

Positive Charges of the Initial C-terminus Domain of Cx32 Inhibit Gap Junction Gating Sensitivity to CO₂

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ABSTRACT Gap junction channels close with CO₂ exposure. To determine whether the carboxy-terminus (CT) of connexin32 (Cx32) participates in gating, the CO₂ sensitivity of channels made of Cx32 or Cx32 mutants was studied by double voltage clamp. In *Xenopus laevis* oocytes expressing Cx32, junctional conductance (G_j) dropped to 85% and 47% of controls with 3- and 15-min CO₂ exposures, respectively. In response to the 15-min exposure to CO₂, pH_i dropped to ~6.4 in 5–7 min and did not decrease further, even with 30-min exposures. CT deletion by 84% did not affect CO₂ sensitivity, but replacement of five arginines (R215, R219, R220, R223, and R224) with asparagines (N) or threonines at the beginning of CT (CT₁) in Cx32 or Cx32 deleted beyond residue 225 greatly enhanced CO₂ sensitivity (with 3-min CO₂ G_j dropped to ~8%). Partial R/N replacement resulted in intermediate CO₂ sensitivity enhancement. R215 is a stronger inhibitor than R219–220, whereas R223–224 may diminish the inhibitory efficiency of R215 and R219–220. Therefore, positive charges of CT₁ reduce the CO₂ sensitivity of Cx32, whereas the rest (>80%) of CT seems to play no role in CO₂-induced gating. The role of presumed electrostatic interactions among Cx32 domains in CO₂-induced gating is discussed.

INTRODUCTION

Gap junctions are the regions of cell contact responsible for direct cell-cell communication by metabolic and electrical signals. Gap junctional communication accounts for synchrony in events such as contraction of the uterus and myocardium, and is believed to play an important role in regulating growth and differentiation (reviewed in Bruzzone et al., 1996). A functional gap junction channel consists of two hemichannels (connexons), one in each cell, composed of hexamers of transmembrane proteins known as connexins (Cx). During the last decade more than a dozen members of the connexin family have been cloned. Connexins contain four transmembrane regions, linked by two extracellular and a cytoplasmic (CL) loop, a short N-terminus (NT), and a C-terminus (CT) of variable length. Connexin sequences are highly conserved aside from CL and CT regions, which vary significantly in both length and composition, being nearly unique to each connexin. The connexin-specific sequences may account for unique functional properties.

Functional gap junction channels are mostly in an open state, but can close in response to changes in cytosolic Ca²⁺ or H⁺ concentration (reviewed in Peracchia et al., 1994), resulting in cell-cell uncoupling. Most cells can be reversibly uncoupled by exposure to 100% CO₂ (Turin and Warner, 1977; Spray et al., 1981a), but the molecular mechanisms of CO₂-induced channel gating are not yet understood. The CT chain has been suggested to play a role in

determining the CO₂ gating sensitivity of Cx43 (Liu et al., 1993), and a ball-and-chain model for CO₂-gating of Cx43 has been proposed (Morley et al., 1996; Ek-Vitorin et al., 1996). In contrast, Cx32 mutants missing >80% of CT are as sensitive to CO₂ as wild-type Cx32 (Werner et al., 1991; Wang et al., 1996b, and present study). Spray and Burt (1990) have proposed that low-pH-induced uncoupling follows protonation of histidine (H) residues. Indeed, a role in determining the CO₂-sensitivity of Cx43 has been attributed to H95 (Ek-Vitorin et al., 1994), a residue located at the beginning of CL in most connexins. Hermans et al. (1996) have provided preliminary evidence indicating that two other H residues of Cx43 (H126 and H142) modulate in opposite ways the uncoupling effect of CO₂. On the other hand, in Cx32 the replacement of H126 with R did not affect the CO₂ sensitivity of Cx32 (Wang and Peracchia, 1996).

Recently, we have used site-directed mutagenesis and chimeric construction techniques for characterizing, in *Xenopus* oocyte pairs, domains of Cx32 and Cx38 potentially involved in CO₂-induced channel gating (Wang et al. 1996a; Wang and Peracchia, 1996). Cx32, the principal rat liver connexin, is poorly sensitive to CO₂, whereas Cx38, the connexin expressed by *Xenopus* embryos, is very sensitive to CO₂. Our data indicate that the second half of CL (CL₂) contains a domain relevant for CO₂ gating sensitivity, because a Cx32 chimera containing CL₂ of Cx38 is as sensitive to CO₂ as Cx38, (Wang and Peracchia, 1996). The NT chain does not appear to be relevant, whereas the potential role of the CT chain could not be tested because the relevant chimeras did not express functional channels (Wang et al., 1996a).

To test the potential role of Cx32's CT in CO₂-induced gating, the present study has evaluated the effects of CT deletions and basic residue mutations. Our data show that whereas 84% CT deletion has no effect, replacement of the

Received for publication 24 February 1997 and in final form 15 May 1997.

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0006-3495/97/08/798/09 \$2.00

five basic residues (R) of the initial CT domain (CT₁), with neutral residues (N or T), dramatically enhances Cx32 sensitivity to CO₂. A preliminary account of these data has been published (Wang et al., 1996b).

MATERIALS AND METHODS

Site-directed mutagenesis

Molecular biology protocols were generally as described by Sambrook et al. (1989). The cDNA of Cx32 (Paul, 1986) was used in the construction of DNA mutants. The strategy used to create all of the Cx32 mutants has been previously described (Wang et al., 1996a). All of the mutants were verified by DNA sequence analysis. The mutants D219, D222, D225, and D225-5R/N were kindly provided by Dr. Rudolf Werner (Werner et al., 1991; Rabadan-Diehl et al., 1994). For mutant sequences see Table 1.

Oligonucleotide sequences

Oligonucleotides were synthesized by a DNA synthesizer (model 393; ABI, Foster City, CA). The sequences used to produce mutants are shown below. Letters in italics and underlined represent mutated nucleotides.

