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CO_2 Sensitivity of Voltage Gating and Gating Polarity of Gap Junction Channels—Connexin40 and its COOH-Terminus-Truncated Mutant

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Abstract. The CO_2 sensitivity of transjunctional voltage (V_j) gating was studied by dual voltage clamp in oocytes expressing mouse Cx40 or its COOH terminus (CT)-truncated mutant (Cx40-TR). V_j sensitivity, determined by a standard V_j protocol (20 mV V_j steps, 120 mV maximal), decreased significantly with exposure to 30% CO_2. The Boltzmann values of control versus CO_2-treated oocytes were: V_0 = 36.3 and 48.7 mV, n = 5.4 and 3.7, and $G_{j\ min}$ = 0.21 and 0.31, respectively. CO_2 also affected the kinetics of V_j-dependent inactivation of junctional current (I_j); the time constants of two-term exponential I_j decay, measured at V_j = 60 mV, increased significantly with CO_2 application. Similar results were obtained with Cx40-TR, suggesting that CT does not play a role in this phenomenon. The sensitivity of Cx40 channels to 100% CO_2 was also unaffected by CT truncation. There is evidence that CO_2 decreases the V_j sensitivity of Cx26, Cx50 and Cx37 as well, whereas it increases that of Cx45 and Cx32 channels. Since Cx40, Cx26, Cx50 and Cx37 gate at the positive side of V_j, whereas Cx45 and Cx32 gate at negative V_j, it is likely that V_j behavior with respect to CO_2-induced acidification varies depending on gating polarity, possibly involving the function of the postulated V_j sensor (NH_2-terminus).

Key words: Cell communication — Connexins — Gap junctions — Voltage gating — Chemical gating — Channel gating — CO_2 — *Xenopus* oocytes

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Introduction

Gap junctions are membrane domains endowed with channels that mediate the cell-to-cell diffusion of small cytosolic molecules. A gap junction channel is formed by the extracellular interaction of two hemichannels (connexons), which in turn are hexamers of connexins. Connexins (Cx) are a family of proteins whose structure comprises 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).

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 largely unclear (reviewed in Peracchia, Wang & Peracchia, 2000a; Harris, 2001; Peracchia, 2004). We have reported that changes in junctional conductance (G_j) induced by cytosolic acidification are more closely related to $[Ca^{2+}]_i$ than to $[H^+]_i$ (Peracchia, 1990ab; Lazrak & Peracchia, 1993), and there is evidence that in some cells channel 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 chemical gate are believed to be distinct, as the former closes the channel rapidly (< 1 ms) but incompletely, leaving a 20–30% residual conductance, whereas the latter closes the channel slowly (8–10 ms) but completely (Bukauskas & Peracchia, 1997). The slow V_j gate and the chemical gate are likely to be the same (Bukauskas & Peracchia, 1997, Peracchia, Wang & Peracchia, 1999, 2000b). Slow and fast V_j gates are in series and each hemichannel appears to have both gates. The

slow gate closes at the negative side of V_j in all connexin channels, whereas the polarity of the fast V_j gating mechanism varies among connexin channels (reviewed in Harris, 2001).

Over the years, chemical and voltage gating of gap junction channels have been studied almost exclusively by testing chemical agents and voltage gradients, respectively, whereas little interest has been devoted to potential effects of voltage on chemical gating, or chemical agents on voltage gating. Over a decade ago, however, CO_2 has been shown to increase the V_j sensitivity of Cx32 channels (Werner et al., 1991). Conversely, in the late 1990's CO_2-induced chemical gating was shown to be reversed by V_j gradients positive at the mutant side of heterotypic channels between Cx32 and various Cx32 mutants, suggesting that the chemical gate is V_j sensitive (Peracchia et al., 1999, 2000b). Similarly, chemical gating was reversed in insect cells by bilateral hyperpolarization (Weingart & Bukauskas, 1998). These observations indicate that careful examination of the effect of chemicals on V_j gates, and of V_j on the chemical gate, may provide important clues on gating mechanisms.

Recently, we have reported that the speed and sensitivity of V_j-dependent inactivation of junctional current (I_j) are increased by CO_2 in both Cx45 (Peracchia et al., 2003a) and Cx32 (Young & Peracchia, 2002; Peracchia et al., 2003b) channels. Interestingly, however, not all connexin channels are affected in the same way by CO_2-induced acidification. In the present study, we report that channels made of mouse Cx40, or its COOH terminus-truncated mutant, decrease in sensitivity and speed of V_j gating with CO_2 application. A preliminary account of these findings has been published (Peracchia, Chen & Peracchia, 2004).

Materials and Methods

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 containing (in mM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5 (pH 7.6 with NaOH). Oocytes at stages V or VI were subsequently defolliculated in 2 mg/ml collagenase (Sigma Chemical, St. Louis, MO) for 80 minutes 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 nanoject apparatus (Drummond, Broomall, PA). The antisense oligonucleotide blocks completely the endogenous junctional communication within 48 hours. 24–72 hours later 46 nl of mouse Cx40 wild-type cRNA (~0.4 μg/μl), or cRNA of a mouse Cx40 mutant whose COOH terminus was truncated beyond residue 248 (Cx40-TR), were injected into oocytes at the vegetal pole and the oocytes were incubated overnight at 18°C. Cx40-TR was prepared by introducing a stop codon in the cDNA of Cx40 wild-type in the same location used by Stergiopoulos et al. (1999), employing the same primer used by this group (Cx40-TR sense: 5′-GAC AAG CAC TAG CTG CCT GGC-3′). 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 withND96. All oocyte pairs were studied electrophysiologically 2–3 hours after pairing.

