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Slow Gating of Gap Junction Channels and Calmodulin

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Abstract. Certain COOH-terminus mutants of connexin32 (Cx32) were previously shown to form channels with unusual transjuctional voltage (V_j) sensitivity when tested heterotypically in oocytes against Cx32 wild type. Junctional conductance (G_j) slowly increased by several-fold or decreases to nearly zero with V_j positive or negative, respectively, at mutant side, and V_j positive at mutant side reversed CO_2-induced uncoupling. This suggested that the CO_2-sensitive gate might be a V_j-sensitive slow gate. Based on previous data for calmodulin (CaM) involvement in gap junction function, we have hypothesized that the slow gate could be a CaM-like pore plugging molecule (cork gating model). This study describes a similar behavior in heterotypic channels between Cx32 and each of four new Cx32 mutants modified in cytoplasmic-loop and/or COOH-terminus residues. The mutants are: ML/NN+3R/N, 3R/N, ML/NN and ML/EE; in these mutants, N or E replace M105 and L106, and N replace R215, R219 and R220. This study also reports that inhibition of CaM expression strongly reduces V_j and CO_2 sensitivities of two of the most effective mutants, suggesting a CaM role in slow and chemical gating.

Key words: Cell communication — Connexins — Gap junctions — Calmodulin — Channel gating — CO_2

Introduction

Gap junctions are cell contact domains that contain channels for direct cell-to-cell exchange of small cytosolic molecules. A gap junction channel is made of two hexameric hemichannels (connexons) composed of connexin proteins. Connexins are intramembrane proteins with four transmembrane domains, two extracellular loops, a cytoplasmic loop (CL), a short NH_2-terminus (NT) and a COOH-terminus (CT) of variable length (reviewed in Peracchia, Lazrak & Peracchia, 1994).

Transjuctional voltage (V_j) gradients and increased $[Ca^{2+}]_i$ or $[H^+]_i$ are known to reduce channel permeability (reviewed in Loewenstein, 1990; Bruzzone, White & Paul, 1996; Peracchia & Wang, 1997), but the molecular mechanisms of channel gating are still largely unknown. V_j and chemical gates are believed to be two distinct gates; the former closes the channel rapidly but incompletely and the latter closes the channel slowly but completely (Bukauskas & Peracchia, 1997).

Recently, we have studied connexin mutants and chimeras, expressed in *Xenopus* oocytes, to identify domains of the rat connexin32 (Cx32) that participate in CO_2-induced (low pH_i) channel gating. Cx32 is expressed in liver, pancreas, kidney, thyroid, and mammary gland, as well as in various cells of the nervous system such as neurons, oligodendrocytes and Schwann cells (reviewed in Bruzzone et al., 1996). Several Cx32 mutations are relevant to the pathogenesis of the X-linked Charcot Marie Tooth demyelinating disease (CMTX; Bergoffen et al., 1993; Ionasescu, Searby & Baritt, 1994; Ressott et al., 1998). CL and the initial segment of CT (CT_1) appear to be relevant to the CO_2 gating sensitivity of Cx32 (Wang et al., 1996; Wang & Peracchia, 1996; Wang & Peracchia, 1997; Wang & Peracchia, 1998*a*), whereas NT (Wang et al., 1996) and the rest of CT (84%, Wang & Peracchia, 1997; Wang & Peracchia, 1998*a*) do not seem to play a role. Based on these data we have proposed that the membrane proximal domain of CT (CT_1), a basic and partly hydrophobic domain, may interact electrostatically and hydrophobically with acidic and hydrophobic residues of the initial CL domain (CL_1), and may inhibit chemical gating by latching CL (Wang & Peracchia, 1998*b*).

To further probe the chemical gating mechanism of

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Cx32, our previous study has tested three Cx32 mutants (Peracchia, Wang & Peracchia, 1999). One mutant (tandem) is a dimer in which two Cx32 monomers are concatenated NT-to-CT. The other two (5R/E and 5R/N) are mutants in which E or N residues replace five CT_1 residues (R215, R219, R220, R223 and R224). The data showed that heterotypic channels obtained by pairing each of these mutants with Cx32 wild-type display an intriguing V_j behavior. In these channels, junctional conductance (G_j) increased with V_j gradients positive at the mutant side and decreased with opposite V_j polarity. Furthermore, V_j positive at the mutant side partially reversed the CO_2-induced G_j drop. These data suggested the existence of a V_j-sensitive slow gate and the possibility that the slow gate and the chemical gate are the same gate (Peracchia et al., 1999). Based on these data we have proposed a model ("cork" gating model) that envisions chemical and slow gating as being mediated by an acidic cytosolic molecule (calmodulin?) that physically blocks the pore (Peracchia et al., 1999, 2000).

The present study reports that several other Cx32 mutants, modified in CL_1 and/or CT_1 residues, display a similar behavior when paired heterotypically with Cx32. The mutants are: ML/NN+3R/N, 3R/N, ML/NN and ML/EE; in these mutants, N or E residues replace M105 and L106, and N residues replace R215, R219 and R220. More significantly, this study shows that the unusual V_j behavior and the CO_2 sensitivity of two of the most effective mutants, tandem (Peracchia et al., 1999) and ML/NN+3R/N is drastically reduced or eliminated when calmodulin (CaM) expression is inhibited with oligonucleotides antisense to CaM mRNA. This treatment was previously shown to inhibit CO_2-induced uncoupling in oocyte pairs expressing Cx38 (Peracchia et al., 1996).

Materials and Methods

Site-Directed Mutagenesis

Molecular biology protocols were generally as described by Sambrook, Fritsch & Maniatis (1989). Cx32 cDNA (Paul, 1986) was used in the construction of DNA mutants. The method used for constructing the Cx32 tandem has been previously described (Peracchia et al., 1999). The strategy employed to create the 3R/N, ML/NN and ML/EE mutants of Cx32 has been reported earlier (Wang & Peracchia, 1997). The mutant ML/NN+3R/N was constructed by replacing the COOH-terminus of the ML/NN mutant with the COOH-terminus of the 3R/N mutant. All of the mutations were verified by digestion of DNA with restriction enzymes and sequence analysis. Oligonucleotides were synthesized by a DNA synthesizer (model 393; ABI, Foster City, CA).

Preparation of cRNA

Wild-type and mutated forms of Cx32 cDNA were subcloned into pBluscript (Stratagene, La Jolla, CA) or pGEM 3Z (Promega, Madison, WI), and used for in vitro synthesis of cRNA. The cRNAs were transcribed from linearized plasmid using T7 or SP6 mMESSAGE mMACHINE (Ambion, Austin, TX) in the presence of the cap analogue m7G(5′)ppp(5′)G (Ambion).

Oocyte Preparation and Microinjection

Oocytes were prepared as previously described (Peracchia et al., 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. Oocytes at stages V or VI were subsequently defolliculated in 2 mg/ml collagenase (Sigma Chemical, St. Louis, MO) in Ca^{2+}-free OR2 for 80 min at room temperature. The defolliculated oocytes were injected with 46nl (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 nanoject apparatus (Drummond, Broomall, PA). The antisense oligonucleotide blocks completely the endogenous junctional communication within 48 hr. 24–72 Hours post-injection, 46 nl of either Cx32 wild-type, ML/NN+3R/N, 3R/N, ML/NN or ML/EE cRNA (0.04, 0.43, 0.02, 0.2 and 1.0 μg/μl, respectively) were injected into oocytes at the vegetal pole and the oocytes were incubated overnight at 18°C. The oocytes were mechanically stripped of their vitelline layer in a hypertonic medium (Peracchia et al., 1996) and heterotypically paired (mutant against Cx32) at the vegetal poles in ND96. Oocyte pairs were studied electrophysiologically 2–3 hr after pairing.

For experiments testing the effect of inhibiting CaM expression, oocytes injected 24 hr earlier with oligonucleotides antisense to Cx38 were injected with oligonucleotides antisense to the CaM mRNAs expressed by *Xenopus* oocytes (46 nl, 1.12 μg/μl) as previously described (Peracchia et al., 1996). 4–6 Hr later and on each of two subsequent days the oocytes were injected with tandem or ML/NN+3R/N cRNA (0.4 and 0.43 μg/μl, respectively). The oocytes were incubated overnight at 18°C, mechanically stripped, heterotypically paired (mutant+anti-CaM against Cx32) and studied electrophysiologically 2–3 hr after pairing.

Uncoupling Protocol

The oocyte chamber was continuously perfused at a flow rate of 0.6 ml/min by a peristaltic pump (Dyamax Mod. 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. Electrical uncoupling of oocyte pairs was induced by a 3–15 min superfusion (0.6 ml/min) of salines continuously gassed with 100% CO_2. A Cl^- free saline (Cl^- replaced with methanesulfonate) was used. The Cl^- free saline contained (in mM): NaOH 75, KOH 10, $Ca(OH)_2$ 4, $Mg(OH)_2$ 5, MOPS 10, adjusted to pH 7.2 with methane-sulfonic acid. As previously reported (Peracchia et al., 1996), the opening of Ca^{2+} activated Cl^- channels during long exposures to 100% CO_2 often causes an increase in membrane current that may interfere with G_j measurement.

