Intracellular Magnesium-Dependent Modulation of Gap Junction Channels Formed by Neuronal Connexin36

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Gap junction (GJ) channels composed of Connexin36 (Cx36) are widely expressed in the mammalian CNS and form electrical synapses between neurons. Here we describe a novel modulatory mechanism of Cx36 GJ channels dependent on intracellular free magnesium ($[Mg^{2+}]_i$). We examined junctional conductance (g_j) and its dependence on transjunctional voltage (V_j) at different $[Mg^{2+}]_i$ in cultures of HeLa or N2A cells expressing Cx36. We found that Cx36 GJs are partially inhibited at resting $[Mg^{2+}]_i$. Thus, g_j can be augmented or reduced by lowering or increasing $[Mg^{2+}]_i$, respectively. Similar changes in g_j and V_j -gating were observed using MgATP or K₂ATP in pipette solutions, which increases or decreases $[Mg^{2+}]_i$, respectively. Changes in phosphorylation of Cx36 or in intracellular free calcium concentration were not involved in the observed Mg²⁺-dependent modulation of g_j . Magnesium ions permeate the channel and transjunctional asymmetry in $[Mg^{2+}]_i$ resulted in asymmetric V_j -gating. The g_j of GJs formed of Cx26, Cx32, Cx43, Cx45, and Cx47 was also reduced by increasing $[Mg^{2+}]_i$, but was not increased by lowering $[Mg^{2+}]_i$; single-channel conductance did not change. We showed that $[Mg^{2+}]_i$ affects both open probability and the number of functional channels, likely through binding in the channel lumen. Finally, we showed that Cx36-containing electrical synapses between neurons of the trigeminal mesencephalic nucleus in rat brain slices are similarly affected by changes in $[Mg^{2+}]_i$. Thus, this novel modulatory mechanism could underlie changes in neuronal synchronization under conditions in which ATP levels, and consequently $[Mg^{2+}]_i$, are modified.

Introduction

Electrical synapses are specialized junctions between neurons formed by clusters of gap junction (GJ) channels that enable direct cell-to-cell transfer of electrotonic potential, signaling molecules, and metabolites. Each GJ channel is formed by two apposed hemichannels (aHCs), each of which is formed by six connexin (Cx) subunits. Neurons in the adult brain, as well as insulin-secreting β -cells in the pancreas, express Cx36 (Condorelli et al., 1998; Söhl et al., 1998; Serre-Beinier et al., 2000). GJs composed of a single Cx isoform generally show a maximum junctional conductance (g_j) at transjunctional voltage (V_j) equal zero and a symmetric g_j decrease with increasing V_j of either polarity. The change in g_j is attributed to the presence in each aHC of two V_j -sensitive gates, a “fast” gate and a “slow” gate

(Bukauskas and Weingart, 1994). Cx36-containing electrical synapses are expressed in many regions of the mammalian CNS, such as the trigeminal mesencephalic (MesV) nucleus, inferior olive, thalamus, hippocampus, cortex, and retina (Connors and Long, 2004), and are thought to promote neuronal synchronization and coordinated activity of various neuronal networks (Bennett and Zukin, 2004).

Normally, the intracellular concentration of free magnesium ($[Mg^{2+}]_i$) is $\sim 10\times$ lower than that of total magnesium (Grubbs, 2002); most of the Mg²⁺ is bound to ATP, and changes in cytosolic ATP concentration ($[ATP]_i$) produce opposite changes in $[Mg^{2+}]_i$ (Lüthi et al., 1999). Under physiological conditions, neuronal $[ATP]_i$ increases during glucose and lactate exposure (Ainscow et al., 2002), and when neuronal activity is reduced during sleep (Dworak et al., 2010). Conversely, ATP levels are reduced by increased neuronal activity during wake periods and hyperactivity (Dworak et al., 2010). Pathological conditions, such as hypoxia, ischemia, and seizures, produce long-lasting depletion of $[ATP]_i$ and elevated $[Mg^{2+}]_i$ (Murphy et al., 1989; Headrick and Willis, 1991; Helpert et al., 1993). In contrast, traumatic brain injury results in a $\sim 50\%$ reduction in $[Mg^{2+}]_i$ for several days (Cernak et al., 1995; Heath and Vink, 1996; Suzuki et al., 1997). Resting brain $[Mg^{2+}]_i$ is also reduced in patients with neurological diseases, such as Parkinson's (Barbiroli et al., 1999) and Alzheimer's (Andrási et al., 2000); or increased in patients with schizophrenia (Hinsberger et al.,

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Table 1. Composition of pipette solutions used in this study

Solution	Free $[Mg^{2+}]$ (mM)	MgCl ₂ (mM)	Free $[Ca^{2+}]$ (nM)	CaCl ₂ (mM)	EGTA (mM)	EDTA (mM)	BAPTA (mM)	KCl (mM)
I	0.01	0.14	25	0.89	5	0.2	2	121.2
II	0.1	0.13	25	0.86	5	0	2	121
III	1	1.26	25	0.83	5	0	2	119.3
IV	5	6.1	25	0.72	5	0	2	112.2
V	10	12	25	0.62	5	0	2	103.5
VI	10	12	0	0	5	0	10	100.4

Concentrations of free Mg^{2+} and Ca^{2+} were calculated using MaxChelator software (see Materials and Methods). In addition to the components indicated, each solution contained (in mM) the following: 10 NaAsp, 5 tetraethylammonium, 5 HEPES. Differences in osmolality were compensated for with different concentrations of KCl, and solutions were titrated to pH 7.2 with KOH.

1997). Together these findings suggest that changes in neuronal $[Mg^{2+}]_i$ during physiological as well as pathological conditions can be sufficient to modulate electrical synapses.

Here we show that Cx36 GJs are inhibited by resting $[Mg^{2+}]_i$ (~ 1 mM) and that g_j can be augmented or reduced by lowering or increasing $[Mg^{2+}]_i$, respectively. We find that intracellular ATP is critical for the Mg^{2+} -dependent modulation of Cx36 GJs and propose that Mg^{2+} is directly involved in channel gating by interacting with a sensorial domain located in the channel lumen. Our results indicate that Mg^{2+} occupancy of Cx36 GJ channels induces a reduction in open probability by increasing sensitivity to V_j -induced closure of fast gates, and stabilization of a closed conformation of slow gates. Finally, we show that Cx36-containing electrical synapses between MesV neurons respond similarly to changes in $[Mg^{2+}]_i$, indicating that this novel Mg^{2+} -dependent modulatory mechanism of Cx36 GJs is relevant for the modification of neuronal electrical transmission.

Materials and Methods

Cell lines and culture conditions. Experiments were performed in HeLa (human cervical carcinoma cells, ATCC CCL2) or N2A (mouse neuroblastoma cells, CCL-131) cells transfected with Cx26, Cx32, Cx36, Cx43, Cx45, or Cx47 wild type or fused with color variants of green fluorescent proteins (EGFP or CFP) attached to the C terminus. We also used Novikoff cells expressing endogenous Cx43. Cells were grown in DMEM supplemented with 8% fetal calf serum, 100 μ g/ml streptomycin, and 100 U/ml penicillin, and maintained at 37°C in humidified air with 5% CO₂. Vectors for transfection and cell lines stably expressing the Cxs used were developed in collaboration with the laboratories of Dr. K. Willecke (Cx36 and Cx47) and Dr. D.W. Laird (Cx43). More details on these issues were published previously (Bukauskas et al., 2000; Teubner et al., 2000, 2001). Phosphomimetic mutants of Cx36 were introduced into wild-type Cx36 (Al-Ubaidi et al., 2000) at Ser110 and Ser293 using the QuikChange Multi Site-directed mutagenesis kit (Agilent). Mutants were subcloned into pEGFP-N1 (Clontech) and transfected into HeLa cells using Lipofectamine 2000 (Invitrogen).

