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Unusual Slow Gating of Gap Junction Channels in Oocytes Expressing Connexin32 or Its COOH-Terminus Truncated Mutant

Camillo Peracchia · Mohammad Salim · Lillian L. Peracchia

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Abstract Gap junction channels are gated by a chemical gate and two transjunctional voltage (V_j)-sensitive gates: fast and slow. Slow V_j gate and chemical gate are believed to be the same. The slow gate closes at the negative side of V_j and is mostly inactive without uncouplers or connexin (Cx) mutations. In contrast, our present data indicate otherwise. Oocytes expressing Cx32 were subjected to series of –100 m*V* V_j pulses (12-s duration, 30-s intervals). Both peak (PK) and steady-state (SS) junctional conductances (G_j), measured at each pulse, decreased exponentially by 50–60% (tau = ~1.2 min). G_jPK dropped more dramatically, such that G_jSS/G_jPK increased from 0.4 to 0.6, indicating a drop in V_j sensitivity. Less striking effects were obtained with –60 m*V* pulses. During recovery, G_j, measured by applying 20 m*V* pulses (2-s duration, 30-s intervals), slowly returned to initial values (tau = ~7 min). With reversal of V_j polarity, G_jPK briefly increased and G_jSS/G_jPK decreased, suggesting that V_j-dependent hemichannel reopening is faster than hemichannel closing. Similar yet more dramatic results were obtained with COOH-terminus truncated Cx32 (Cx32-D225), a mutant believed to lack fast V_j gating. The data indicate that the slow gate of Cx32 is active in the absence of uncouplers or mutations and displays unusual V_j behavior. Based on previous evidence for direct calmodulin (CaM) involvement in chemical/slow gating, this may also be CaM-mediated.

C. Peracchia (✉) · M. Salim · L. L. Peracchia
Department of Pharmacology and Physiology, University of Rochester School of Medicine, 601 Elmwood Ave., Rochester, NY 14642-8711, USA
e-mail: camillo_peracchia@urmc.rochester.edu

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Introduction

Gap junctions are discrete plasma membrane specializations that enable cell-to-cell diffusion of small cytosolic molecules. This form of cell-cell communication is mediated by channels that span two adjacent plasma membranes and a narrow extracellular space (gap). Each channel is formed by the extracellular interaction of two hemichannels (connexons), each a hexamer of connexin (Cx) proteins (reviewed in Peracchia, 1980). Connexins contain four transmembrane domains, two extracellular loops, a cytoplasmic loop, a short NH_2 terminus and a COOH terminus of variable length. The sequences of cytoplasmic loops and COOH termini vary significantly among members of the connexin family, whereas other domains are relatively well conserved (reviewed in Peracchia, Lazrak & Peracchia, 1994).

Gap junction channels are gated by transjunctional voltage (V_j; Spray, Harris & Bennett, 1981a) and increased $[Ca^{2+}]_i$ (Loewenstein, 1966; Rose & Loewenstein, 1975) or $[H^+]_i$ (Turin & Warner, 1977; Spray, Harris & Bennett, 1981b) via molecular mechanisms still poorly defined (reviewed in Harris, 2001; Peracchia, 2004). V_j gradients activate two types of gates: fast and slow. Chemical uncouplers activate a chemical gate that behaves identically to the slow V_j gate in terms of kinetics and efficiency (Bukauskas & Peracchia, 1997; Bukauskas et al., 2002). Furthermore, although the chemical gate and the slow V_j gate are usually referred to as separate gates, they are likely to be the same gate.

The electrical behavior of individual gap junction channels can be monitored by double whole-cell clamp in

small cells. Cytosolic acidification of small cells subjected to moderate V_j gradients allows one to distinguish the behavior of the chemical/slow gate from that of the fast V_j-sensitive gate (Bukauskas & Peracchia, 1997). The fast V_j gate flickers rapidly between the open and residual states, whereas the chemical/slow gate undergoes slow transitions between open and closed states (Bukauskas & Peracchia, 1997).

The chemical/slow gate is believed to be mostly inactive in the absence of chemical uncouplers as it appears only sporadically in single-channel records of Cx32wt (Oh et al., 1997). In contrast, it manifest itself widely in a variety of heterotypic channels generated by pairing oocytes expressing wild-type Cx32 with oocytes expressing Cx32 mutants (Peracchia, Wang & Peracchia, 1999, 2000a,b). In the present study, we report some novel features that slow gating activity in homotypic channels made of either wild-type Cx32 or a COOH-terminus deleted Cx32 mutant (Cx32-D225). This mutant has been shown to lack the fast component of V_j-sensitive gating (Revilla, Castro & Barrio, 1999). Similarly, COOH-terminus truncation has been shown to eliminate fast V_j gating (loss of residual conductance state), while leaving intact slow V_j gating, in both Cx40 (Anumonwo et al., 2001) and Cx43 (Revilla et al., 1999; Moreno et al., 2002) channels.

