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Is the Voltage Gate of Connexins CO_2-sensitive? Cx45 Channels and Inhibition of Calmodulin Expression

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Abstract. The sensitivity of Cx45 channels to CO_2, transjunctional voltage (V_j) and inhibition of calmodulin (CaM) expression was tested in oocytes by dual voltage clamp. Cx45 channels are very sensitive to V_j and close with V_j preferentially by the slow gate, likely to be the same as the chemical gate. With a CO_2-induced drop in junctional conductance (G_j), both the speed of V_j-dependent inactivation of junctional current (I_j) and V_j sensitivity increased. With 40-mV V_j-pulses, the τ of single exponential I_j decay reversibly decreased by ~40% during CO_2 application, and $G_{j\ steady\ state}/G_{j\ peak}$ decreased multiphasically, indicating that both kinetics and V_j sensitivity of chemical/slow V_j gating are altered by changes in $[H^+]_i$ and/or $[Ca^{2+}]_i$. CaM expression was inhibited with oligonucleotides antisense to CaM mRNA. With 15 min CO_2, relative junctional conductance (G_{jt}/G_{jt0}) dropped to 0% in controls, but only by ~17% in CaM-antisense oocytes. Similarly, V_j sensitivity was significantly lessened in CaM-antisense oocytes. The data indicate that both the speed and sensitivity of V_j-dependent inactivation of the junctional current of Cx45 channels are affected by CO_2 application, and that CaM plays a key role in channel gating.

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

Introduction

Gap junctions are plasma membrane domains that contain channels specialized for direct cell-to-cell exchange of small cytosolic molecules. A cell-cell channel is formed by the extracellular interaction of two hemichannels (connexons), which in turn are hexamers of connexin proteins. Connexins (Cx) are a family of intramembrane 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 (rev. in Peracchia, Lazrak & Peracchia, 1994).

Gap junction channels are known to close in response to 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), but the molecular mechanisms of channel gating are poorly understood (rev. in Peracchia, Wang & Peracchia, 2000b; Harris, 2001). We have reported that junctional conductance (G_j) is more closely related to $[Ca^{2+}]_i$ than to $[H^+]_i$ (Peracchia, 1990a,b; Lazrak & Peracchia, 1993), and there is evidence that channel gating is sensitive to nearly physiological $[Ca^{2+}]_i$ (Lazrak & Peracchia, 1993; Lazrak et al., 1994; Enkvist & Mc Carthy, 1994; Giaume & Venance, 1996; Crow, Atkinson & Johnson, 1994; Mears et al., 1995), although higher concentrations may be needed to uncouple cardiac myocytes (Delage & Délèze, 1998). There is evidence for two V_j-sensitive gates: fast and slow V_j gate. Fast V_j gate and chemical gate are believed to be distinct gates, 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, 2000c).

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Chemical gating may involve calmodulin (CaM) activation. The CaM role in channel gating was first proposed based on the ability of CaM antagonists to prevent cell uncoupling (Peracchia, Bernardini & Peracchia, 1981, 1983; Peracchia, 1984, 1987). This was also supported by in vitro evidence for CaM binding to connexins (Hertzberg & Gilula, 1981; Van Eldick et al., 1985; Zimmer et al., 1987; Török, Stauffer & Evans, 1997; Elvira & Villalobo, 1997; Diez, Elvira & Villalobo, 1998). The CaM hypothesis was further strengthened by evidence that inhibition of CaM expression eliminates the CO_2 gating sensitivity of Cx38 channels in *Xenopus* oocytes (Peracchia et al., 1996). This treatment also eliminated both chemical gating and slow V_j gating of heterotypic channels between Cx32 and Cx32 mutants (Peracchia et al., 2000c). A CaM role in chemical gating was also suggested by evidence for a dramatic effect of a CaM mutant with increased Ca^{2+} sensitivity (CaMCC) on both chemical and fast V_j gating of Cx32 channels expressed in *Xenopus* oocytes (Peracchia et al., 2000a), and by preliminary evidence for CaM-Cx32 colocalization at junctional sites (Peracchia et al., 2000a).

The present study has tested the effect of CO_2 on the V_j sensitivity of Cx45 channels. Over a decade ago, CO_2 has been shown to increase the V_j sensitivity of Cx32 channels expressed in oocytes (Werner et al., 1991), but this interesting observation has not led to subsequent detailed studies; we believe that this phenomenon should be studied in detail as it might provide new tools for understanding gating mechanisms at the molecular level. In addition, the present study has evaluated the role of CaM in chemical and V_j gating by monitoring the effect of inhibiting CaM expression on CO_2-induced electrical uncoupling of *Xenopus* oocyte pairs expressing Cx45. Channels made of Cx45 are very sensitive to V_j and CO_2 (Veenstra et al., 1992; Hermans et al., 1995; Moreno et al., 1995; Steiner & Ebihara, 1996; Barrio et al., 1997), and are unique among connexin channels because they close with V_j preferentially by means of the slow V_j gate (Bukauskas et al., 2002). Cx45 is expressed in various tissues including the cardiac conductive system, Schwann cells, oligodendrocytes, osteoblasts, myometrial and vascular smooth muscle, ovarian granulosa cells, skin, and olfactory system.