In vitro electrophysiological measurements. Experiments were performed in a modified Krebs'–Ringer's solution containing the following (in mM): 140 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, 2 CsCl, 1 BaCl₂, 5 glucose, 2 pyruvate, 5 HEPES, pH 7.4. Recording pipettes (3–5 M Ω) were filled with standard pipette solution containing the following (in mM): 140 KCl, 10 NaAsp, 1 MgCl₂, 0.26 CaCl₂, 2 EGTA, 5 HEPES, pH 7.2. To study the effect of $[Mg^{2+}]_i$, from 0.01 to 10 mM, we used pipette solutions containing different concentrations of MgCl₂ (Table 1) and applied the web-based MaxChelator software to calculate free ionic concentrations (www.stanford.edu/~cpatton/webmaxc.htm). To study the effect of divalents other than Mg^{2+} , we used pipette solutions containing the following (in mM): 140 KCl, 10 NaAsp, 5 HEPES, pH 7.2, with or without 2 mM XCl₂ of X divalent. For simultaneous electrophysiological and fluorescence recordings, cells were grown on glass coverslips and transferred to an experimental chamber mounted on the stage of an inverted microscope (Olympus IX70) equipped with a fluorescence imaging sys-

tem. Cells were perfused with modified Krebs'–Ringer's solution at room temperature. Junctional conductance (g_j) was measured using a dual whole-cell voltage-clamp system. Briefly, each cell of a pair was voltage-clamped independently with a separate patch-clamp amplifier (EPC-8, HEKA). By stepping the voltage in cell-1 (V_1) and keeping the voltage in cell-2 (V_2) constant, we generated a transjunctional voltage ($V_j = \Delta V_1$), and the corresponding junctional current (I_j) was measured as the negative of the current change in cell-2, $I_j = -\Delta I_2$; I_j has the same polarity as the voltage step in cell-1. Thus, g_j was obtained from the equation $g_j = I_j/V_j$. Signals were acquired and analyzed using custom-made software (Trexler et al., 1999) and an analog-to-digital converter from Molecular Devices.

Brain-slice preparation and ex vivo electrophysiological measurements. A minimum number of animals were killed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and according to the Institutional Animal Care and Use Committee of Albert Einstein College of Medicine. Transverse brainstem slices (250 μ m thick) were prepared from male or female Sprague Dawley rats (age, postnatal day 14–18). Slices were obtained using a vibratome (DTK Microslicer) and placed in cold sucrose solution containing the following (in mM): 248 sucrose, 2.69 KCl, 1.25 KH₂PO₄, 26 NaHCO₃, 10 glucose, 2 CaCl₂, and 2 MgSO₄. The slices were then transferred to an incubation chamber filled with sucrose solution at room temperature and incubated for 60 min. The sucrose solution was slowly replaced by physiological solution containing (in mM): 124 NaCl, 2.69 KCl, 1.25 KH₂PO₄, 26 NaHCO₃, 10 glucose, 2 CaCl₂, and 2 MgSO₄. Sections were kept at room temperature in the physiological solution until they were transferred into the recording chamber. The recording chamber, mounted on an upright microscope stage (Nikon Eclipse E600), was continuously perfused with physiological solution (1–1.5 ml/min) at room temperature. Whole-cell patch recordings were performed under visual control using infrared differential interference contrast optics (IR-DIC). MesV neurons were identified on the basis of their location, large spherical somata, and characteristic electrophysiological properties in response to both depolarizing and hyperpolarizing current pulses (Curti et al., 2012). Recording pipettes (6–12 M Ω) were filled with intracellular solution containing the following (in mM): 140 K-gluconate, 3 MgCl₂, 0.2 EGTA, 10 HEPES, pH 7.2. Free Mg^{2+} was adjusted to 0.01 mM by adding 4 mM EDTA, or to 5 mM by adding 2 mM MgCl₂. Simultaneous recordings were made using a Multiclamp 700B amplifier (Molecular Devices), acquired and analyzed using Igor software (Wave Metrics).

Estimates of the junctional conductance between MesV neurons (G_j) were calculated following Bennett, 1966 and Parker et al., 2009:

$$G_j = \frac{R_{\text{transfer}}}{(R_{\text{input1}} \times R_{\text{input2}}) - (R_{\text{transfer}})^2}$$

where R_{input1} and R_{input2} represent the input resistance of cell-1 and cell-2, respectively, and R_{transfer} represents the transfer resistance between coupled cells. The R_{transfer} is defined as the amplitude of the voltage response measured in cell-1 or cell-2, divided by the amplitude of the current step injected into cell-2 or cell-1, respectively. To calculate G_j in current-clamp configuration, hyperpolarizing current pulses (-300 pA) of 200–400 ms duration were injected into one cell and the resulting voltage deflections were measured in both cells. A total of 5–20 single responses were averaged to improve the signal-to-noise ratio, and for each coupled pair the mean G_j was calculated as the average from the values in both directions. In a linear system, R_{transfer} is equal in both directions. These estimates assume a simple two-neuron model with passive membrane properties coupled directly by a single domain isopotential on each side. Voltage dependence of G_j is assumed to be negligible, and additional pathways via coupled dendrites or adjacent coupled neurons are excluded. These assumptions are reasonable for Cx36 GJs (Srinivas et al., 1999; Teubner et al., 2000; Moreno et al., 2005) and anatomy of MesV neurons (Curti et al., 2012).

Fluorescence imaging and magnesium transfer studies. Fluorescence signals were acquired using an ORCA digital camera (Hamamatsu) with UltraVIEW software for image acquisition and analysis (PerkinElmer Life Sciences). For magnesium transfer studies, the tetrapotassium salt of

Figure 1. Mg^{2+} -dependent modulation of g_j and V_j -gating in HeLa cells expressing Cx36-EGFP GJs. **A, C**, Bottom, Dynamics of g_j (normalized to initial g_j value) during repeated V_j ramps (+20 to -20 mV and 1.3 s in duration; top traces) at $[Mg^{2+}]_p = 0.01$ (**A**) and 5 mM (**C**). **B, D**, g_j - V_j dependence (normalized to initial g_j value at $V_j = 0$) obtained during 50-s-long V_j ramps (from 0 to -100 mV; middle traces) derived from data shown in **A** and **C**, respectively; the numbers on the g_j - V_j plots correspond to numbers on g_j traces in **A** and **C**. Fitted curves shown in color were obtained using the S16SM. Gray lines show fitted curves obtained from control g_j - V_j plots ($[Mg^{2+}]_p = 1$ mM). **E**, Concentration-response relation of g_j (normalized to initial g_j value) as a function of $[Mg^{2+}]_p$; $EC_{50} \approx 0.45$ mM. Averaged data are shown in colored circles. Data of individual experiments are shown in gray triangles. Roman numerals correspond to different solutions shown in Table 1. **F**, Averaged g_j - V_j dependencies (normalized to g_j value at $V_j = 0$) obtained at different $[Mg^{2+}]_p$ (curves in black) were fitted using a S16SM (g_j - V_j plots in color). Roman numerals correspond to different Mg^{2+} concentrations shown in **E**. Parameters obtained after fitting are shown in Table 2. **G-I**, Open probabilities of fast and slow gates in α and β aHCs ($P_{oF,\alpha}$, $P_{oS,\alpha}$, $P_{oF,\beta}$, $P_{oS,\beta}$, respectively) depending on V_j were calculated using parameters obtained in **F** for $[Mg^{2+}]_p = 0.01$ (**G**), 1 (**H**), and 10 mM (**I**).

the magnesium fluorescent probe (50 μ M), Mag-Fluo-4 (Invitrogen), was introduced into cell-1 of a pair through a patch pipette with modified standard pipette solution without $MgCl_2$. Pipette solution for cell-2 was modified with addition of 9 mM $MgCl_2$. Typically, breaking into cell-1 was followed by an increased fluorescence intensity in cell-1 (FI_1). After reaching steady state in cell-1, the patch in cell-2 was opened and the changes in FI_1 and g_j were measured. The rate of FI_1 changes [(arbitrary units per minute (AU/min))] was calculated as the difference between FI_1 at the moment of breaking into cell-2 and FI_1 after 2 min.

Data analysis. The analysis and statistics were performed using SigmaPlot software. Averaged data are reported as the means $\pm$ SEM. Means for each group were compared using unpaired Student's t test.

Results

Intracellular magnesium-dependent modulation of junctional conductance and voltage-gating of Cx36 GJ channels

To understand the influence of Mg^{2+} on Cx36-mediated electrical coupling, we studied changes in g_j and g_j - V_j dependence at different $[Mg^{2+}]_i$ in pairs of HeLa cells expressing Cx36 wild type, and HeLa or N2A cells expressing Cx36 tagged with EGFP (Cx36-EGFP) using dual whole-cell patch clamp. We changed $[Mg^{2+}]_i$ using pipette solutions containing from 0.01 to 10 mM free Mg^{2+} ($[Mg^{2+}]_p$) with compositions (Table 1) determined using the Maxchelator program (see Materials and Methods). The pipette solutions also contained 5 mM EGTA and 2 mM BAPTA to buffer free Ca^{2+} concentration at 25 nM. Basal g_j was measured with small-amplitude V_j ramps (+20 to -20 mV; duration, 1.3 s), and g_j - V_j dependence was measured with high-amplitude V_j ramps (from 0 to -100 mV; duration, 50 s). When both pipettes contained $[Mg^{2+}]_p = 0.01$ mM, g_j of Cx36 GJs increased after patch opening (Fig. 1A), and became less V_j -sensitive than in control condition with $[Mg^{2+}]_p = 1$ mM (Fig.

