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Chemical Gating of Heteromeric and Heterotypic Gap Junction Channels

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Abstract. Gap junction channels contain two hemichannels (connexons), each being a connexin (Cx) hexamer. In cells expressing multiple connexins, heteromeric connexons are believed to form, whereas cell pairs expressing different connexins generate heterotypic channels. To define gating behavior of heteromeric and heterotypic channels, CO_2-induced gating was tested in *Xenopus* oocyte pairs expressing Cx32, or 5R/N (Cx32 mutant), as well as in pairs in which one oocyte (mx) expressed a 50/50 mixture of Cx32 and 5R/N and the other either the mixture (mx), Cx32 (32) or 5R/N (R/N). In 5R/N, replacement of 5 C-terminus arginines with asparagines greatly increased CO_2 sensitivity. In response to 3 and 15 min CO_2 exposures, junctional conductance (G_j) decreased to 85% and 47%, in 32–32 pairs, and to 7% and 0.9%, in R/N-R/N pairs, respectively. In mx-mx and mix-32 pairs, G_j decreased to similar values (33% and 35%, respectively) with 15 min CO_2. The sensitivity of mx-R/N pairs was similar to that of heterotypic 32-R/N pairs, as G_j dropped to 36% and 38%, respectively, with 3 min CO_2. Monoheteromeric (mx-32 and mx-R/N) and biheteromeric (mx-mx) channels behaved as if Cx32 were dominant, suggesting that hemichannel sensitivity is not an average of the sensitivities of its connexin monomers. In contrast, heterotypic channels behaved as if the two hemichannels of a cell-cell channel had no influence on each other.

Key words: Cell communication — Connexin — Cell junctions — Channels — Chemical gating

Introduction

Gap junctions are plasma membrane differentiations specialized for direct cell-to-cell transmission of metabolic and electrical signals. A gap junction channel is made of two hemichannels (connexons) composed of hexamers of connexin (Cx) proteins. Connexins are believed to contain four transmembrane regions, linked by two extracellular and a cytoplasmic (inner) loop, a short N-terminal chain and a C-terminal chain of variable length. Connexin sequences are highly conserved aside from cytoplasmic loop and C-terminus regions.

Gap junction channels are known to close in response to changes in cytosolic Ca^{2+} or H^+ concentration (rev. in Loewenstein, 1990; Peracchia, Lazrak & Peracchia, 1994; Bruzzone, White & Paul, 1996), but the molecular mechanisms of channel gating are still unclear (rev. in Peracchia & Wang, 1997). Recently, we have used site-directed mutagenesis, chimeric construction techniques and *Xenopus* oocyte expression system, to identify domains of Cx32 and Cx38 potentially involved in CO_2-induced (low pH_i) channel gating (Wang et al., 1996; Wang & Peracchia, 1996; Wang & Peracchia, 1997*b*). Cx32, the principal rat liver connexin, is much less sensitive to CO_2 than Cx38, the connexin expressed by *Xenopus* embryos. Our data indicate that the second half of the inner loop (IL_2) contains a domain relevant for CO_2 gating sensitivity, whereas the N-terminus chain does not seem to play a role (Wang et al., 1996; Wang & Peracchia, 1996). Deletion of over 80% of the C-terminus (CT) chain of Cx32 does not affect CO_2 sensitivity (Werner et al., 1991; Wang & Peracchia, 1997*b*), whereas replacement of 5 basic residues (arginines, R: R215, R219, R220, R223 and R224) of the initial 17 residue C-terminus domain (C_1) with neutral residues (asparagines, N, or threonines, T; mutants 5R/N and 5R/T, respectively) dramatically increases Cx32 sensitivity to CO_2, indicating that somehow the positively charged nature of C_1 makes it function as a domain that inhibits chemical gating (Wang & Peracchia, 1997*b*).

A question that has not been addressed yet is whether chemical gating requires a cooperative interaction

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among the connexins forming a connexon, and between the connexons forming a cell-cell channel. To address these questions, the present study has attempted to generate heteromeric connexons (composed of different connexins) and heterotypic cell-cell channels (made of two connexons each expressing a different connexin) with wild-type Cx32 and its 5R/N mutant. The drastic difference in CO_2 sensitivity between Cx32 and its 5R/N mutant, in spite of their almost identical amino acid sequence, made these two connexins ideal for this type of study.

The data suggest either that cooperatively among homologous connexin monomers of a connexon may be important for efficient chemical gating or that gating involves connexon interaction with an accessory molecule. In contrast, two connexons forming a cell-cell channel appear not to influence each other's chemical gating sensitivity. A preliminary account of this study has been published (Wang & Peracchia, 1997*a*).