Data from the present study indicate that exposure to CO_2 affects both speed and sensitivity of V_j-dependent inactivation of junctional current. This is demonstrated by a decrease in time constant (τ) of G_j decay and a change in the ratio between G_j steady state ($G_{j\ ss}$) and $G_{j\ peak}$, indicating that V_j gating of Cx45 channels is affected by changes in $[H^+]_i$ and/or $[Ca^{2+}]_i$. In addition, inhibition of CaM expression drastically reduces both chemical and V_j gating sensitivities of Cx45 channels.

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. Twenty-four to seventytwo hours post-injection, 46 nl of Cx45 wild-type cRNA (~0.4 μg/μl) 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 hypertonic medium (Peracchia et al., 1996) and paired at the vegetal poles in conical wells of culture dishes (Falcon Products, Becton Dickinson Labware, Franklin Lakes, NJ) filled with ND96. Oocyte pairs were studied electrophysiologically 2–3 hours after pairing.

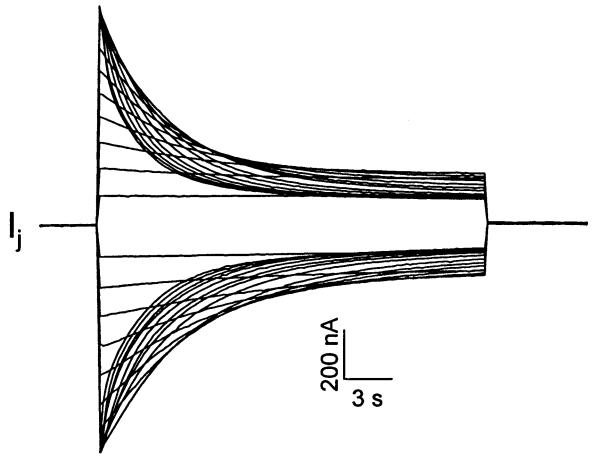
For experiments testing the effect of inhibition of CaM expression, oocytes previously injected with oligonucleotides antisense to Cx38 were injected with oligonucleotides antisense to the two CaM mRNAs expressed in *Xenopus* oocytes (46 nl, 1.12 μg/μl), as previously described (Peracchia et al., 1996). Twentyfour hours later the oocytes were injected with Cx45 cRNA. The oocytes were incubated overnight at 18°C, mechanically stripped, paired and studied electrophysiologically 2–3 hours 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 either 3-min or 15-min superfusions (0.6 ml/min) of ND96 continuously gassed with 100% CO_2 (ND96-CO_2). The perfusion was switched from ND96 to ND96-CO_2 simultaneously with the beginning of the trial. Due to the dead space of the perfusion system, the CO_2 solution reached the oocytes ~2.5 min after the beginning of the trial.

Measurement of Junctional Conductance in Oocyte Pairs

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 initially voltage-clamped individually by 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



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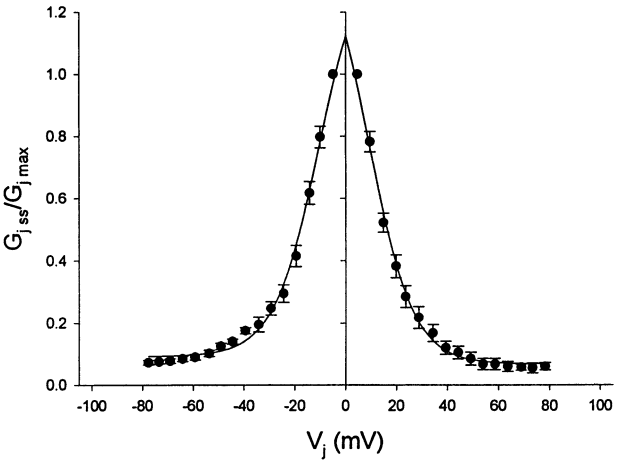

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Fig. 1. Response of junctional current (I_j) to transjunctional voltage (V_j) pulses in *Xenopus* oocyte pairs expressing Cx45 (*A*). The oocytes were tested with Protocol #1: voltage steps of −5 mV (−80 mV V_j maximum) and 25 s duration applied every 45 s to either oocyte of the pair while maintaining the other at −20 mV. Cx45 channels are highly sensitive to V_j, as I_j decays with time for $V_j >$ ±5 mV. (*B*) Plots of the relationship between $G_{j\,ss}/G_{j\,max}$ and V_j demonstrate the high V_j sensitivity of Cx45 channels. The Boltzmann values are: V_0 = 9.7 mV, η = 2.8, $G_{j\,min}$ = 0.08 and $G_{j\,max}$ = 1.47 (n = 4).