1B). In contrast, $[Mg^{2+}]_p = 5$ mM in both pipettes led to a reduction in g_j (Fig. 1C) and increased V_j -sensitivity compared with control (Fig. 1D). In both cases, the g_j reached steady state within ~ 20 min. A steady-state g_j - $[Mg^{2+}]_p$ curve normalized to initial g_j values (Fig. 1E) shows a half maximal effective concentration (EC_{50}) of 0.45 mM. All g_j - V_j relations shown in Figure 1B,D were fitted using a stochastic 16-state model (S16SM) of voltage gating (Paulauskas et al., 2012) containing in series for each aHC two V_j -sensitive gates, a fast gate and a slow gate (Bukauskas and Weingart, 1994).

In the S16SM, fast gates have an open state with conductance $\gamma_{F,open}$ and a "closed" or residual state with conductance $\gamma_{F,res} > 0$. Meanwhile, slow gates have an open state with conductance $\gamma_{S,open}$ and a closed state with zero conductance ($\gamma_{S,closed} = 0$). Therefore, the channel can occupy one of the 16 possible states made by the combination of four states of the fast gates with four states of the slow gates. For simplicity we assume that $\gamma_{F,open} = \gamma_{S,open}$, and that conductance of the fully open channel $\gamma_{open} = \gamma_{F,open}/4 = \gamma_{S,open}/4$. The behavior of each gate is characterized by Boltzmann equilibrium constants between open and residual/closed states, for fast ($K_{F,o \leftrightarrow res} = e^{A_F(-\Pi \cdot V_F - V_{F,o})}$) or slow ($K_{S,o \leftrightarrow c} = e^{A_S(-\Pi \cdot V_S - V_{S,o})}$) gates, where A (A_F and A_S) characterize the maximal steepness of changes in open probability (P_{oF} and P_{oS}) as a function of voltage across the gate (V_F and V_S), V_o ($V_{F,o}$ and $V_{S,o}$) is the voltage across the gate at which its probability to be in the open state is 0.5, and Π is a gating polarity (+1 or -1). Thus, the S16SM allowed us to estimate gating parameters characterizing sensitivity to V_j for each gate and the number of operational/functional channels (N_F). In addition, the S16SM uses an exponential function to describe rectification over voltage (e.g., $\gamma_{F,res} = \gamma_{F,res,0} e^{-V_F/R_F}$, where $\gamma_{F,res,0}$ is $\gamma_{F,res}$ at $V_F = 0$), therefore allowing

Table 2. Parameters of voltage-gating estimated with S16SM

Figure (Cx)	Fast gate					Slow gate			
	A_f (mV ⁻¹)	$V_{f,o}$ (mV)	$\gamma_{f,open}$ (pS)	$\gamma_{f,res}$ (pS)	$R_{f,res}$ (mV)	A_s (mV ⁻¹)	$V_{s,o}$ (mV)	$\gamma_{s,open}$ (pS)	$\gamma_{s,closed}$ (pS)
1F (Cx36)									
Solution I	0.041	122	24	0.6		0.100	154	24	0
Solution II	0.031	87	24	0.7		0.062	158	24	0
Solution III	0.037	41	24	0.5		0.085	77	24	0
Solution V	0.045	12	24	0.6		0.084	68	24	0
3H (Cx47)									
Solution I	0.30	40	220	11.2	296	0.99	131	220	0
Solution III	0.20	34	220	7.0	322	0.10	124	220	0
Solution IV	0.14	27	220	6.2	272	0.09	103	220	0
Solution V	0.16	21	220	6.2	210	0.11	86	220	0

Parameters were obtained from the fitting of g_j – V_j data shown in Figures 1F and 3H using the stochastic 16-state model of GJ channels (S16SM).

an estimate of rectification coefficients for the conductive states of fast ($R_{F,open}$ and $R_{F,res}$) and slow ($R_{S,open}$) gates. V_j across the GJ channel is the sum of voltages across all gates, $V_j = V_{F,\alpha} + V_{S,\alpha} + V_{F,\beta} + V_{S,\beta}$, where α and β stand for the two aHCs. Closing one gate changes the voltage across the other three gates in series, and this affects the probability of the state's changing over a discreet time interval. The residual conductance of the GJ channel (γ_{res}) typically is approximately one-fifth of γ_{open} (Bukauskas and Verselis, 2004), which is measured when one of fast gates is in the closed/residual state and three other gates in series are in the open state ($1/\gamma_{res} = 1/\gamma_{F,res} + 1/\gamma_{F,open} + 2/\gamma_{S,open}$). Values of A and V_o for fast and slow gates estimated during the fitting process allowed calculation of open probabilities for each gate in α and β aHCs ($P_{oF,\alpha}$, $P_{oS,\alpha}$, $P_{oF,\beta}$, and $P_{oS,\beta}$) and the probability of a GJ channel to reside in a fully open state (P_o ; 4 gates in the open state) as a function of V_j . Fitting of averaged experimental g_j – V_j dependence normalized to g_j values at $V_j = 0$ (Fig. 1F, black lines; we assumed symmetry of the g_j – V_j relation around $V_j = 0$; fitted curves are in colors) revealed that with reduction of $[Mg^{2+}]_p$, $V_{F,o}$ and $V_{S,o}$ increased while A_F and A_S remained relatively constant (Table 2). V_o and A for both fast and slow gates, and $\gamma_{F,res}$ were set as free parameters during the fitting process (Table 2). Figure 1G–I shows open probability of each gate as functions of V_j derived from g_j – V_j plots obtained at different $[Mg^{2+}]_p$ and shown in Figure 1F. At $[Mg^{2+}]_p = 0.01$ mM (Fig. 1G), all gates remain open (P_o close to unity) over the entire V_j range. At $[Mg^{2+}]_p = 1$ mM (Fig. 1H), $P_{oF,\alpha}$ and $P_{oF,\beta}$ showed enhanced sensitivity to V_j with a value of 0.8 at $V_j = 0$, and $P_{oS,\alpha}$ and $P_{oS,\beta}$ remain close to unity at $V_j = 0$. Therefore, on average, only 64% of functional GJ channels have all four gates in the open state at $[Mg^{2+}]_p = 1$ mM and $V_j = 0$. At $[Mg^{2+}]_p = 10$ mM (Fig. 1I), $P_{oF,\alpha}$ and $P_{oF,\beta}$ show even higher sensitivity to V_j corresponding to the shift of $P_{oF,\alpha}$ and $P_{oF,\beta}$ plots along the V_j axis, but $P_{oS,\alpha}$ and $P_{oS,\beta}$ did not change very much. As a result, P_o at $V_j = 0$ was reduced to ~ 0.36 . Thus, P_o at $[Mg^{2+}]_i = 10$ mM was ~ 2.8 -fold less than P_o at 0.01 mM, and changes in $P_{oF,\alpha}$ and $P_{oF,\beta}$ caused by changes in V_o account for $\sim 80\%$ of the changes in g_j as a function of $[Mg^{2+}]_i$.

Figure 2. Differences in $[Mg^{2+}]_i$ -dependent modulation of g_j in HeLa or N2A cells expressing Cx26, Cx32, Cx36, Cx43, Cx45, or Cx47; changes in $[Ca^{2+}]_i$ are not involved in $[Mg^{2+}]_i$ -dependent modulation of Cx36. All data represent mean g_j (normalized to initial g_j value). **A**, Normalized g_j measured in HeLa Cx36-EGFP cell pairs using pipette solutions containing 10 mM free Mg^{2+} and 2 mM BAPTA (free $Ca^{2+} = 25$ nM) or 10 mM BAPTA (free $Ca^{2+} \approx 0$). **B**, **C**, Normalized g_j measured using pipette solutions containing 0.01 (**B**) or 5 mM free Mg^{2+} (**C**) in HeLa Cx36WT, HeLa Cx36-EGFP, or N2A Cx36-EGFP cell pairs. **D**, **E**, Normalized g_j measured using pipette solutions containing 0.01 (**D**) or 5 mM (**E**) of free Mg^{2+} in HeLa cells expressing Cx26, Cx32, Cx36, Cx43, Cx45, or Cx47. Numbers of cell pairs are indicated within columns; * $p < 0.05$; ns, nonsignificant p values.