Materials and Methods

Site-directed Mutagenesis

Molecular biology protocols were generally as described by Sambrook, Fritsch and Maniatis (1989). Cx32 cDNA was used in the construction of DNA mutants. The strategy employed to create the 5R/N mutant of Cx32 has been previously described (Wang et al., 1996). The mutant was verified by DNA sequence analysis.

Oligonucleotide Sequences

Oligonucleotides were synthesized by a DNA synthesizer (model 393; ABI, Foster City, CA). The sequence used to produce the 5R/N mutant is shown below. Letters in italics represent mutated nucleotides:

5′-CCTTGCGGGAGGGCGGATTGGA*GTTGTT*CTGAGC*GTTGTT*

Preparation of cRNA

Wild-type and mutated forms of Cx32 cDNA were subcloned into pBluescript (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 mMESSAGE mMACHINE (Ambion, Austin, TX) in the presence of the cap analogue m7G(5′)ppp(5′)G (Ambion).

Oocyte Preparation and Microinjection

Oocytes were prepared as previously described (Peracchia et al., 1996). Briefly, adult female *Xenopus laevis* frogs were anesthetized with 0.3% tricaine (MS-222) and the oocytes were surgically removed from the abdominal incision. The oocytes were placed in ND 96 medium. Oocytes at stages V or VI were subsequently defolliculated in 2 mg/ml collagenase (Sigma Chemical, St. Louis, MO) in Ca^{2+}-free OR2 for 80 min at room temperature. The defolliculated oocytes were injected with 46nl of antisense oligonucleotide (0.25 μg/μl) complementary to endogenous *Xenopus* Cx38:

5′-GCTTTAGTAATTCCCATCCTGCCATGTTTC-3′

(commencing at nt −5 of Cx38 cDNA sequence, Barrio et al., 1991), by means of a Drummond nanoject apparatus (Drummond, Broomall, PA). The antisense oligonucleotide blocks completely the endogenous junctional communication within 48 hr. 48–72 hours post-injection, 46 nl of either wild-type, mutated, or a 50/50 mixture of the two cRNAs (0.03–0.04 μg/μl total) were injected into oocytes at the vegetal pole and the oocytes were incubated overnight at 18°C. The oocytes were mechanically stripped of their vitelline layer in a hypertonic medium (Wang et al., 1996) and paired at the vegetal poles in ND 96. Oocyte pairs were studied electrophysiologically 0.5–2 hr 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, 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 3–15 min superfusion (0.6 ml/min) of salines continuously gassed with 100% CO_2. A Cl^--free saline (Cl^- replaced with methanesulfonate) was used. The Cl^--free saline contained (in mM): NaOH 75, KOH 10, $Ca(OH)_2$ 4, $Mg(OH)_2$ 5, MOPS 10, adjusted to pH 7.2 with methanesulfonic acid. As previously reported (Peracchia et al., 1996) the opening of Ca^{2+}-activated Cl^- channels during exposure to 100% CO_2 causes an increase in membrane currents that may interfere with measurements of junctional current. In all cases the perfusion of the CO_2-gassed solution was started at the beginning of the experiment; the solution reached the oocytes 3 min later.

Measurement of Gap Junctional Conductance in Oocyte Pairs

A standard double voltage clamp procedure (Spray, Harris & Bennett, 1981) was used in all of the experiments. Following the insertion of a current and a voltage microelectrode in each of the two oocytes, both oocytes were initially voltage clamped to the same holding potential, 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-sec duration every 30 sec to oocyte 1, while maintaining V_2 at V_m, thus, $V_j = V_1$. The negative feedback current (I_2), injected by the clamp amplifier in oocyte 2 for maintaining V_2 constant at V_m, was used for calculating the junctional conductance (G_j), as it is identical in magnitude to the junctional current (I_j), but of opposite sign ($I_j = -I_2$); $G_j = I_j/V_j$ (Ohm's law). Pulse generation and data acquisition were performed by means of a computer equipped with pClamp software (Axon Instrument, Foster City, CA) and Labmaster TL-1 A/D-D/A interface (Axon).

Calculation of Theoretical Frequency of Heteromeric Connexon Types

Gap junction channels can be heterotypic (made of two connexons each composed of a different connexin) or homotypic (made of two con-

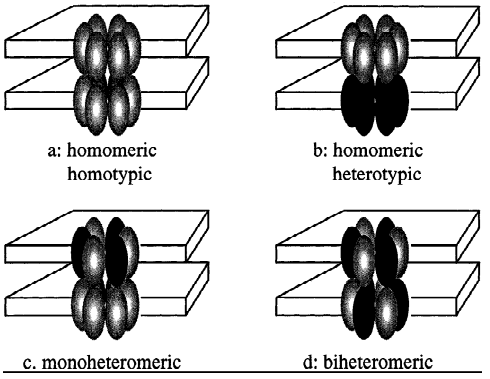

Fig. 1. A cell-cell channel can be homotypic (*a*, made of two connexons expressing the same connexin) or heterotypic (*b*, made of two connexons each expressing a different connexin). In turn, connexons can be homomeric (*a* and *b*, made of the same connexin) or heteromeric (*c* and *d*, composed of different connexins). Therefore, cell-cell channels can be homomeric-homotypic (*a*), homomeric-heterotypic (*b*), monoheteromeric (*c*, one connexon heteromeric and the other homomeric) and biheteromeric (*d*, both connexons heteromeric).