Materials and Methods

Oocyte Preparation and Microinjection

Oocytes were prepared as previously described (Peracchia, Wang & Peracchia, 1996). Briefly, adult female *Xenopus laevis* frogs were anesthetized with 0.3% tricaine (MS-222) and the oocytes were surgically removed from the abdominal incision. The oocytes were placed in ND96 medium, containing (in mM) NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 5 (pH 7.6 with NaOH). Oocytes at stages V or VI were subsequently defolliculated in 2 mg/ml collagenase (Sigma, St. Louis, MO) for 80 min at room temperature in nominally Ca^{2+}-free OR2 solution, containing (in mM) NaCl 82.5, KCl 2, $MgCl_2$ 1, HEPES 5 (pH 7.6 with NaOH). The defolliculated oocytes were injected with 46 nl (0.25 μg/μl) of antisense oligonucleotide complementary to endogenous *Xenopus* Cx38: 5′-GCTTTAGTAATTCCCATCCTGCCATGTTTC-3′ (commencing at nt -5 of Cx38 cDNA sequence; Barrio et al., 1991), by means of a Drummond (Broomall, PA) nanoject apparatus. The antisense oligonucleotide blocks the endogenous junctional communication within 24 h. Then, 24–72 h later, 46 nl of cRNA (~0.4 μg/μl) of either wild-type rat Cx32 or Cx32-D225, a rat Cx32 mutant whose COOH terminus was truncated beyond residue 225, was prepared by introducing a stop codon by polymerase chain reaction (Werner et al., 1991). The oocytes were incubated overnight at 18°C. The oocytes were mechanically stripped of their vitelline layer in hypertonic medium (Peracchia et al., 1996) and paired at the vegetal poles in conical wells of culture dishes (Falcon Products Becton Dickinson Labware, Franklin Lakes, NJ) filled with ND96. All oocyte pairs were studied electrophysiologically 2–3 h after pairing.

Measurement of Junctional Conductance

The oocyte chamber was continuously perfused at a flow rate of 0.6 ml/min by a peristaltic pump (Dyamax RP-1; Rainin Instrument, Woburn, MA). The superfusion solution was ejected by a 22-gauge needle placed near the edge of the conical well containing the oocyte pair. The level of the solution in the chamber was maintained constant by continuous suction. All experiments were performed using the standard double-voltage clamp procedure for measuring junctional conductance (G_j) (Spray et al., 1981a). Following insertion of a current and a voltage microelectrode in each oocyte, both oocytes were individually clamped by two oocyte clamp amplifiers (OC-725C; Warner Instrument, Hamden, CT) to the same holding potential, $V_{m1} = V_{m2}$ (–40 mV), so that no junctional current would flow at rest ($I_j = 0$).

For measuring G_j and V_j sensitivity, V_j gradients were created by imposing a series of negative voltage pulses (V_1) of either –100 or –60 mV and 12-s duration, repeated at 30-s intervals to oocyte 1, while maintaining V_2 (in oocyte 2) at V_m, thus $V_j = V_1$. The negative feedback current (I_2), injected by the clamp amplifier in oocyte 2 for maintaining V_2 constant at V_m, was used for calculating G_j as it is identical in magnitude to the junctional current (I_j) but of opposite sign ($I_j = -I_2$); $G_j = I_j/V_j$. Pulse generation and data acquisition were performed by means of pCLAMP v. 8.2.0.232 software (Axon Instruments, Foster City, CA) and the DigiData 1322A interface (Axon). I_j and V_j were measured with Clampfit (Axon) and the data plotted with SigmaPlot (SPSS, Chicago, IL). The time constant (tau) of G_j decay and the ratio G_j steady-state over G_j peak (G_jSS/G_jPK) were calculated by fitting each I_j curve to a single exponential function, following baseline correction (Clampfit). For measuring G_j during recovery, following the series of –100 mV V_j pulses, voltage pulses (V_1) of 20 mV and 2-s duration, repeated at 30-s intervals, were applied to oocyte 1, while maintaining V_2 at V_m. Membrane current (I_m) was measured in the pulsed oocyte ($I_m = I_1 - I_j$).

Results

Effect of Repeated V_j Pulses of –100 mV on G_j and V_j Sensitivity of Cx32wt and Cx32-D225 Channels