The remaining $\sim 20\%$ can be explained by changes in N_F , where channels may enter into a Mg^{2+} -occupied (long-lived) closed conformation of the slow gate. To test whether the decrease in coupling under high $[Mg^{2+}]_i$ depends on changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), we enhanced the buffering capacity of the pipette solution by increasing BAPTA to 10 mM (Table 1, Solution VI), which reduced $[Ca^{2+}]_p$ close to zero. We did not find significant differences in g_j decay under high $[Mg^{2+}]_i$ conditions (10 mM) with normal/control (25 nM) or close to zero $[Ca^{2+}]_p$ (Fig. 2A). HeLa cells expressing Cx36 and Cx36-EGFP exhibited similar Mg^{2+} -dependent modulation of g_j (Fig. 2B,C). However, neuroblastoma cells expressing Cx36-EGFP (N2A-Cx36-EGFP) showed higher increase in g_j than HeLa cells expressing Cx36-EGFP when exposed to 0.01 mM $[Mg^{2+}]_p$ (Fig. 2B). In summary, Cx36 GJs exhibited a “run-up” or “run-down” in g_j when $[Mg^{2+}]_p$ in both pipette solutions was lower

Figure 3. $[Mg^{2+}]_i$ -dependent modulation of g_j and V_j -gating of GJs formed by other Cxs expressed in CNS does not affect single-channel conductance. **A, C, E**, Changes of I_j in response to repeated 50-s-long V_j ramps from 0 to -100 mV and intermediate small-amplitude steps (-15 mV) using $[Mg^{2+}]_p = 5$ mM in Novikov (A), HeLa Cx45 (C), and HeLa Cx47-EGFP (E) cell pairs. **B, D, F**, First (purple) and last (cyan) g_j - V_j relations (normalized to initial g_j value at $V_j = 0$) obtained from experiments shown in A, C, and E, respectively. Last g_j - V_j relation normalized to g_j value at $V_j = 0$ (gray) is also shown for comparison. **G**, Concentration-response relation of g_j (normalized to initial g_j value) as a function of $[Mg^{2+}]_p$ for Cx47; $EC_{50} \approx 2.8$ mM. Averaged data are shown in colored circles; roman numerals correspond to different pipette solutions shown in Table 1. **H**, Averaged g_j - V_j dependence (normalized to g_j at $V_j = 0$) obtained at different $[Mg^{2+}]_p$ for Cx47; roman numerals correspond to different $[Mg^{2+}]$ shown in G and Table 1. Experimental g_j - V_j plots shown in black were fitted using the S16SM; calculated values of gating parameters are shown in Table 2, and the best-fitting g_j - V_j plots are shown in colors that correspond to colors of circles in G. **I, J**, Open probabilities of fast and slow gates in α and β aHCs ($P_{oF,\alpha}$, $P_{oS,\alpha}$, and $P_{oF,\beta}$, $P_{oS,\beta}$, respectively) depending on V_j were calculated using parameters obtained in H for $[Mg^{2+}]_p = 0.01$ (I) and 10 mM (J). **K**, I_j record of Cx43-CFP GJs obtained at $V_j = -80$ mV. **L**, Histogram from data in K shows a series of peaks separated by $\sim 96.5 \pm 8$ pS. **M**, Single-channel conductance for Cx47-EGFP obtained in response to a V_j step of -52 mV. The histogram shows peaks for the closed state, substate ($\gamma_s = 11.2 \pm 2.3$ pS), and open state ($\gamma_o = 49 \pm 5.6$ pS). The arrowhead indicates a slow transition from the closed state to open state, and the arrow indicates a fast transition from the open state to the substate. **N**, I_j trace from a HeLa Cx47-EGFP cell pair in response to 1.5-s-long V_j ramps from 90 to -90 mV using $[Mg^{2+}]_p = 5$ mM after 32, 37, and 42 min of recording.

or higher than ~ 1.3 mM, respectively, and we assume that 1.3 mM is the resting $[Mg^{2+}]_i$ in HeLa cells under our experimental conditions (Fig. 1E). Changes in $[Mg^{2+}]_i$ have a marked effect on $V_{F,o}$, which affects mainly P_o and, to a small degree, N_F .

Junctional conductance of GJ channels formed of Cx26, Cx32, Cx43, Cx45, or Cx47 is also reduced by increase in intracellular magnesium

To compare Cx36 to other isoforms with respect to the effects of $[Mg^{2+}]_i$, we studied Mg^{2+} -dependent modulation of g_j in HeLa cells expressing the following: Cx43 (α -group); Cx26 and Cx32

(β -group); and Cx45 and Cx47 (γ -group). In contrast to Cx36 (δ -group), $[Mg^{2+}]_p = 0.01$ mM did not increase g_j above initial values in all other tested Cxs (Fig. 2D). However, $[Mg^{2+}]_p = 5$ mM reduced g_j in all tested Cxs (Fig. 2E). To compare Cx36 to other Cxs expressed in the CNS with respect to g_j - V_j dependence at different $[Mg^{2+}]_i$, we chose Cx43, Cx45, and Cx47, which are expressed in astrocytes, neurons, and oligodendrocytes, respectively. Similar to the effect on Cx36, high $[Mg^{2+}]_p$ increased sensitivity to V_j in Cx43- and Cx47-expressing cells (Figs. 3A,B, 4E,F); increased V_j -sensitivity was not observed in Cx45-expressing cells (Fig. 3C,D). Increase in $[Mg^{2+}]_p$ to 10 mM de-

creased g_j for Cx47 (normalized to initial values) to 0.08 (Fig. 3G). Steady-state g_j - V_j relationships (normalized to g_j at $V_j = 0$) at different $[Mg^{2+}]_p$ for Cx47 (Fig. 3H, black lines) were fitted using the S16SM (Fig. 3H, colored lines); all parameters are shown in Table 2. Elevation in $[Mg^{2+}]_i$ moderately increased A_F and the maximal steepness of g_j -versus- V_j changes, and markedly decreased $V_{F,0}$, as is reflected in narrowing of the (flat topped) bell-shaped g_j - V_j plot (Fig. 3F,H). However, P_{oF} and P_{oS} at $V_j = 0$ remained constant and close to unity for all $[Mg^{2+}]_p$, as reflected in the flat top of the g_j - V_j relation (Fig. 3I,J). Therefore, all changes in g_j observed at $V_j = 0$ under different $[Mg^{2+}]_i$ are due to a reduction in N_F and not to changes in parameters of V_j -gating. In contrast to g_j of the Cx36 GJ, g_j of the Cx26, Cx32, Cx43, Cx45, and Cx47 GJs showed spontaneous “run down” even at $[Mg^{2+}]_p = 0.01$ mM (Fig. 2D). Therefore all data in the g_j - $[Mg^{2+}]_p$ dependence for Cx47 are below unity (Fig. 3G). This decay in g_j of Cx47 was prevented by adding 3 mM K_2ATP to solutions with low $[Mg^{2+}]_p$ (data not shown), indicating that stability of these GJs depends on ATP as observed for GJs formed between cardiomyocytes (Sugiura et al., 1990; Verrecchia et al., 1999).