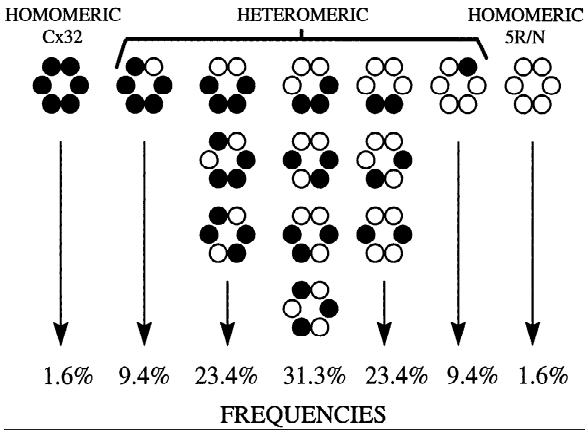

Fig. 2. Assuming no restrictions in the capacity of wild-type Cx32 and mutant 5R/N connexins to associate with each other in forming hexameric connexons, by co-injecting oocytes with equal amounts of wild-type (Cx32) and mutant (5R/N) cRNA, one would expect to generate seven groups of connexon types, expressing with the above frequencies. Expression frequencies were calculated by the following binomial equation: $p^r q^{n-r}\ n!/r!(n\text{-}r)!$ where p is the incorporation probability of the wild-type subunit, q is the incorporation probability of the mutant type subunit, and n is the number of subunits in a connexon (six) taken r subunits at a time.

nexons composed of the same connexin). In addition, connexons can be heteromeric (composed of different connexins, Falk & Gilula, 1996; Brink et al., 1997) or homomeric (made of the same connexin) (rev. in Peracchia & Wang, 1997). Thus, a channels can be homomeric-homotypic (Fig. 1*A*), homomeric-heterotypic (Fig. 1*B*), monoheteromeric (Fig. 1*C*, one connexon heteromeric and the other homomeric), biheteromeric (Fig. 1*D*, both connexons heteromeric), etc. When two connexins are coexpressed in a cell, seven groups of connexon types are likely to be generated (Fig. 2), if one assumes unrestricted connexin association in connexon formation. The expression frequency of each group of connexon types (Fig. 2) were calculated by the following binomial equation:

$$p^r q^{n-r} \frac{n!}{r!(n-r)!}$$

where p is the incorporation probability of the wild-type subunit, q is the incorporation probability of the mutant type subunit, and n is the number of subunits in a connexon (six) taken r subunits at a time. A value of 0.5 was used for both p and q because, based on the observation that oocytes injected with mRNAs of either wild-type Cx32 or its mutant 5R/N display comparable G_j values following the same length of pairing time (Table), there is no reason to believe that these connexins differ drastically in expression efficiency.

Table. Conductances developed by oocyte pairs expressing homomeric-homotypic, biheteromeric, monoheteromeric and homomeric-heterotypic channels

Type	Pairing time (hr)	G_j (μS, mean ± SD)	G_j (μS, max.)	G_j (μS, min.)	n
32-32	0.5	4.2 ± 1.3	27.5	0.4	26
R/N-R/N	0.5	3.8 ± 1.3	9.8	0.3	8
mx-mx	0.5	7.2 ± 2.3	17.7	0.8	8
mx-32	0.5	7.9 ± 2.6	19.2	1.0	7
mx-R/N	0.5	5.3 ± 2.0	12.9	1.0	6
32-R/N	0.5	6.1 ± 2.9	22.7	0.8	7

Results

CO_2 Sensitivity of Cx32 or 5R/N Homomeric-Homotypic Channels

Oocyte pairs expressing homomeric-homotypic Cx32 channels (32–32), developed coupling soon after pairing; within half an hour after pairing, junctional conductance (G_j) averaged 4.2 μS (*see* the Table). Cx32 was weakly sensitive to CO_2. With a 3-min exposure to 100% CO_2 G_j decreased to 85 ± 5% (mean ± SE, $n = 7$) of the initial value, and with a 15 min exposure to 47 ± 4.8% (mean ± SE, $n = 16$) (Fig. 3*A*). G_j decreased with CO_2 at a maximum rate of ~9%/min (Fig. 3*A*) and recovered to pretreatment values at a maximum rate of 8–11%/min (Fig. 3*A*). The onset of G_j recovery was always rather abrupt (Fig. 3*A*).