Measurement of Junctional Conductance and Uncoupling Protocols

The oocyte chamber was continuously perfused at a flow rate of 0.6 ml/min by a peristaltic pump (Dyamax Model RP-1, Rainin Instrument Co. Inc., 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 of the experiments were performed using the standard double-voltage-clamp procedure for measuring G_j (Spray et al., 1981a). Following the 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 Corp., Hamden, CT) to the same holding potential, $V_{m1} = V_{m2}$ (usually -20 mV), so that no junctional current would flow at rest ($I_j = 0$). For measuring junctional conductance (G_j) and CO_2 sensitivity, a V_j gradient was created by imposing a voltage step (V_1) to oocyte # 1 while maintaining V_2 at V_m, thus, $Vj = V_1$. 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 DigiData 1200 interface (Axon). I_j and V_j were measured with Clampfit (Axon) and the data were plotted with SigmaPlot (SPSS Inc., Chicago, IL).

For testing the effect of CO_2 on G_j two protocols were used. In CO_2-Protocol # 1, electrical uncoupling was induced by 3 or 15 min supervisions (0.6 ml/min) of salines gassed with 100% CO_2 and G_j was measured by applying V_j gradients of +20 mV (2 s duration) every 30 s to one oocyte, while maintaining the other oocyte at V_m. In CO_2-Protocol #2, electrical uncoupling was induced by 12–60 min supervisions (0.6 ml/min) of salines gassed with 30% CO_2 and G_j was measured by applying V_j gradients of −40 mV (12 s duration) every 30 s to one oocyte, while maintaining the other oocyte at V_m. In the latter protocol, the time constants (τ) of G_j decay and the ratio G_j steady-state ($G_{j\ ss}$) over G_j peak ($G_{j\ ss}/G_{j\ peak}$), in the presence and absence of CO_2, were calculated by fitting each I_j curve to a two-term exponential function (τ_1 and τ_2), following baseline correction (Clampfit, Axon). $G_{j\ ss}$ was obtained from the exponential fit (parameter "C" of Clampfit, Axon).

For studying voltage dependence of G_j in the presence and absence of 30% CO_2 a standard V_j-Protocol was used. Each oocyte was first voltage clamped at -20 mV. Voltage steps of -20 mV (120 mV V_j maximum) and 25 s duration were applied every 45 s to either oocyte of the pair, while maintaining the other at -20 mV. The voltage-insensitive junctional conductance ($G_{j\ max}$) was calculated using I_j values elicited by $V_j = -20$ mV, since at this V_j there is no I_j decay. To illustrate the relationship between steady-state G_j ($G_{j\ ss}$) and V_j the ratio $G_{j\ ss}/G_{j\ max}$ was plotted with respect to V_j.

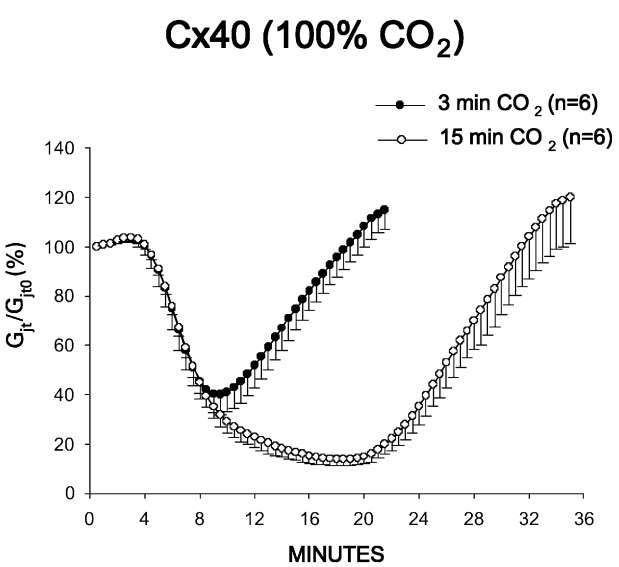

Fig. 1. Sensitivity of junctional conductance (G_j) to 100% CO_2 in Cx40 channels. Time course of the ratio G_{jt}/G_{jt0} (where G_{jt} and G_{jt0} are G_j at times t and t = 0, respectively). G_j sensitivity was determined by applying the CO_2-Protocol #1. With 3 and 15 min exposures to 100% CO_2, G_j decreases to 39.9 ± 8.1% and 13.9 ± 2.5% (mean ± SE, n = 6), respectively, at a maximum rate of ~16% min^{-1}. G_j recovers to near control values at a maximum rate of ~8% min^{-1} with either 3 or 15 min exposures to CO_2.