With the application of series of V_j pulses of –100 mV, both G_jPK and G_jSS junctional conductances decreased significantly in gap junctions made of either Cx32wt or Cx32-D225 channels (Fig. 1). This is clearly seen in tracings of junctional current (I_j; Fig. 1a,b). G_jPK decreased to 46.3 ± 1.3% (mean ± standard error [SE], n = 13) and 33.1 ± 2.6% (mean ± SE, n = 12) in Cx32wt and Cx32-D225, respectively, following single exponential functions with time constants (tau) of 1.17 and 1.18 min, respectively (Fig. 1c). G_jSS decreased by a lesser fraction than G_jPK: down to 62.1 ± 4.6% (mean ± SE, n = 13) and 63.8 ± 4.6% (mean ± SE, n = 12) in Cx32wt and Cx32-D225, respectively (*data not shown*). The different percent drop between G_jPK and G_jSS resulted in an increase in the G_jSS/G_jPK ratio from 0.38 ± 0.006 to 0.61 ± 0.01 (mean ± SE, n = 13) in Cx32wt and from 0.28 ± 0.009 to 0.54 ± 0.01 (n = 11) in Cx32-D225 (Fig. 1d), a 60% and a 93% increase, respectively. The changes in G_jSS/G_jPK, which reflect a drop in V_j sensitivity, followed single exponential growths with taus of 1.97 and 2.8 min in Cx32 and Cx32-D225, respectively (Fig. 1d). The increase in G_jSS/G_jPK is clearly seen in superimposed I_j curves sampled at the beginning and the end of the series of V_j pulses and normalized to peak amplitude (Fig. 1a,b, insets). The initial G_j, measured at peak current in the first –100 mV pulse of each series of pulses, was 0.16 ± 0.1 μS (mean ± standard deviation [SD], n = 13) with Cx32wt and 0.45 ± 0.64 μS (mean ± SD, n = 12) with Cx32-D225. Similar changes in G_jPK and G_jSS/G_jPK were obtained with initial G_j values as high as 10 μS (Cx32wt) and 2.4 μS (Cx32-D225) or as low as 0.08 μS (Cx32wt) and 0.05 μS (Cx32-D225).

The kinetics of I_j inactivation at the pulse changed slightly with repeated application of V_j pulses. This change was more pronounced in Cx32-D225 than in Cx32wt

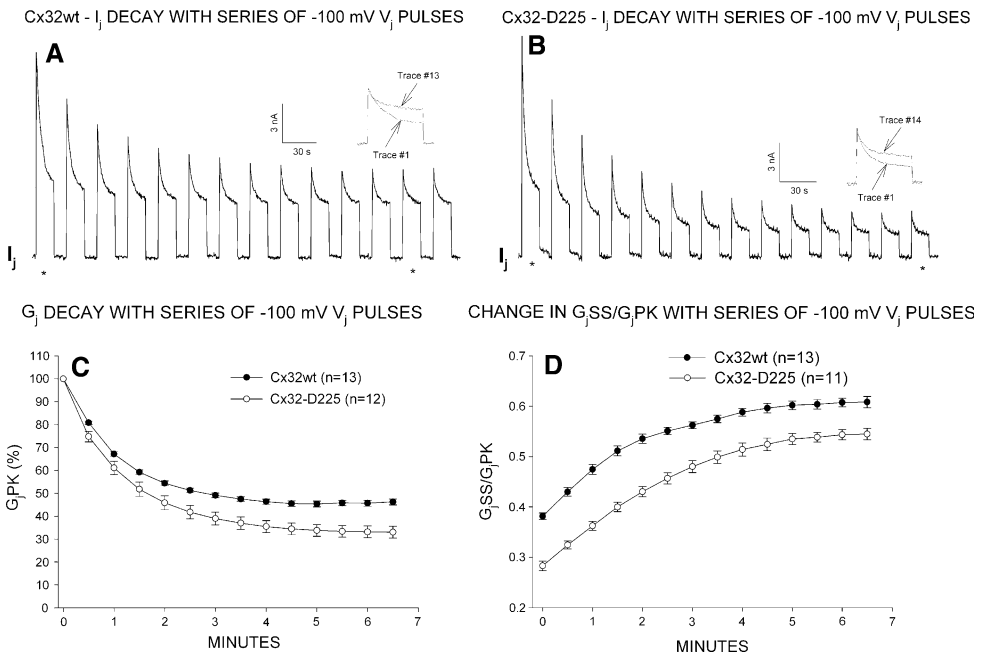

Fig. 1 Slow decay of G_jPK and G_jSS in oocyte pairs expressing either Cx32wt or its COOH-terminus truncated mutant (Cx32-D225) subjected to repeated exposure to –100 mV V_j gradients (12-s duration, 30-s intervals). (**a, b**) Tracings of junctional current (I_j) recorded from oocytes expressing Cx32wt and Cx32-D225, respectively. Note the progressive drop in I_jPK and, to a lesser extent, I_jSS, which is less pronounced in oocytes expressing Cx32wt (**a**) than in those expressing Cx32-D225 (**b**). The drop in junctional current reflects an average drop in G_jPK to 46.3 ± 1.3% (mean ± SE, n = 13) and 33.1 ± 2.6% (mean ± SE, n = 12) in Cx32wt and Cx32-D225, respectively, which follows a single exponential function with time constants (tau) of 1.17 and 1.18 min, respectively (**c**). G_jSS decreases to 62.1 ± 4.6% (mean ± SE, n = 13) and 63.8 ± 4.6% (mean ± SE, n = 12) in Cx32wt and Cx32-D225, respectively (*data not shown*). Because of a larger drop in G_jPK than in G_jSS, the ratio G_jSS/G_jPK increases from 0.38 ± 0.006 to 0.61 ± 0.01 (mean ± SE, n = 13) in Cx32wt and from 0.28 ± 0.009 to 0.54 ± 0.01 (mean ± SE, n = 11) in Cx32-D225 (**d**), 60% and 93% increases, respectively. The increase in G_jSS/G_jPK follows single exponential growths (tau = 1.97 and 2.8 min in Cx32 and Cx32-D225, respectively) (**d**). The increase in G_jSS/G_jPK is obvious in superimposed I_j traces sampled at the beginning (**a** and **b,** trace 1) and the end (**a** and **b,** traces 13 and 14, respectively) of the series of V_j pulses and normalized to peak amplitude