(I_j = 0). A V_j gradient was created by imposing +20 or ±40 mV voltage steps (V_1) of either 2 s or 12 s duration 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 the 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 v. 2000 (SPSS Inc., Chicago, IL). The time constant (τ) of G_j decay and the ratio $G_{j\,ss}/G_{j\,peak}$ with application of V_j pulses (±40 mV, 12s duration), in the presence and absence of CO_2, were calculated by fitting each I_j curve to a standard single exponential function, following baseline correction (Clampfit, Axon). $G_{j\,ss}$ was obtained from the τ fit (parameter "C" of Clampfit, Axon).

For studying voltage dependence of G_j, each oocyte was first voltage clamped at −20 mV. Two protocols were tested. Protocol #1: voltage steps of −5 mV (80 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. Protocol #2: same as protocol #1, except that voltage steps of −20 mV (120 mV V_j maximum) were used. Due to the high V_j sensitivity of Cx45 channels, the first protocol was used for testing Cx45 channels expressed in control oocytes, whereas the second protocol was used most often for testing Cx45 channels expressed in oocytes previously injected with oligonucleotides antisense to CaM, which have low V_j sensitivity. 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. The curve was fitted to a two-state Boltzmann distribution of the form: $(G_{j\,ss} - G_{j\,min})/(G_{j\,max} - G_{j\,ss}) = \exp[-A(V_j - V_0)]$, where V_0 is the V_j value at which G_j is one half the value of $G_{j\,max} - G_{j\,min}$, $G_{j\,max}$ 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.

Results

V_J Sensitivity

Cx45 channels display high V_j sensitivity, characterized by drastic, exponential I_j decay with time with $V_j >$ ±5 mV (Fig. 1*A*). The high sensitivity to V_j is obvious in plots of the relationship between $G_{j\,ss}/G_{j\,max}$ and V_j (Fig. 1*B*). The Boltzmann values are: V_0 = 9.7 mV, η = 2.8, $G_{j\,min}$ = 0.08 and $G_{j\,max}$ = 1.47 (n = 4).

CO_2 Sensitivity of $G_{j\,peak}$

The CO_2 sensitivity of $G_{j\,peak}$, which reflects the fraction of channels closed by the CO_2 treatment, as $G_{j\,peak}$ is virtually V_j-insensitive, was tested by establishing ±40-mV V_j steps of 12 s duration at 30-s intervals during superfusion with ND96 gassed with 100% CO_2 (0.6 ml/min). In view of the high V_j sensitivity of Cx45 channels, this pulse protocol enabled us to study the effect of CO_2 both on G_j (determined by the amplitude of $I_{j\,peak}$) and on V_j sensitivity (*see* below).

With 3-min exposures to CO_2, $G_{j\,peak}$ reversibly decreased by ~50%, from an initial value of 7.8 ± 1.1 μS (mean ± SE, n = 10) to a minimum value of 4.8 ± 1.2 μS (Fig. 2*A*). The time course of $G_{j\,peak}$ was biphasic, as $G_{j\,peak}$ increased at first by ~17%, to 9.2 ± 1.4 μS, before dropping (Fig. 2*A*).

With 15-min exposures to CO_2, $G_{j\,peak}$ decreased to ~0 μS, from an initial value of 2.6 ± 0.8 μS (mean ± SE, n = 5; Fig. 2*B*). Also in this case, $G_{j\,peak}$ increased by ~17%, to 3.03 ± 0.85 μS, before dropping (Fig. 2*B*). G_j dropped at a maximum rate of ~25%/min.