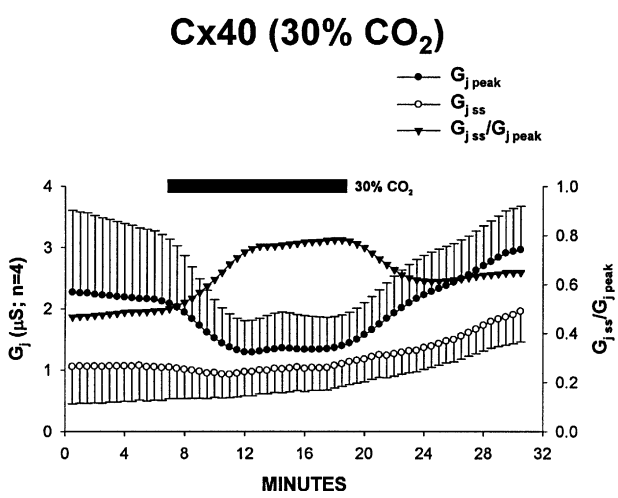

Fig. 2. Effect of 30% CO_2, on Cx40 channels. To determine the time needed to reach steady state, oocytes were superfused for 12 min with salines gassed with 30% CO_2 and both $G_{j\ peak}$ and $G_{j\ ss}$ were measured by applying the CO_2-Protocol #2. Steady state is reached in < 10 minutes. At steady state, $G_{j\ peak}$ has decreased from 2.3 ± 1.3 µS to 1.3 ± 0.5 µS (mean ± SE, n = 4), whereas $G_{j\ ss}$ does not change significantly and increases progressively during recovery. As a result of these changes, $G_{j\ ss}/G_{j\ peak}$ increases to ~0.78 from initial values of ~0.47. During recovery, $G_{j\ ss}/G_{j\ peak}$ remains slightly higher (~0.65) than before CO_2 exposure, probably because pH_i has not yet returned to control values.

The curve was fitted to a two-state Boltzmann distribution of the form: $(G_{j\ ss}\text{-}G_{j\ min})/(G_{j\ max}\text{-}G_{j\ ss}) = \exp[-A(V_j\text{-}V_0]$, where V_0 is the V_j, value at which G_j is one half the value of $G_{j\ max}\text{-}G_{j\ min}$, $G_{j\ max}$ is G_j at V_j = 0 mV and $G_{j\ min}$ is the theoretical minimum normalized G_j. $A = \eta q/kT$ is a constant expressing voltage sensitivity in terms of number of equivalent gating charges, η, moving through the entire applied field, where q is the electron charge, k is the Boltzmann constant and T is the temperature in °K. The time constants (τ_1 and τ_2) of G_j decay were calculated as described above.

Results

Cx40 Channels

G_j Sensitivity to 100% CO_2

The G_j sensitivity to CO_2 of channels made of Cx40 was measured by applying the CO_2 Protocol #1. With 3 and 15 min exposures to 100% CO_2, G_j decreased to 39.9 ± 8.1% and 13.9 ± 2.5% (mean ± SE, n = 6; Fig. 1), respectively, at a maximum rate of ~16%/min (Fig. 1). G_j recovered to near control values at a maximum rate of ~8% min^{-1} with either 3 or 15 min exposures to CO_2 (Fig. 1).

Effect of CO_2 on V_j Sensitivity

In order to test the effect of CO_2 on V_j sensitivity, the standard V_j protocol was applied before and during exposure to 30% CO_2. The use of 30% rather than 100% CO_2 enabled us to achieve steady-state conditions while maintaining sufficient coupling for accurately measuring G_j and V_j.

To determine the time needed to reach steady-state conditions, oocyte pairs were superfused for 12 minutes with salines gassed with 30% CO_2, and both $G_{j\ peak}$ and $G_{j\ ss}$ were measured applying the CO_2 protocol #2. Steady state was reached in < 10 minutes (Fig. 2). At steady state, $G_{j\ peak}$ had reversibly decreased from 2.3 ± 1.3 µS to 1.3 ± 0.5 µS (mean ± SE, n = 4, Fig. 2*A*), whereas $G_{j\ ss}$ did not change significantly during CO_2 exposure, and increased progressively during the recovery period (Fig. 2). As a result of these changes, the ratio $G_{j\ ss}/G_{j\ peak}$ increased to ~0.78 from initial values of ~0.47 (Fig. 2). In the recovery period, the ratio $G_{j\ ss}/G_{j\ peak}$ remained slightly higher (~0.65) than before CO_2 exposure. A likely reason for it is that pH_i may not have yet returned to control values. Indeed, we have previously reported that pH_i recovers slowly following exposure to CO_2 (Wang & Peracchia, 1998).

For evaluating the effect of 30% CO_2 on V_j sensitivity, oocyte pairs were first tested for V_j-sensitivity by the standard V_j protocol in the absence of CO_2 (Fig. 4*A* and *C*, and Table 1). Then, they were superfused with 30% CO_2 and monitored by applying the CO_2-Protocol #2 until steady-state conditions were attained (Fig. 3). During this period, $G_{j\ peak}$ dropped by ~50%, whereas $G_{j\ ss}$ decreased minimally