Oocyte pairs expressing homomeric-homotypic 5R/N channels (R/N-R/N), developed coupling soon after pairing as well; within half an hour after pairing, the mean G_j was 3.8 μS (*see* Table). Channels made of 5R/N, a mutant in which all of the five arginine (*R*) residues (R215, R219, R220, R223 and R224) present in the initial 17 residue segment (C_1) of the Cx32s C-terminal chain were replaced with asparagines (N) were

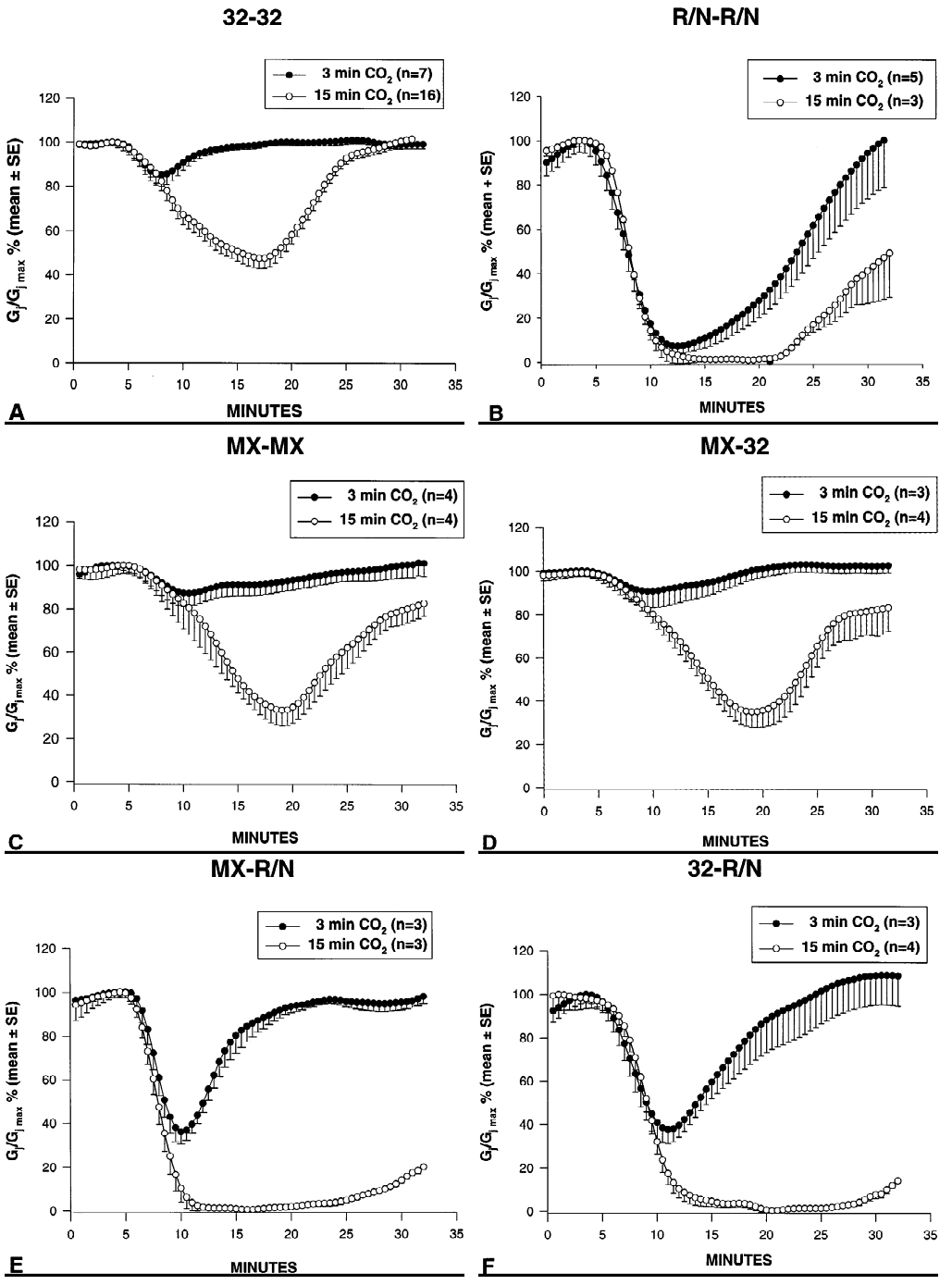

Fig. 3. Time course of normalized G_j ($G_j/G_{j\max}$, 100% being the control, pretreatment, value) in *Xenopus* oocyte pairs exposed to 100% CO_2. The oocyte pairs expressed either homomeric-homotypic channels, composed of wild-type Cx32 (*A*, 32–32) or mutant 5 R/N (*B*, R/N-R/N), biheteromeric channels (*C*, mx-mx), monoheteromeric channels (*D*, mx-32; *E*, mx-R/N) or heterotypic channels (*F*, 32-R/N). In 32–32 pairs (*A*), G_j decreased to 85 ± 5% (mean ± SE, $n = 7$) and to 47 ± 4.8% (mean ± SE, $n = 16$), with 3 min and 15 min CO_2 exposures, respectively, at a maximum rate of ~9%/min. The onset of G_j recovery was rather abrupt (*A*). G_j recovered to pretreatment values at a maximum rate of 8–11%/min. In R/N-R/N pairs (*B*), G_j decreased to 7.12 ± 3.4% (mean ± SE, $n = 5$) and to nearly 0% ($n = 3$), with 3 min and 15 min CO_2 exposures, respectively, at a maximum rate of ~20%/min. G_j recovered at a maximum rate of ~8%/min. In mx-mx pairs (*C*), G_j decreased to 87.2 ± 6% (mean ± SE, $n = 4$) and to 33.1 ± 7.1% (mean ± SE, $n = 4$) with 3-min and 15-min CO_2 exposures, respectively, at a maximum rate of ~8.3%/min. In mx-32 pairs (*D*), G_j decreased to 90.7 ± 7.4% (mean ± SE, $n = 4$) and 34.7 ± 7% (mean ± SE, $n = 3$), with 3-min and 15-min CO_2 exposures, at a maximum rate of ~6%/min. In mx-R/N pairs (*E*), G_j decreased to 36.1 ± 5.6% (mean ± SE, $n = 3$) and to nearly 0% ($n = 3$) with 3-min and 15-min exposures, respectively, at a maximum