(5R/N):

5'-CCTTGC~~GGG~~GAGGGCGGATTGGAGTTGTTCTGAGCGTTGTT

(5R/T):

5'-ACGGCCTGTGCCACCACTGCTCAGAC-
CACCTCCAATCCGCCCTCCGCAAG

(5R/T):

5'-GTGGTCTGAGCAGTGGTGGCACAGGC-
CGTGATGATGAGGTACACCACCTCCG

(4R/N):

5'-GAGCGTTGTTTGGCACAGGCCCGGATGATGAGGTACACCAC

(3R/N #1):

5'-GATTGGAGTTGTTCTGAGCACGGCGGCACAGGCGTTGATGATG

(3R/N #2):

5'-GCCGATTGGAGCGGCGCTGAGCGTTGTTTGGCACAGGC

(2R/N):

5'-TTGGAGCGGCGCTGAGCATTGTTTGGCACAGGCCCGGATGAT

(1R/N):

5'-AGCACGGCGGGCACAGGCGTTGATGATGAGGTACACCAC

Table 1 Sequences of Cx32 C-ter mutants

Cx32	208	EVVYLIIRACARRAQRNSNPP
5R/N	208	***** N *** NN ** NN ****
5R/T	208	***** T *** TT ** TT ****
4R/N	208	***** R *** NN ** NN ****
3R/N #1	208	***** N *** RR ** NN ****
3R/N #2	208	***** N *** NN ** RR ****
2R/N	208	***** R *** NN ** RR ****
1R/N	208	***** N *** RR ** RR ****
D225	208	***** R *** RR ** RR */225
D225 5R/N	208	***** N *** NN ** NN */225
D222	208	***** R *** RR ***/222
D219	208	***** R *** R */219

Preparation of cRNA

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

Oocyte preparation and microinjection

Oocytes were prepared as described previously (Peracchia et al., 1996). Briefly, adult female *Xenopus* frogs were anesthetized with 0.3% tricaine (MS-222) and the oocytes were surgically removed from the abdominal incision. The oocytes were placed in ND 96 medium. Oocytes at stages V or VI were subsequently defolliculated in 2 mg/ml collagenase (Sigma Chemical Co., St. Louis, MO) in Ca²⁺-free OR2 for 80 min at room temperature. The defolliculated oocytes were injected with 46 nl of 0.25 μg/μl of antisense oligonucleotide complementary to endogenous *Xenopus* Cx38: 5'-GCTTTAGTAATCCCATCCTGCCATGTTTC-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 completely blocks the endogenous junctional communication within 48 h. Forty-six nl of either wild-type or mutated cRNA (0.02–0.1 μg/μl) were injected into oocytes at the vegetal pole, and the oocytes were incubated overnight at 18°C 48–72 h post-injection. The oocytes were mechanically stripped of their vitelline layer in a hypertonic medium (Peracchia et al., 1996) and paired at the vegetal poles in ND 96. Oocyte pairs were studied electrophysiologically 0.5–2 h after pairing.

Uncoupling protocol

The oocyte chamber was continuously perfused at a flow rate of 0.6 ml/min by a peristaltic pump (Micro Perpex, Pharmacia LKB Biotechnology Inc., Piscataway, NY). 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- to 15-min superfusion (0.6 ml/min) of salines continuously gassed with 100% CO₂. Either ND96 or 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 methanesulfonic acid. The Cl⁻-free saline was used in all of the experiments, aside from a few early experiments on oocytes expressing Cx32 wild-type that have not been used for the statistical comparisons of junctional sensitivity to CO₂. As previously reported (Peracchia et al., 1996) the opening of Ca²⁺-activated Cl⁻ channels during exposure to 100% CO₂ causes an increase in membrane current that may interfere with junctional current measurement.

Measurement of gap junctional conductance in oocyte pairs

All the experiments were performed using the standard double voltage clamp procedure for measuring junctional conductance (G_j; Spray et al., 1981b), using a previously published protocol (Peracchia et al., 1996). Briefly, microelectrodes were pulled from borosilicate glass capillaries, 1.2 mm (OD), 0.68 mm (ID) (Kwik fill, W-P Instruments, Inc., New Haven, CT), by means of a Brown-Flaming micropipette puller (Sutter Instruments Co., San Francisco, CA). The microelectrodes, filled with a 3 M KCl solution, had a low tip resistance (0.5–1 MΩ in ND96), such that even in oocyte pairs with significantly different initial junctional conductance (G_j, Table 2) similar percent drops in G_j with CO₂ were attained. The bath was grounded with a silver-silver chloride reference electrode connected to the superfusion chamber via an agar bridge. After the insertion of a current and a voltage microelectrode in each of the two oocytes, both oocytes were

Table 2. Initial junctional conductances developed by oocyte pairs expressing Cx32 mutants

Type	Pairing Time (hr)	G_j (μS , mean \pm SD)	G_j (μS , max.)	G_j (μS , min.)	n
Wild-type Cx32	0.5	4.2 \pm 1.3	27.5	0.4	26
5R/N	0.5	3.8 \pm 1.3	9.8	0.3	8
5R/T	0.5	0.7 \pm 0.3	2.1	0.3	6
4R/N	0.5	3.8 \pm 0.8	7.2	0.5	10
3R/N #1	0.5	1.1 \pm 0.4	3.9	0.5	8
3R/N #2	0.5	9.7 \pm 3.6	30.6	0.3	11
2R/N	0.5	22.5 \pm 5.3	47.0	0.4	9
1R/N	0.5	14.6 \pm 2.9	22.3	0.9	6
D225	0.5	6.0 \pm 2.7	31.8	0.4	11
D225 5R/N	0.5	2.0 \pm 0.8	3.9	0.7	11
D222	0.5	8.6 \pm 2.7	17.7	0.2	6
D219	0.5	3.1 \pm 0.9	7.2	0.7	8

initially voltage clamped to the same holding potential (V_m), similar to their resting membrane potential, so that no junction current would flow at rest ($I_j = 0$ pA). A V_j gradient was created by imposing a +20 mV voltage step (V_1) of 2 s duration every 30 s 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 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 a computer equipped with pClamp software (Axon Instruments, Inc., Foster City, CA) and Labmaster TL-1A/D-D/A interface (Axon Instr. Inc.).