Single-channel conductance of Cx43 and Cx47 GJs is not affected by high intracellular magnesium

Previous studies of Cx36 GJs have shown that γ_{open} is very small compared with those of GJs formed of other Cxs (Srinivas et al., 1999; Teubner et al., 2000; Moreno et al., 2005), making the study of changes in γ_{open} under different $[Mg^{2+}]_i$ impracticable. Thus, we tested whether γ_{open} of GJ channels formed by Cxs with relatively high γ_{open} (Cx43 and Cx47) were modified by 5 mM $[Mg^{2+}]_p$ (Table 1, Solution IV). Experiments were performed in HeLa cells expressing Cx43-CFP or Cx47-EGFP. The γ_{open} values of Cx43 and Cx47 under high $[Mg^{2+}]_p$ did not differ significantly from those previously reported under control conditions (Moreno et al., 1994; Bukauskas et al., 2000; Teubner et al., 2001). We found that, at $[Mg^{2+}]_p = 5$ mM, γ_{open} of Cx43 and Cx47 GJ channels was 96.5 ± 8 pS (Fig. 3K,L), and 49 ± 5.6 pS (Fig. 3M), respectively. In the experiment shown in Figure 3K, initial g_j at time 0 was ~ 40 nS, corresponding to ~ 400 open Cx43 GJ channels. High $[Mg^{2+}]_p$ reduced g_j and ~ 36 min later no more than three GJ channels were open simultaneously. Similarly, in the experiment shown in Figure 3N, initial g_j was ~ 6 nS, corresponding to ~ 120 open Cx47 GJ channels. High $[Mg^{2+}]_p$ reduced g_j , and, after 32, 37, and 42 min, maximum numbers of channels open were seven, three, and one, respectively. In summary,

Figure 4. Intracellular K_2ATP and $MgATP$ have opposite effects on g_j and V_j -gating in HeLa cells expressing Cx36-EGFP GJ channels. **A**, **C**, I_j 's recorded during repeated 35-s-long V_j ramps from 0 to -100 mV. Brief voltage steps of -10 mV were used to measure g_j in between V_j ramps. Pipette solutions contained 10 mM K_2ATP (**A**) or $MgATP$ (**C**). **B**, **D**, First (purple) and last (cyan) g_j - V_j relations (normalized to initial g_j value at $V_j = 0$) measurements obtained from experiments shown in **A** and **C**, respectively. **E**, **F**, First (purple) and last (cyan) g_j - V_j relations (normalized to initial g_j at $V_j = 0$) obtained using the same V_j protocol as in **A**. Pipette solutions contained 1.5 mM EDTA (**E**) or 5 mM $MgCl_2$ (**F**). In all g_j - V_j plots, we assumed that the g_j - V_j relation for $V_j > 0$ was the mirror image of that for $V_j < 0$ (i.e., symmetrical around $V_j = 0$) and the last g_j - V_j plots normalized to g_j at $V_j = 0$ (gray) are also shown for comparison. **G**, Changes in normalized g_j for the different compositions of the pipette solutions shown at the bottom. Values were obtained from the ratios of the steady-state final g_j to the initial g_j . Numbers of independent experiments are shown in the histogram bars; * $p < 0.05$; *** $p < 0.001$; ns, nonsignificant p values.

reduction in conductance at $V_j \approx 0$ of Cx43 and Cx47 GJs by $[Mg^{2+}]_p = 5$ mM results from a reduction of N_F without changes in γ_{open} .

Intracellular ATP-dependent modulation of junctional conductance and voltage-gating of Cx36 GJs

Adding K_2ATP to the pipette solution, which reduces free $[Mg^{2+}]_p$, or adding $MgATP$ to the pipette solution, which in-

Figure 5. Permeation of Cx36 or Cx47 GJ channels by Mg^{2+} ions. **A, B**, Mag-fluo-4 (MF4) fluorescence intensity measured in cell-1 (FI_1) of HeLa Cx36-EGFP (**A**) and HeLa Cx47-EGFP (**B**) cell pairs increased after opening the patch in cell-1 (arrow). After FI_1 reached a plateau (normalized to this value), pipette-2 was opened (arrowhead) and FI_1 again increased. Pipette-1 contained 50 μM MF4 and zero $MgCl_2$, and pipette-2 contained 10 mM $MgCl_2$ (top diagram). The g_j measurements were started after patch opening in cell-2 (top). FI_1 and g_j decreased after bath application of the GJ blocker octanol (1 mM); decrease in FI_1 is ascribable to loss of Mg^{2+} into pipette-1. **C, D**, Rates of FI_1 changes in HeLa Cx36-EGFP (gray; $n = 12$) and HeLa Cx47-EGFP (black; $n = 15$) cell pairs measured after patch opening in cell-2 and plotted over g_j (**C**) or the calculated number of open channels (**D**). Gray and black lines are linear regressions for Cx36-EGFP ($R^2 = 0.71$) and Cx47-EGFP ($R^2 = 0.9$) data, respectively.

creases free $[Mg^{2+}]_p$ (Lüthi et al., 1999), replicated results obtained with low or high free $[Mg^{2+}]_p$, respectively (Table 1, solutions) (Fig. 1). Indeed, adding 10 mM K_2ATP to pipette solution with $MgCl_2 = 1$ mM (see Materials and Methods) increased g_j (Fig. 4A, G) and decreased its sensitivity to V_j (Fig. 4B). Similar results were obtained when $[Mg^{2+}]_p$ was reduced by adding the Mg^{2+} chelator EDTA (Fig. 4E). In contrast, addition of 10 mM $MgATP$, which increases free Mg^{2+} (Lüthi et al., 1999), decreased g_j (Fig. 4C, G) and increased its sensitivity to V_j (Fig. 4D). Similar results were obtained by adding 5 mM $MgCl_2$ (Fig. 4F). These results show that low free Mg^{2+} conditions obtained by adding K_2ATP , or high free Mg^{2+} conditions obtained by adding $MgATP$, have a marked effect on g_j . Addition of 1.5 mM BAPTA to solutions with K_2ATP or $MgATP$ to minimize changes in $[Ca^{2+}]_p$ showed no differences (Fig. 4G). In summary, we attribute the effect of ATP on g_j of Cx36 GJs mainly to its capacity to modify $[Mg^{2+}]_i$.

Magnesium ions permeate Cx36 and Cx47 GJ channels

To determine whether Mg^{2+} permeates GJ channels and discard the hypothesis of a physical occlusion of the channel pore, we used a cell-impermeant fluorescent Mg^{2+} indicator (Mag-Fluo-4

or MF4) in HeLa Cx36-EGFP or Cx47-EGFP cell pairs. First, we opened the patch in cell-1 (Fig. 5A, B, arrows) with a pipette containing 50 μM MF4 and nominally zero $[Mg^{2+}]_p$. Fluorescence intensity in cell-1 (FI_1), increased to a plateau, presumably corresponding to a low $[Mg^{2+}]_i$ due to loss into pipette-1 and basal fluorescence of MF4. Then, we opened the patch in cell-2 (Fig. 5A, B, arrowheads) connecting it to a pipette containing 10 mM $MgCl_2$ and measured FI_1 and g_j (top) determined by applying repeated small-amplitude V_j ramps (same as in Fig. 1A). FI_1 rapidly increased indicating flux of Mg^{2+} from cell-2 to cell-1. When the GJ blocker octanol was applied, g_j and FI_1 decreased due to the closure of GJs between cell-1 and cell-2 and diffusion of Mg^{2+} into pipette-1 (Fig. 5A, B). For different pair of cells, the rate of increase of FI_1 (measured in AUs for the first 2 min after breaking into cell-2) was proportional to g_j for both Cx36 and Cx47 and approximately half as fast per unit g_j for Cx36 as for Cx47 (Fig. 5C). The same flux data were plotted as a function of the number of open channels obtained by dividing g_j by γ_{open} of Cx36 [although we could not reliably measure γ_{open} for Cx36, we used the published value of 6 pS (Moreno et al., 2005)] and Cx47 [$\gamma_{open} = 55$ pS (Teubner et al., 2001)]; for these values of γ_{open} , the per channel flux for Cx36 was approximately one-twentieth of that for Cx47 (Fig. 5D). In summary, Cx36 and Cx47 GJ channels are permeable to Mg^{2+} , and the Mg^{2+} flux per nS g_j for Cx47 was approximately twice that for Cx36. For comparison, the ratio of γ_{open} of Cx36 to γ_{open} of Cx47, which are presumably K^+ -dominated, is $\sim 1:10$.

Transjunctional asymmetry of free magnesium ions results in asymmetric voltage-gating of Cx36 GJs

Since Mg^{2+} ions permeate Cx36 GJ channels, V_j applied to GJs with differing Mg^{2+} concentration on the two sides will alter the Mg^{2+} distribution within the channel through ionophoresis. Relative positivity in the cell with lower $[Mg^{2+}]_i$ will decrease the Mg^{2+} occupancy of the channel. If the sensorial domain for Mg^{2+} is located within the channel lumen, relative positive V_j on the lower $[Mg^{2+}]_i$ side should decrease V_j -gating sensitivity of fast gates and increase P_{of} . The opposite changes should occur with relative negativity in the cell with lower $[Mg^{2+}]_i$. In homotypic Cx36 GJs, with a gradient of $[Mg^{2+}]_p$ (0.01 mM in pipette-1 and 5 mM in pipette-2), relative positive V_j on the lower $[Mg^{2+}]_i$ side increased g_j , while relative negative V_j on the lower $[Mg^{2+}]_i$ side decreased g_j (Fig. 6A, B). The changes in g_j were independent of which pipette received the voltage (Fig. 6D, E). Decrease in g_j during relative negativity on the lower $[Mg^{2+}]_i$ side implies that the electric field increases Mg^{2+} occupancy of Cx36 channels, while increase in g_j during relative positivity on the same side implies that the field decreases Mg^{2+} occupancy. Thus, transjunctional asymmetry of $[Mg^{2+}]_i$ resulted in an asymmetric g_j - V_j

relationship, probably involving sensitivity to both V_j and Mg^{2+} . Asymmetric g_j - V_j dependence could also be achieved by having MgATP or K_2 ATP in one pipette to increase or reduce $[Mg^{2+}]_i$, respectively, on one side of the junction (Fig. 6C,F).