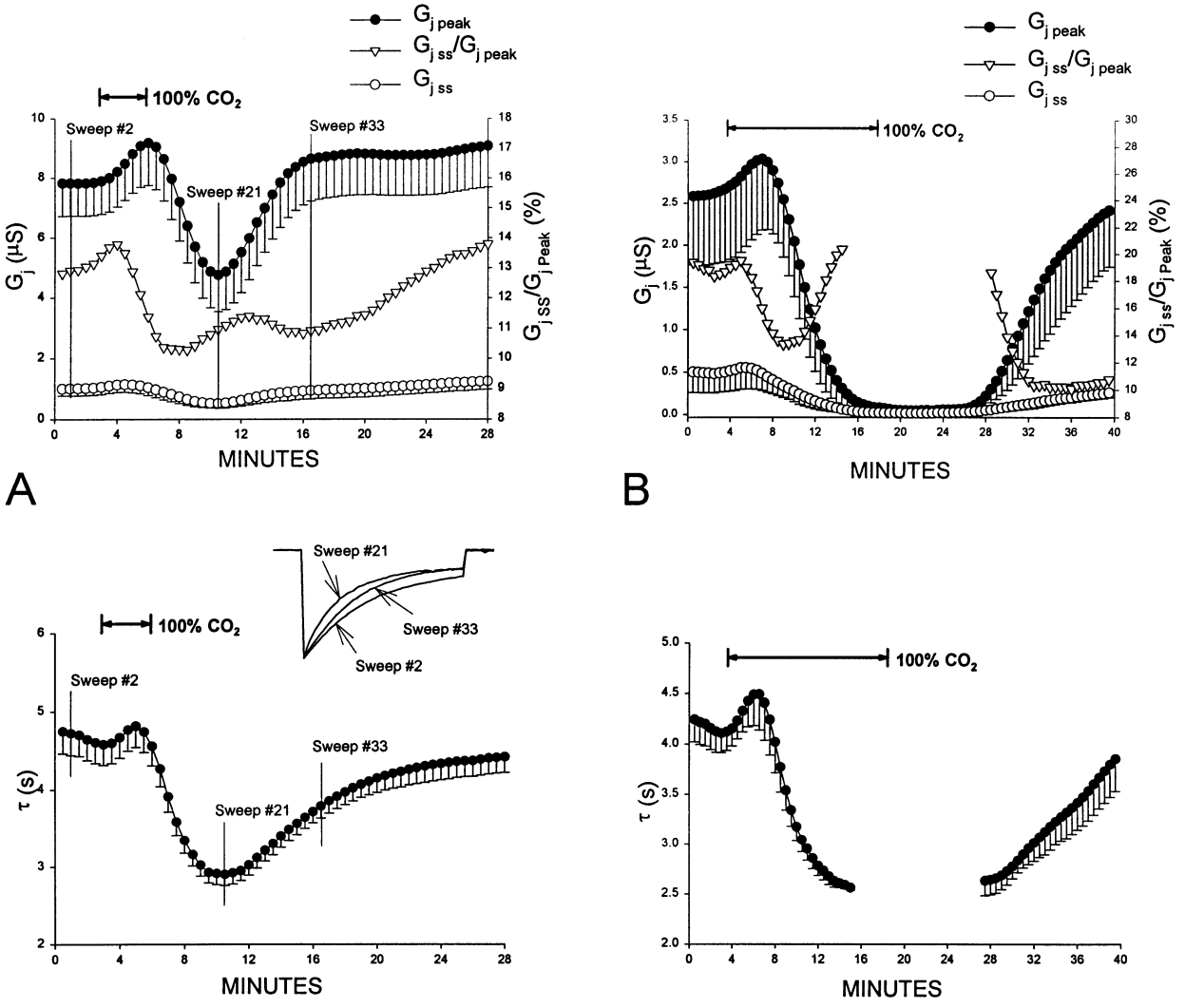


Fig. 2. Time course of junctional conductance (G_j), ratio $G_{j\ ss}/G_{j\ peak}$, and time constant (τ) of single-exponential I_j- decay with exposure to 100% CO_2 for either 3 min (*A, C*) or 15 min (*B, D*). G_j was measured by applying 40-mV V_j pulses (12 s duration, 30 s intervals). With 3 min CO_2, $G_{j\ peak}$ reversibly decreased from 7.8 ± 1.1 μS. (mean ± SE, $n = 10$) to 4.8 ± 1.2 μS (*A*). The time course of $G_{j\ peak}$ was biphasic, with $G_{j\ peak}$ increasing to 9.2 ± 1.4 μS before dropping (*A*). With 15 min CO_2, $G_{j\ peak}$ decreased to ~0 μS, from 2.6 ± 0.8 μS (mean ± SE, $n = 5$), at a maximum rate of ~25%/min (*B*), following an initial rise to 3.03 ± 0.85 μS. With CO_2, τ decreased by 39.0% and 39.6% with 3-min (*C*) and 15-min (*D*) CO_2, respectively (*see* representative sweeps #2, #21 and #33, normalized to $I_{j\ peak}$, in the inset of *C*), following a transient increase. The time course of τ matched reasonably well that of $G_{j\ peak}$, but the transient rise in τ and the onset of τ drop preceded those of $G_{j\ peak}$ by ~1 min. The time course of $G_{j\ ss}/G_{j\ peak}$ was multiphasic. $G_{j\ ss}/G_{j\ peak}$ dropped significantly after a transient increase, but, as uncoupling developed it increased substantially (*A* and *B*), eventually recovering slowly. Similar results were observed with −40 mV pulses (*data not shown*), indicating lack of participation of V_m-sensitive gating.

CO_2 Sensitivity of V_j Gating

To determine whether V_j gating is sensitive to the CO_2 treatment, both the time constant (τ) of single-exponential G_j-decay and the ratio $G_{j\ ss}/G_{j\ peak}$ were measured. The speed of channel closing with 40-mV V_j steps increased reversibly during CO_2 exposure, as τ decreased by 39% and 39.6% with 3 min (Fig. 2*C*) and 15 min (Fig. 2*D*) CO_2, respectively (compare representative sweeps #2, 21 and 33 in the inset of Fig. 2*C*). Significantly, τ increased slightly at first before dropping (Fig. 2*C* and *D*). The time course of τ matched reasonably well with that of $G_{j\ peak}$, but the brief increase in τ and the onset of τ drop preceded those of $G_{j\ peak}$ by ~1 min for both 3-min (Fig. 2*A* and *C*) and 15-min (Fig. 2*B* and *D*) CO_2 exposures. Similar results were obtained in preliminary experiments testing 20-mV V_j steps; in these

experiments, τ reversibly decreased from ~9 s to ~4 s, with 15-min CO_2 applications (~56% drop; *data not shown*).