Measurement of Gap Junctional Conductance in Oocyte Pairs

All of the experiments were performed using the standard double voltage clamp procedure for measuring G_j (Spray, Harris & Bennett, 1981). Following the insertion of a current and a voltage microelectrode in

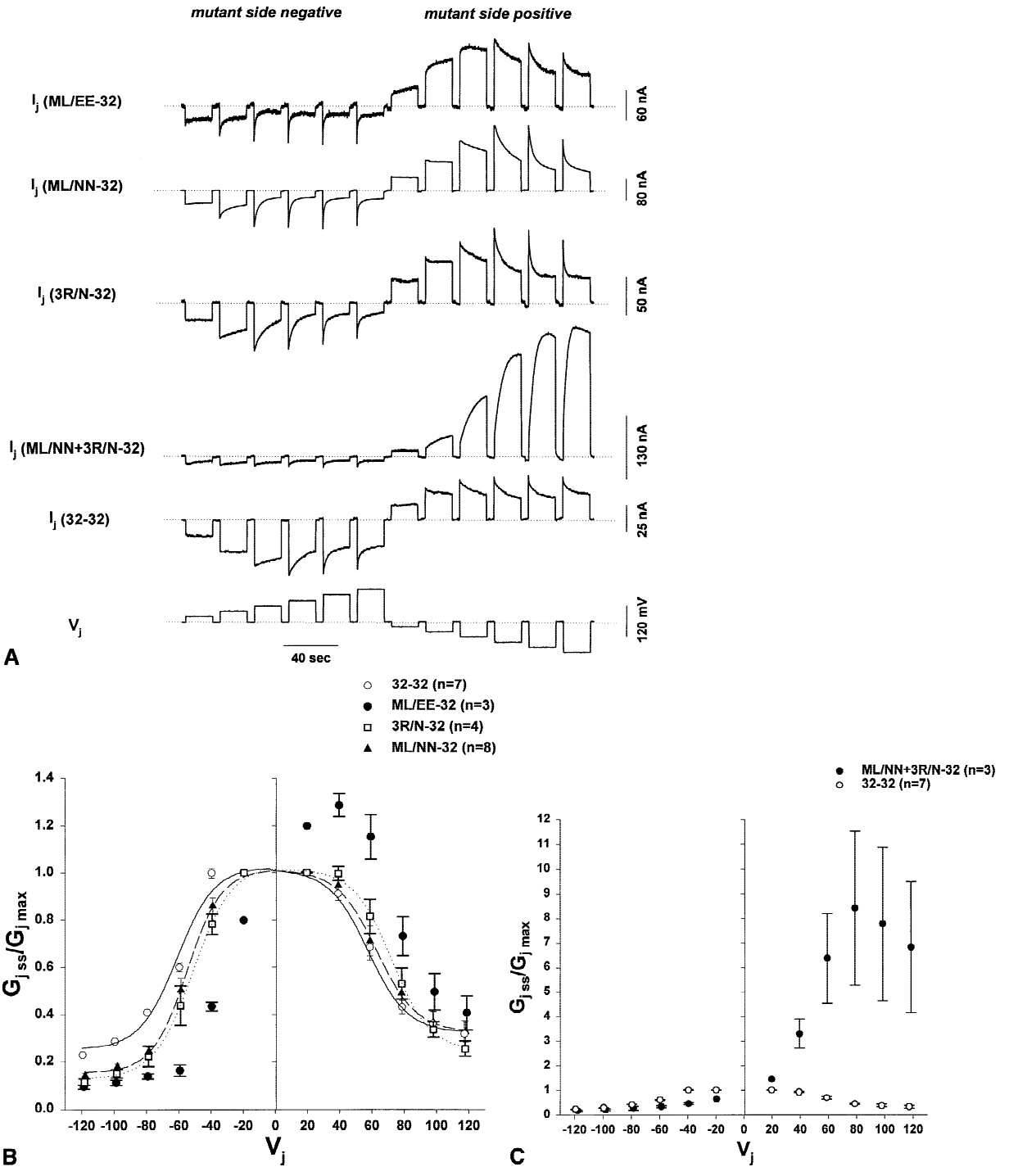


Fig. 1. Response of junctional current (I_j) to transjunctional voltage (V_j) pulses in *Xenopus* oocyte pairs expressing homotypic Cx32 channels (32-32) or heterotypic channels between Cx32 wild-type and each of four Cx32 mutants: ML/NN+3R/N-32, 3R/N-32, ML/NN-32 and ML/EE-32. 32-32 channels (*A*) display a characteristic sensitivity to V_j, as I_j decays exponentially with time for V_j's > ±40 mV. In contrast, mutant-32 channels display a unique I_j/V_j behavior (*A*). This is most pronounced with the ML/NN+3R/N mutant (*A*). With ML/NN+3R/N side negative (*A*, left trace), as V_j is increased from 20 to 120 mV initial and final I_j progressively decrease to very low values, and V_j sensitivity seems present even at V_j = 20 mV. With ML/NN+3R/N side positive (*A*, right trace) I_j progressively increases to high values, and I_j increases rather than decreases from the initial I_j; only with V_js of 100–200 mV a more conventional behavior starts developing, with I_j reaching a peak followed by exponential decay. The other three mutants (3R/N, ML/NN and ML/EE) have a similar behavior, but the I_j asymmetry between positive and negative V_j protocols is less pronounced (*A*). The asymmetrical I_j/V_j behavior of mutant-32 channels is obvious in plots of normalized $G_j(G_{jss}/G_{jmax})$ versus V_j (*B* and *C*). ML/NN+3R/N-32 channels have a very asymmetrical I_j/V_j behavior (*C*), whereas with ML/NN-32 or 3R/N-32 channels the I_j/V_j asymmetry is minimal (*B*). Note that the double mutation (ML/NN+3R/N) generates a very asymmetrical I_j/V_j behavior (*A* and *C*), whereas individual mutations (ML/NN or 3R/N) result in minimal asymmetry (*A* and *B*). The asymmetrical behavior suggests that V_j gradients negative or positive at the mutant side of the channels, progressively closes or opens, respectively, an increasing number of channels.