channels. The time constant of the single exponential I_j decay increased from 2.56 ± 0.09 to 2.69 ± 0.08 s (mean ± SE, n = 13) in Cx32wt and from 1.70 ± 0.08 to 1.89 ± 0.04 s (mean ± SE, n = 12) in Cx32-D225 (Fig. 2a). The kinetics of I_j inactivation fully recovered to initial values with G_j recovery (*data not shown*).

Membrane current (I_m), measured in pulsed oocytes, did not change significantly during the repeated application of V_j pulses in oocytes expressing Cx32wt but decreased significantly in those expressing Cx32-D225 (Fig. 2b). At the end of a series of V_j pulses, I_m was virtually the same (101.9 ± 2.4%, mean ± SE, n = 13) with Cx32wt, but it dropped to 84.1 ± 5.1% (mean ± SE, n = 12) with Cx32-D225 (Fig. 2b) channels, following a single exponential decay with tau = 3.04 min (Fig. 2b).

Following the series of –100 m*V* V_j pulses, G_j, measured by passing small V_j pulses (20 m*V*, 2-s duration), slowly recovered, usually reaching values greater than the initial ones (Fig. 2c). G_j recovery followed single exponential growths with taus of 7.17 and 8.8 min in Cx32wt and Cx32-D225 channels, respectively (Fig. 2c).

Effect of Repeated V_j Pulses of –60 m*V* on G_j and V_j Sensitivity of Cx32wt and Cx32-D225 Channels

With the application of series of V_j pulses of –60 m*V*, G_jPK decreased slightly in both Cx32wt and Cx32-D225 gap junctions (Fig. 3a). G_jPK decreased to 82.1 ± 3.7% (mean ± SE, n = 4) and 78.8 ± 7.0% (mean ± SE, n = 6) in Cx32wt and Cx32-D225, respectively, following single exponential functions with time constants (tau) of 2.6 and 2.9 min, respectively (Fig. 3a). G_jSS decreased minimally such that the ratio G_jSS/G_jPK increased from 0.69 ± 0.02 to 0.71 ± 0.006 (mean ± SE, n = 4) in Cx32wt and from 0.63 ± 0.07 (mean ± SE, n = 6) to 0.67 ± 0.05 (mean ± SE, n = 6) in Cx32-D225 (Fig. 3b), increases of 3% and 6%, respectively.

Effect of Reversal of V_j Pulse Polarity on G_j and V_j Sensitivity of Cx32wt and Cx32-D225 Channels

To test the effect of V_j polarity reversal, immediately after the series of V_j pulses (–100 m*V*) applied to oocyte 1