To test whether V_j -gating asymmetry caused by a $[Mg^{2+}]_i$ gradient is reversible, we performed an experiment in which pipette-2 containing relatively high $[Mg^{2+}]_p$ (MgATP, 3 mM) was replaced by a pipette containing low $[Mg^{2+}]_p$ (K_2 ATP, 5 mM), and then replaced again by a pipette containing the original solution, high $[Mg^{2+}]_p$ (MgATP, 3 mM) (Fig. 7A). Pipette-1 contained standard pipette solution ($MgCl_2 = 1$ mM) throughout the experiment. During the first 8 min, g_j decreased, resulting in a g_j - V_j asymmetry with somewhat higher sensitivity to V_j at negativity on the side with standard pipette solution (Fig. 7Ba). After the first exchange of pipette-2, g_j increased and V_j -gating asymmetry reversed very quickly (Fig. 7Bb). After the second exchange of pipette-2, g_j decreased and V_j -gating asymmetry reversed again very quickly (Fig. 7Bc). In summary, V_j has a strong effect on the Mg^{2+} -dependent modulation, suggesting a presence within the channel lumen of a sensorial domain for Mg^{2+} .

The capability of V_j steps of alternating polarity to induce closures and openings of Cx36 GJ channels under a transjunctional concentration gradient of Mg^{2+} in tens of seconds (Fig. 6A,D) and reversal of g_j - V_j asymmetry by exchange of pipette solutions (Fig. 7) indicate that the Mg^{2+} -dependent effect on g_j is reversible. It is most likely that g_j changes are caused by direct Mg^{2+} effects on channel function rather than through post-translational modifications (no ATP in pipette solutions) or insertion/removal of channels from the junctional plaque. Furthermore, if Cx36 has one or more cytoplasmic Mg^{2+} -binding sites, then the Mg^{2+} -dependent closure of one aHC exposed to high $[Mg^{2+}]_i$ would result in P_o close to zero. We did not observe a comparable reduction in g_j at $V_j = 0$ between experiments with symmetric ($[Mg^{2+}]_p = 5$ mM in both pipettes) or asymmetric ($[Mg^{2+}]_p = 5$ mM in pipette-1; 0.01 mM in pipette-2) Mg^{2+} conditions, suggesting that Mg^{2+} sensorial domain is not located in the cytoplasmic side of the channel. Together, these results indicate that the effect of Mg^{2+} is completely reversible and suggest that the site or sites of action of Mg^{2+} are in the channel pore-lining residues where V_j 's affect the ionic occupancy of the channel, through ionophoresis, and possibly binding affinity.

High intracellular magnesium stabilizes a closed conformation of Cx36 GJ channels

To test whether $[Mg^{2+}]_i$ affects the recovery of g_j after closing Cx36 GJ channels by V_j -gating, we examined changes in g_j after applying V_j steps in symmetrical $[Mg^{2+}]_p = 5$ mM (Fig. 8A). V_j -gating at high Mg^{2+} induced a fast reduction in g_j followed by a slow continuous decay. The recovery of g_j after V_j steps showed fast and slow components (Fig. 8A), presumably due to the opening of fast and slow gates, respectively. However, the recovery of g_j did not reach values of an

Figure 6. Transjunctional asymmetry of $[Mg^{2+}]_i$ causes asymmetric V_j gating of homotypic Cx36-EGFP GJs. **A, D**, Transjunctional asymmetry in $[Mg^{2+}]_i$ (see diagrams at the top of **B** and **E** for free Mg^{2+} concentration in pipette solutions and stimulation site) caused asymmetry in V_j -gating with decrease in g_j for relative negativity on the low $[Mg^{2+}]_p$ side. V_j steps (± 80 mV) of opposite polarities produced opposite effect on g_j . Small-amplitude repeated V_j ramps (± 20 mV, same as in Fig. 1A) were used to measure g_j between V_j steps. **B, E**, g_j - V_j relations (normalized to g_j value at $V_j = 0$) measured by applying long (60 s) V_j ramps from 0 to +100 and -100 mV. Relative positivity on the high $[Mg^{2+}]_p$ side decreased g_j . **C, F**, Asymmetric concentration of MgATP (**C**, top diagram) or K_2 ATP (**F**, top diagram) was associated with asymmetry of g_j - V_j dependence (normalized to g_j value at $V_j = 0$).

averaged curve of g_j decay obtained in the absence of V_j steps (Fig. 8A, dashed line). These results indicate that increasing V_j -gating and the Mg^{2+} occupancy by V_j -dependent ionophoresis in Cx36 GJ channels increased the speed of the Mg^{2+} -dependent reduction in g_j . Moreover, at $[Mg^{2+}]_p = 5$ mM, the values of g_j obtained using V_j steps to accelerate the decay were smaller than those obtained at the same $[Mg^{2+}]_p$ without using V_j steps, suggesting that high Mg^{2+} could stabilize a closed conformation, possibly of slow gates. We were not able to assess g_j recovery after V_j -gating in low $[Mg^{2+}]_p$ due to the lack of V_j -sensitivity (Fig. 1).

To close Cx36 GJ channels at $V_j = 0$, we used the chemical uncoupler decanol (0.5 mM) and examined g_j recovery during washout at different $[Mg^{2+}]_p$ (Fig. 8B). These experiments were normalized to g_j values after reaching the steady state ($g_{j,ss}$) at each $[Mg^{2+}]_p$ (~ 20 min after opening patches), and time 0 was adjusted to the beginning of decanol perfusion. Decanol was applied until g_j neared zero and then washed out with normal bath solution. Full recovery of g_j was reached only at $[Mg^{2+}]_p = 0.01$ mM (Fig. 8Ba). Recovery of g_j was $\sim 50\%$ at $[Mg^{2+}]_p = 1$ mM (Fig. 8Bb) and $\sim 25\%$ at $[Mg^{2+}]_p = 5$ mM (Fig. 8Bc). A possible explanation for these results is that binding of Mg^{2+} in the Cx36 GJ channels stabilizes a closed conformation, explaining the low recovery of g_j after closing the channels with V_j -gating or chemical-gating under high $[Mg^{2+}]_p$.

A phosphomimetic mutant of Cx36 shows magnesium-dependent modulation of junctional conductance similar to Cx36 wild type

Function of Cx36 GJs strongly depends on phosphorylation of two intracellular serine residues, which are phosphorylated by CaMKII, cGMP-dependent protein kinase, and cAMP-dependent protein kinase (Mitropoulou and Bruzzone, 2003;

Figure 7. Fast reversal of asymmetric g_j – V_j dependence by reversal of transjunctional gradient of $[Mg^{2+}]_i$. **A**, Changes in I_j during consecutive 35-s-long V_j ramps from 0 to -100 mV and from 0 to 100 mV. Initially, cell-1 was loaded with a control/standard pipette solution ($MgCl_2$, 1 mM) and cell-2 contained $MgATP$ (**Aa**). From ~ 9 to 14 min after onset, pipette-2 was carefully detached and replaced with a pipette containing K_2ATP (**Ab**) reversing the Mg^{2+} gradient. From ~ 25 to 30 min after onset, pipette-2 was replaced with a pipette containing $MgATP$ (**Ac**) reversing the Mg^{2+} gradient once again. **B**, g_j – V_j plots (normalized to initial g_j value at $V_j = 0$) from ramp pairs in (**A**) designated with numbers 1 and 2 (**Ba**), 3 and 4 (**Bb**), and 5 and 6 (**Bc**).