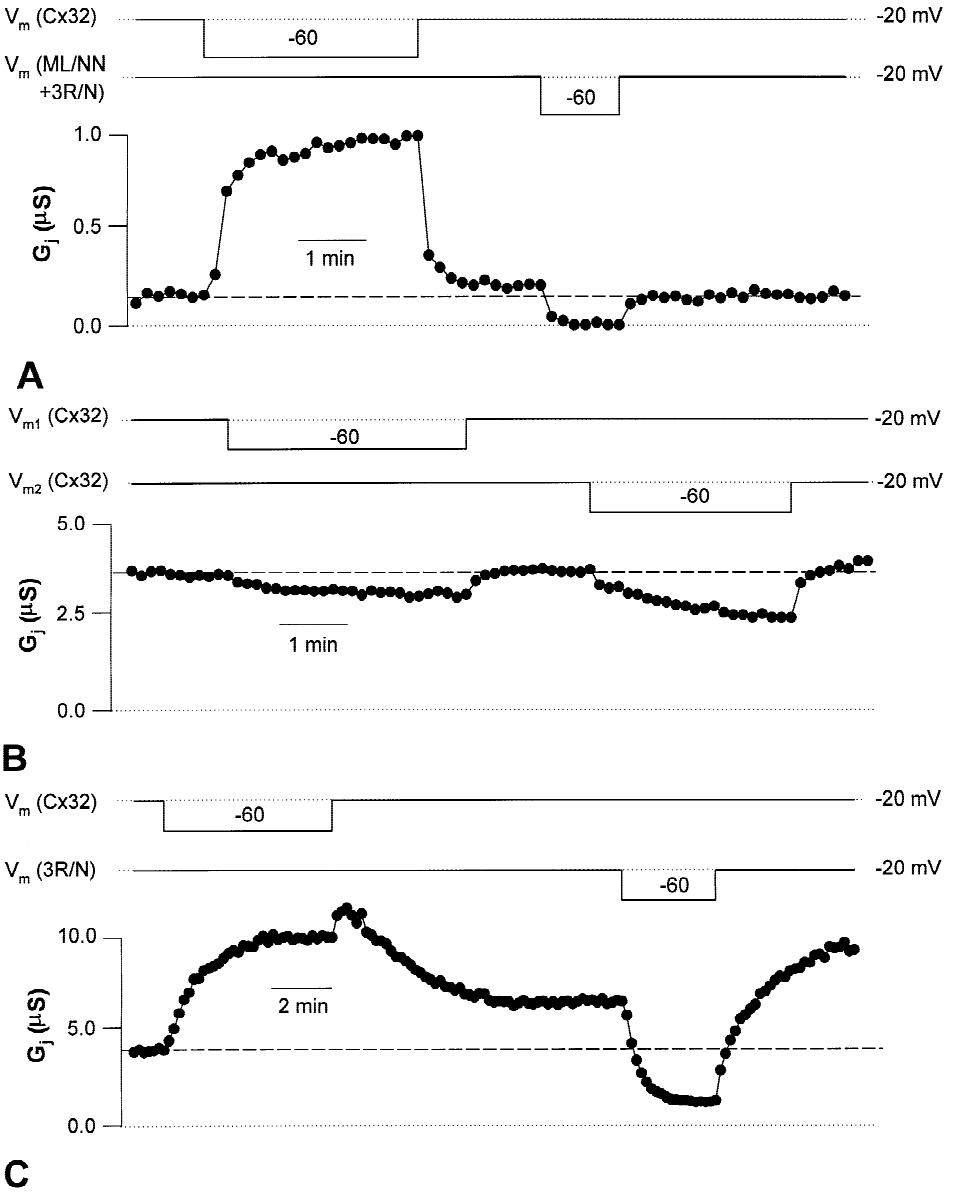


Fig. 2. G_j response to steady state V_j gradients in ML/NN+3R/N-32 (*A*), 32-32 (*B*), 3R/N-32 (*C* and *D*), ML/NN-32 (*E* and *F*) and ML/EE-32 (*G*) channels; G_j was measured by the usual pulse protocol (+20 mV voltage pulses of 2 sec duration applied to one oocyte every 10 sec). Exposure of ML/NN+3R/N-32 channels to $V_j = 40$ mV (mutant side positive) reversibly increases G_j by 386 ± 181% (mean ± SD, $n = 21$, *A*) with $\tau = 0.25$ ± 0.1 min (mean ± SD, $n = 21$), whereas exposure to $V_j = 40$ mV (ML/NN+3R/N side negative) decreases G_j exponentially to 14.8 ± 5.6% (mean ± SD, $n = 9$) of control values with $\tau = 0.07 \pm 0.02$ min (mean ± SD, $n = 6$). In contrast, exposure of 32-32 channels to 40 mV V_j gradients of either polarity decreases G_j to 67.7 ± 8.2% (mean ± SD, $n = 7$, *B*); G_j drops rapidly in the first 10–20 sec (*B*, more obvious with the second V_j application) and then slowly over 4–5 min. Rapid drop and slow decay may result from sequential closure of fast and a slow V_j gates, respectively. The behavior of the other mutant-32 channels (*C*–*G*) was similar but less pronounced than that of ML/NN+3R/N-32 (*A*). Exposure to $V_j = 40$ mV (mutant side positive) increases G_j by 64.4 ± 20.2% (mean ± SD, $n = 6$) with $\tau = 0.68 \pm 0.39$ min (mean ± SD, $n = 6$) in 3R/N-32 (*C* and *D*), by 27.2 ± 16.2% (mean ± SD, $n = 11$) with $\tau = 0.23 \pm 0.12$ min (mean ± SD, $n = 9$) in ML/NN-32 (*E* and *F*), and by 167.1 ± 51.8% (mean ± SD, $n = 8$) with $\tau = 0.23 \pm 0.1$ min (mean ± SD, $n = 8$) in ML/EE-32 (*G*). $V_j = 40$ mV (mutant side negative) decreases G_j exponentially to 19.8 ± 5.6% (mean ± SD, $n = 5$) of control values, with $\tau = 0.36 \pm 0.13$ min (mean ± SD, $n = 3$) in 3R/N-32 (*C* and *D*), to 35.9 ± 11% (mean ± SD, $n = 12$) with $\tau = 0.7 \pm 0.46$ min (mean ± SD, $n = 12$) in ML/NN-32 (*E* and *F*), and to 31.1 ± 7% (mean ± SD, $n = 7$), with $\tau = 0.15 \pm 0.06$ min (mean ± SD, $n = 3$) in ML/EE-32 (*G*). In 3R/N-32 and ML/NN-32 channels, with return to $V_j = 0$ from $V_j = 40$ mV (mutant side positive) G_j increases abruptly before dropping (*C* and *E*). This is not observed when V_j is reversed from positive to negative at mutant side (*D* and *F*). In contrast, with ML/NN+3R/N-32 and ML/EE-32 channels the abrupt increase in G_j is not observed either in the transition from $V_j = 40$ mV (mutant side positive) to $V_j = 0$ (*A* and *G*) or with V_j reversal from positive to negative at mutant side (*data not shown*).

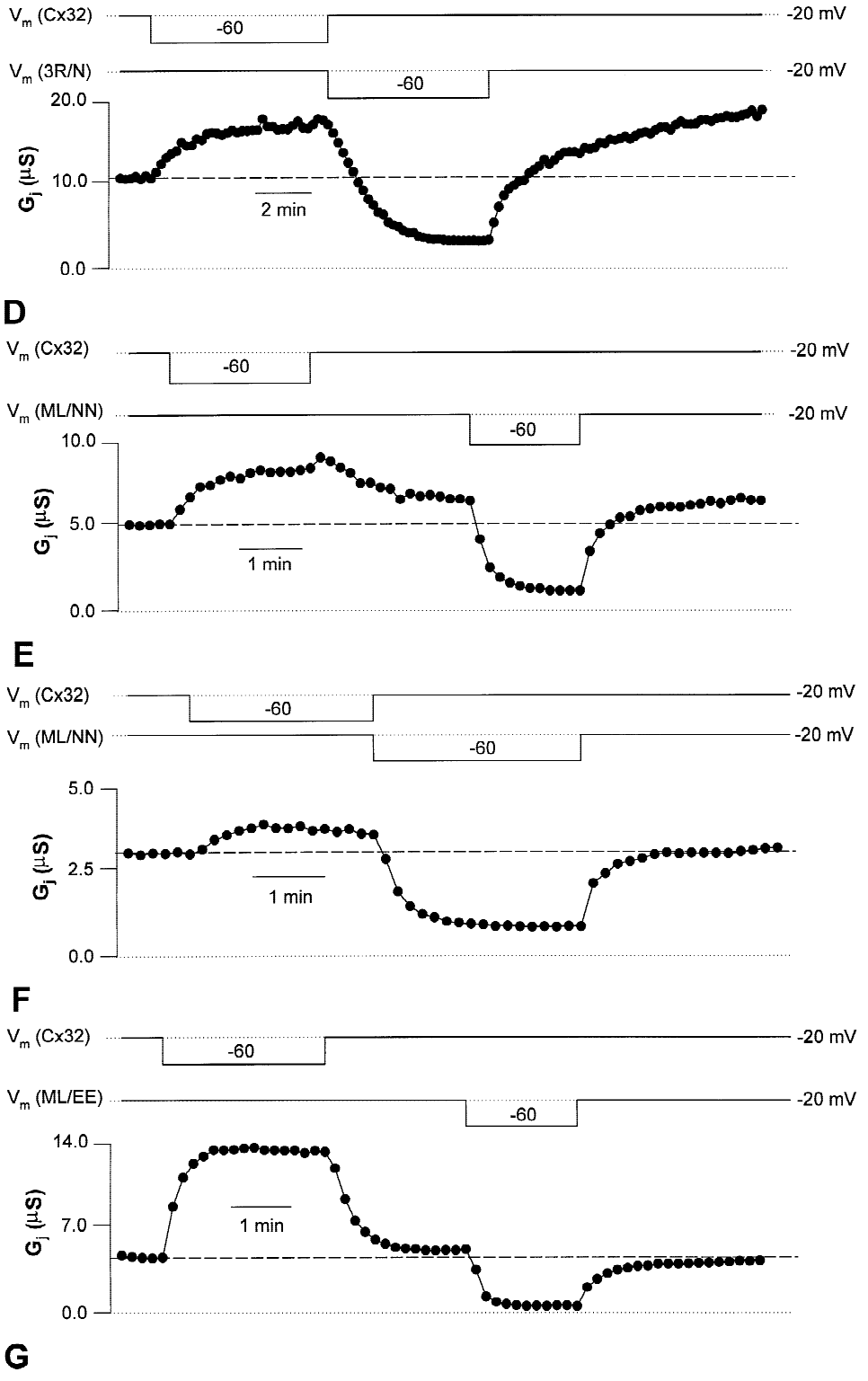
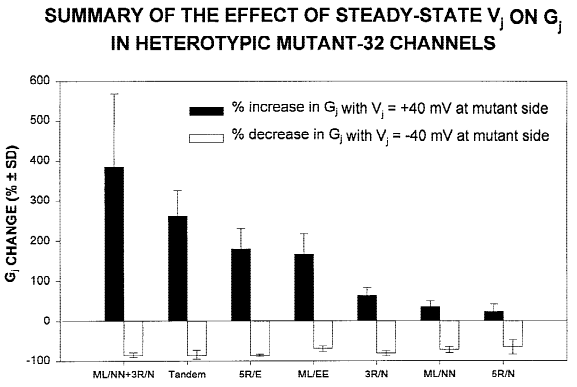
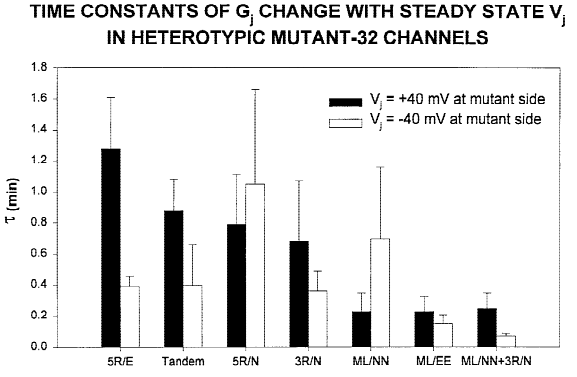


Fig. 2. *Continued.*

each oocyte, both oocytes were initially voltage clamped individually with two oocyte clamp amplifiers (OC-725C, Warner Instrument, Hamden, CT) to the same holding potential, $V_{m1} = V_{m2}$ (usually −20 mV), so that no junctional current would flow at rest ($I_j = 0$). A V_j gradient was created by imposing a +20 mV voltage step (V_1) of 2 sec duration every 10 or 30 sec to oocyte 1, while maintaining V_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



A



B

Fig. 3. Comparison of the effect of steady-state V_j on G_j in all the mutant-32 channels tested in this and a previous study (Peracchia, Wang & Peracchia, 1999) in terms of magnitude (*A*) and kinetics (τ, *B*) of G_j change with V_j = 40 mV of either polarity. Note that CT mutations (5R/E, 5R/N and 3R/N) seem to result in longer τ than CL (ML/NN and ML/EE) and combined CL-CT mutations (ML/NN+3R/N). This is true with both positive and negative V_j for all the mutants except for ML/NN which has an unusually long τ at negative V_j.

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 Clampex 8.0 software (Axon Instruments, Foster City, CA) and DigiData 1200 interface (Axon). I_j and V_j were measured with Clampfit 8.0 (Axon) and the data were plotted with SigmaPlot v. 5.0 (SPSS, Chicago, IL).

For studying voltage dependence of junctional conductance, each oocyte of the pair was first voltage clamped at −20 mV. Voltage steps of 20 mV (±120 mV maximum) and 25 sec duration were then applied every 45 sec to either oocyte of the pair, while maintaining the other at −20 mV. To illustrate the relationship between steady-state $G_j(G_{jss})$ and V_j, the normalized $G_j(G_{jss}/G_{jmax})$ was plotted with respect to V_j. The curve was fitted to a two-state Boltzmann's distribution of the form: $(G_{jss} - G_{jmin})/(G_{jmax} - G_{jss}) = exp[-A(V_j - V_0)]$, where V_0 is the V_j value at which G_j is one half the value of $G_{jmax} - G_{jmin}$, G_{jmax} is G_j at V_j = 0 mV and G_{jmin} is the theoretical minimum normalized G_j. $A = nq/kT$ is a constant expressing voltage sensitivity in terms of number of equivalent gating charges, n, moving through the entire applied field, where q is the electron charge, k is the Boltzmann constant and T is the temperature in °K.

Results

Sensitivity to Transjunctional Voltage Pulses

Whereas homotypic Cx32 junctions (32–32) displayed a characteristic sensitivity to V_j, with I_j decaying almost exponentially with time for $V_j > \pm 40$ mV (Fig. 1*A*), ML/NN+3R/N-32 channels displayed a unique I_j–V_j behavior (Fig. 1*A*). With ML/NN+3R/N side negative, as V_j was increased in 20 mV steps from 20 to 120 mV the initial and final I_j progressively decreased to very low conductance values, and the channels appeared to be V_j sensitive even at the lowest V_j. With ML/NN+3R/N side positive, as V_j was increased in 20 mV steps from 20 to 120 mV I_j progressively increased to high values, and I_j recorded at the end of the pulse was greater than the initial I_j. Only at the largest V_j gradients (100–120 mV) a more conventional behavior started developing, with I_j reaching a peak followed by exponential decay (Fig. 1*A*).