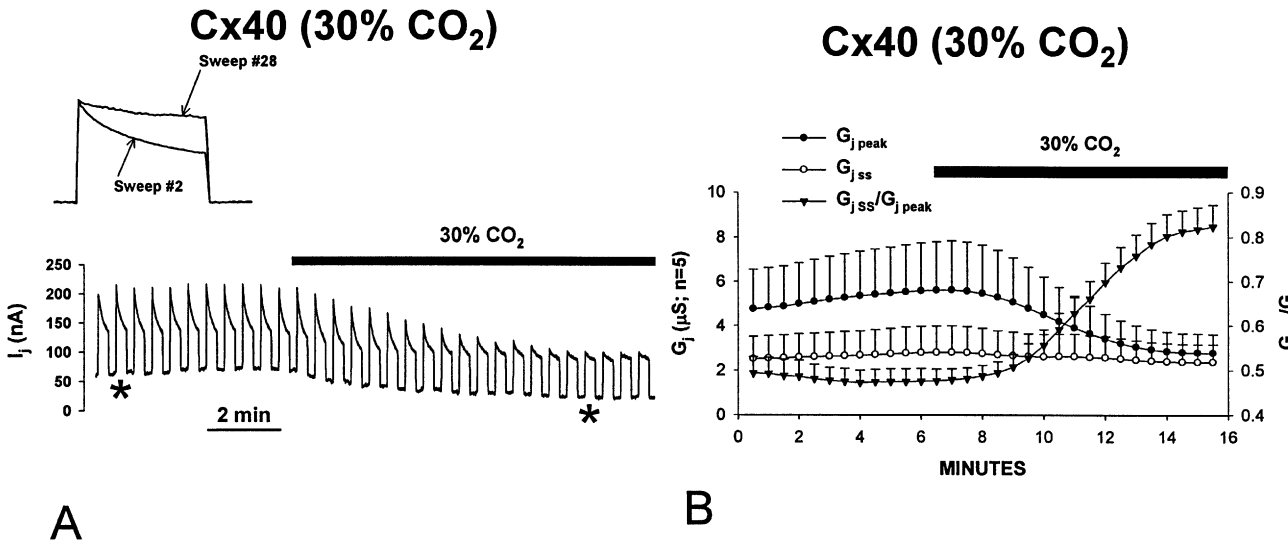

Fig. 3. Effect of 30% CO_2 on V_j sensitivity or Cx40 channels. The oocytes were first tested for V_j sensitivity by the standard V_j protocol in the absence of CO_2 (Fig. 4*A* and *C*). Then, they were superfused with 30% CO_2 and monitored up to steady state (*A* and *B*) by applying CO_2 Protocol #2. During this period, $G_{j\ peak}$ drops by ~50%, whereas $G_{j\ ss}$ decreases minimally (*A* and *B*), the result being an increase in $G_{j\ ss}/G_{j\ peak}$ from 0.5 ± 0.04 to 0.82 ± 0.05 (mean ± SE, $n = 5$, *B*). The increase in $G_{j\ ss}/G_{j\ peak}$ is clearly demonstrated by the progressive change in junctional current (I_j; *A* and inset, normalized to peak current). Note that the time course of $G_{j\ peak}$ corresponds closely to that of $G_{j\ ss}/G_{j\ peak}$ (*B*). After reaching steady state, the CO_2 superfusion was continued for 45–60 minutes, during which time the oocytes were retested with the standard V_j protocol (Fig. 4*B* and *C*).

(Fig. 3*A* and *B*), the result being an increase in $G_{j\ ss}/G_{j\ peak}$ from 0.5 ± 0.04 to 0.82 ± 0.05 (mean ± SE, $n = 5$, Fig. 3*B*). The drop in $G_{j\ ss}/G_{j\ peak}$ is clearly demonstrated by the progressive change in junctional current (I_j; Fig. 3*A* and inset) during CO_2 exposure.

After reaching steady state (Fig. 3), the CO_2 superfusion was continued for as long as 45–60 minutes, during which time the oocytes were retested with the standard V_j protocol (Fig. 4*B* and *C*). At steady-state conditions, in 30% CO_2, the channels displayed a significant decrease in V_j sensitivity (Fig. 4*B* and *C*) with respect to controls (absence of CO_2; Fig. 4*A* and *C*). In plots of the relationship between $G_{j\ ss}/G_{j\ max}$ and V_j (Fig. 4*C*), the Boltzmann values were: $V_0 = 36.3$ m*V*, $\eta = 5.4$ and $G_{j\ min} = 0.21$, in the absence of CO_2 ($n = 11$), and $V_0 = 48.7$ mV, $\eta = 3.7$ and $G_{j\ min} = 0.31$, in the presence of CO_2 ($n = 9$)(*see* Table 1). The Boltzmann values obtained in the absence of CO_2 are comparable to those previously reported for Cx40 channels expressed in oocytes (Hennemann et al., 1992; Ebihara, 1993; Bruzzone et al., 1993; Anumonwo et al. 2001), HeLa (Traub et al., 1994; Bukauskas et al., 1995), N2A (Beblo et al., 1995) or Jeg3 (Hellmann, Winterhager & Spray, 1996) cells.

The speed of V_j gating was also affected by exposure to 30% CO_2. The kinetics of I_j inactivation was best fit by a two-term exponential function (τ_1 and τ_2). Both τ_1 and τ_2 increased with exposure to 30% CO_2. At $V_j = 60$ mV, τ_1 increased from 1.92 ± 0.22 s to 5.99 ± 0.51 s (mean ± SE, $n = 7$), and τ_2 from 0.41 ± 0.04 s to 0.87 ± 0.2 s (mean ± SE, $n = 7$). V_j sensitivity and kinetics returned to control values after prolonged CO_2 washout (*data not shown*).