Measurement of intracellular hydrogen ion concentration

$[\text{H}^+]_i$ measurements were performed with the I^3 Calcium Imaging System (Intracellular Imaging Inc., Cincinnati, OH). For measuring $[\text{H}^+]_i$, the fluorescein derivative pH indicator BCECF (B-1151, Molecular Probes Inc., Eugene, OR) was injected into control oocytes in amounts sufficient to reach an intra-oocyte concentration of ~ 100 μM . After the injection, the oocytes were placed in the same conical wells used for electrophysiology, modified by replacing the plastic floor with a glass coverslip. The conical wells were mounted on the stage of a Nikon TMS microscope equipped for epifluorescence and the oocytes were superfused with ND96, with or without 100% CO_2 bubbling, as previously described. Specimen observation and light measurements were performed with a Nikon Fluore 10x objective. Light from a 300-W Xenon arc illuminator passed through a computer-controlled filter changer and shutter unit containing 440- and 490-nm band pass filters and liquid light guide. Light emitted by the oocytes was collected by an integrating CCD video camera with a microscope-interfacing relay lens. Pairs of images at the two wavelengths were collected at 3-s intervals and $[\text{H}^+]_i$ was computer-calculated online (p5-90, Gateway 2000, North Sioux City, SD) by dividing the short-wavelength by the long-wavelength image, after subtraction of the respective backgrounds. Calibration curves were generated by ratioing droplets of 0.1 M phosphate buffers (pH 8, 7.5, 7, 6.5, 6) containing 13 μM BCECF.

RESULTS

Effect of CO_2 on junctional conductance and $[\text{H}^+]_i$ in oocytes expressing Cx32

Oocytes expressing the rat liver connexin, Cx32, developed sufficient coupling for accurate measurement of junctional conductance (G_j) soon after pairing; 30 min after pairing, G_j was usually ~ 4 μS (Table 2). Cx32 was weakly sensitive to CO_2 . With a 3-min exposure to 100% CO_2 , G_j decreased to $85 \pm 5\%$ (mean \pm SE, $n = 7$) (see Fig. 3 B); and with a 15-min exposure to $47 \pm 5\%$ (mean \pm SE, $n = 16$) (see

Figs. 1 and 3 B). Similar drops in G_j were observed in oocyte pairs displaying different initial G_j ranging from 0.4 μS to 27.5 μS (Table 2). G_j decreased with CO_2 at a maximal rate of $\sim 9\%/min$ (Fig. 1) and recovered to pretreatment values at a maximal rate of 8–11%/min (Fig. 1). The onset of G_j recovery was always rather abrupt (Fig. 1). Longer CO_2 treatments did not significantly increase the uncoupling magnitude (Fig. 2 A).

In response to 15-min exposures to CO_2 , $[\text{H}^+]_i$ increased in 5–7 min from 26.3 ± 1.2 nM, $\text{pH}_i = 7.58$, to 421.8 ± 0.4 (mean \pm SE, $n = 11$), $\text{pH}_i = 6.38$, at the maximum rate of $\sim 25\%/min$, and remained at that level until the end of the CO_2 exposures (Fig. 2 B). Longer exposures to CO_2 did not result in further $[\text{H}^+]_i$ increase, as $[\text{H}^+]_i$ remained at a virtually steady value of 412 ± 0.7 nM (mean \pm SE, $n = 12$), $\text{pH}_i = 6.38$, until the end of the CO_2 exposures (Fig. 2 B). After either 15- or 30-min CO_2 exposures, $[\text{H}^+]_i$ recovered following single exponential decays with time constants (τ) of 7 and 8.1 min, respectively. In a previous study we have reported that pH_i decreases with 3-min CO_2 exposures from 7.63 ± 0.115 to 6.54 ± 0.113 (mean \pm SE, $n = 18$, Peracchia et al., 1996). Note that the kinetics of G_j and $[\text{H}^+]_i$ differ significantly from each other; $[\text{H}^+]_i$ rose rapidly and reached its maximum in 5–7 min (Fig. 2 B); whereas G_j decreased, slowly reaching its minimum in 12–15 min (Figs. 1 and 2 A). Since pH_i reached a steady state well before the end of 15-min exposures to 100% CO_2 (Fig. 2 B), and G_j did not decrease significantly more with CO_2 exposures longer than 15 min (Fig. 2 A), exposures to

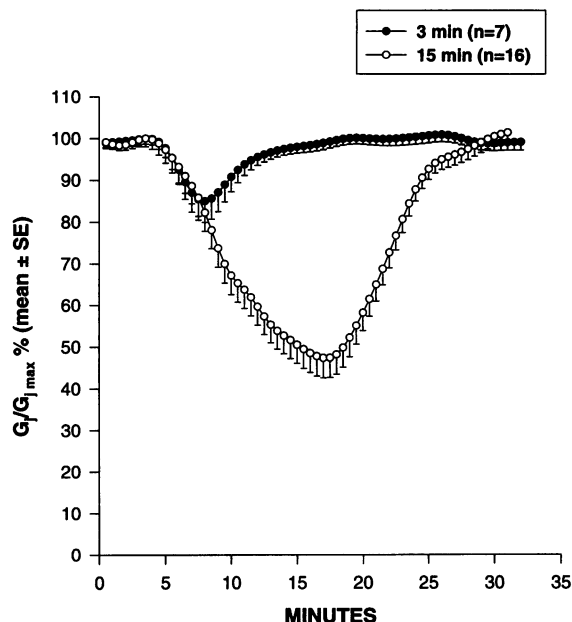


FIGURE 1 Time course of G_j in *Xenopus* oocyte pairs, expressing wild-type Cx32, exposed to 100% CO_2 . With a 3-min exposure to CO_2 , G_j decreases to $85 \pm 5\%$ (mean \pm SE) and with a 15-min exposure to $47 \pm 5\%$ (mean \pm SE), at a maximal rate of $\sim 9\%/min$. G_j recovers to pretreatment values at a maximal rate of 8–11%/min. The onset of G_j recovery is rather abrupt.