The other three mutants displayed a similar behavior, although the I_j asymmetry between positive and negative V_j protocols was significantly less pronounced. The asymmetrical I_j/V_j behavior of mutant-32 channels is clearly demonstrated by plotting normalized $G_j(G_{jss}/G_{jmax})$ versus V_j (Fig. 1*B* and *C*). Note that the double mutation (ML/NN+3R/N) generates a very asymmetrical I_j/V_j behavior (Fig. 1*A* and *C*), whereas with individual mutations (ML/NN or 3R/N) the I_j/V_j asymmetry is minimal (Fig. 1*A* and *B*). Consistent with previous data from other mutant-32 channels (Peracchia et al., 1999) this intriguing voltage behavior suggested that V_j gradients negative or positive at the mutant side of the channels, progressively decreased or increased, respectively, the number of functional channels. This idea was tested by subjecting the oocytes to steady state V_j gradients either positive or negative at mutant side (*see below*).

Effect on G_j of Steady-State V_j

In oocyte pairs expressing ML/NN+3R/N-32 channels, the establishment of V_j = 40 mV (ML/NN+3R/N side positive) increased G_j almost exponentially by 386 ± 181% (mean ± SD, n = 21) with τ = 0.25 ± 0.1 min (mean ± SD, n = 21, Fig. 2*A*); G_j was measured by the conventional protocol: +20 mV pulses of 2 sec duration applied to one oocyte every 10 sec. With the reestablishment of V_j = 0 mV from V_j = 40 mV (ML/

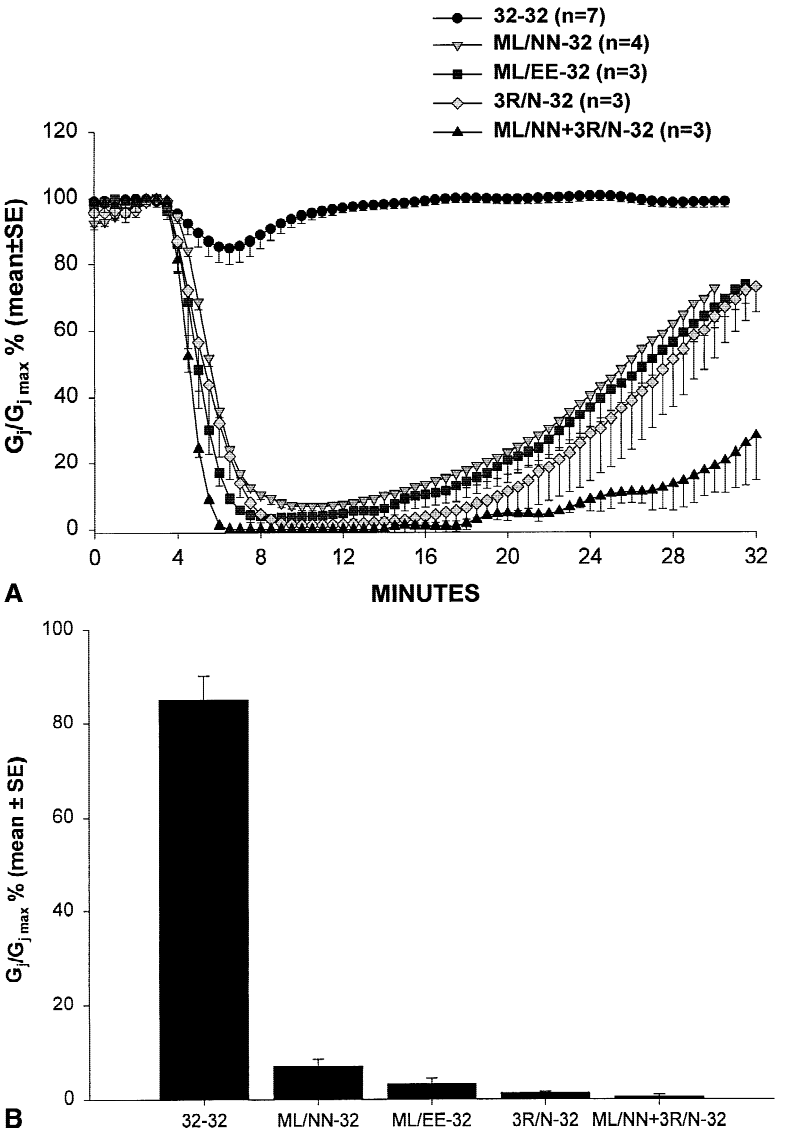


Fig. 4. G_j sensitivity to 3 min exposure to 100% CO_2 of 32-32, ML/NN+3R/N-32, 3R/N-32, ML/NN-32 and ML/EE-32 channels (*A* and *B*). With 32-32 channels G_j decreases to only 85 ± 5% (mean ± SE, $n = 7$, *A* and *B*) of the control value (100%) at a maximum rate of ~9%/min. All of the heterotypic channels are more CO_2-sensitive than 32-32 channels (*A* and *B*). G_j drops to 0.27 ± 0.5% (mean ± SE, $n = 3$) in ML/NN+3R/N-32, to 1.16 ± 0.37% (mean ± SE, $n = 3$) in 3R/N-32, to 6.89 ± 1.56% (mean ± SE, $n = 4$) in ML/NN-32, and to 3.18 ± 1.29% (mean ± SE, $n = 3$) in ML/EE channels (*A* and *B*). With ML/NN+3R/N-32 channels G_j drops very fast (54%/min; *A*); this makes them the most CO_2 sensitive channels tested thus far in our lab. The other mutant-32 channels uncoupled with the following rates (*A*): 3R/N-32 = 28.6%/min, ML/NN-32 = 33%/min, and ML/EE-32 = 43.5%/min. Thus, based on rate and magnitude of uncoupling the CO_2 sensitivity of all of these channels ranks as follows (in decreasing order): ML/NN+3R/N-32 > ML/EE-32 ⩾3R/N-32 > ML/NN-32 > 32-32.

NN+3R/N side positive) G_j returned fairly rapidly to control values. In oocyte pairs initially clamped at V_j = 0 mV, the establishment V_j = 40 mV (ML/NN+3R/N side negative) decreased G_j exponentially to 14.8 ± 5.6% (mean ± SD, $n = 9$) of control values, following exponential decays with $\tau = 0.07 \pm 0.02$ min (mean ± SD, $n = 6$).

In contrast, in homotypic 32–32 junctions the application of 40 mV V_j gradients to either oocyte decreased G_j to 67.7 ± 8.2% (mean ± SD, $n = 7$, Fig. 2*B*). Upon V_j application G_j dropped rapidly in the first 10–20 sec (Fig. 2*B*, more obvious with the second V_j application) and then slowly over the following 4–5 min, eventually reaching steady state. The rapid drop and the slow decay may result from the sequential closure of the conventional (fast) V_j gate and a slow V_j gate, respectively.

The other mutant-32 channels behaved as ML/NN+3R/N-32 channels, but G_j did not change as dramatically. Exposure to V_j = 40 mV (mutant side positive) increased G_j by 64.4 ± 20.2% (mean ± SD, $n = 6$) with $\tau = 0.68 \pm 0.39$ min (mean ± SD, $n = 6$) in 3R/N-32 channels (Fig. 2*C* and *D*), by 27.2 ± 16.2% (mean ± SD, $n = 11$) with $\tau = 0.23 \pm 0.12$ min (mean ± SD, $n = 9$) in ML/NN-32 channels (Fig. 2*E* and *F*), and by 167.1 ± 51.8% (mean ± SD, $n = 8$) with $\tau = 0.23 \pm 0.1$ min (mean ± SD, $n = 8$) in ML/EE-32 channels (Fig. 2*G*). In oocyte pairs initially clamped at V_j = 0 mV, the establishment of V_j = 40 mV (mutant side negative) decreased G_j exponentially to 19.8 ± 5.6% (mean ± SD, $n = 5$) of control values, with $\tau = 0.36 \pm 0.13$ min (mean ± SD, $n = 3$) in 3R/N-32 channels (Fig. 2*C* and *D*), to 35.9 ± 11% (mean ± SD, $n = 12$) with $\tau = 0.7 \pm 0.46$ min (mean ± SD, $n = 12$) in ML/NN-32 channels (Fig. 2*E* and *F*), and to 31.1 ± 7% (mean ± SD, $n = 7$), with