Table 1. Boltzmann parameters of voltage gating in the presence and absence of CO_2

	V_0 (mV)	η	$G_{j\ min}$	n
Cx40	36.3	5.4	0.21	11
Cx40-TR	35.4	5.9	0.23	16
Cx40 (30% CO_2)	48.7	3.7	0.31	9
Cx40-TR (30% CO_2)	49.6	4.3	0.28	9

COOH Terminus-truncated Cx40 Channels (Cx40-TR)

G_j *Sensitivity to 100% CO_2*

The G_j sensitivity to CO_2 of channels made of Cx40-TR was very similar to that of Cx40 channels. With 3 and 15 min exposures to CO_2, G_j, measured by the CO_2-Protocol #1, decreased to 43.1 ± 3.8% and 18.6 ± 6% (mean ± SE, $n = 6$; Fig. 5), respectively, at a maximum rate of ~14% min^{-1}. G_j recovered to near control values at maximum rates of ~7.4% min^{-1} and ~5.7% min^{-1}, with 3 and 15 min exposures to CO_2, respectively (Fig. 5).

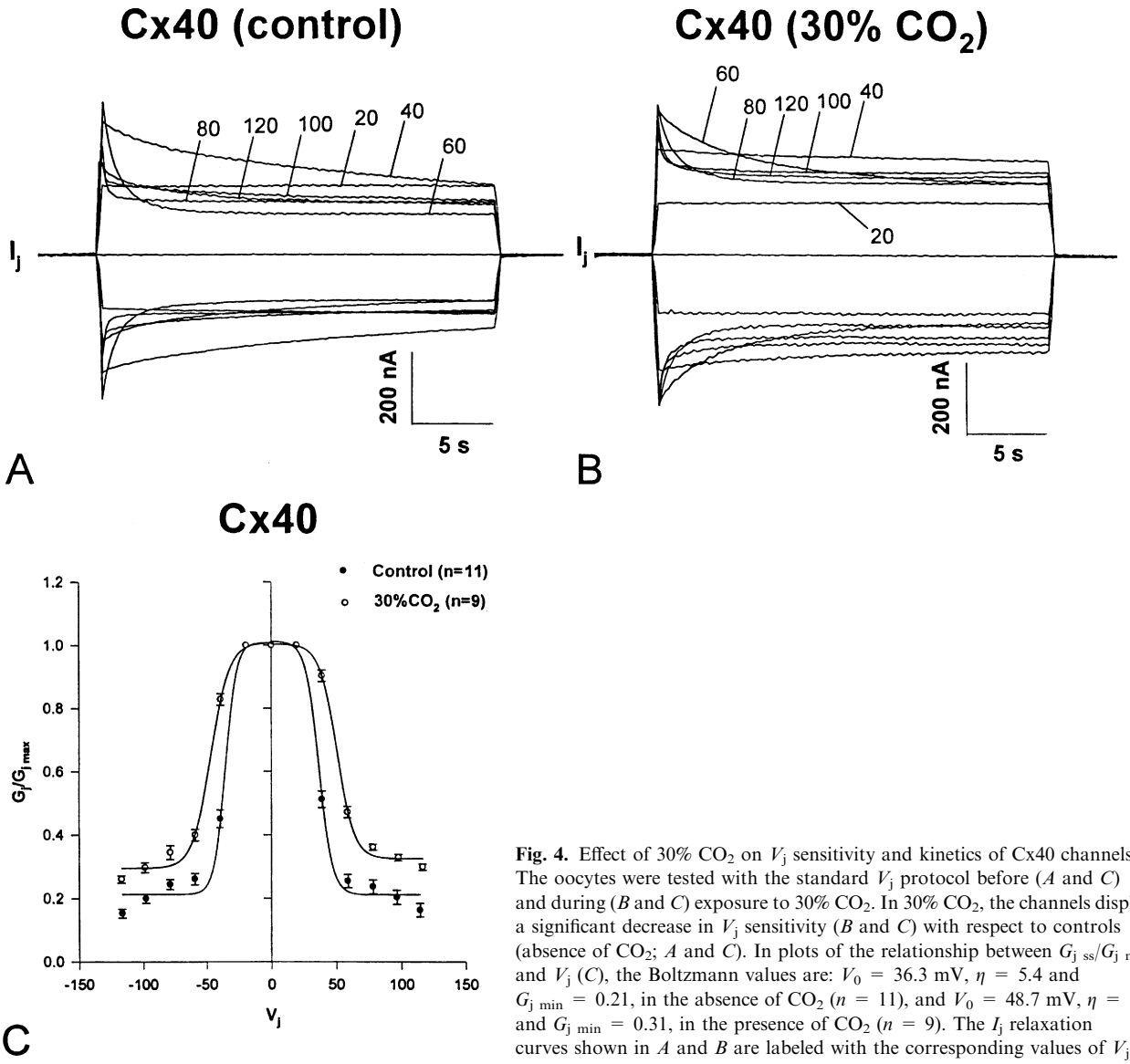

Fig. 4. Effect of 30% CO_2 on V_j sensitivity and kinetics of Cx40 channels. The oocytes were tested with the standard V_j protocol before (*A* and *C*) and during (*B* and *C*) exposure to 30% CO_2. In 30% CO_2, the channels display a significant decrease in V_j sensitivity (*B* and *C*) with respect to controls (absence of CO_2; *A* and *C*). In plots of the relationship between $G_{j\,ss}/G_{j\,max}$ and V_j (*C*), the Boltzmann values are: $V_0 = 36.3$ mV, $\eta = 5.4$ and $G_{j\,min} = 0.21$, in the absence of CO_2 ($n = 11$), and $V_0 = 48.7$ mV, $\eta = 3.7$ and $G_{j\,min} = 0.31$, in the presence of CO_2 ($n = 9$). The I_j relaxation curves shown in *A* and *B* are labeled with the corresponding values of V_j.