In contrast, the time course of $G_{j\ ss}/G_{j\ peak}$ was multiphasic, matching only partially those of τ and $G_{j\ peak}$. $G_{j\ ss}/G_{j\ peak}$ dropped significantly following an initial increase, but, as uncoupling developed, it increased transiently, eventually recovering slowly to control values (Fig. 2*A* and *B*). The same changes in $G_{j\ ss}/G_{j\ peak}$ and τ during CO_2 were observed with either +40 mV or −40 mV pulses (*data not shown*), indicating that they are not likely to reflect a change in the behavior of gates sensitive to membrane potential (V_m).

Expression Efficiency of Functional Cx45 Channels after Inhibition of CaM Expression

Inhibition of CaM expression decreased the expression of functional Cx45 channels. Within 2–3 h after pairing, in oocytes injected with Cx45 cRNA (24 h earlier), G_j was 3.79 ± 1.06 μS (mean ± SE, $n = 22$). In contrast, in oocytes injected with oligonucleotides antisense to CaM mRNAs (48 h earlier) and with Cx45 cRNA (24 h earlier), G_j was 1.25 ± 0.3 μS (mean ± SE, $n = 9$).

V_J Sensitivity after Inhibition of CaM Expression

Inhibition of CaM expression drastically reduced the V_j sensitivity of Cx45 channels. Whereas with normal CaM expression I_j decayed with time for V_j values greater than ±5 mV (Fig. 1*A*), following inhibition of CaM expression, I_j did not decay with V_j values lower than ±40 mV (Fig. 3*A*). The τs, measured at $V_j = 100$ mV and $V_j = 120$ mV V_j, were 13.9 ± 1.8 s (mean ± SE, $n = 13$) and 11.0 ± 0.7 s (mean ± SE, $n = 13$), respectively. The low V_j sensitivity of Cx45 channels expressed after CaM inhibition is obvious in plots of the relationship between $G_{j\ ss}/G_{j\ max}$ and V_j (Fig. 3*B*). The Boltzmann values are: $V_0 = 75.5$ mV, $\eta = 1$, $G_{j\ min} = 0.49$ and $G_{j\ max} = 1.03$ ($n = 10$).

CO_2 Sensitivity after Inhibition of CaM Expression

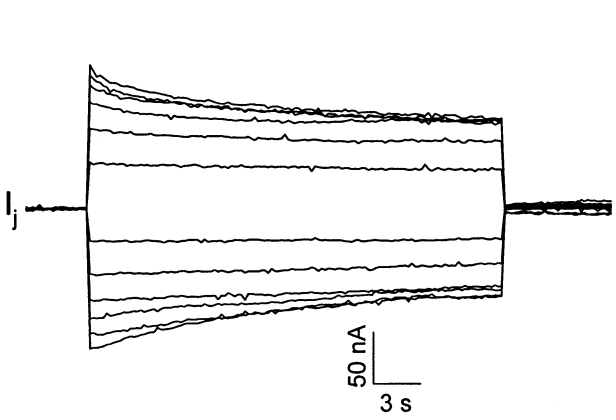
The CO_2 sensitivity of Cx45 channels was markedly reduced by inhibition of CaM expression. With 15-min application of CO_2 G_j dropped reversibly by 17.3% ($n = 5$) at a maximum rate of ~3.3%/min (Fig. 4). Significantly, the increase in G_j that preceded the G_j drop in control oocytes (Fig. 2*A* and *B*) was not observed with inhibition of CaM expression (Fig. 4).

Discussion

This study reports the effects of CO_2 on chemical and V_j gating of Cx45 channels, expressed either in wild-type *Xenopus* oocytes or in oocytes in which CaM expression was previously inhibited with oligonucleotides antisense to CaM mRNAs. The data show that CO_2 alters both kinetics and sensitivity of V_j gating, and that inhibition of CaM expression significantly decreases the gating sensitivity of Cx45 channels to CO_2 and V_j. Evidence for loss of V_j and CO_2 sensitivity of gating with inhibition of CaM expression confirms previous data on Cx38 (Peracchia et al., 1996) and Cx32 mutant channels (Peracchia et al., 2000c).