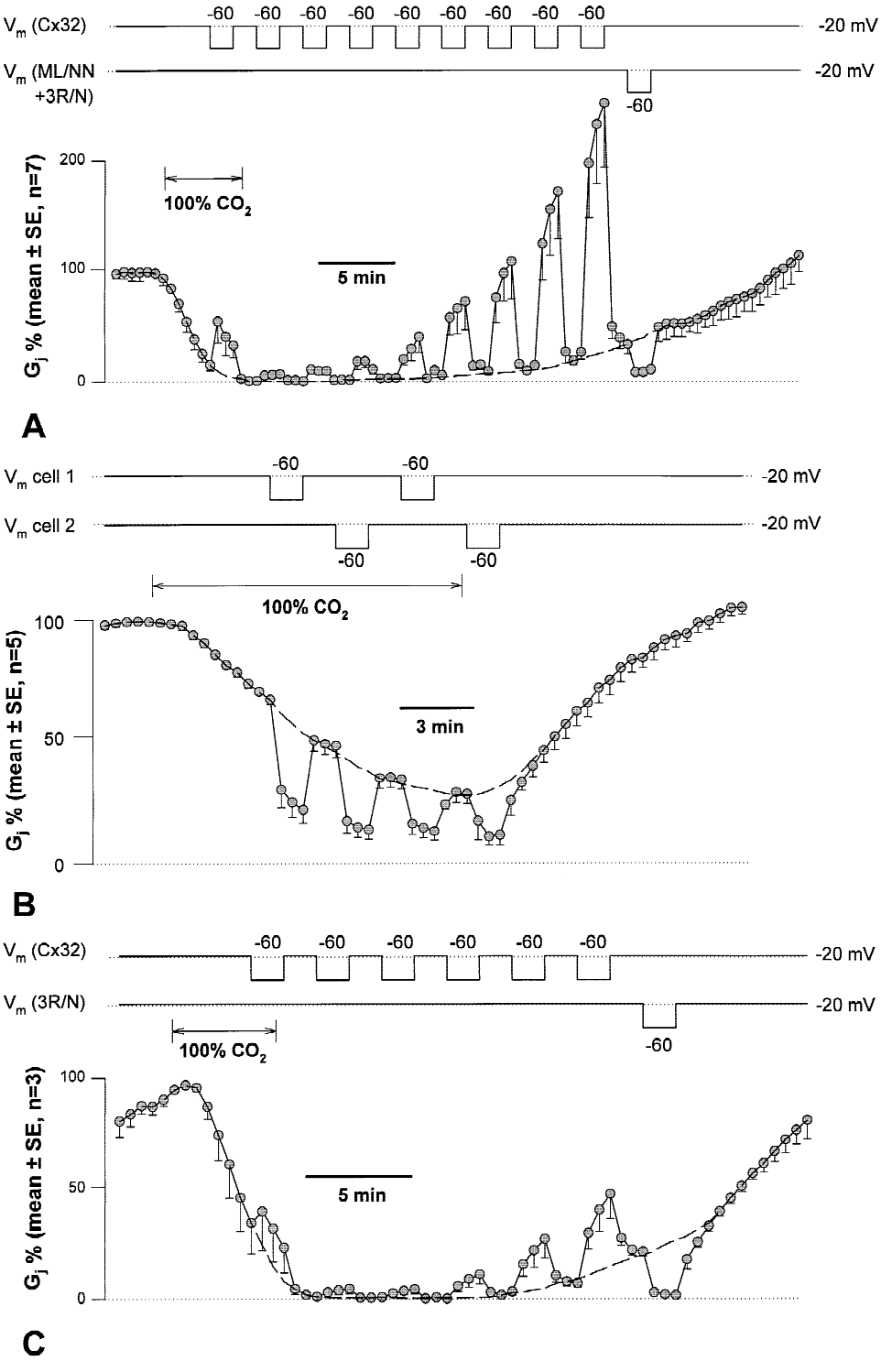


Fig. 5. Effect of V_j on G_j during exposure to 100% CO_2 in oocyte pairs expressing ML/NN+3R/N-32 (*A*), 32-32 (*B*), 3R/N-32 (*C*), ML/NN = 32 (*D*) or ML/EE-32 (*E*) channels. In mutant-32 channels, G_j, reduced to low values by CO_2 at $V_j = 0$, increases significantly and reversibly with the application of V_j gradients positive at the mutant side (*A*, *C–E*). With similar V_j gradients, G_j increases less at maximal uncoupling. This reduced effect of V_j on G_j at maximum uncoupling suggests that most of the channels might be locked in closed state. Thus, there might be two closed states: one V_j reversible and the other V_j irreversible. V_j negative at the mutant side dramatically and reversibly reduces G_j to very low values (*A*, *C–E*). In contrast, with 32-32 channels V_j gradients of either polarity significantly decrease G_j (*B*); the magnitude of G_j drop (*B*) suggest that V_j affects both fast and slow V_j gates. The dashed lines in *A–E* indicate the predicted G_j time course in the absence of V_j gradients.

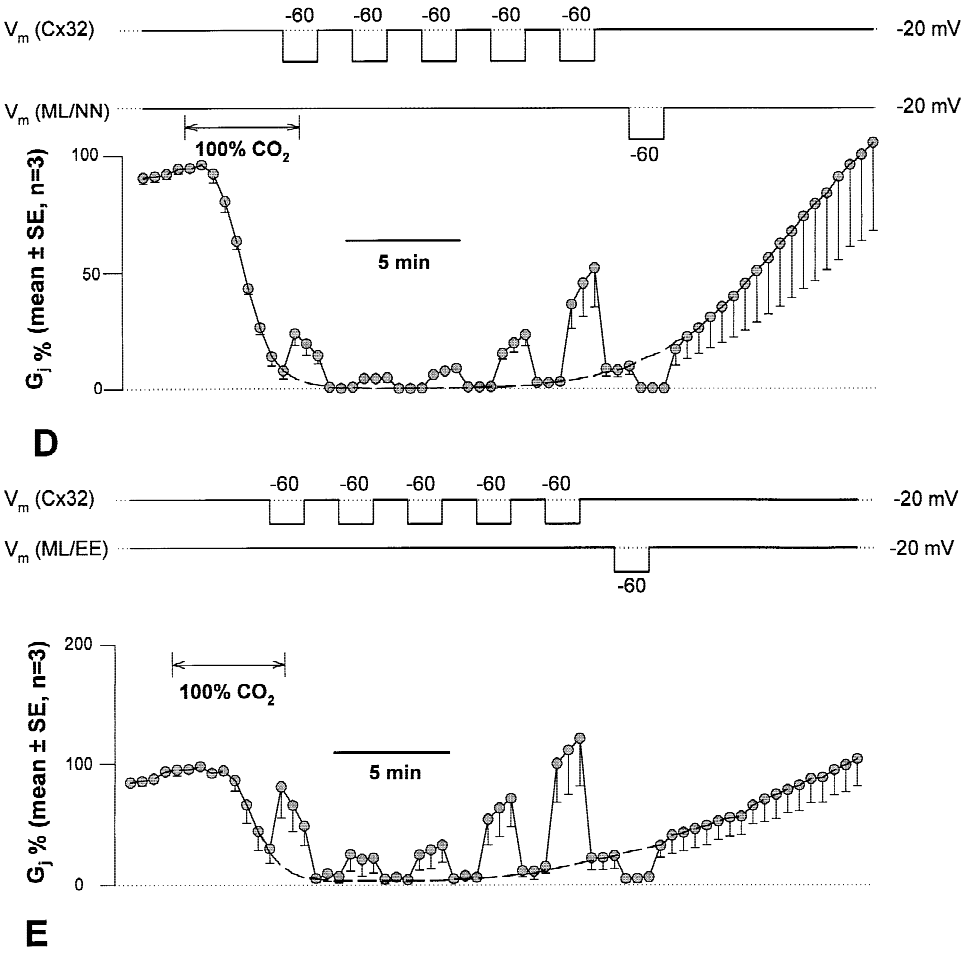


Fig. 5. *Continued.*

τ = 0.15 ± 0.06 min (mean ± SD, n = 3) in ML/EE-32 channels (Fig. 2*G*).

With 3R/N-32 and ML/NN-32 channels, upon return to V_j = 0 from V_j = 40 mV (mutant side positive) G_j increased abruptly at first, before dropping (Fig. 2*C* and *E*). This, however, was not observed when V_j was reversed from positive to negative at mutant side (Fig. 2*D* and *F*). In contrast, with ML/NN+3R/N-32 and ML/EE-32 channels the abrupt increase in G_j was not observed either in the transition from V_j = 40 mV (mutant side positive) to V_j = 0 (Fig. 2*A* and *G*) or with V_j reversal from positive to negative at mutant side (*data not shown*).

Figure 3 compares the effect of V_j on G_j for all the mutant-32 channels tested in this and a previous study (Peracchia et al., 1999) in terms of magnitude (Fig. 3*A*) and τ (Fig. 3*B*). Note that CT mutations (5R/E, 5R/N and 3R/N) seem to result in longer τ than CL (ML/NN and ML/EE) and combined CL-CT mutations (ML/NN+3R/N). This is true for both positive and negative V_j for all except ML/NN which shows an unusually long τ at negative V_j.

CO_2 Sensitivity

Homotypic 32-32 channels were weakly sensitive to CO_2, as previously reported (Wang et al., 1996). With a 3 min exposures to CO_2 G_j decreased to 85 ± 5% (mean ± SE, n = 7; Fig. 4*A* and *B*), at a maximum rate of ~9%/min. Mutant-32 channels were much more sensitive to CO_2 than 32-32 channels (Fig. 4*A* and *B*). With 3 min exposures to CO_2 G_j dropped to 0.27 ± 0.5% (mean ± SE, n = 3) in ML/NN+3R/N-32, to 1.16 ± 0.37% (mean ± SE, n = 3) in 3R/N-32, to 6.89 ± 1.56% (mean ± SE, n = 4) in ML/NN-32, and to 3.18 ± 1.29% (mean ± SE, n = 3) in ML/EE channels (Fig. 4*A* and *B*).

With ML/NN+3R/N-32 channels G_j decreased at a very fast rate (54% G_j drop per min; Fig. 4*A*), which makes these channels the most CO_2 sensitive gap junction channels tested thus far in our lab. The other mutant-32 channels uncoupled with the following rates (Fig. 4*A*): 3R/N-32 = 28.6%/min, ML/NN-32 = 33%/min, and ML/EE-32 = 43.5%/min. Thus, based on rate and magnitude of uncoupling the CO_2 sensitivity of all of these channels ranked as follows (in decreasing order):

ML/NN+3R/N-32 > ML/EE-32 > 3R/N-32 > ML/NN-32 > 32-32.

Effect on G_j of Steady-State V_j During CO_2-Induced Uncoupling

Oocyte pairs expressing mutant-32 or 32-32 channels were subjected to V_j gradients (40 mV) of either polarity during exposure to 100% CO_2. In mutant-32 channels, G_j, reduced to very low values by exposure to CO_2 at $V_j = 0$ mV, increased dramatically and reversibly with V_j gradients that made the ML/NN+3R/N (Fig. 5*A*), 3R/N (Fig. 5*C*), ML/NN (Fig. 5*D*) or ML/EE (Fig. 5*E*) side positive by 40 mV. With the same V_j gradient, G_j increased more during uncoupling or recovery from uncoupling than when uncoupling was maximal, but the effect of positive V_j was clearly detectable even when full uncoupling was maintained for 5–10 min (*see* Fig. 5*A* and *C-E:* 2nd, 3rd and 4th V_j applications). These data indicate that V_j is able to open some of the mutant-32 channels that had been closed by the CO_2 treatment.