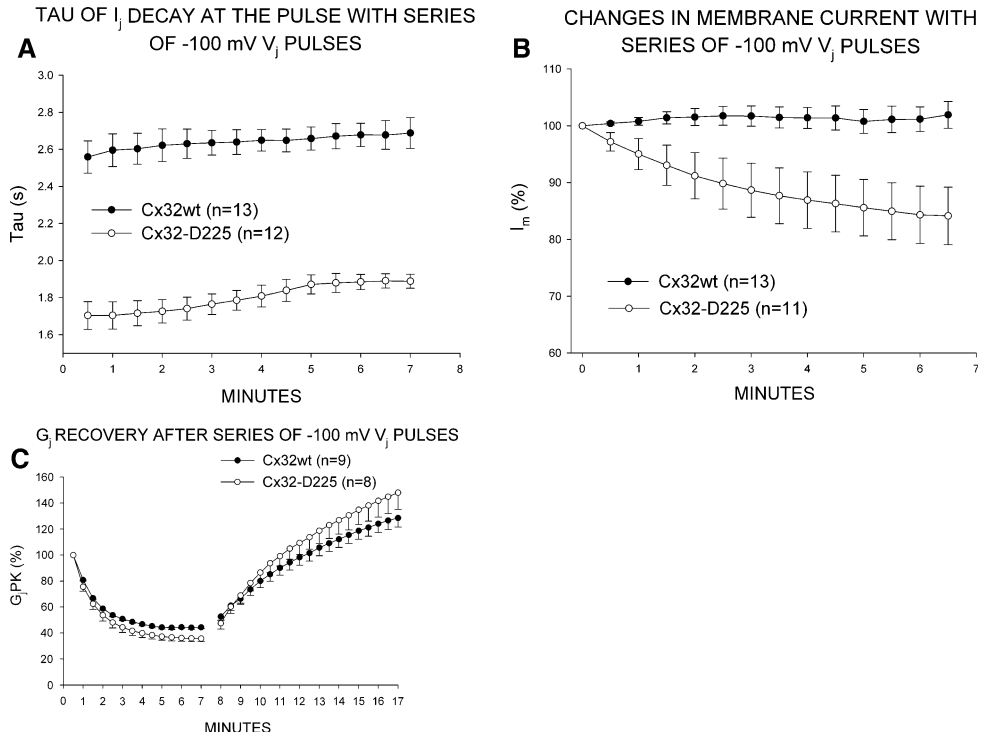

Fig. 2 (**a**) Time constant of I_j inactivation at the pulse with repeated application of V_j pulses in oocytes expressing either Cx32wt or Cx32-D225. Tau increases minimally with Cx32wt and slightly more with Cx32-D225, changing from 2.56 ± 0.09 to 2.69 ± 0.08 s (mean ± SE, n = 13) in Cx32wt and from 1.70 ± 0.08 to 1.89 ± 0.04 s (mean ± SE, n = 12) in Cx32-D225. (**b**) Changes in membrane current (I_m). I_m measured in pulsed oocytes does not change significantly during repeated application of V_j pulses in oocytes expressing Cx32wt but decreased significantly in those expressing Cx32-D225. At the end of the series of V_j pulses, I_m increases by only ~1.9% (n = 13) with Cx32wt, but drops by ~16% (n = 12) with Cx32-D225, following a single exponential decay with tau = 3.04 min. (**c**) G_j recovery following series of –100 m*V* V_j pulses. G_j, measured by passing small V_j pulses (20 m*V*, 2-s duration), slowly recovers, usually to higher than initial values, following single exponential growths with taus = 7.17 and 8.8 min with Cx32wt and Cx32-D225, respectively

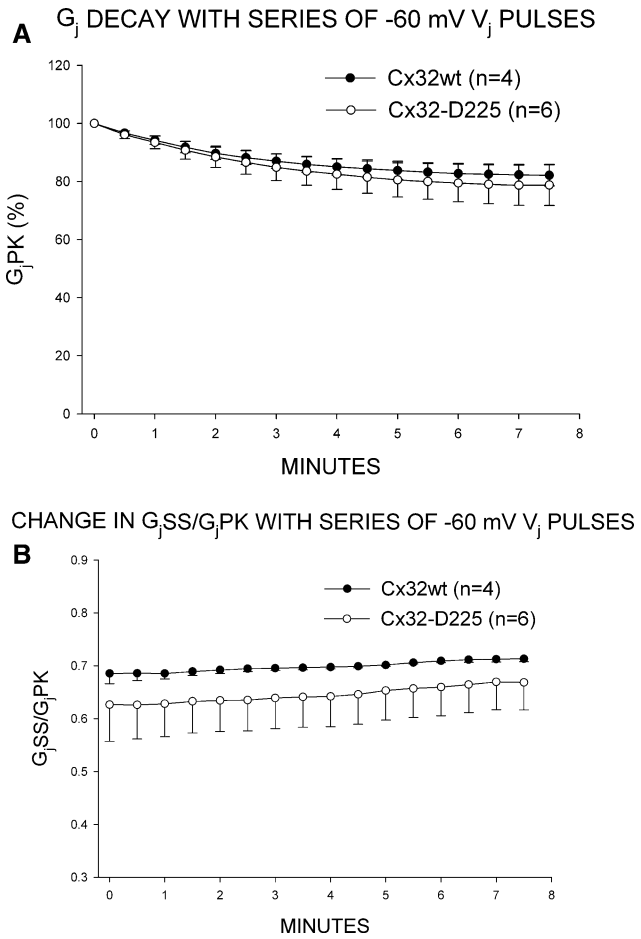

Fig. 3 Application of series of V_j pulses of –60 mV has a small effect on G_jPK (**a**) and G_jSS/G_jPK (**b**). G_jPK decreases to 82.1 ± 3.7% (mean ± SE, n = 4) with Cx32wt and to 78.8 ± 7.0% (mean ± SE, n = 6) with Cx32-D225 following single exponential decays with tau = 2.6 and 2.9 min, respectively (**a**). The ratio G_jSS/G_jPK increases from 0.69 ± 0.02 to 0.71 ± 0.006 (mean ± SE, n = 4) with Cx32wt and from 0.63 ± 0.07 (mean ± SE, n = 6) to 0.67 ± 0.05 (mean ± SE, n = 6) with Cx32-D225 (**b**), 3% and 6% increases, respectively