Cx45 channels are very sensitive to V_j, as their conductance is affected by V_j gradients as low as ±5 mV or lower (Veenstra et al., 1992; Moreno et al., 1995; Barrio et al., 1997). In contrast to most of the other connexins, however, in Cx45 channels the gate activated by V_j appears to be preferentially the slow gate. The fast V_j gate is also present in Cx45 channels, but relatively large V_j gradients are required for its activation (Bukauskas et al., 2002). In contrast, the slow gate is sensitive to even the smallest V_j gradients. Indeed, data from heterotypic Cx45/Cx43-EGFP channels indicate that a number of channels are closed by the slow gate even at $V_j = 0$ (Bukauskas et al., 2002). This explains why in the relationship between $G_{j\ ss}/G_{j\ max}$ and V_j the value of $G_{j\ ss}/G_{j\ max}$ at $V_j = 0$, extrapolated by the Boltzmann fit, is greater than one (Fig. 1*B*). The slow gating activity of Cx45 channels was demonstrated at the single-channel level by evidence for slow and complete channel closure in response to small V_j gradients (Elenes et al., 2001; Bukauskas et al., 2002), a behavior typical of both the slow gate and the chemical gate (Bukauskas & Peracchia, 1997), which indeed are believed to be the same gate (Bukauskas & Peracchia, 1997; Peracchia et al., 1999, 2000c). Similar behavior was observed in Cx45 hemichannels (Valiunas, 2002). In all connexin channels studied, the slow gate always closes at the negative side of V_j, whereas the gating polarity of the fast V_j gate varies among connexins. Significantly, aside from Cx45 channels, the function of the slow gate is usually hidden in the absence of chemical uncouplers, but it manifests itself in various disparate channels made of connexin mutants (Suchyna et al., 1993; Purnick et al., 2000; Peracchia et al., 1999, 2000c). The fact that wild-type Cx45 channels spontaneously manifest the function of the slow gate (Bukauskas et al., 2002) makes this connexin quite unique and an excellent tool for further attempts at understanding the molecular basis of chemical and slow gating.

CO_2 application affected in different ways the kinetics of the V_j gate as well as its V_j sensitivity. The ~40% drop in τ indicates that CO_2-induced uncou-



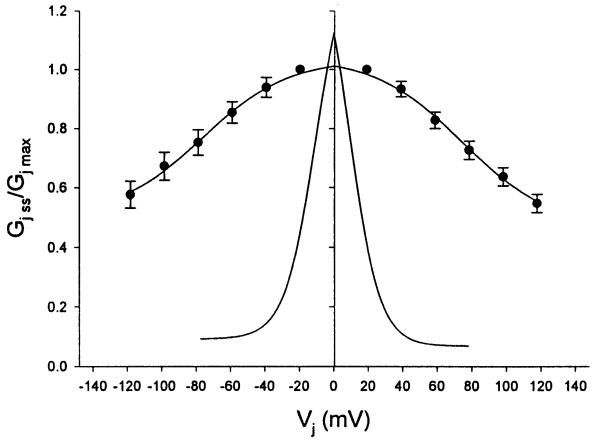


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Fig. 3. Response of junctional current (I_j) to transjunctional voltage (V_j) pulses in *Xenopus* oocyte pairs expressing Cx45 after inhibition of CaM expression (*A*). The oocytes were tested with Protocol #2: voltage steps of −20 mV (−120 mV V_j maximum) and 25 s duration applied every 45 s to either oocyte of the pair while maintaining the other at −20 mV. With inhibition of CaM expression, I_j did not decay with V_j values lower than ±40 mV (*A*). The τs, measured at 100 and 120 mV V_j, were 13.9 ± 1.8 s (mean ± SE, $n = 13$) and 11.0 ± 0.7 s (mean ± SE, $n = 13$), respectively (*B*). The reduced V_j sensitivity with inhibition of CaM expression is demonstrated by the relationship between $G_{j\,ss}/G_{j\,max}$ and V_j (*filled circles*). The Boltzmann values are: $V_0 = 75.5$ mV, $\eta = 1$, $G_{j\,min} = 0.49$ and $G_{j\,max} = 1.03$ ($n = 10$). For comparison, the Boltzmann fit of control Cx45 channels (Fig. 1*B*) is also plotted.

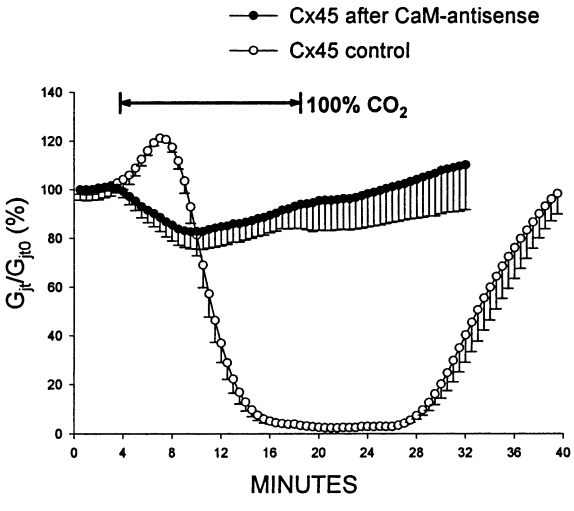

Fig. 4. Time course of the ratio G_{jt}/G_{jt0} (where G_{jt} and G_{jt0} are G_j at times t and $t = 0$, respectively) in oocytes expressing Cx45 channels after inhibition of CaM expression, and exposed to 100% CO_2 for 15 min. G_j was measured by applying 20-mV V_j pulses (2 s duration, 30 s intervals). With inhibition of CaM expression, G_j dropped reversibly by 17.3% ($n = 5$) at a maximum rate of ~3.3%/min (*filled circles*). Significantly, the increase in G_j that precedes the G_j drop in Cx45 control oocytes (oocytes not injected with oligonucleotides antisense to CaM mRNA; *empty circles*) did not occur with inhibition of CaM expression.