In contrast, V_j gradients that made the mutant side negative dramatically and reversibly reduced G_j virtually to zero (Fig. 5*A* and *C–E*). When a similar protocol was tested on 32-32 channels, the application of 40 mV V_j gradients of either polarity always resulted in significant G_j drop (Fig. 5*B*).

Effect of Inhibition of CaM Expression on Sensitivity to V_j Pulses

For testing the effect of inhibition of CaM expression on V_j and CO_2 sensitivity, the two most effective Cx32 mutants (tandem and ML/NN+3R/N, Fig. 3*A*) were expressed in oocytes previously injected with oligonucleotides antisense to CaM, and the oocytes were heterotypically paired 24–48 hr later with oocytes expressing Cx32 wild type. CaM mRNA was previously shown to be completely and irreversibly degraded within 5 hr after oocyte injection of oligonucleotides antisense to CaM mRNA (Peracchia et al., 1996). The paired oocytes were tested with the standard V_j protocol (V_j steps of 20 mV, ±120 mV max., and 25 sec duration, applied every 45 sec).

Figure 6 shows that the asymmetrical V_j behavior of tandem-32 (Fig. 6*A*, and *see* Peracchia, Wang & Peracchia, 1999) and ML/NN+3R/N-32 (Fig. 6*C*) is virtually abolished or greatly reduced, respectively, following inhibition of CaM expression. Indeed, the plots of normalized G_j (G_j/G_{jmax}) versus V_j for 32-32 and tandem-32 (anti-CaM) channels are almost superimposible (Fig. 6*B*), weren't for a minimal asymmetry of tandem-32 behavior with V_j positive at tandem side (Fig. 6*B*).

Effect of Inhibition of CaM Expression on Sensitivity of G_j To Steady-State V_j

Tandem and ML/NN+3R/N mutants, expressed in oocytes previously injected with oligonucleotides antisense to CaM mRNA and tested heterotypically 24–48 hr with Cx32 wild type, were subjected to a steady-state $V_j = 40$ mV of either polarity. The establishment of $V_j = 40$ mV (mutant side positive) increased G_j by 44.7 ± 64.6% (mean ± SD, $n = 24$) and 37.3 ± 41.5% (mean ± SD, $n = 18$) for tandem-32 (Fig. 7*A*) and ML/NN+3R/N-32 (Fig. 7*B*), respectively. This shows that inhibition of CaM expression drastically reduces the effect of V_j on mutant-32 channels, because in controls (absence of CaM inhibition) G_j increased by 262 ± 64% (mean ± SD, $n = 4$) and 386 ± 181% (mean ± SD, $n = 21$) for tandem-32 (Fig. 7*A* and see Peracchia, Wang & Peracchia, 1999) and ML/NN+3R/N-32 (Fig. 7*B*), respectively. The progressive reduction of the effect of V_j on G_j over time is shown in Fig. 7*C* and *D* for tandem-32 and ML/NN+3R/N-32, respectively.

Similarly, the establishment of $V_j = 40$ mV (mutant side negative) decreased G_j only to 53.5 ± 16.7% (mean ± SD, $n = 15$) and 66.3 ± 19.8% (mean ± SD, $n = 13$) for tandem-32 (Fig. 7*A*) and ML/NN+3R/N-32 (Fig. 7*B*). In contrast, in controls G_j decreased to 16 ± 10% (mean ± SD, $n = 4$) and 14.8 ± 5.6% (mean ± SD, $n = 9$) for tandem-32 (Fig. 7*A*, and *see* Peracchia et al., 1999) and ML/NN+3R/N-32 (Fig. 7*B*), respectively.

The same results were obtained in preliminary experiments testing the mutant 5R/E (Peracchia et al., 1999), expressed in oocytes injected with CaM antisense and tested heterotypically with Cx32 wild type. With 5R/E-32 channels, $V_j = 40$ mV (mutant side positive) increased G_j by 15.3 ± 22% (mean ± SD, $n = 4$), whereas in controls (absence of CaM inhibition) it increased G_j by 182 ± 50% (mean ± SD, $n = 5$). Similarly, $V_j = 40$ mV (mutant side negative) decreased G_j to 25.3 ± 13% (mean ± SD, $n = 3$), whereas in controls (absence of CaM inhibition) it decreased G_j to 14.8 ± 3.3% (mean ± SD, $n = 4$).

Effect of Inhibition of CaM Expression on G_j Sensitivity to V_j during CO_2-Induced Uncoupling

Tandem and ML/NN+3R/N mutants, expressed in oocytes previously injected with oligonucleotides antisense to CaM mRNA and heterotypically paired 24–48 hr later with oocytes expressing Cx32, were subjected to V_j gradients (40 mV) of different polarity during exposure to 100% CO_2. Whereas in controls G_j, reduced to very low values by exposure to CO_2 at $V_j = 0$ mV, increased dramatically and reversibly with V_j gradients that made

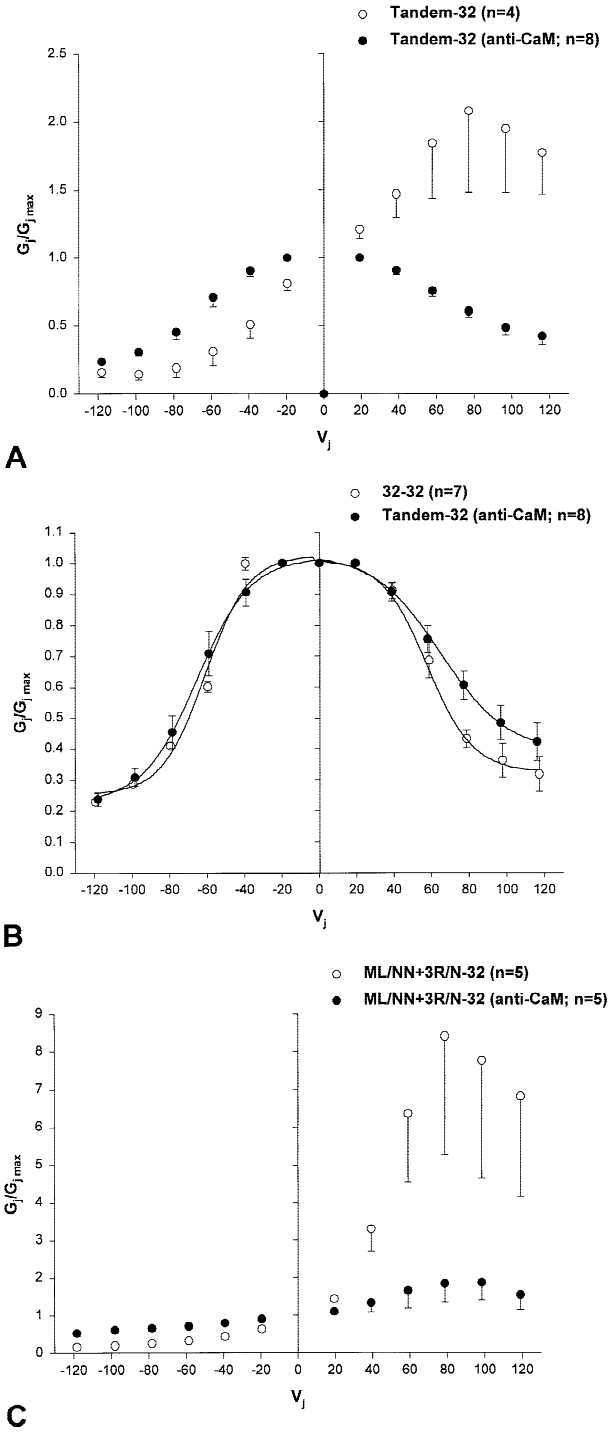


Fig. 6. Effect of inhibition of CaM expression on channel sensitivity to V_j pulses, presented as normalized G_j *vs.* V_j. Tandem and ML/NN+3R/N mutants were expressed in oocytes previously injected with oligonucleotides antisense to CaM, heterotypically paired 24–48 hrs later with oocytes expressing Cx32, and tested with the conventional V_j protocol: V_j steps of 20 mV, ±120 mV maximum and 25 sec duration applied every 45 sec. Note that the asymmetrical V_j behavior of tandem-32 (*A*, and *see*: Peracchia, Wang & Peracchia, 1999) and ML/NN+3R/N-32 (*C*) is virtually abolished or greatly reduced, respectively, with inhibition of CaM expression. Tandem-32 (anti-CaM) and 32-32 channels behave almost identically (*B*), weren't for a slight asymmetry of tandem-32 at V_j positive at tandem side (*B*).

the tandem or ML/NN+3R/N side positive by 40 mV (Figs. 8*A* and 5*A*, respectively), following inhibition of CaM expression V_j positive at mutant side decreased G_j in tandem-32 channels (Fig. 8*B*) and had virtually no effect on ML/NN+3R/N channels (Fig. 8*C*).

Similarly to controls (Figs. 8*A* and 5*A*) V_j negative at mutant side decreased G_j in both tandem-32 (Fig. 8*B*) and ML/NN+3R/N-32 (Fig. 8*C*) channels. With both mutants the effect of CO_2 on G_j was minimal following inhibition of CaM expression (Fig. 8*B* and *C*). This confirms data previously reported on oocytes expressing Cx38 (Peracchia et al., 1996).