Ouyang et al., 2005; Patel et al., 2006; Alev et al., 2008; Kothmann et al., 2009). Furthermore, phosphatases are highly dependent on $[Mg^{2+}]_i$ (Merlevede et al., 1984). Therefore, we tested whether changes in phosphorylation of Cx36 might be involved in the observed Mg^{2+} -dependent changes of g_j . For these tests, we used mutants of Cx36 in which serines 110 and 293 were replaced by aspartates, which resemble negatively charged phosphoserine residues. These “phosphomimetic” mutants are locked in a pseudophosphorylated state that cannot be dephosphorylated, thus allowing study of Mg^{2+} -dependent modulation of g_j independently of phosphorylation or dephosphorylation at these sites. We found that combined point mutations, S110D and S293D, exhibited slightly more increase in g_j at low $[Mg^{2+}]_i$ and slightly less decay of g_j at high $[Mg^{2+}]_i$. However, in neither case were the values significantly different from those measured in Cx36. In summary, these phosphomimetic mutants behave similarly to the wild-type Cx36, suggesting that changes in phosphorylation of at least these two serine residues are not involved in the Mg^{2+} -dependent modulation of g_j .

Modulation of junctional conductance by different divalent cations in Cx36 GJ channels

To rule out a possible nonspecific effect of surface charge screening and test the specificity of the effects of Mg^{2+} ions on Cx36, we examined g_j using pipette solutions containing divalent cations from alkaline earth metals (Ca^{2+} or Ba^{2+}) or transition metals

(Mn^{2+} , Cd^{2+} , or Zn^{2+}). Because EGTA and BAPTA are not good buffers for all these divalents (Patton et al., 2004), we prepared solutions without EGTA or BAPTA and compared results with a control solution of nominally zero divalents (see Materials and Methods). The control solution increased g_j to ~ 2.5 -fold of initial g_j (Fig. 9). All divalents at a concentration of 2 mM decreased g_j (Fig. 9A, B), but with different times to reach 5% of initial g_j (Fig. 9A, C), indicating that nonspecific screening of charges in the membrane surface and/or cytoplasmic side of the Cx36 protein does not play a major role in this inhibition. Because 2 mM Zn^{2+} produced the fastest inhibition, we used solutions with 0.2 mM Zn^{2+} to compare the degree of inhibition and time to reach the steady state. Interestingly, 0.2 mM Zn^{2+} , like 2 mM Zn^{2+} , almost completely blocked g_j (Fig. 9A, B), but took five times longer to reach 5% of initial g_j (Fig. 9A, C). In summary, all examined divalent cations strongly inhibited conductance of Cx36 GJs but with different kinetics. These results suggest that divalent cations act through a relatively nonspecific negatively charged binding site in Cx36 rather than through surface charge screening.

Intracellular magnesium-dependent modulation of junctional conductance in neurons of the MesV nucleus

To test whether native electrical synapses expressing Cx36 are sensitive to changes in $[Mg^{2+}]_i$, we examined changes in the

strength of electrical coupling by measuring junctional conductance (G_j) between pairs of MesV neurons at low or high $[Mg^{2+}]_i$. The MesV nucleus is formed by the somata of primary afferents originating in jaw-closing muscles whose cell bodies are located within the CNS rather than peripheral ganglia (Nagy et al., 1986). These large somata (Fig. 10A) are electrically coupled through Cx36-containing somato-somatic GJs (Curti et al., 2012). We recorded from pairs of MesV under current-clamp configuration, and G_j was indirectly estimated using interleaved hyperpolarizing current steps on each cell (see Materials and Methods) (Fig. 10B). When recording with a solution containing $[Mg^{2+}]_p = 0.01$ mM in both pipettes, we observed a progressive increase in G_j following patch opening (Fig. 10C). In contrast, solutions containing $[Mg^{2+}]_p = 5$ mM produced a progressive reduction of G_j (Fig. 10C). Averaged G_j (normalized to initial values) showed that low $[Mg^{2+}]_i$ led to a $18 \pm 4\%$ increase in G_j after ~ 10 min of patch opening, whereas high $[Mg^{2+}]_i$ produced a $21 \pm 3\%$ reduction (Fig. 10D). Longer-lasting (~ 30 min) experiments with 5 mM $[Mg^{2+}]_i$ showed further reductions in G_j ($>30\%$; data not shown). Thus, native Cx36-containing electrical synapses exhibited similar sensitivity to $[Mg^{2+}]_i$, suggesting that this mechanism could operate *in vivo*.

Discussion

Although effects of divalent cations on GJ channels have long been recognized (Loewenstein, 1967; Déléze and Loewenstein,

Figure 8. Recovery of g_j after decrease induced by V_j -gating or chemical-gating depends on $[Mg^{2+}]_i$. **A**, Bottom, Dynamics of g_j recovery (normalized to initial g_j value) after V_j steps (-80 mV; top) at $[Mg^{2+}]_p = 5$ mM. Small-amplitude repeated V_j ramps (± 21 mV, 1.3 s; inset) were used to measure g_j before and after V_j steps. Dashed line represents averaged decay in g_j in the absence of V_j steps using pipette solutions with $[Mg^{2+}]_p = 5$ mM. **B**, Dynamics of g_j recovery normalized to steady-state g_j value ($g_{j,ss}$) before decanol (0.5 mM) application to induce uncoupling at $[Mg^{2+}]_p = 0.01$ mM (**Ba**), 1 mM (**Bb**), and 5 mM (**Bc**). Small-amplitude repeated V_j ramps (same as in **A**) were used to measure g_j . Gray dashed lines show levels of g_j recovery during washout from decanol.

1976; Spray et al., 1982; Noma and Tsuboi, 1987; Vera et al., 1996; Matsuda et al., 2010), the mechanisms by which Mg^{2+} modulates Cx36 GJs have not been studied. Here we demonstrate that control/resting levels of $[Mg^{2+}]_i$ (~ 1 mM) maintain Cx36 GJ channels expressed in heterologous systems and neurons of the MesV nucleus partially inhibited and that cell–cell coupling is modulated by changes in $[Mg^{2+}]_i$. We showed that the Mg^{2+} -dependent modulation of g_j is caused by changes of P_o and N_F (Fig. 1G–I). In Cx36, the Boltzmann parameters A_F and A_S remained relatively constant during changes in $[Mg^{2+}]_i$ (Table 2), indicating that changes in P_o at $V_j = 0$ were mainly due to changes in $V_{F,o}$, and that the gating charge of the V_j sensor was not modified by changes in $[Mg^{2+}]_i$. g_j approached zero at $[Mg^{2+}]_p = \sim 10$ mM, while maximal values of g_j in HeLa cells were reached by decreasing $[Mg^{2+}]_p$ to 0.01 mM or less (Fig. 1E). Similar effects on g_j were achieved by adding to the pipette solution K_2ATP or $MgATP$ (Fig. 4), which reduced or increased $[Mg^{2+}]_i$, respectively. These data suggest that ATP affects g_j mainly through its capacity to modulate free $[Mg^{2+}]_i$ (Lüthi et al., 1999). In our studies, $[Ca^{2+}]_i$ was strongly buffered at low values (~ 25 nM)

Figure 9. Divalent cations decrease g_j of Cx36 GJs. **A**, Dynamics of g_j (normalized to initial g_j value) changes for different divalent cations. **B**, Average g_j (normalized to initial g_j values) from experiments using pipette solution containing the following: nominally zero divalents ($n = 5$); 2 mM free Mg^{2+} ($n = 3$), Ca^{2+} ($n = 5$), Ba^{2+} ($n = 5$), Mn^{2+} ($n = 6$), Cd^{2+} ($n = 4$), Zn^{2+} ($n = 3$); 0.2 mM free Zn^{2+} ($n = 4$). **C**, Average time for g_j to undergo 95% of the change from initial to virtual steady-state conductance after beginning dual whole-cell voltage clamp.

with BAPTA and EGTA, indicating that Mg^{2+} ions, not Ca^{2+} ions, were involved in the observed changes in g_j .