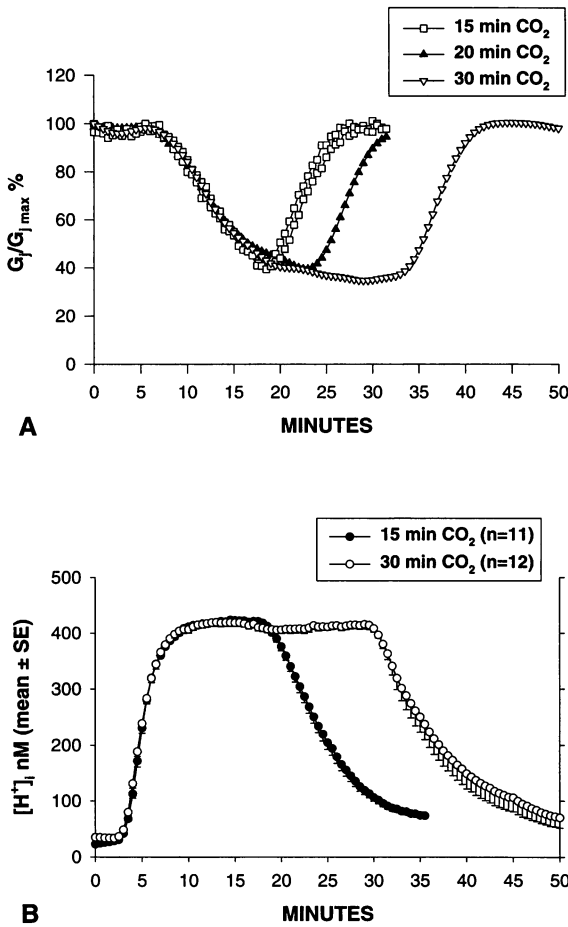


FIGURE 2 Time course of G_j (A) and $[H^+]_i$ (B) in *Xenopus* oocytes exposed to 100% CO_2 for 15–30 min. CO_2 treatments longer than 15 min only minimally increase the magnitude of G_j drop resulting from 15-min exposures (A). Note the consistency of uncoupling and recoupling rates in spite of the different durations of CO_2 exposure. With either 15 or 30-min exposures to CO_2 , $[H^+]_i$ increases to similar plateau values at similar maximal rates (25%/min) in 5–7 min (B). With 15-min exposures to CO_2 , $[H^+]_i$ increased from 26.3 ± 1.2 nM, $pH_i = 7.58$, to 421.8 ± 0.4 nM (mean \pm SE, $n = 11$), $pH_i = 6.38$. With 30-min exposures to CO_2 , $[H^+]_i$ increased from 34.4 ± 1.05 nM, $pH_i = 7.46$, to 412.0 ± 0.7 nM (mean \pm SE, $n = 12$), $pH_i = 6.38$. $[H^+]_i$ remained at that level until the end of the CO_2 exposures. After either 15- or 30-min CO_2 exposures, $[H^+]_i$ recovered following single exponential decays with time constants (τ) of 7 and 8.1 min, respectively.

CO_2 longer than 15 min were not routinely included in our protocol.

Effect of carboxy-terminus deletion on CO_2 gating sensitivity

Cx32 mutants deleted by up to 84% of CT expressed functional channels as efficiently as Cx32, their initial G_j values ranging from 3.1 μS to 8.6 μS (Table 2). Deletion of most of the CT chain did not affect CO_2 sensitivity, as all of the three deletion mutants tested (D225, D222, and D219) behaved very similarly to Cx32 in uncoupling magnitude with either 15-min (Fig. 3, A and B) or 3-min (Fig. 3 B) expo-

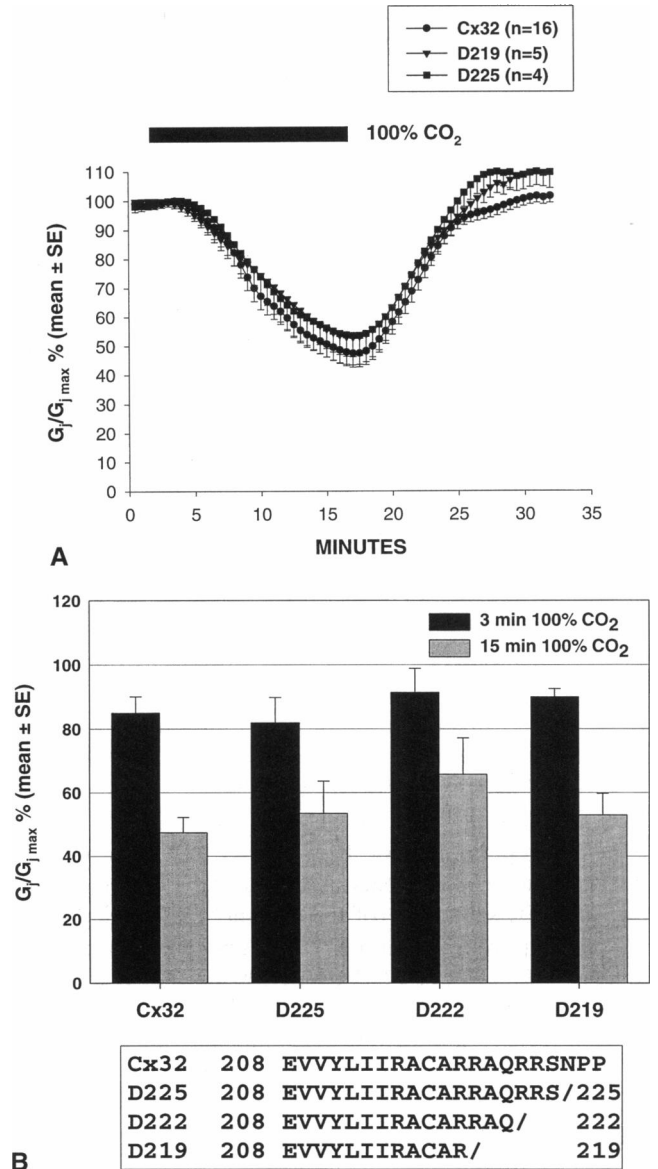


FIGURE 3 Decrease of G_j in *Xenopus* oocyte pairs, expressing wild-type Cx32 or Cx32 deleted of over 80% of CT, with exposure to 100% CO_2 for either 15-min (A and B) or 3-min (B). Note that deletion of CT by over 80% (D225, D222, and D219) does not affect CO_2 sensitivity, as in both wild-type and mutated Cx32 G_j drops by approximately the same amount (A and B) with the same uncoupling and recoupling rates (A). With 3-min CO_2 , G_j dropped to $82 \pm 8\%$ (mean \pm SE, $n = 3$), $91 \pm 7\%$ (mean \pm SE, $n = 4$), and $90 \pm 3\%$ (mean \pm SE, $n = 4$) with D225, D222, and D219, respectively, and with 15-min CO_2 , to $53.5 \pm 10\%$ (mean \pm SE, $n = 4$), $65 \pm 11\%$ (mean \pm SE, $n = 3$), and $53 \pm 7\%$ (mean \pm SE, $n = 5$) with D225, D222, and D219, respectively.