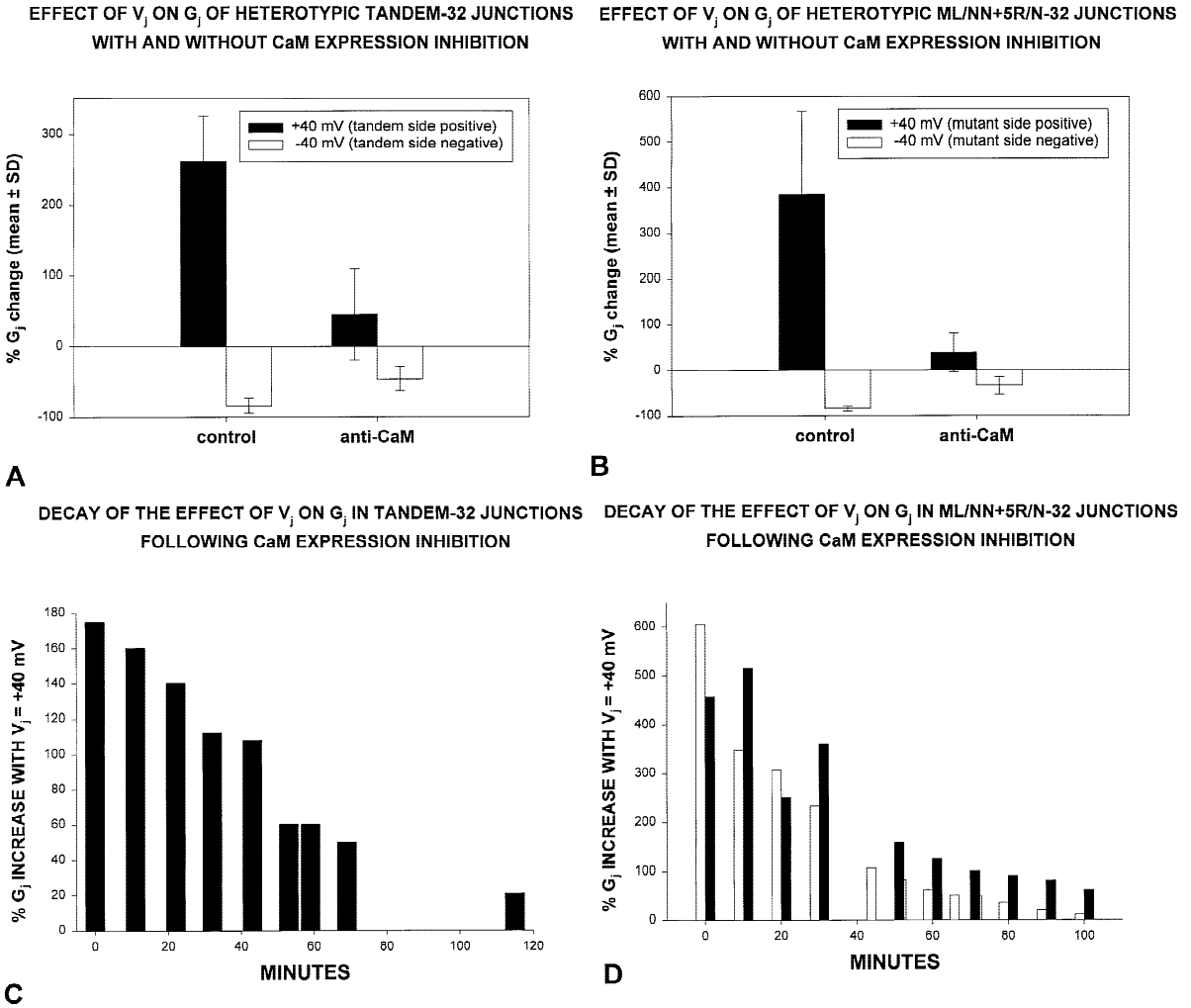


Fig. 7. Effect of inhibition of CaM expression on channel sensitivity to steady-state $V_j = \pm 40$ mV. Tandem and ML/NN+3R/N mutants, expressed in oocytes previously injected with oligonucleotides antisense to CaM mRNA and heterotypically paired 24–48 hr later with oocytes expressing Cx32, were subjected to a steady-state $V_j = 40$ mV, either positive or negative at mutant side. $V_j = 40$ mV (mutant side positive) increases G_j by 44.7 ± 64.6% (mean ± SD, $n = 24$) and 37.3 ± 41.5% (mean ± SD, $n = 18$) in tandem-32 (*A*) and ML/NN+3R/N-32 (*B*), respectively. In contrast, in controls (absence of CaM inhibition) G_j increases by 262 ± 64% (mean ± SD, $n = 4$) and 386 ± 181% (mean ± SD, $n = 21$) for tandem-32 (*A* and *see* Peracchia, Wang, & Peracchia, 1999) and ML/NN+3R/N-32 (*B*), respectively. The progressive reduction of the effect of V_j on G_j over time is shown in *C* and *D* for tandem-32 and ML/NN+3R/N-32 (two oocyte pairs), respectively. Similarly, $V_j = 40$ mV (mutant side negative) decreases G_j only to 53.5 ± 16.7% (mean ± SD, $n = 15$) and 66.3 ± 19.8% (mean ± SD, $n = 13$) for tandem-32 (*A*) and ML/NN+3R/N-32 (*B*), respectively, whereas in controls (absence of CaM inhibition) it decreases G_j to 16 ± 10% (mean ± SD, $n = 4$) and 14.8 ± 5.6% (mean ± SD, $n = 9$) for tandem-32 (*A*, and *see* Peracchia, Wang & Peracchia, 1999) and ML/NN+3R/N-32 (*B*), respectively.

Discussion

This study reports that oocyte pairs expressing heterotypic channels between Cx32 mutants and Cx32 wild type display a unique V_j behavior and increased CO_2 sensitivity. With V_j positive at the mutant side, G_j dramatically increases, whereas with V_j negative G_j decreases to nearly zero. Furthermore, V_j positive at mutant side reopens channels closed by 100% CO_2, whereas V_j negative closes virtually all the channels. Significantly, the effect of these mutations is virtually eliminated with inhibition of CaM expression.

The V_j behavior of these channels made of CL and/or CT mutants is similar to that of channels made of tandem and other CT mutants (5R/E and 5R/N) of Cx32 (Peracchia, Wang & Peracchia, 1999), suggesting that it is not a peculiar characteristic of CT mutations. As proposed for tandem, 5R/E and 5R/N mutants (Peracchia et al., 1999), this behavior is believed to reflect a channel gating mechanism mediated by a V_j-sensitive slow gate,

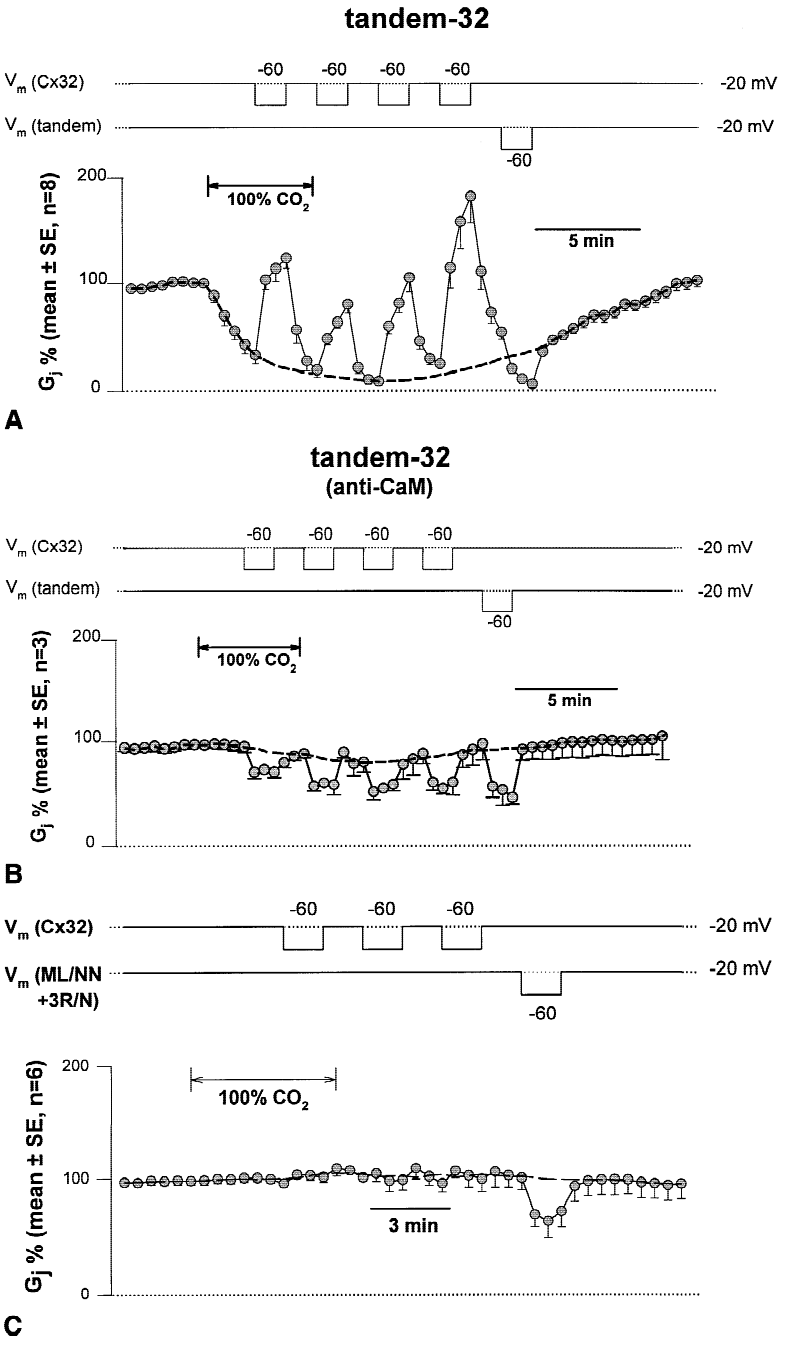


Fig. 8. Effect of inhibition of CaM expression on channel sensitivity to steady-state V_j during CO_2-induced uncoupling. Tandem and ML/NN+3R/N mutants, expressed in oocytes previously injected with oligonucleotides antisense to CaM mRNA and heterotypically paired 24–48 hr later with oocytes expressing Cx32, were exposed to V_j gradients (40 mV) of different polarity during exposure to 100% CO_2. In controls (absence of CaM inhibition) G_j, reduced by CO_2 at $V_j = 0$ mV, increases dramatically with V_j positive at tandem (*A*) or ML/NN+3R/N (Fig. 5*A*) side. In contrast, following CaM inhibition V_j positive at mutant side decreases G_j in tandem-32 (*B*) and has virtually no effect on ML/NN+3R/N channels (*C*). Similarly to controls (*A* and Fig. 5*A*) V_j negative at mutant side decreases G_j in both tandem-32 (*B*) and ML/NN+3R/N (*C*) channels. With both mutants the effect of CO_2 on G_j is minimal with inhibition of CaM expression (*B* and *C*), as reported earlier for oocytes expressing Cx38 (Peracchia et al., 1996).

distinct from the conventional (fast) V_j gate. Based on the V_j behavior of these mutant channels in the presence of CO_2 we have proposed that the chemical gate is V_j sensitive and that the chemical gate and slow V_j gate are the same (Peracchia et al., 1999). Voltage sensitivity of chemical gating was also reported in insect cells, but insect channels were sensitive to V_m rather than V_j (Weingart & Bukauskas, 1998). The slow gate may also be active in Cx32 wild type, because in homotypic 32-32 channels the G_j decay with steady-state V_j has a fast and a slow component, suggesting that also in these channels both fast and slow V_j gates are activated. Moreover, with 32-32 channels V_j gradients of 40 mV, applied during CO_2 exposure, decrease G_j much more than one would predict if the fast V_j gate were the only player, because the fast V_j gate of Cx32 is relatively insensitive to V_j gradients <50–60 mV.