Effect of 30% CO_2 on V_j Sensitivity Monitored at Different V_j Values

Cx40-TR channels, tested by the standard V_j protocol displayed a V_j sensitivity virtually identical to that of Cx40 channels both in the absence and presence of 30% CO_2 (*compare* Figs. 4*A–C* and 6*A–C*). As seen with Cx40 channels, the V_j sensitivity of Cx40-TR channels decreased significantly with exposure to 30% CO_2 (Fig. 6*B* and *C*), as compared to control conditions (absence of CO_2; Fig. 6*A* and *C*). In plots of the relationship between $G_{j\,ss}/G_{j\,max}$ and V_j (Fig. 6*C*), the Boltzmann values were: $V_0 = 35.4$ mV, $\eta = 5.9$ and $G_{j\,min} = 0.23$ in the absence of CO_2 ($n = 16$), and $V_0 = 49.6$ mV, $\eta = 4.3$ and $G_{j\,min} = 0.28$ in the presence of 30% CO_2 ($n = 9$) (*see* Table 1). The kinetics of I_j inactivation were also indistinguishable from those of Cx40 channels both in the presence and absence of 30% CO_2 (*data not shown*).

Discussion

This study reports the effects of CO_2 on chemical and V_j gating of channels made of Cx40 or its COOH-terminus truncated mutant (Cx40-TR). The data show that CO_2 decreases the V_j gating sensitivity of both Cx40 and Cx40-TR channels, and alters the kinetics of V_j gating. In addition, the data show that COOH-terminus (CT) truncation beyond residue 248 (82% CT deletion) does not significantly alter the CO_2 sensitivity of both chemical gating and V_j gating,

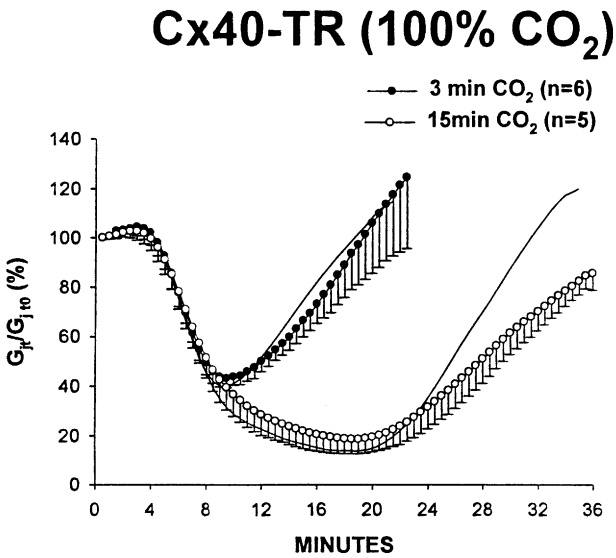

Fig. 5. Effect of 100% CO_2 on G_j sensitivity of COOH terminus-truncated Cx40 channels (Cx40-TR), tested by applying the CO_2-Protocol #1. Time course of the ratio G_{jt}/G_{jt0} (where G_{jt} and G_{jt0} are G_j at times t and $t = 0$, respectively). The G_j sensitivity to CO_2 of Cx40-TR channels is very similar to that of Cx40 channels. With 3 and 15 min exposures to CO_2, G_j decreases to 43.1 ± 3.8 % and 18.6 ± 6% (mean ± SE, $n = 6$), respectively, at a maximum rate of ~ 14% min^{-1}. G_j recovers to near control values at maximum rates of ~7.4% min^{-1} and ~5.7% min^{-1} with 3 and 15 min exposures to CO_2, respectively. For comparison, the effect of 100% CO_2 on $G_{jt}/G_{j\ t0}$ of Cx40 channels is also plotted (*line plots*).

indicating that most of Cx40's CT does not play a role in these phenomena.

The effect of CO_2-induced cytosolic acidification on voltage gating is intriguing and complex. In experiments testing voltage sensitivity to V_j gradients of 40 mV, $G_{j\ peak}$ dropped by ~50% with 30% CO_2, while $G_{j\ ss}$ changed minimally. Therefore, the increase in $G_{j\ ss}/G_{j\ peak}$, which reflects a drop in V_j sensitivity, results mostly from the drop in $G_{j\ peak}$. Since the drop in $G_{j\ peak}$ reflects the fraction of channels closed by CO_2, one could think that the drop in V_j sensitivity and the CO_2-induced chemical gating might be mechanistically linked. Although this possibility cannot be discarded, we are more inclined to believe that the temporal correspondence between chemical gating and drop in V_j sensitivity might be simply a coincidence. Chemical uncoupling and V_j gating sensitivity to CO_2 are likely to be independent from each other because there are reasons to believe that the latter involves fast V_j gating, which is generally thought to be independent from the chemical gating mechanism.

Presently, at least three major gating mechanisms have been described: chemical gating and both fast and slow V_j gating. Fast and slow V_j gates are likely to be distinct, as the former closes the channel rapidly (<1 ms) but incompletely, leaving a 20–30% residual conductance, whereas the latter closes the channel slowly (8–10 ms) but completely (Bukauskas & Peracchia, 1997). In contrast, the slow V_j gate and the chemical gate are likely to be the same for a number of reasons: at the single-channel level they have the same opening and closing kinetics (Bukauskas & Peracchia, 1997); there is evidence that the chemical gate is sensitive to V_j (Peracchia, et al., 1999); and inhibition of calmodulin (CaM) expression virtually eliminates both chemical and slow V_j gating (Peracchia, et al., 2000b). In both Cx40 and Cx40-TR channels, the V_j-dependent decay of I_j is best fit by a two-term exponential function, indicating the presence of both slow and fast gating components. Both of their time constants (τ_1 and τ_2) are CO_2 sensitive, as they increase significantly with CO_2. This indicates that CO_2 reduces the speed as well as the sensitivity of V_j gating.