(Fig. 4a, left trace), a similar series of V_j pulses (–100 mV) was applied to oocyte 2 (Fig. 4a, right trace). As seen in the current record (Fig. 4a), both peak and steady-state I_j increased significantly with respect to the last few pulses of the previous series. This corresponded to an increase in G_jPK from 0.059 ± 0.02 μS (last pulse of the first series) to 0.069 ± 0.01 μS (first pulse of the new series) in Cx32wt and from 0.087 ± 0.02 to 0.12 ± 0.02 μS in Cx32-D225 (Fig. 4b). G_jSS increased less than G_jPK (*data not shown*), resulting in a decrease in G_jSS/G_jPK from 0.61 ± 0.008 to 0.49 ± 0.01 in Cx32wt (Fig. 4c) and from 0.55 ± 0.02 to 0.37 ± 0.01 in Cx32-D225 (Fig. 4c). Within 5–7 min, both G_jPK and G_jSS, as well as G_jSS/G_jPK, returned to values comparable to those recorded at the end of the earlier series of V_j pulses (Fig. 4a–c, right traces).

Discussion

The data show that in oocyte pairs expressing Cx32wt or its COOH-terminus truncated mutant (Cx32-D225) repeated exposure to relatively large V_j gradients progressively reduces both G_jPK and G_jSS. Cx32wt and Cx32-D225 channels behave similarly, but the effect of V_j is greater with Cx32-D225 channels. The ratio G_jSS/G_jPK increases with both connexins, indicating that repeated exposure to V_j pulses also decreases the V_j sensitivity of these channels.

The slow drop in G_jPK suggests that most of the voltage-activated gates remain closed during the interval between two pulses, a period of ~18 s. Indeed, the delay in recovering channel open state is likely to be of the order of minutes rather than seconds because G_j recovery after a series of large V_j pulses follows a single exponential growth with time constants ranging from 7 to 9 min. Could such a slow phenomenon involve the actual retrieval, and subsequent reinsertion, of channels from gap junction plaques? While this possibility cannot be entirely discarded, it is very unlikely because no data for voltage-dependent channel formation and/or retrieval have been reported. Thus, most likely this is a channel gating phenomenon.

Gap junction channels are gated by V_j gradients and increased $[Ca^{2+}]_i$ or $[H^+]_i$, via mechanisms still poorly understood (reviewed in Harris, 2001; Peracchia, 2004). Changes in G_j induced by cytosolic acidification are more closely related to $[Ca^{2+}]_i$ than to $[H^+]_i$ (Peracchia, 1990a,b; Lazrak & Peracchia, 1993), and there is evidence that in some cells gating is sensitive to nearly physiological $[Ca^{2+}]_i$, probably via calmodulin (CaM) activation (reviewed in Peracchia, 2004). At least two V_j-sensitive gates have been identified: fast and slow. The fast V_j gate and the chemical gate are distinct (Bukauskas & Peracchia, 1997; Oh et al., 1997), whereas the slow V_j gate and the chemical gate are likely to be the same (Bukauskas & Peracchia, 1997; Peracchia et al., 1999, 2000a,b). Slow and fast V_j gates are in series, and each hemichannel has both gates. The slow gate closes at the negative side of V_j in all connexin channels, whereas the polarity of fast V_j gating varies among connexin channels (reviewed in Harris, 2001; Bukauskas et al., 2002).

In the absence of single-channel data, which cannot be obtained from oocyte pairs, one can only guess which of these two gates might be involved. However, the fast V_j gate is unlikely to play a role because this phenomenon is fully manifested, even to a larger extent, in Cx32-D225 channels. Truncation of the COOH termini of Cx32 and Cx43 has been reported to eliminate the fast component of V_j gating in paired oocytes (Revilla et al., 1999). At the single-channel level, this was clearly demonstrated in small cells expressing COOH-terminus truncated Cx40