pling increases the closing speed of the V_j gate. Indeed, since the time course of τ is somewhat similar to that of $G_{j\,peak}$, it is likely that the cytosolic factors that induce channel closure also affect the speed of V_j gating. In view of our previous data indicating that the time course of uncoupling by cytosolic acidification reflects more closely an increase in $[Ca^{2+}]_i$ than in $[H^+]_i$ (Peracchia, 1990ab; Lazrak & Peracchia, 1993; Peracchia et al., 1996), it is possible that the increase in closing speed is a Ca^{2+} effect. In turn, since CaM is likely to be involved in chemical and slow gating (Peracchia et al., 2000a,c and data from the present study), this phenomenon could result from conversion of apo-CaM to Ca^{2+}-CaM.

The changes in $G_{j\,ss}/G_{j\,peak}$ indicate that the CO_2 application affects not only the kinetics of the V_j gate but also its V_j sensitivity. However, the time course of $G_{j\,ss}/G_{j\,peak}$ was multiphasic and did not match that of τ and $G_{j\,peak}$. With CO_2 application, initially V_j sensitivity increased significantly (reflected by a drop in $G_{j\,ss}/G_{j\,peak}$), but as uncoupling developed, transiently it reversed its course, eventually recovering to control values at a much slower rate than τ and $G_{j\,peak}$. This behavior may suggest that the V_j sensitivity of the V_j gate is influenced by more than one factor during CO_2 exposure. Perhaps, changes in both $[Ca^{2+}]_i$ and $[H^+]_i$ play a role in this phenomenon. Since a rise in $[H^+]_i$ precedes a rise in $[Ca^{2+}]_i$ (Peracchia et al., 1996), it is possible that protonation of the gate and/or accessory molecules increases the gate's V_j sensitivity, whereas Ca^{2+} binding to either of these components may reduce it.

Alternatively, it is possible that CO_2-induced changes in $[Ca^{2+}]_i$ and/or $[H^+]_i$ favor substates or the residual state of single-channel conductance (γ_j). In this case, CO_2 might generate two interconvertible

channel populations: one that flickers with V_j from open state to closed state and another that flickers from substate or residual state to closed state. However, the conversion from open state to substate or residual state of progressively larger fractions of channels would result in sizable decrease in $G_{j\ peak}$ whose time course should match with those of $G_{j\ ss}/G_{j\ peak}$ and τ. This certainly was not the case for $G_{j\ ss}/G_{j\ peak}$, and even though the time course of τ was somewhat similar to that of $G_{j\ peak}$, τ peaked ~1 min earlier than $G_{j\ peak}$ and recovered at significantly slower rate than $G_{j\ peak}$. A conversion from open state to substate or residual conductance state was not observed in single-channel records of Cx43 channels exposed to CO_2 at different V_j values (Bukauskas & Peracchia, 1997). However, whether it occurs with Cx45 channels cannot be ruled out yet, as this hypothesis can only be tested in small cells at the single-channel level. Another possibility is that there are two different pools of Cx45 channels with different kinetics and various degrees of pH and V_j sensitivities. This also can only be determined in single-channel experiments.

Cx45 channels have also been shown to be mildly sensitive to the inside-outside voltage (V_m, Barrio et al., 1997). Therefore, theoretically the changes in τ and $G_{j\ ss}/G_{j\ peak}$ could reflect an effect of CO_2 on the V_m gate. However, this is unlikely because the same changes in τ and $G_{j\ ss}/G_{j\ peak}$ were obtained with hyperpolarizing (−40 mV) and depolarizing (+40 mV) pulse protocols.

A sizable increase in V_j sensitivity with CO_2 application was previously reported in oocytes expressing wild-type Cx32 (Werner et al., 1991). We have recently confirmed these data by monitoring both τ and $G_{j\ ss}/G_{j\ peak}$ of Cx32 channels during CO_2 (Young & Peracchia, 2002). However, the behavior of Cx32 differs from that of Cx45, as with CO_2 the $G_{j\ ss}/G_{j\ peak}$ of Cx32 channels decreased monophasically with a time course reasonably close to that of uncoupling. The difference between Cx32 and Cx45 may be related to the type of gate involved, which is likely to be the fast V_j gate for Cx32 and the slow V_j gate for Cx45 channels. Even though the mechanism by which CO_2 affects V_j gating sensitivity and kinetics is still unclear, this phenomenon should be explored in detail, as it might provide useful tools for understanding further the key players of both chemical and voltage sensitive gating mechanisms. While our data indicate that the slow V_j gate of Cx45 channels is affected by CO_2, they do not exclude the possibility of pH-gating independent of V_j-gating. However, the pH gate and the slow gate are most likely the same gate.