Aside from the significant diversity among the mu-

tants tested in this and the previous study (Peracchia et al., 1999) in magnitude of V_j effect on G_j, there are also significant differences in the kinetics of G_j change. Based on the τ values, tandem and CT mutants (5R/E, 5R/N and 3R/N) have slower kinetics than either the CL mutants (ML/NN, ML/EE) or the mutant with combined CL and CT mutations (ML/NN+3R/N). The reason for this phenomenon is unclear. If indeed the slow gating involves the pore plugging action of an accessory molecule ("cork" gating model; Peracchia et al., 2000), this could mean that the gating molecule (CaM?) moves faster in and out of the pore with CL than CT mutations.

With two of the mutants (3R/N and ML/NN) an abrupt increase in G_j was observed upon return to $V_j = 0$ from V_j positive at the mutant side. This was previously noticed with tandem-32, 5R/E-32 and 5R/N-32 channels (Peracchia et al., 1999), and was believed to reflect the activity of the fast V_j gate of Cx32. Since the fast V_j gate of Cx32 closes with negative potentials (Verselis, Ginter & Bargiello, 1994), the abrupt increase in G_j may mark the reopening of the fast V_j gate of Cx32. Indeed, the increase in G_j is absent with V_j reversal from positive to negative at mutant side, as the opening of the fast V_j gate of Cx32 is masked by the closing of the fast V_j gate of the mutant hemichannel. However, the faster closing kinetics of the slow gate with V_j reversal may also contribute in masking the opening of the fast Cx32 gate. This may be the reason why with mutants displaying shorter τ (ML/NN+3R/N and ML/EE) the abrupt increase in G_j is not observed.

Mutant-32 channels were more sensitive to CO_2 than homotypic 32-32 channels, suggesting that these mutations facilitate the chemical gating mechanism either by weakening CL_1-CT_1 interactions or by rendering the pore more accessible to a gating element. Significantly, the CO_2 sensitivity of mutant-32 channels matches well the magnitude of slow gating asymmetry. The channels most sensitive to slow V_j gating (ML/NN+3R/N-32) are also the most sensitive to CO_2 (with 3 min CO_2, G_j drops to 0% at a maximum rate of 54%/min). Similarly, the least V_j-sensitive channels (5R/N-32) are also the least CO_2 sensitive (with 3 min CO_2, G_j drops to 37.5% at a maximum rate of 21%/min; Peracchia et al., 1999). This rule applies to all of the mutant-32 channels except for tandem-32, which are relatively more sensitive to V_j than to CO_2 (Peracchia et al., 1999). The general correspondence between CO_2 and slow V_j sensitivities is consistent with the idea that chemical gate and slow V_j gate are the same.

As previously reported (Peracchia et al., 1999), there are several reasons to believe that slow gate and fast V_j gate are distinct gates. One is the much slower kinetics of the slow gate. Another is the fact that negative V_j's as low as 40 mV close most of the mutant-32 channels, whereas for the fast V_j gate a residual conductance of 20–25% remains even with V_j gradients as high as 120 mV (Bukauskas & Peracchia, 1997). The distinct function of the two gates is also revealed by the abrupt increase in G_j with return to $V_j = 0$ from V_j positive at the mutant side, which marks the reopening of the fast V_j gate, whereas the close relationship between slow gate and chemical gate is demonstrated by evidence that positive V_j reopens channels closed by CO_2. Finally, most relevant additional evidence is provided by the behavior of heterotypic channels between Cx26 wild-type a Cx26 mutant (4pos/E) in which, as in 5R/E, all of the four basic residues of CT were mutated to E (R215E, K220E, K222E and R223E); 4pos/E-26 channels behaved as 5R/E-32 channels when exposed to V_j (Peracchia, Wang & Peracchia, 2000), in spite of the fact that the fast V_j gates of Cx26 and Cx32 are sensitive to opposite V_j polarities (Verselis et al., 1994).

The idea that slow gate and chemical gate are the same is further supported by the observation that the unusual behavior of the most sensitive mutant-32 channels (ML/NN+3R/N-32 and tandem-32) is virtually normalized by inhibition of CaM expression. In oocytes previously injected with oligonucleotides antisense to CaM mRNA, these mutants lost almost completely their asymmetrical V_j sensitivity and became virtually insensitive to CO_2, as previously observed in oocytes expressing Cx38 (Peracchia et al., 1996). These two mutants were selected for this study because they displayed the most pronounced asymmetry of slow V_j gating sensitivity. Although ML/NN+3R/N is a fairly conventional mutant that is not expected to alter significantly the connexon architecture, this may be questionable for the tandem. As previously discussed (Peracchia et al., 1999), the architecture of tandems in connexons is hard to predict and in the absence of structural information the possibility that only one of the two monomers of a tandem is normally inserted in the membrane cannot be ruled out. However, this is of little relevance to the message of this and the previous study (Peracchia et al., 1999), because our work was not aimed at correlating the behavior of tandem channels to the tandem structure; the tandem was used primarily as a tool. In any event, since the tandem performed like six other more conventional mutants and was able to make heterotypic channels it is unlikely that its conformation is that unusual. Furthermore, in addition to tandem and ML/NN+3R/N, preliminary data indicate that with inhibition of CaM expression also heterotypic channels between the mutant 5R/E (Peracchia et al., 1999) and Cx32 almost completely lose both their asymmetrical V_j sensitivity and their abnormal V_j behavior during CO_2 treatment.

The possible participation of CaM in channel gating was previously suggested by evidence that CaM inhibitors (Peracchia et al., 1981, 1983; Peracchia, 1984, 1987) and inhibition of CaM expression (Peracchia et al., 1996) prevent CO_2-induced uncoupling. This was also supported by data for CaM binding to Cx32 (Hertzberg &

Gilula, 1981; Van Eldik et al., 1985; Zimmer et al., 1987; Török, Stauffer & Evans, 1997). Two potential CaM binding sites have been identified in Cx32: one at the NH_2-terminus and the other at the base of the COOH-terminus (Peracchia, 1988). The CaM binding capacity of synthetic peptides matching identified COOH-terminal sequences of Cx32, Cx38 and Cx43 has been demonstrated by spectrofluorometry and circular dichroism spectroscopy (Girsch & Peracchia, 1992; Peracchia & Shen, 1993) and by the ability of a fluorescent CaM derivative to interact with the identified NH_2- and COOH-terminal domains of Cx32 (Török, Stauffer & Evans, 1997).

Based on past and present evidence for CaM involvement in connexin function we have proposed a model ("cork" gating model) that envisions chemical gating as the result of interaction between CaM or a CaM lobe and the cytoplasmic mouth of the channel (Peracchia et al., 1999, 2000). Evidence from the V_j behavior of mutant-32 channels suggests that the gating mechanisms may involve the slow displacement in and out of the pore of a sizable negatively charged molecule. Since the CaM lobes and the cytoplasmic mouth of the channel (Perkins, Goodenough & Sosinsky, 1997) are ~25Å in diameter and have opposite charge characteristics, a direct interaction between the two structures is conceivable. In Cx32 the deletion of 84% of the COOH-terminus does not affect CO_2 gating (Werner et al., 1991; Wang & Peracchia, 1997), so that a "ball-and-chain" model as that proposed for Cx43 (Liu et al., 1993; Ek-Vitorin et al., 1996; Morley, Taffet & Delmar 1996) is unlikely.

In conclusion, data on chemical and voltage gating characteristics of Cx32 mutants expressed heterotypically with Cx32 wild type indicate that V_j gradients activate a slow gating mechanism distinct from the conventional V_j gating mechanism. The slow gate opens at relatively positive V_j and closes at negative V_j, following exponential courses with long time constants. In addition, V_j positive at the mutant side reopens channels closed by CO_2, suggesting that chemical gate and slow V_j gate are the same. The slow gate could be a pluglike acidic cytosolic molecule (cork-gating model; Peracchia et al., 2000). Based on the finding that blockage of CaM expression strongly inhibits both slow gating and CO_2-induced gating, and based on the size and surface charge of CaM lobes and channel mouth, CaM is likely to be the gating element. This is not unreasonable because in recent years the direct involvement of CaM in the function of many other membrane channels has been demonstrated (Saimi, Ling & Kung, 1994; Menegazzi et al., 1996; Lan, Brereton, Barritt, 1998; Fanger et al., 1999; Lee et al., 1999; Xia et al., 1998; Zühlke et al., 1999; Peterson et al., 1999; Qin et al., 1999). Evidence for CaM participation in both slow V_j-sensitive gating and CO_2-induced gating of gap junction channels confirms the original hypothesis for a pivotal role of cytosolic free-Ca^{2+} in gap junction regulation (Loewenstein, 1996; Rose & Loewenstein, 1975).

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Note Added in Proof

Recent evidence for a drastic increase in chemical gating sensitivity of homotypic Cx32 channels with expression of CaMCC (a CaM mutant with higher overall Ca^{2+} binding affinity), for an effect of CaMCC on V_j gating and for the colocalization of CaM and Cx32 at junctional sites, indicates that CaM is involved in chemical gating of Cx32 channels through a direct interaction with the connexin. Peracchia, C., Sotkis, A., Wang, X.G., Peracchia, L.L., Persechini, A. 2000. Calmodulin directly gates gap junction channels. *J. Biol. Chem.* **34:**26220–26224