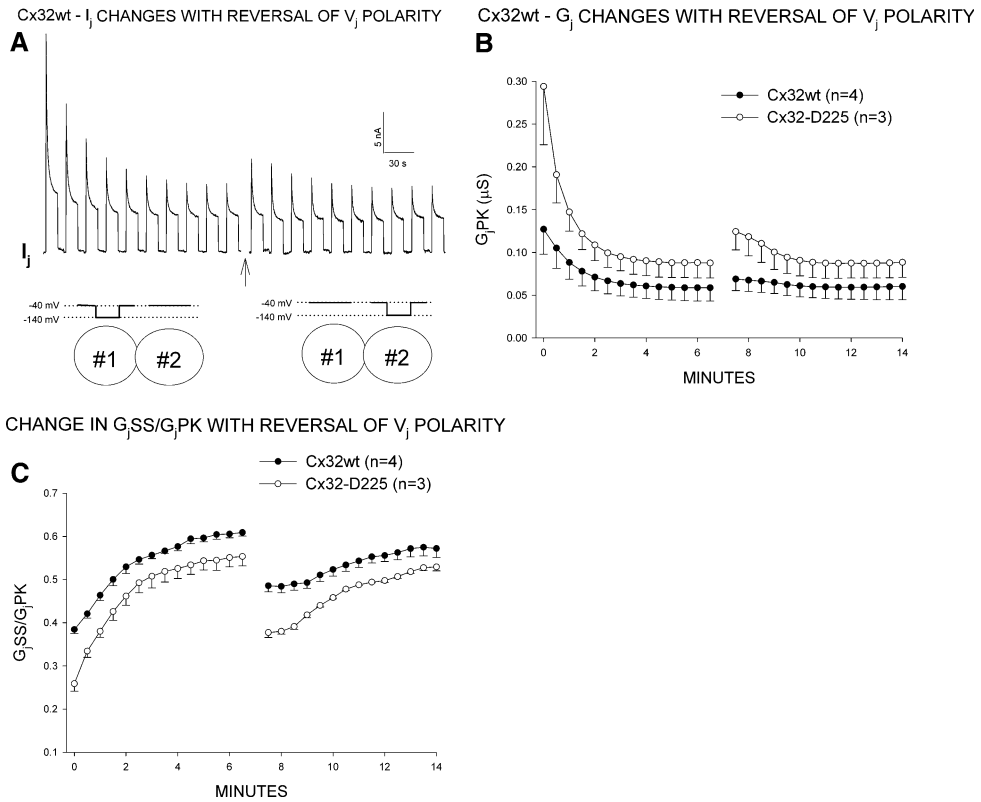

Fig. 4 Effect of V_j polarity reversal. Series of V_j pulses (–100 m*V*) applied to oocyte 1 (**a,** *left trace*) were immediately followed by similar series of V_j pulses (–100 m*V*) applied to oocyte 2 (**a,** *right trace*). The I_j record shows that in the first pulse of the second series (**a,** *arrow*) both peak and steady-state I_j are substantially greater than those of the last pulse of the first series. As average, G_jPK increases from 0.059 ± 0.02 μS (last pulse of the first series) to 0.069 ± 0.01 μS (first pulse of the second series) in Cx32wt (**b**) and from 0.087 ± 0.02 μS to 0.12 ± 0.02 μS in Cx32-D225 (**b**). G_jSS increased less than G_jPK (*data not shown*), resulting in a decrease in G_jSS/G_jPK from 0.61 ± 0.008 to 0.49 ± 0.01 in Cx32wt (**c**) and from 0.55 ± 0.02 to 0.37 ± 0.01 in Cx32-D225 (**c**). Within 5–7 min both G_jSS and G_jPK as well as G_jSS/G_jPK return to values comparable to those recorded at the end of the earlier series of V_j pulses (**a–c,** *right traces*)

(Anumonwo et al., 2001) or Cx43 (Moreno et al., 2002). Neither slow V_j gating nor chemical gating was affected by COOH-terminus truncation (Anumonwo et al., 2001; Moreno et al., 2002), whereas loss of fast V_j gating was demonstrated by absence of the residual conductance state (Anumonwo et al., 2001; Moreno et al., 2002). Although the slow gating behavior of COOH-terminus truncated Cx32 (Cx32-D225) was not tested at the single-channel level, by analogy the similar disappearance of the fast V_j gating component in COOH-terminus truncated Cx32 and Cx43 (Revilla et al., 1999) suggests that both of these connexin mutants have lost the fast V_j gating. Therefore, it is reasonable to believe that the phenomenon we have observed reflects the behavior of the slow V_j gate. Oh et al. (1997) first reported single-channel evidence of sporadic slow gating events in Cx32wt channels, but this type of gating is rare in wild-type connexin channels in the absence of chemical uncouplers, while it is known to manifest itself spontaneously in heterotypic channels between various Cx32 mutants and Cx32wt (Peracchia, 2004; Peracchia et al., 1999, 2000a,b).

Evidence from the present study suggests that the slow gate is V_j-dependent and fully functional in wild-type connexin channels even in the absence of uncoupling treatments and has very long opening and closing times. Indeed, the time course of the slow, reversible drop in G_jPK is reminiscent of that seen in cell-cell uncoupling by cytosolic acidification (Wang & Peracchia, 1996) or other means. At the single-channel level, channels close and reopen one by one rather than in bulk during low pH_i-induced uncoupling (Bukauskas & Peracchia, 1997), indicating that the induction time needed to convert a channel's functional state from open to closed, and vice versa, is relatively long. This may be the same for the slow V_j gating phenomenon as with application of V_j the channels appear to close individually or a few at a time and reopen in similar fashion with return to zero V_j.