The idea that the slow V_j gate and the chemical gate are the same is further supported by the observation that inhibition of CaM expression with antisense oligonucleotides greatly reduces both chemical and V_j sensitivities of Cx45 channels. This treatment was previously shown to permanently degrade CaM's mRNA within five hours (Peracchia et al., 1996). Significantly, with inhibition of CaM expression, a residual V_j sensitivity remained, although greatly reduced. A possibility is that with this treatment most, but not all, of the channels lost the V_j gate; indeed, also chemical gating was not entirely abolished, as with CO_2 G_j dropped by 17.3%. Alternatively, this residual V_j sensitivity may reflect a greatly reduced V_j sensitivity of the slow V_j gate. In any event, this issue can only be clarified at the single-channel level.

Inhibition of CaM expression also appears to eliminate the early rise in G_j that precedes the G_j drop in control Cx45 oocytes (Fig. 4). The reason for the CO_2-induced G_j rise, which incidentally is seen in most connexin channels, especially in Cx43 channels (Peracchia, unpublished), is totally unclear, but the fact that it is eliminated by inhibition of CaM expression suggests a CaM involvement. Recently, activation of Ca^{2+}/CaM kinase II has been shown to increase the junctional conductance of goldfish Mauthner cells (Pereda et al., 1998) and mouse astrocytes (Pina-Benabou et al., 2001). Based on these data one may speculate that an early increase in $[Ca^{2+}]_i$ at the initial stages of CO_2 treatment may activate Ca^{2+}/CaM kinase II, resulting in a G_j rise, whereas a subsequent larger increase in $[Ca^{2+}]_i$ may close the channels by direct CaM participation in the gating mechanism. However, there is no evidence for a role of Ca^{2+}/CaM kinase II in Cx45 channel function, and mechanisms other than that connexin phosphorylation may very well be involved.

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; Peracchia et al., 2000c) 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 et al., 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 NH_2- and COOH-terminal domains of Cx32 (Török et al., 1997), and preliminary immunocytochemical evidence for CaM-Cx32 association was reported in HeLa cells (Peracchia et al., 2000a). Ca^{2+}-independent CaM binding to Cx32 was also shown to prevent Cx32 proteolysis by m-calpain (Elvira &

Villalobo, 1997) and phosphorylation by EGF receptor tyrosine kinase (Diez et al., 1998).

The idea of CaM-mediated gating was also supported by data from oocytes expressing CaMCC, a CaM mutant with an overall Ca-sensitivity significantly higher than that of wild-type CaM, due to the replacement of the N-terminal EF hand pair by a duplication of the C-terminal pair (Persechini et al., 1996). CaMCC greatly enhanced chemical gating sensitivity of Cx32 only when it was expressed before Cx32, suggesting that CaM associates with Cx before gap junction formation (Peracchia et al., 2000a). The intimate relationship between CaMCC and Cx32 was confirmed by a large reduction in V_j sensitivity (Peracchia et al., 2000a). CaM has also been shown to play a role in gap-junction assembly, as it appears to enable the oligomerization of connexins into connexon hemichannels (Ahmad et al., 2001). This may be the reason why with inhibition of CaM expression the formation of functional Cx45 channels decreased to $\sim$1/3 of controls.

A different gating mechanism, named "particle-receptor" model, has been proposed for Cx43 and Cx40 channels (rev. in Delmar et al., 2000). This model envisions gating as the result of interaction between a distal COOH-terminus domain and a receptor domain likely to be part of the cytoplasmic loop (rev. in Delmar et al., 2000). Consistent with this model, at least for Cx43, may be recent *in vitro* evidence from experiments combining resonant mirror technology, enzyme-linked sorbent assay and nuclear magnetic resonance (NMR), which demonstrate an interaction between CT (res. 255–382) and a peptide corresponding to the second half of the cytoplasmic loop (res. 119–144), which is enhanced by low pH (Duffy et al., 2002). The particle-receptor model, however, may only be applicable to few connexins because, aside from Cx43, COOH-terminus deletion was reported to be partially effective in Cx40 channels, minimally in Cx37, and ineffective in Cx45 and Cx32 channels (Werner et al., 1991; Wang & Peracchia, 1997; Stergiopoulos et al., 1999), and obviously in channels made of Cx26, whose COOH-terminus is very short (18 res.).

In conclusion, the data show that exposure to CO_2 of oocyte pairs expressing Cx45 channels alters the kinetics and V_j sensitivity of V_j gating as uncoupling develops. In addition, as previously shown with other connexins, both chemical and V_j sensitivities of Cx45 channels are significantly reduced by inhibition of CaM expression. The latter confirms previous evidence for a role of CaM in gap junction channel gating. Direct CaM participation in the regulation of connexin channels would not be surprising because in recent years CaM has been shown to be directly involved in the regulation of a number of other membrane channels (reviewed in Saimi & Kung, 2002).

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