With mild cytosolic acidification, histidine (H) residues are likely to be protonated, resulting in the addition of positive charges to the relevant domains of Cx40 and/or accessory proteins. The sequence of Cx40 wild-type preceding the CT truncation contains a total of ten H residues: H15 and H17 in NT, H74 in OL1, H95, H98 and H118 in CL, H183 and H192 in OL2, and H228 and H248 in CT; there are three more H residues in CT beyond the truncation (residues 249–358), but they can be disregarded because Cx40-TR channels behaved just like Cx40 channels in V_j- and chemical-gating sensitivity to CO_2. In CaM, there is only one histidine residue, H108. An in vitro study has reported an increase in molecular asymmetry and alpha-helix content of CaM with severe acidification, as well as a change to a more extended shape (Török et al., 1992). However, whether protonation of H residues and/or conformational changes in CaM are involved in the mechanism by which acidification affects V_j gating is unclear.

Interestingly, not all connexins are equally affected by CO_2-induced acidification. Whereas CO_2 decreases the V_j sensitivity of Cx40 (this study), Cx26 (Peracchia et al., 2003b), Cx50 and Cx37 (Peracchia, unpublished) channels, it increases both V_j sensitivity and gating speed of Cx32 (Werner et al., 1991; Young & Peracchia, 2002; Peracchia et al., 2003b) and Cx45 (Peracchia et al., 2003a) channels. This suggests that there may be two distinct groups of connexins behaving in opposite ways in response to CO_2 acidification. The opposite behavior is likely to reflect the gating polarity of the connexin. The fast V_j gates are believed to close at the positive side of V_j (positive gaters) in some connexin channels and at the negative side (negative gaters) in others. Cx26, Cx37, Cx38, Cx40, Cx46 and Cx50 are believed to be positive gaters, whereas Cx30, Cx32, Cx43 and Cx45 are thought to be negative gaters (reviewed in Harris, 2001). Indeed, Cx32 and Cx45, whose V_j sensitivities increase with