rate of ~23%/min. In homomeric-heterotypic 32-R/N pairs (*F*), G_j decreased to 37.5 ± 6.4% (mean ± SE, $n = 3$), and to nearly 0% ($n = 4$) with 3-min and 15-min CO_2 exposures, respectively, at a maximum rate of ~21%/min. Note that mx-mx (*C*) and mx-32 (*D*) pairs are as sensitive to CO_2 as 32–32 pairs (*A*), and that mx-R/N pairs (*E*) are as sensitive to CO_2 as the homomeric-heterotypic 32-R/N pairs (*F*).

much more sensitive to CO_2 than wild-type Cx32. In R/N-R/N oocyte pairs G_j dropped to 7.1 ± 3.4% (mean ± SE, $n = 5$) of initial values at a maximum rate of ~20%/min with 3 min exposures to 100% CO_2 (Fig. 3*B*), and to nearly 0% ($n = 3$) with 15 min exposures (Fig. 3*B*). G_j recovered at a maximum rate of ~8%/min.

CO_2 Sensitivity of Biheteromeric and Monoheteromeric Channels

In oocyte pairs in which each oocyte was injected with equal amounts of Cx32 and 5R/N cRNAs (mx-mx) G_j averaged 7.2 μS (*see* Table) within half an hour since pairing. With a 3-min exposures to 100% CO_2 G_j decreased to 87.2 ± 6% (mean ± SE, $n = 4$) (Figs. 3*C* and 4*A*) of its initial value, and with 15 min exposures to 33.1 ± 7.1% (mean ± SE, $n = 4$) (Figs. 3*C* and 4*B*). G_j decreased with CO_2 at a maximum rate of ~8.3%/min (Fig. 3*C*) and recovered to pretreatment values at a maximum rate of 5–7%/min (Fig. 3*C*). Similar results were obtained with oocyte pairs in which one expressed a 50/50 mixture of Cx32 and 5R/N and the other Cx32 (mx-32). In mx-32 pairs G_j averaged 7.9 μS (*see* Table). In these pairs, 3-min CO_2 exposures reduced G_j to only 90.7 ± 7.4% (mean ± SE, $n = 4$) (Figs. 3*D* and 4*A*) and 15-min exposures to 34.7 ± 7% (mean ± SE, $n = 3$) (Figs. 3*D* and 4*B*), at a maximum rate of ~6%/min. Note that the sensitivity of mx-mx (Figs. 3*C* and 4) and mx-32 (Figs. 3*D* and 4) oocyte pairs is similar to that of homomeric-homotypic 32–32 pairs (Figs. 3*A* and 4) for both 3 min and 15 min CO_2 exposures.