sure to CO_2 , as well as in both uncoupling and recoupling rates (Fig. 3 A). With 3-min exposure to CO_2 , G_j dropped to $82 \pm 8\%$ (mean \pm SE, $n = 3$), $91 \pm 7\%$ (mean \pm SE, $n = 4$), and $90 \pm 3\%$ (mean \pm SE, $n = 4$), with D225, D222, and D219, respectively (Fig. 3 B), and with 15-min CO_2 , to $53.5 \pm 10\%$ (mean \pm SE, $n = 4$), $65 \pm 11\%$ (mean \pm SE, $n = 3$), and $53 \pm 7\%$ (mean \pm SE, $n = 5$), with D225, D222, and D219, respectively (Fig. 3, A and B).

Effect of arginine mutation to polar-uncharged residues in the initial domain of CT (CT₁) on CO₂ gating sensitivity

The initial 17-residue segment (CT₁) of the Cx32's CT chain is a basic and hydrophobic domain, as it contains five arginines (R), eight hydrophobic residues, three polar-uncharged, and one acidic (Table 1). To test the potential relevance of the positive charges of CT₁ in CO₂ gating sensitivity of Cx32, the R residues were mutated individually or in groups to polar-uncharged residues. All of the mutants tested expressed functional channels efficiently, their initial G_j values ranging from 0.7 μ S to 22.5 μ S (Table 2).

Replacement of all of the five R residues with asparagines (N) strongly enhanced the CO₂ sensitivity of both full-length Cx32 (5R/N, Figs. 4 A and 6) and D225 (D225-5R/N, Figs. 4 B and 6). With 3-min exposures to 100% CO₂, G_j dropped to $7.12 \pm 3.39\%$ (mean \pm SE, $n = 5$) of initial values at a maximum rate of $\sim 18\%/min$ in oocytes expressing 5R/N (Fig. 4 A), to $8.28 \pm 5.35\%$ (mean \pm SE, $n = 4$) at a maximal rate of $\sim 17\%/min$ in those expressing D225-5R/N (Fig. 4 B), and to nearly 0% in both types with 15-min CO₂ exposures (Fig. 6). Similar results were obtained with replacement of R with T residues (5R/T, Fig. 6). With 5R/T, G_j dropped to $7.1 \pm 2.7\%$ (mean \pm SE, $n = 6$) with 3-min CO₂, and to nearly 0% with 15-min CO₂ (Fig. 6).

Partial replacement of R with N residues resulted in intermediate enhancements of CO₂ sensitivity (Figs. 5 and 6). The CO₂ sensitivity of the mutants increased progressively in the following order (from least to most sensitive mutant): 4R/N, 3R/N #1, 2R/N, 1R/N, and 3R/N #2 (Table 1 and Figs. 5 and 6). The increase in sensitivity was more gradual with 15-min than with 3-min CO₂ exposures, as G_j dropped to $38.5 \pm 7.2\%$ (mean \pm SE, $n = 5$) with 4R/N (Figs. 5 A and 6), to $16.1 \pm 4.5\%$ (mean \pm SE, $n = 4$) with 3R/N #1 (Figs. 5 B and 6), to $8.4 \pm 2.3\%$ (mean \pm SE, $n = 5$) with 2R/N (Figs. 5 C and 6), to $1.3 \pm 0.2\%$ (mean \pm SE, $n = 3$) with 1R/N (Figs. 5 D and 6), and to $0.5 \pm 0.05\%$ (mean \pm SE, $n = 4$) with 3R/N #2 (Figs. 5 E and 6). With 3-min CO₂ exposures, a significant increase in CO₂ sensitivity was obvious only with 3R/N #1 and 3R/N #2 (Fig. 5 B and E; Fig. 6). The maximum uncoupling rate with 15-min exposures to CO₂ also gradually increased among the mutants in an order similar to that of the uncoupling magnitude. At the two extremes were the rates of 4R/N ($\sim 7\%/min$, Fig. 5 A), which was similar to those of Cx32 (Fig. 1), D225, D219 (Fig. 3 A), and D222 (data not shown), and that of 3R/N #2 ($\sim 20\%/min$, Fig. 5 E(B), which was similar to those of 5R/N (Fig. 4 A) and D225-5R/N (Fig. 4 B).

DISCUSSION

The data show that positively charged residues located in the initial (17 residue-long) domain (CT₁) of the carboxy-terminus chain of Cx32 have an inhibitory action on CO₂ gating sensitivity, whereas the rest of CT does not appear to