Our data show that high $[Mg^{2+}]_i$ also reduces g_j of GJ channels formed of Cx26, Cx32, Cx43, Cx45, and Cx47 (Fig. 2E). However, we did not observe an increase in g_j for these Cxs at $[Mg^{2+}]_i = 0.01$ mM (Fig. 2D). Thus, Cx36 was the only Cx examined that exhibited a substantial inhibition of GJ channels at control/resting levels of $[Mg^{2+}]_i$. This suggests that changes in $[Mg^{2+}]_i$ under physiological or pathological conditions can modulate preferentially Cx36-mediated cell–cell coupling. The γ_{open} of Cx43 and Cx47 did not change under enhanced $[Mg^{2+}]_p$ (Fig. 3L,M) demonstrating that Mg^{2+} -mediated decrease in g_j for these Cxs was not due to reduction in γ_{open} . GJs formed of both Cx36 and Cx47 are permeable to Mg^{2+} (Fig. 5); thus, V_j may modify the Mg^{2+} occupancy of the channel by ionophoresis and also by changing the on and off rates of Mg^{2+} binding. We conclude that Mg^{2+} acts inside the Cx36 channel lumen based on the following facts: (1) Mg^{2+} permeates the channel (Fig. 5); (2) transjunctional gradients of $[Mg^{2+}]_i$ caused asymmetric g_j – V_j dependence (Figs. 6, 7); and (3) the averaged $g_{j,ss}$ at $V_j = 0$ observed using transjunctional asymmetric $[Mg^{2+}]_i$ (5 mM in pipette-1; 0.01 mM in pipette-2) is higher than averaged $g_{j,ss}$ obtained under symmetric high $[Mg^{2+}]_i$ (5 mM). Mg^{2+} -dependent changes in g_j were too fast to be explained by an insertion or removal of channels during V_j 's of different polarities (Fig. 6) and after changes in $[Mg^{2+}]_i$ (Fig. 7). Furthermore, we

Figure 10. Mg^{2+} -dependent modulation of G_j in pairs of MesV neurons. **A**, IR-DIC image of a pair of electrically coupled MesV neurons during dual whole-cell patch clamp. **B**, Simultaneous current-clamp recordings from a pair of electrically coupled MesV neurons; arrows indicate the direction of the spread of electrotonic potential. Voltage traces were recorded from cell-1 and cell-2 (V_1 and V_2 , respectively) during 300 ms hyperpolarizing current steps of -300 pA injected either in cell-1 or in cell-2 (I_1 and I_2 , respectively). **C**, Time course of changes in mean G_j (normalized to initial values) at $[Mg^{2+}]_p = 0.01$ (gray) and 5 mM (black). Each point represents an average from five independent experiments. **D**, Mean percentage changes of G_j from initial values after 12 min of patch openings with $[Mg^{2+}]_p = 0.01$ (gray) and 5 mM (black). Numbers of cell pairs are indicated within columns; $*p < 0.05$.

did not detect changes in the size or fluorescence intensity of Cx36-EGFP junctional plaques (data not shown).

The Mg^{2+} occupancy of Cx36 GJ channels reduces P_{oF} (Fig. 1) by favoring transitions of fast gates into a closed state, and also appears to stabilize a long-lived closed conformation of slow gates (Fig. 8), which in the S16SM is reflected in a reduction of N_F . Low $[Mg^{2+}]_i$ conditions allowed for a full recovery of g_j from uncoupling induced by chemical-gating (Fig. 8B). However, in the presence of high $[Mg^{2+}]_i$, the recovery of g_j from uncoupling induced by chemical-gating or V_j -gating was markedly reduced (Fig. 8A,B). These results suggest that Mg^{2+} stabilizes the closed conformation of the slow gate mediated by chemical uncouplers or V_j . The relative slow kinetics of the Mg^{2+} -dependent changes in g_j at $V_j = 0$ (Fig. 1; ~ 20 min to reach steady state) could be the combined results of a preferential binding of Mg^{2+} to a closed state ($P_o = 0.64$ at $[Mg^{2+}]_p = 1$ mM and $V_j = 0$) and a low rate of unbinding (off rate) leading to the stabilization of a closed channel conformation. In addition, Cx36 GJ channels may require coordinated binding of Mg^{2+} to more than one binding site to stabilize the closed channel conformation. The effect of Mg^{2+} on the reduction of N_F is reversible (Figs. 6, 7), and channels can become operational/functional again in low $[Mg^{2+}]_i$, which suggests a stochastic release of Mg^{2+} from binding site(s). Mg^{2+} -dependent modulation of g_j was not affected by phosphomimetic mutations of Cx36 at residues S110 and S293. We conclude that changes in phosphorylation at those positions are not necessary for the observed Mg^{2+} -dependent modulation of g_j ; however, we do

not rule out the possibility that phosphorylation of Cx36 could modify coupling at different $[Mg^{2+}]_i$. In addition, the fact that asymmetry in g_j - V_j dependence can be reversed in the same cell pair by exchanging pipettes with different $[Mg^{2+}]_p$ (Figs. 6, 7), and the fact that openings or closures can be consecutively induced by V_j steps of different polarity over relative short times using pipette solutions without ATP (Fig. 6A,D), strongly suggest that ATP-dependent post-translational modifications, such as phosphorylation, are not involved in the Mg^{2+} -dependent modulation of Cx36 GJ channels. The inhibition of Cx36 GJ channels could also be induced by divalent cations other than Mg^{2+} , and each divalent exhibited a different kinetics of g_j decay (Fig. 9). These differences suggest that nonspecific screening of surface charges in the Cx36 protein or membrane phospholipids is unlikely to be a primary mechanism of Mg^{2+} -dependent changes in g_j . Importantly, Cx36 GJ channels showed high sensitivity to $[Zn^{2+}]_i$, which plays an important role in CNS and pancreatic β -cells (Frederickson et al., 2000; Slepchenko and Li, 2012).

Finally, Cx36-containing electrical synapses between MesV neurons showed similar, although smaller, Mg^{2+} -dependent changes in cell-to-cell coupling compared with those observed in HeLa and N2A cells (Fig. 10C), suggesting that this mechanism could operate in the brain. The differences in the magnitude of the Mg^{2+} effect could be explained by differences between MesV neurons and heterologous expression systems, such as intrinsic buffer capacity, resting levels of $[Mg^{2+}]_i$, Cx36 levels of expression, and the presence of another Cx [based on residual coupling between MesV neurons in Cx36 knock-out mice (Curti et al., 2012)]. Nonetheless, electrical synapses between MesV neurons showed a clear and significant bidirectional modulation in G_j ($\sim \pm 20\%$) dependent on $[Mg^{2+}]_i$ (Fig. 10D), which is consistent with results obtained in HeLa and N2A cells. Furthermore, the magnitude of changes in MesV neurons is likely to be of physiological relevance, since long-term depression producing an equivalent reduction in G_j between neurons of the thalamic reticular nucleus has been suggested to produce changes in neuronal synchronization (Landisman and Connors, 2005).

In conclusion, the function of Cx36 GJ channels is strongly modulated by changes in $[Mg^{2+}]_i$. Effects of intracellular ATP on Cx36 GJs result, at least in part, from modulation of $[Mg^{2+}]_i$. We showed that (1) a substantial fraction of Cx36 GJ channels are inhibited even at resting levels of $[Mg^{2+}]_i$ (~ 1 mM); (2) Cx36 GJ channels are permeable to Mg^{2+} ions, which can enter the channel and interact with pore-lining residues; and (3) the Cx36 GJ channel lumen contains a sensorial domain for divalent cations that upon binding induces a reduction in P_o and stabilization of a closed channel conformation. Thus, physiological conditions where cytosolic ATP decreases (e.g., increased neuronal activity during prolonged waking periods) or pathological conditions, such as hypoxia, ischemia or seizures, which lead to increased $[Mg^{2+}]_i$, might reduce neuronal synchronization via reduction

in the strength of electrical synapses formed of Cx36 (Fig. 11). On the other hand, physiological conditions where cytosolic ATP increases (e.g., reduced neuronal activity during sleep period) or pathological conditions, such as traumatic brain injury, might induce an increase in Cx36-mediated gap junctional intercellular communication (GJIC) and neuronal synchronization (Fig. 11). In addition, increased synchronization of inhibitory neurons could lead to a reduction in synchronous activity of excitatory neurons. Modulation in the rates of binding or unbinding of Mg^{2+} through modification of the binding site(s) by physiological stimuli, such as post-translational modifications, lipophilic neuromodulators, and changes in pH_i , could be a source for fast control of Cx36-mediated GJIC and synchronization through electrical synapses.

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Figure 11. Diagram illustrating relation between ATP/ADP ratio and $[Mg^{2+}]_i$ and the effect on Cx36 GJ channels. During sleep and reduced neuronal activity, ATP/ADP ratio is increased by mitochondrial metabolism of glucose and lactate coming from capillaries and surrounding glia, respectively. During wake period and enhanced neuronal activity, ATP/ADP ratio is decreased. Changes in ATP have a direct effect on $[Mg^{2+}]_i$, leading to changes in Cx36-dependent GJIC. Pathological conditions, such as hypoxia, ischemia, and seizures may result in decreased GJIC, while traumatic brain injury may lead to increased GJIC. GLUT, glucose transporter; MCT, monocarboxylate transporter.

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