Oocyte pairs in which one of the two oocytes expressed a 50/50 mixture of Cx32 and 5R/N and the other 5R/N (mx-R/N) were significantly more sensitive to CO_2 (Figs. 3*E* and 4) than mx-mx (Figs. 3*C* and 4) or mx-32 (Figs. 3*D* and 4), but not quite as sensitive as the homomeric-homotypic R/N-R/N pairs (Figs. 3*B* and 4). In mx-R/N pairs G_j averaged 5.3 μS (*see* Table). With 3-min exposures to CO_2 G_j decreased to 36.1 ± 5.6% (mean ± SE, $n = 3$) (Figs. 3*E* and 4*A*), and with 15-min exposures to nearly 0% ($n = 3$) (Figs. 3*E* and 4*B*), at a maximum rate of ~23%/min. Interestingly, the sensitivity of mx-R/N oocyte pairs (Figs. 3*E* and 4) was similar to that of homomeric-heterotypic 32-R/N pairs (Figs. 3*F* and 5) for both 3-min and 15-min CO_2 exposures.

CO_2 Sensitivity of Heterotypic Channels

Oocyte pairs in which one of the two expressed Cx32 and the other 5R/N (32-R/N) developed coupling soon after pairing to the same degree as homotypic 32–32 and R/N-R/N pairs; within half an hour after pairing, junctional conductance (G_j) averaged 6.1 μS (*see* Table). These oocytes displayed a CO_2 sensitivity (Figs. 3*F* and 5) similar to that of mx-R/N pairs (Figs. 3*E* and 4). With 3-min exposures to CO_2 G_j decreased to 37.5 ± 6.4% (mean ± SE, $n = 3$) (Figs. 3*F* and 5), and with 15-min exposures to nearly 0% ($n = 4$) (Figs. 3*F* and 5), at a maximum rate of ~21%/min.

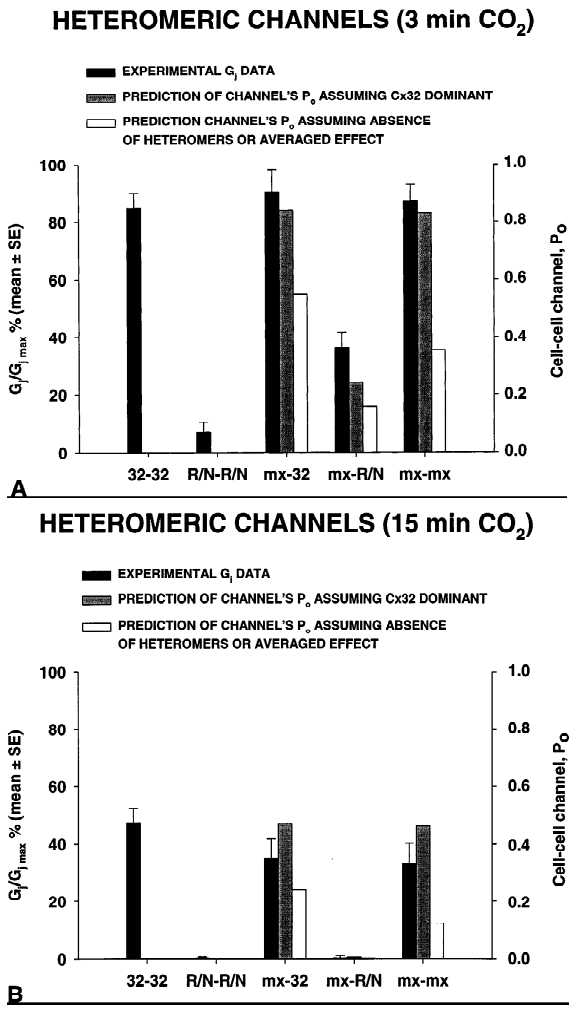

Fig. 4. Maximum G_j drop from control values (100%) with 3-min (*A*) and 15-min (*B*) CO_2 treatments in *Xenopus* oocyte pairs expressing homomeric-homotypic Cx32 (32–32), homomeric-homotypic 5R/N (R/N-R/N), monoheteromeric (mx-32, mx-R/N) and biheteromeric (mx-mx) cell-cell channels (black bars). Gray bars represent the normalized cell-cell channel open probability (P_O) predicted by assuming that the CO_2 sensitivity of heteromeric hemichannels reflects entirely the sensitivity of wild-type Cx32. White bars represent the P_O predicted by assuming either that heteromeric hemichannels do not form or that the CO_2 sensitivity of heteromeric hemichannels is a weighted average of the sensitivity of the two components (Cx32 and 5R/N). It is clear that the behavior of monoheteromeric (mx-32 and mx-R/N) and biheteromeric (mx-mx) channels (black bars) corresponds more closely to the first prediction (gray bars). This indicates both that heteromeric hemichannels form and that their gating sensitivity is not a weighted average of the sensitivities of the connexin monomers that they contain, but rather that Cx32 has a dominant effect on the gating behavior of heteromers. This would mean that the presence of even one Cx32 monomer in a hexamer is sufficient to inhibit the sensitivity of the other five 5R/N monomers.