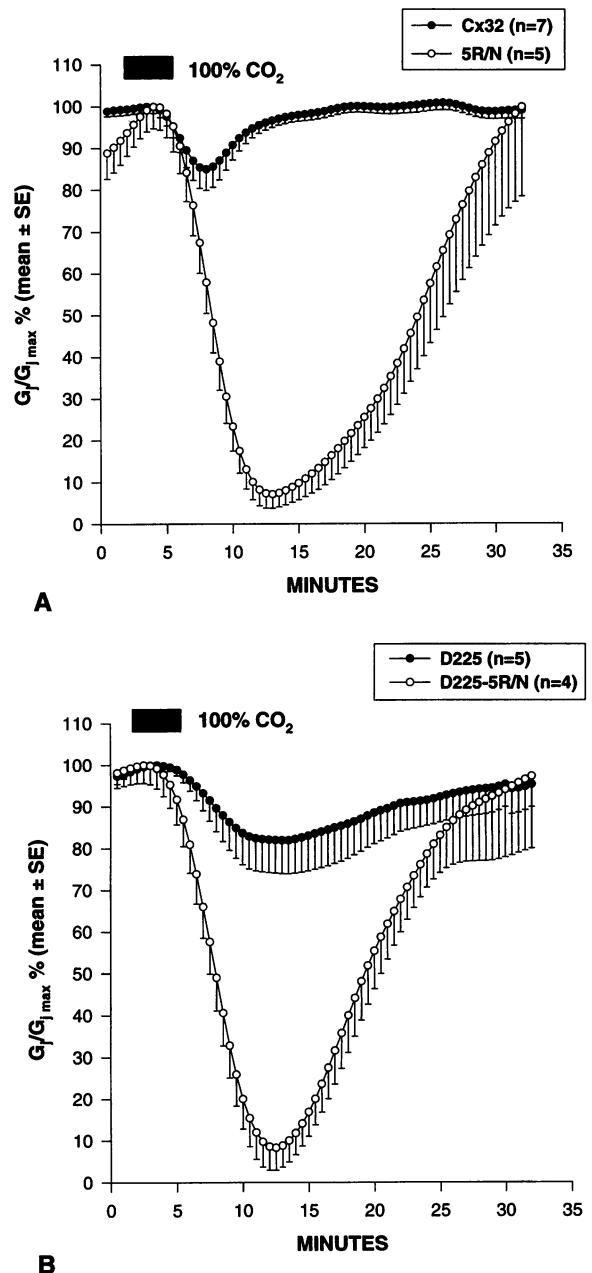
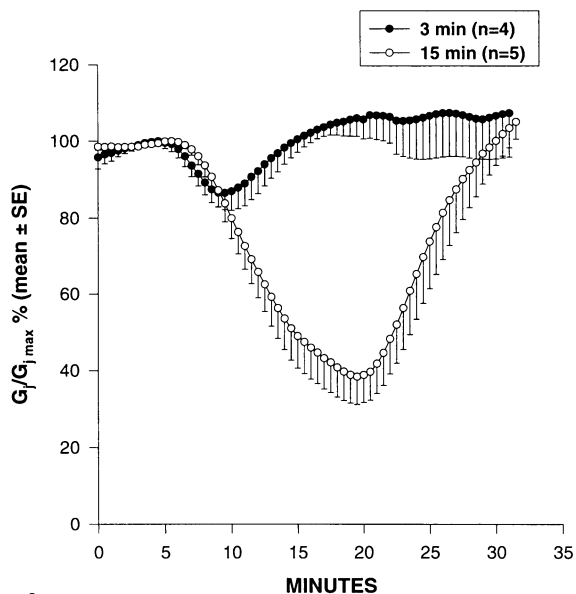
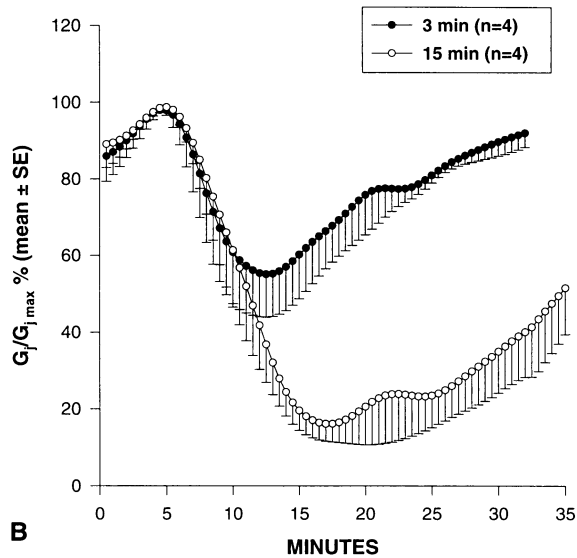


FIGURE 4 CO₂ gating sensitivity of mutants in which five arginines (R) were replaced with asparagines (N) at CT₁ in either wild-type Cx32 (5R/N) (A) or Cx32 deleted at residue 225 (D225-5R/N) (B). Note that the R/N replacement strongly enhances the CO₂ sensitivity of both 5R/N (A) and D225-5R/N (B) with respect to controls: Cx32 (A) and D225 (B), respectively. With 3-min exposures to 100% CO₂, G_j dropped to $7.1 \pm 3.4\%$ (mean \pm SE, $n = 5$) of initial values at a maximal rate of $\sim 18\%/min$ in oocytes expressing 5R/N (A) and to $8.3 \pm 5.3\%$ (mean \pm SE, $n = 4$) at a maximum rate of $\sim 17\%/min$ in those expressing D225-5R/N (B).

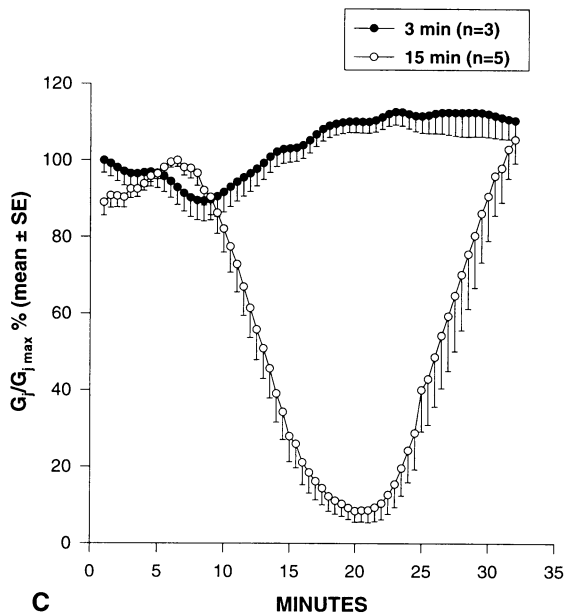
influence CO₂ sensitivity. This conclusion is based on a comparison of magnitude and rate of G_j decrease with exposure to 100% CO₂ between oocyte pairs expressing wild-type Cx32 and oocyte pairs expressing mutants in which either the R residues of CT₁ were replaced with N or T residues, or CT was deleted by as much as 84%.



