4. Variation of second relaxation time constant (τ_2) as a function of number of channels (N) incorporated in lipid bilayer at different voltages, (a) +80 mV, (b) −80 mV, (c) +60 mV and (d) −60 mV. The average data points are taken from the same five experiments as of Fig. 3 (±standard deviations). The experimental conditions remained the same as in Fig. 2.

that no other non-connexin protein is present (Fig. 1B). In this regard we would like to mention that Cx26 is usually expressed with other members of the family, typically with Cx32. Both of these two connexins Cx32 and Cx26 coexist in the same gap junction plaque in liver [37–41]. Reports are also available on the conduction of current by both Cx26 and Cx32 [42,43]. Henceforth our result shows the particular connexin bands, which are expected from rat liver.

Fig. 2 demonstrates representative current traces of rat liver multichannels in bilayer membrane. Fig. 2a,b show the channel kinetics at +60 mV having different number of functional hemichannels along with their corresponding relaxation. Fig. 2c designate a 0 mV trace as a control. We have considered that the channel current comprises of the full open state as well as the sub-states. It is obvious in both the current traces of 2a and 2b that the multichannels are opening and closing in a cumulative way in different conducting states. This cumulative opening and closing of multichannels prompted us to look into the channel–channel interaction, which could be responsible for their collective behaviour in multichannel assemblies. With an aim to understand this, we analyzed the relaxation of the multichannel current traces from different experimental data recorded at different applied potentials in BLM. While fitting the experimental data of multichannel relaxation in exponential equation, we found triple exponentials leading to three time constants τ_1, τ_2, τ_3 gives a better fit with the lowest chi square value (current decay fittings in Fig. 2a and b). From this we conclude that there exists grossly three major modes of relaxation. Regarding the interpretation of τ_1, τ_2, τ_3, we offer the following suggestions.

(i) As per our analysis, the value of the first relaxation time τ_1 lies below 5 ms, and does not significantly change with the number of channels incorporated (N) (Fig. 3). Accordingly, $\tau_1$is attributed to the interaction of the channel proteins of the lipid bilayer to ionic environment.

(ii) The value of second relaxation τ_2 lies below 50 ms and has been observed not to change significantly with the number of channels incorporated (N) at different voltages as demonstrated in Fig. 4. Consid-

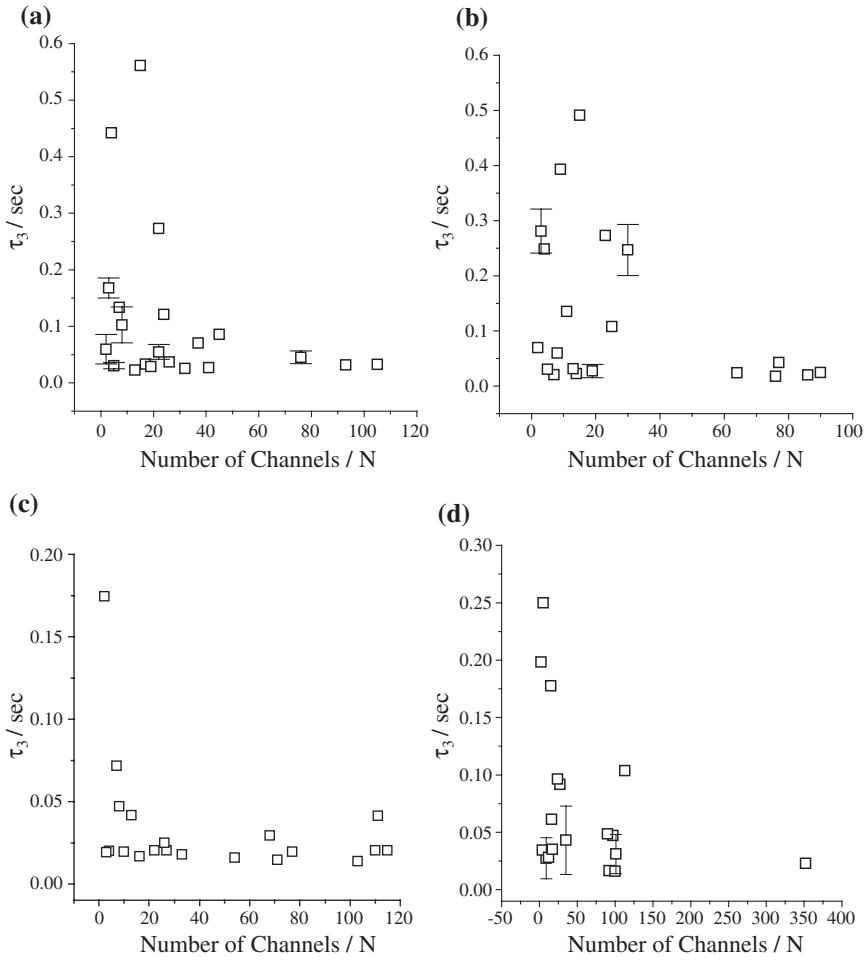

Fig. 5. Variation of relaxation time (τ_3), as a function of number channels (N) incorporated in a lipid bilayer membrane at different voltages, (a) +80 mV, (b) −80 mV, (c) +60 mV and (d) −60 mV. The data points are taken from the same five experiments as of Fig. 3. The experimental details are similar to that of Fig. 2.

ering the point that the relaxation of lipid bilayer membrane is expected to be slower than that of the channel in the aqueous solvent τ_2 is attributed to the relaxation of lipid bilayer structure.