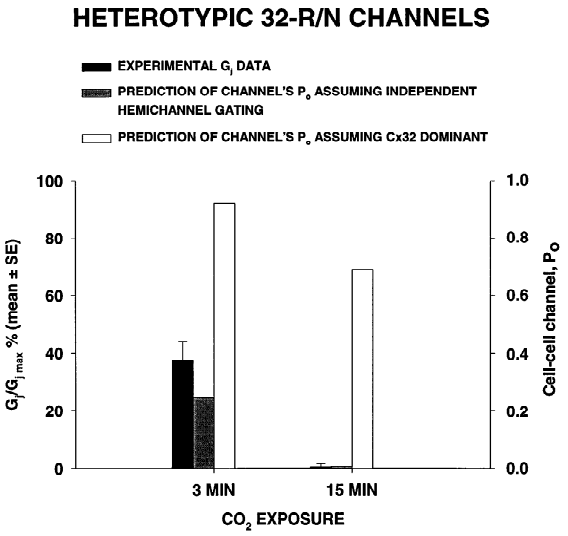

Fig. 5. Maximum G_j drop from control values (100%) with 3-min and 15-min CO_2 treatments in *Xenopus* oocyte pairs expressing homomeric-heterotypic (32-R/N) channels (black bars). Gray bars represent the normalized cell-cell channel open probability (P_O) predicted by assuming that the CO_2 sensitivity of a heterotypic channel reflects the individual gating properties of each of the two hemichannels (independent hemichannel gating). White bars represent the P_O predicted by assuming that the gating sensitivity of the hemichannel made of wild-type Cx32 has a dominant effect on cell-cell channel gating. The sensitivity of heterotypic 32-R/N channels corresponds more closely to the first prediction (gray bars), indicating that two hemichannels forming a cell-cell channel do not influence each other's CO_2 gating sensitivity.

Discussion

This study has tested the gating sensitivity to CO_2-induced acidification of homomeric-homotypic, monoheteromeric, biheteromeric, and homomeric-heterotypic cell-cell channels formed in *Xenopus* oocyte cell pairs expressing either wild-type Cx32, its more CO_2-sensitive 5R/N mutant, or 50/50 mixtures of Cx32 and 5R/N. The data show that heteromeric channels are less sensitive to CO_2 than one would predict based on the assumption that connexins participate in hemichannel gating independently of each other. In contrast, the sensitivity of heterotypic channels is similar to that predicted by assuming that the two connexons forming a cell-cell channel behave independently of each other.

Since many cells express more than one connexin, it is generally believed that heteromeric connexons may form. However, firm evidence for heteromeric connexons is still lacking. Recently, the presence of heteromeric connexons in cells expressing Cx43 and Cx37 has been indirectly implied by the unexpected behavior of the resulting gap junction channels in terms of conductance and voltage sensitivity (Brink et al., 1997). Evidence for the capacity of different connexins to oligomerize into hexameric connexons has also been reported (Falk & Gilula, 1996), but this preliminary study did not demonstrate that heteromeric connexons form functional channels.

In our study, proof that heteromeric channels form is lacking as well, but there are reasons to believe that they do form. We have not used different connexins, as in previous studies, but rather wild-type Cx32 and its mutant (5R/N), that only differs from Cx32 by having 5 R residues replaced by N residues at C_1. One would predict that the almost perfect sequence match between Cx32 and 5R/N would enable them to oligomerize with each other easier than two different connexins would. Cx32 and 5R/N express channels very well and with equal efficiency, after the same pairing time, following oocyte injections of equal cRNA concentrations. If heteromeres had formed but their channels were nonfunctional, the coexpression of Cx32 and 5R/N should have resulted in much lower junctional conductance values, after the same pairing time, than with expression of either one in homomeric conditions. Furthermore, the CO_2 gating sensitivity of oocytes injected with a mixture of Cx32 and 5R/N mRNAs is different (much lower) than that predicted by assuming that only homomeric hemichannel types (Cx32 hexamers and 5R/N hexamers), rather than heteromers, had formed.

The data show that coexpression of Cx32 and 5R/N results in heteromeric channels with CO_2 gating sensitivities much lower than expected, and more similar to that of Cx32 than to that of 5R/N. To evaluate the meaning of this observation, we have compared our data to predictions of cell-cell channel gating sensitivity based on different assumptions. Since both hemichannels of a cell-cell channel have gates, and only one hemichannel gate needs to close for a cell-cell channel to close, one can predict the fraction of closed cell-cell channels by knowing the fraction of closed hemichannels in each oocyte, and vice versa. As an example, let's say that with a certain CO_2 treatment 50% of the hemichannels of each oocyte remain open, i.e., open hemichannel probability, $P_0 = 0.5$; then, if hemichannels gate independently, cell-cell channel open probability is: $P_0^2 = 0.25$, i.e., 75% of the cell-cell channels are closed. In our case, since we know that for example in homotypic R/N-R/N pairs CO_2 closes ~93% of the cell-cell channels (~7% of the channels remain open), we can predict that ~26.5% of the hemichannels of each oocyte are open at any given time, as $\sqrt{0.07} = 0.265$. This means that if only the hemichannels of one oocyte were closing, G_j would drop by ~73.5%, rather than by 93%. By knowing the hemichannel open probability for homomeric Cx32 and 5R/N channels following either 3-min or 15-min CO_2 treatment, we can predict the open hemichannel probability in cells coexpressing Cx32 and 5R/N based on different assumptions.