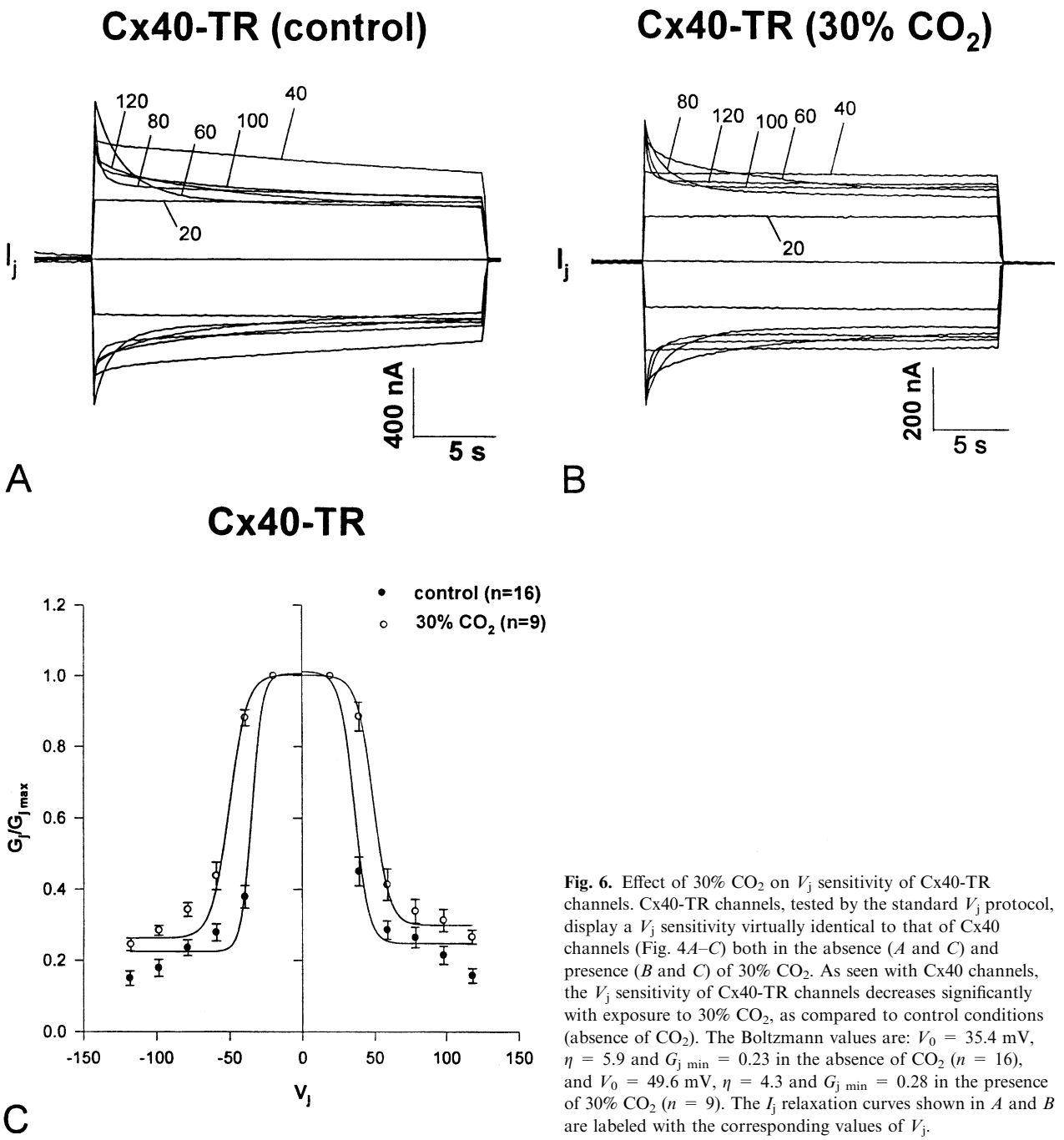

Fig. 6. Effect of 30% CO_2 on V_j sensitivity of Cx40-TR channels. Cx40-TR channels, tested by the standard V_j protocol, display a V_j sensitivity virtually identical to that of Cx40 channels (Fig. 4*A–C*) both in the absence (*A* and *C*) and presence (*B* and *C*) of 30% CO_2. As seen with Cx40 channels, the V_j sensitivity of Cx40-TR channels decreases significantly with exposure to 30% CO_2, as compared to control conditions (absence of CO_2). The Boltzmann values are: $V_0 = 35.4$ mV, $\eta = 5.9$ and $G_{j\,min} = 0.23$ in the absence of CO_2 ($n = 16$), and $V_0 = 49.6$ mV, $\eta = 4.3$ and $G_{j\,min} = 0.28$ in the presence of 30% CO_2 ($n = 9$). The I_j relaxation curves shown in *A* and *B* are labeled with the corresponding values of V_j.

CO_2, are negative gaters, whereas Cx40, Cx26, Cx50 and Cx37 whose sensitivity decreases, are positive gaters. Therefore, it is very likely that the type of CO_2-induced V_j behavior varies among connexin channels, depending on V_j gating polarity.

The molecular basis of gating polarity is still only partly understood, but there is good evidence that charged residues at the initial segment of NT play a role (Verselis et al., 1994). A standing hypothesis envisions an NT domain as the voltage sensor, located within the channel's mouth (Verselis, Ginter & Bargiello, 1994; Oh et al., 2000; Purnick et al., 2000a, b). Positive and negative gaters would have an acidic or a basic residue at the initial sequence of NT, respectively. With the establishment of a V_j gradient, the NT of positive gaters would move away from the channel's mouth (toward the cytoplasm) at the positive side of V_j, enabling channel gating by another connexin domain or an accessory molecule. Conversely, in negative gaters NT would move away from the channel's mouth at the negative side of V_j. Cx40, which is a positive gater (Hennemann et al., 1992),

would be so by virtue of an acidic residue in the third position (aspartate, D3), which is present in a similar location in other positive gaters (Cx26, Cx37, Cx38, Cx46 and Cx50), but absent in negative gaters (Cx30, Cx32 and Cx45), except in Cx43.

Based on this idea and the likelihood that CO_2-induced acidification results in histidine protonation, how could we explain the drop in V_j sensitivity of Cx40 channels with acidification? A possibility is that the acidic residue of NT (D3) is located near an H residue. D3 and H residues would only interact electrostatically at low pH_i (positively charged histidine). The electrostatic D-H interaction would hinder the V_j-dependent displacement of NT from the channel's mouth, so that at low pH_i larger V_j gradients would be needed for NT displacement. The reverse would be true for negative gaters. Among the ten H residues of Cx40 mentioned above, potential players could be the well-conserved H17, H74, H95 and H98 residues. This hypothesis is presently being tested by point mutation.

Truncation of CT by 82% (beyond residue 248) had no effect on any parameter tested in this study. This seems to eliminate any potential role of much of CT in the effect of CO_2 on V_j gating sensitivity and speed. At the single-channel level, CT 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 (Moreno et al., 2002) channels expressed in small cells. However, in agreement with our data, Anumonwo et al. (2001) have reported that Cx40-TR channels, expressed in oocytes, are indistinguishable from Cx40 wild-type channels in V_j gating sensitivity and kinetics, indicating that in this expression system both fast and slow gating components are preserved in Cx40-TR. CT truncation did not decrease the effectiveness of CO_2 on chemical gating either. In contrast, a previous study reported a drastic drop in CO_2 sensitivity of Cx40-TR channels (Stergiopulous et al., 1999). The reason for this discrepancy is unclear; perhaps it relates to different protocols used for acidifying the oocytes.

In conclusion, this study shows that cytosolic acidification induced by superfusion with solutions gassed with 30% CO_2 decreases the V_j gating sensitivity of channels made of mouse Cx40 or its COOH-terminus (CT)-truncated mutant (Cx40-TR), and slows their V_j gating kinetics. In addition, the data show that CT truncation beyond residue 248 does not significantly alter the CO_2 sensitivity of chemical gating. Evidence from this and previous work indicates that there may be two distinct groups of connexins whose V_j sensitivities respond in opposite ways to cytosolic acidification. There are reasons to believe that this may be related to the difference in gating polarity between the two groups. If this were the case, the postulated voltage sensor domain (NT) might play a role in the mechanism by which CO_2 affects voltage-gating sensitivity and kinetics.

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