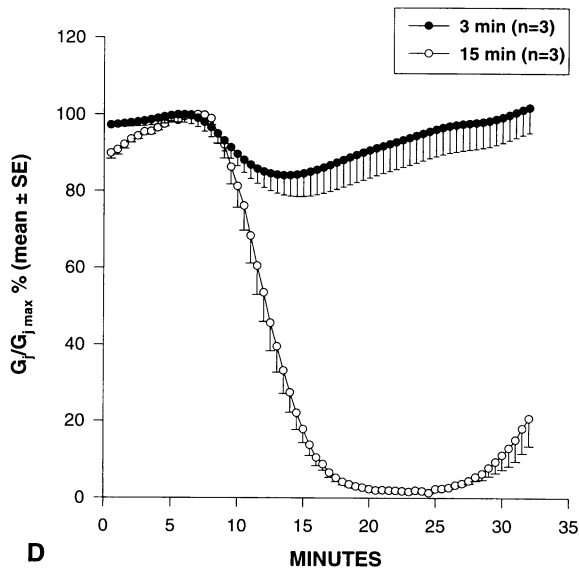
A



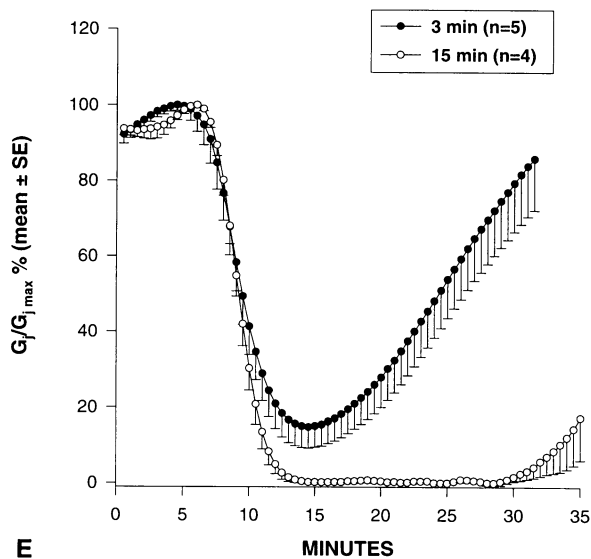
B



C



D



E

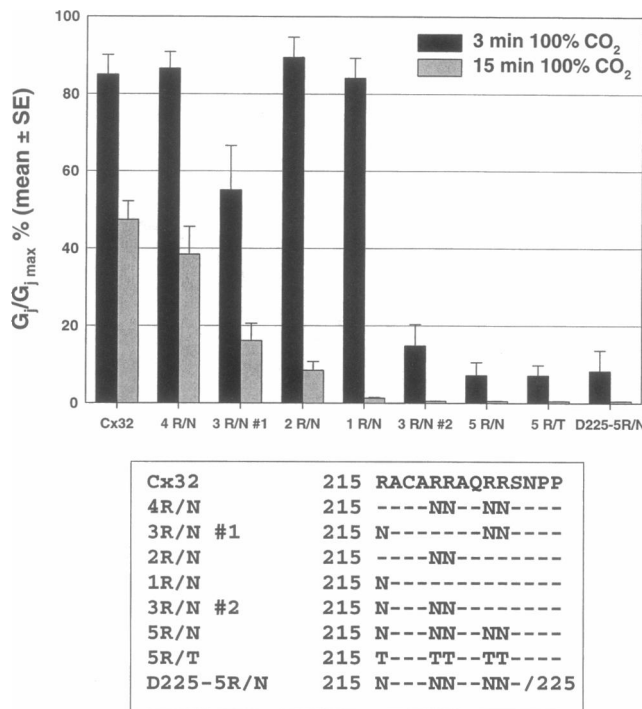


FIGURE 6 Summary of the effect of either partial or total replacement of R residues with N or T residues, in CT₁, on normalized G_j with either 3-min or 15-min exposures to CO₂ (100% G_j = control, pretreatment, G_j value). Note that the replacement of all of the 5 R with N or T residues greatly increases the CO₂ sensitivity of Cx32, whereas partial R/N replacement results in intermediate CO₂ sensitivities. R215 is the strongest inhibitor, because the sensitivity of 4R/N is almost as low as that of wild-type Cx32 and because 1R/N is almost as sensitive as 5R/N (at least with 15-min exposure to CO₂). Conversely, it is clear that R219–220 inhibit less than R215, because 3R/N #1 is more sensitive to CO₂ than 4R/N. R223–224 seem to have no inhibitory power at all, because 3R/N #2 is virtually as sensitive as 5R/N; in contrast, they appear to diminish the inhibitory efficiency of both R215 and R219–220, because 2R/N and 1R/N are more sensitive to 15-min exposures to CO₂ than 4R/N and 3R/N #1, respectively.

The kinetics of G_j and $[H^+]_i$ differed significantly from each other. In oocytes expressing Cx32, G_j decreased slowly with CO₂ at a maximal rate of $\sim 9\%/min$, whereas $[H^+]_i$ increased rapidly at the maximal rate of $\sim 25\%/min$, such that G_j minima lagged behind $[H^+]_i$ maxima by several minutes. This is consistent with previous studies showing that, with cytosolic acidification, G_j and pH_i kinetics match poorly (Pressler, 1989; Peracchia, 1990a; Lazrak and Peracchia, 1993; Liu et al., 1993; Peracchia et al., 1996), whereas G_j follows more closely the $[Ca^{2+}]_i$ kinetics (Peracchia, 1990a, b; Lazrak and Peracchia, 1993; Peracchia et al., 1996). Indeed, our recent preliminary data (unpublished observation) on oocytes expressing Cx32 injected with Cal-

cium Green-2 as an intracellular calcium indicator and exposed to 100% CO₂ for 15 min, have confirmed data for a close correlation between G_j and $[Ca^{2+}]_i$ previously obtained with oocytes expressing Cx38, injected with fura-C₁₈ and exposed to CO₂ for 3 min (Peracchia et al., 1996).

The observation that CT deletion by as much as 84% does not change the CO₂ sensitivity of Cx32 confirms previous data (Werner et al., 1991; Rabadan-Diehl et al., 1994) and indicates that most of CT does not participate in the CO₂-induced gating mechanism. In this, Cx32 may be different from Cx43, where two domains, one in the mid-portion and the other at the very end of CT, were found to play an important role in CO₂-induced gating (Liu et al., 1993; Ek-Vitorin et al., 1996; Morley et al., 1996).