Two different predictions were considered. The first forecasts CO_2 sensitivity based on the assumption that heteromeric hemichannels do not form and, therefore, that the drop in G_j with CO_2 represents the average of the sensitivity of two homomeric hemichannel types (with equal chance of expression), one composed of Cx32 hexamers and the other of 5R/N hexamers. Indeed, the same drop in G_j would be expected if heteromeric channels had formed (as described in Fig. 2) and the CO_2 sensitivity of the heteromeric hemichannels were a weighted average of the sensitivities of the two components (Cx32 and 5R/N). The second prediction forecasts CO_2 sensitivity based on the assumption that heteromeric hemichannels form and that their sensitivity reflects entirely the sensitivity of Cx32. Since 1.6% of the hemichannels are expected to be homomeric 5R/N hemichannels (Fig. 2), in this case our calculation assumes that 1.6% of the hemichannels are as sensitive as 5R/N and 98.4% are as sensitive as Cx32. As shown in Fig. 4, the behavior of monoheteromeric and biheteromeric channels corresponds more closely to the second prediction. This indicates both that heteromeric hemichannels form and that their gating sensitivity is not a weighted average of the sensitivities of the connexin monomers that they contain, but rather that Cx32 has a dominant effect on the gating behavior of heteromeric connexons. This would mean that the presence of even one Cx32 monomer per hexamer is sufficient to inhibit the sensitivity of the other five 5R/N monomers.

These data suggest at least two possible interpretations. One is that connexin cooperativity is needed for efficient hemichannel gating. The other is that an accessory molecule gates the channel by interacting with the connexon, and that the presence of even one set of C_1 positive charges per connexon is sufficient for inhibiting this interaction. The latter possibility could be consistent with previous evidence for the participation in chemical gating of soluble intermediates such as calmodulin (Peracchia, 1988; Peracchia et al., 1996).

Does wild-type Cx32 have a dominant effect on the CO_2 sensitivity of heterotypic 32-R/N cell-cell channels as well? To test this hypothesis we have compared the data from 32-R/N channels with two predictions. One forecasts CO_2 sensitivity based on the assumption that two hemichannels of a cell-cell channel do not influence each others' CO_2 gating sensitivity (independent hemichannel gating). The other forecasts CO_2 sensitivity based on the assumption that the hemichannel containing wild-type Cx32 has a dominant effect on the sensitivity of the 32-R/N cell-cell channel. Figure 5 shows that the sensitivity of heterotypic 32-R/N channels corresponds more closely to the first prediction, indicating that the two hemichannels of a cell-cell channel may not influence each other in CO_2 gating sensitivity. Although we have not performed a detail evaluation of voltage sensitivity, preliminary data indicate that neither R/N-R/N channels nor any of the heteromeric and heterotypic channels tested differ significantly in transjunctional voltage sensitivity from channel made of wild-type Cx32 (Wang et al., 1996; Wang & Peracchia, 1996).

In conclusion, Cx32 and its mutant 5R/N have dramatically different gating sensitivities to 100% CO_2, the former being much less sensitive than the latter. Heteromeric hemichannels generated by Cx32 and 5R/N have a low CO_2-gating sensitivity, similar to that of homomeric Cx32 hemichannels. Thus, Cx32 seems to have a dominant effect on the behavior of heteromeric hemichannels, even when only one Cx32 monomer is present in the hexameric connexon. Since the only difference between Cx32 and its 5R/N mutant is the absence, in the latter, of 5 positive charges at the initial C-terminus domain (C_1), the data indicate that the presence of just one charged C_1 domain per hemichannel is sufficient for inhibiting gating. The low sensitivity of heteromeric hemichannels suggests that efficient gating may require either cooperativity among all the monomers of a hexameric connexon or the interaction of accessory molecules with the connexon. In contrast, the behavior of heterotypic (32-R/N) cell-cell channels is similar to that predicted by assuming independent gating of the two hemichannels of a cell-cell channel. This suggests that the inhibitory influence of wild-type Cx32 on CO_2 gating sensitivity does not extend (across the gap) to the partner hemichannel of a cell-cell channel.

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