The effect of V_j pulses on G_j decay was more pronounced in Cx32-D225 channels. Since in Cx32 both fast and slow V_j gates close at the negative side of V_j, with COOH-terminus truncation the mechanism involved in slow gate closure may not be hindered by that of the fast

gate, likely to be absent in Cx32-D225. Perhaps in some of the Cx32wt channels the earlier closure of the fast gate reduces the strength of the electric field to levels insufficient to activate the slow gate. Obviously, this would not occur in the absence of the fast gate (Cx32-D225), so a larger fraction of channels would be closed by the slow gate. Alternatively, COOH terminus removal may increase the sensitivity of the slow gate by eliminating potential intramolecular interactions.

The increase in G_jSS/G_jPK could reflect a drop in V_j sensitivity of the operational channels, but there is no evidence in the literature that repeated exposure to V_j pulses affects V_j sensitivity. Instead, it is likely that not all of the operational channels are equally sensitive to V_j. For example, if we assume that only 60% of a channel population is sensitive to a V_j gradient of 100 mV, with progressive V_j pulses the fraction of channels that remain open would be expected to keep increasing, resulting in a progressive increase in G_jSS/G_jPK. This is also more likely because the increase in G_jSS/G_jPK is not accompanied by major changes in the time constant of I_j decay at the pulse. Indeed, a change in V_j sensitivity, e.g., a shift in the current-voltage relationship to higher V_j values, would be accompanied by a sizable change in the kinetics of I_j inactivation. A decrease in the number of operational channels with the same V_j sensitivity of the preexisting operational channels is also unlikely because in this case the decrease in G_jPK would not be accompanied by a decrease in G_jSS/G_jPK; this ratio would remain the same.

During the series of 100 mV V_j pulses, I_m slowly decreased by ~16% with Cx32-D225 channels but did not significantly change with Cx32wt channels. Since both connexins are likely to express hemichannels in the plasma membrane as well as cell-cell channels, a possibility is that a resting potential (V_m) of –40 mV is sufficient to maintain Cx32wt, but not Cx32-D225, hemichannels mostly in a closed state. With application of –100 mV pulses, the resulting V_m of –140 mV is likely to progressively close most of the Cx32-D225 hemichannels, resulting in an increase in membrane resistance. Indeed, the long time constant of the I_m drop (~3 min) seems consistent with the behavior of Cx32-D225 channels.

With reversal of V_j polarity, both peak and steady-state G_j increased slightly, with respect to the last few pulses of the previous series, before dropping. Since both fast and slow V_j gates of Cx32 close at the negative side of V_j (reviewed in Harris, 2001; Peracchia, 2004), with reversal of V_j polarity the closed hemichannel gates, now at the positive side of V_j, will start opening, while those at the negative side of V_j will start closing. Naturally, if opening and closing kinetics were identical, one would expect no change in G_jPK and G_jSS between the last pulse of the earlier series and the first pulse of the new series. The fact that G_jPK and G_jSS are greater after V_j polarity reversal may indicate that positive V_j is more effective and faster at inducing hemichannel opening than negative V_j is at closing them. Significantly, G_jSS/G_jPK decreased substantially when the application of negative V_j pulses was switched from one (oocyte 1) to the other (oocyte 2) of the pair. A possible reason for this apparent increase in V_j sensitivity is that the hemichannels of oocyte 2 still have normal V_j sensitivity as they have not been subjected to repeated negative V_j pulses (e.g., 60% of the hemichannel population is V_j-sensitive, as in the previous example), whereas most of the hemichannels of oocyte 1 belong to the residual, relatively V_j-insensitive population.

Based on previous work, which reported that heterotypic channels between the Cx32 mutants and Cx32wt almost completely lost their slow gating behavior following inhibition of CaM expression (Peracchia et al., 2000), it is possible that CaM is also involved in the slow gating phenomenon here described. The possible participation of CaM in chemical/slow gating was previously suggested by evidence that CaM inhibitors and CaM expression inhibition prevent CO_2-induced uncoupling (reviewed in Peracchia, 2004) and by data showing dramatic changes in chemical and V_j gating behavior with expression of CaM mutants (Peracchia et al., 2000a,b). Based on these data, we have proposed a model that envisions chemical/slow gating as the result of interaction between a CaM lobe and the cytoplasmic mouth of the channel (''cork'' gating model; Peracchia et al., 2000b). Perhaps the repeated application of a large negative V_j gradients locks the negatively charged CaM lobe in the channel vestibule (positively charged), stabilizing the closed state for extensive periods of time by means of electrostatic interactions.

In conclusion, this study shows that the junctional conductance of gap junction channels made of Cx32wt and its COOH terminus truncated mutant slowly and reversibly decreases by over 50% with repeated application of relatively large transjunctional voltage gradients. Since with deletion of the COOH-terminus domain fast voltage gating is believed absent, this phenomenon is likely to involve the slow gating activity of these channels, previously believed to be largely inactive in wild-type connexin channels in the absence of chemical uncouplers. Based on earlier data, CaM may play a role in the slow gating mechanism.

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