(iii) The third time constant or τ_3 has variable values from 15 to 600 ms. Since, its values are changing in different experiments having different numbers of reconstituted channels (N), we have attributed it to the channel-to-channel interactions on the bilayer lipid membrane. This is supposed to help a channel most to behave in a coordinated fashion and delay the current decay with time significantly.

As per our observations τ_3 shows a sharp decline in the values with the increasing number of functional channel (N) in different voltages (Fig. 5). It strongly demonstrates that given the experimental set-up and conditions, τ_3 decreases exponentially with increasing N. This decaying (exponential) trend in the τ_3-N plot leads to the conclusion that in the present case, gap junction shows cooperativity due to channel–channel mutual interaction. Accordingly, the process of relaxation should speed up with increasing number of channel (N). This has been shown in Fig. 6, where the current amplitude corresponding to the third relaxation mode is increasing with N.

The present paper deals with some facts behind the cooperativity of rat liver gap junction channels and its relation with the number of channels in a multichannel cluster in BLM. It may be noted here that the basis of channel–channel collectivity is their mutual interaction (direct or indirect). The latter would depend on the number of participating channels. If the channels are cooperative in interaction then the process of relaxation speeds up and the relaxation constant decreases. It has been earlier reported that relaxation time for multichannel gap junction is lower than that of the single-channel in HeLa cells [28]. Our result of decrease of relaxation time with increase in number of channels is consistent with the previous findings. Moreover, our findings give quantification for the aforesaid self-regulation.

We highlight that the collective behaviour of these membrane channels, an example of self-regulation and organization, has very significant consequences in cellular processes. In a cell-to-cell junction, whether it is embryonic or neuronal, there are a number of gap junction channels. In order to maintain the functional stability of the cellular

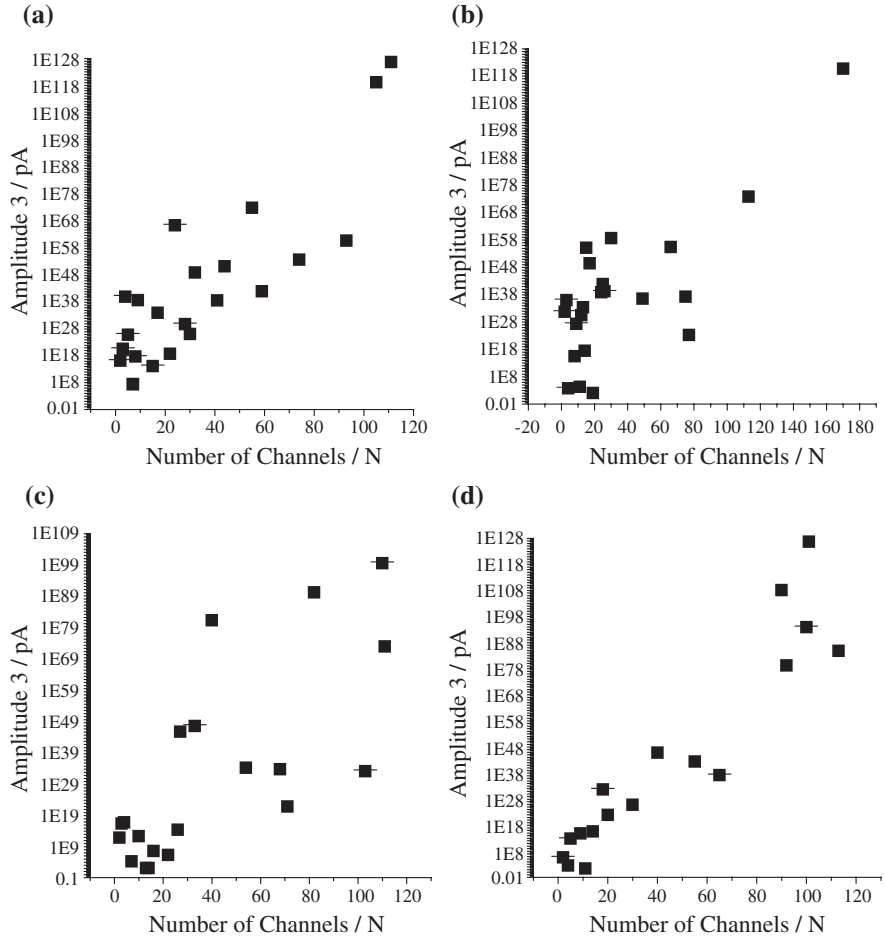

Fig. 6. Variation of the amplitude of the third mode of relaxation (Amplitude 3) with the number of channels (N) at different voltages, (a) +80 mV, (b) −80 mV, (c) +60 mV and (d) −60 mV. The graphs show that the amplitude increases with the increasing N. The experimental details remained the same as in Fig. 4.

system it is not desirable that all the channels function individually or independently. Rather, a self-regulation in the total channel operation is required. And this is achieved by a mutual cooperative interaction among the neighbouring channel proteins.

In conclusion, the present studies demonstrate that the collective behaviour of rat liver gap junction (Cx32) multi-channels, as measured by relaxation constant τ_3 for channel–channel interaction, depends on the number of functional channels open in a particular experiment. Most importantly, they display cooperativity in gating both at positive and negative potentials.

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