Replacement of all of the five R residues of CT₁ with N or T residues resulted in a great increase in CO₂ sensitivity of Cx32, whereas partial R/N replacement resulted in intermediate CO₂ sensitivities. This indicates that the R residues of CT₁ differ in inhibitory power. It is quite clear that R215 is the strongest inhibitor, because the sensitivity of 4R/N, in which all but R215 have been replaced, is almost as low as that of wild-type Cx32, and because the reverse mutation, 1R/N, in which only R215 is replaced, is almost as sensitive as 5R/N (at least with 15-min exposure to CO₂). Conversely, it is obvious that R219–220 have lower inhibitory power than R215, because 3R/N #1 is more sensitive to CO₂ than 4R/N. R223–224 seem to have no inhibitory power at all, because 3R/N #2 is virtually as sensitive as 5R/N. In contrast, R223–224 appear to diminish the inhibitory efficiency of both R215 and R219–220, because 2R/N and 1R/N are more sensitive to 15-min exposures to CO₂ than 4R/N and 3R/N #1, respectively. In summary, R215 seems to have a greater inhibitory power than R219–220, whereas R223–224 seem to partly counteract the inhibitory activity of both R215 and R219–220. More work will be needed to determine potential differences in inhibitory power between R219 and R220, and to establish whether R223, R224, or both are involved in reducing the inhibitory power of R215 and of R219–220.

Curiously, with mutants testing partial R/N replacement the G_j behavior with 3-min exposure to CO₂ did not always match that with 15 min CO₂, as an obvious increase in sensitivity was only observed with mutants 3R/N #1 and 3R/N #2. A possible reason for this is that in some mutants the onset of G_j drop may be slightly delayed, such that the full impact of increased CO₂ sensitivity is only revealed by longer CO₂ exposures. Due to the low tip resistance of the microelectrodes used in our experiments, differences in initial G_j did not affect the maximum percent drop in G_j with CO₂. For example, with mutant 2R/N G_j dropped in

FIGURE 5 CO₂ gating sensitivity of mutants in which arginines (R) were replaced individually or in groups with asparagines (N) in CT₁ of Cx32. Partial R/N replacement resulted in intermediate enhancements of CO₂ sensitivity. With 15-min exposures to CO₂, G_j dropped to $38.5 \pm 7.2\%$ (mean \pm SE, $n = 5$) with 4R/N (A), to $16.1 \pm 4.5\%$ (mean \pm SE, $n = 4$) with 3R/N #1 (B), to $8.4 \pm 2.3\%$ (mean \pm SE, $n = 5$) with 2R/N (C), to $1.3 \pm 0.2\%$ (mean \pm SE, $n = 3$) with 1R/N (D), and to $0.5 \pm 0.05\%$ (mean \pm SE, $n = 4$) with 3R/N #2 (E). With 3-min CO₂ exposures, a significant increase in CO₂ sensitivity was obvious only with mutant 3R/N #2 (E). The maximum uncoupling rate with 15-min exposures to CO₂ also gradually increased among the mutants in the same order: 7%/min (4R/N) (A), 10%/min (3R/N #1), (B), 11%/min (2R/N) (C), 14%/min (1R/N) (D), and 20%/min (3R/N #2) (E).

response to 15-min exposures to CO₂, to 7%, 10%, and 7% in oocyte pairs with initial G_j values of 47.0, 26.3, and 11.5 μS, respectively; similarly, with mutant 3R/N #2 G_j dropped, in response to 3-min exposures to CO₂, to 3%, 4%, 0%, and 7% in oocyte pairs with initial G_j values of 0.3, 21.9, 30.6, and 1.1 μS, respectively.

Our observation of a great increase in CO₂ sensitivity with R to N replacement contrasts with a previous study that reported no difference in CO₂ sensitivity between wild-type Cx32 and mutant D225-5R/N (Rabadan-Diehl et al., 1994). At this stage, we do not have an obvious answer for the reason of this discrepancy. Since the work of Rabadan-Diehl et al. (1994) was not specifically focused on differences in CO₂ sensitivity among mutant connexins, it is possible that CO₂ sensitivity was not studied in detail as much as in our study. Alternatively, differences in uncoupling protocols between the two laboratories may have accounted for data discrepancy.

The present findings, together with our previous data (Wang and Peracchia, 1996; Wang et al., 1996a), indicate that two cytoplasmic domains of Cx32: the initial segment of CT (CT₁) and the second half of CL (CL₂) are relevant for CO₂-induced gating. In view of the fact that: 1) CL₂ and CT₁ are positively charged in most connexins, 2) the inhibitory action of CT₁ depends on its positive charges, and 3) the only cytoplasmic domain with negative charges is the first half of the cytoplasmic loop (CL₁), not considering acidic residues of CT that are lost in our deletion mutants without consequences to CO₂-gating, a possibility is that open and closed channel states depend on competitive electrostatic interactions among CL₁, CL₂, and CT₁. Thus, CL₁-CL₂ interaction could result in a closed channel state, whereas CL₁-CT₁ interaction would maintain the channel in an open state.

Somewhat puzzling is the apparent contradiction between the data on Cx32 and those on Cx43 (Liu et al., 1993; Ek-Vitorin et al., 1996; Morley et al., 1996). Whereas in Cx32 most of CT appears to be irrelevant, and its initial regions (CT₁) to act as a gating inhibitor, in Cx43 middle- and end-regions of CT appear to be gating mediators. Indeed, the CT end of Cx43 is believed to act as the gating particle of a gating mechanism that would resemble that of the ball-and-chain model proposed for K⁺-channels (Armstrong, 1966; Zagotta et al., 1990). In Cx32 this model is very unlikely primarily because of the normal gating behavior of the CT deletion mutants, but also because the CT chain of Cx32 does not contain sequences even remotely similar to that of the proposed gating particle of Cx43. In any event, at this stage one can only conclude that Cx32 and Cx43 may be gated by different molecular mechanisms.

In conclusion, based on data from Cx32 mutants expressed in *Xenopus* oocyte pairs, this study indicates that positively charged residues of the initial domain of the CT chain have an inhibitory effect on the gating sensitivity of Cx32 to cytosolic acidification by CO₂ exposure. Aside from this domain, most of the CT chain appears to have no influence on CO₂ sensitivity of Cx32, as mutants in which

CT is deleted by as much as 84% have CO₂ gating sensitivities indistinguishable from that of wild-type Cx32.

The authors are grateful to Dr. Eric C. Beyer (Washington University) for providing the cDNA clone for the rat liver connexin32, to Dr. Rudolf Werner (University of Miami) for providing the cDNA for mutants D225, D225-5R/N, D222, and D219, and to Lillian M. Peracchia for excellent technical help.

This study was supported by National Institutes of Health Grant GM20113.

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