

Intercellular channels in teleosts: functional characterization of two connexins from Atlantic croaker

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Abstract Gap junction channels, composed of protein subunits termed connexins, are believed to play a critical role in the process of oocyte differentiation and maturation. We have used the paired *Xenopus* oocyte assay to characterize functionally two connexin genes, connexin-32.2 and connexin-32.7, recently cloned from the ovary of the Atlantic croaker (*Micropogonias undulatus*), a species that has emerged as a useful model to study the process of maturation of the ovarian follicle. We have found that, while both connexin proteins were expressed at comparable levels in *Xenopus* oocytes, only one, connexin-32.2, was functionally competent to induce the formation of intercellular channels. Connexin-32.2 channels exhibited voltage-dependent closure that was similar to, but distinct from that of previously characterized mammalian connexins. In addition, the silent connexin-32.7 was unable to functionally interact with connexin-32.2, either in heterotypic channels or as dominant negative inhibitor. Because connexin-32.2 expression is strikingly regulated during oocyte maturation, these data provide further evidence for a role of intercellular channels in the control of oocyte–follicular cell interactions.

Key words: Gap junction; Intercellular communication; *Xenopus*; Oocyte; Voltage

1. Introduction

The programmed switches of cellular activities in embryonic and adult organisms require the co-ordinated response of groups of cells to appropriate stimuli. One way to achieve tighter cellular control is by regulating the direct exchange of ions, metabolites and other messenger molecules between adjacent cells through the intercellular channels clustered at gap junctions [1]. These pathways result from the assembly of two half-channels, or connexons, that align in the extracellular space to delineate a hydrophilic channel bridging the cytoplasm of two contacting cells [2]. The structural subunits of connexons, termed connexins, constitute a multigene family of proteins that have been characterized in a variety of molecular and physiological studies [3,4]. A crucial role of direct cell-to-cell

coupling through intercellular channels has been demonstrated in several dynamic processes both in excitable (reviewed in [1,3,5]) and non-excitable tissues (reviewed in [1,6,7]).

An interesting case where signalling through intercellular channels appears to be of importance in ensuring timely cellular responses is the differentiation and maturation of the ovarian follicle in vertebrates [8–11]. However, several important issues remain to be addressed. For example, while some evidence indicates that abrogation of coupling between follicular cells is required for the onset of oocyte maturation [12,13], other studies suggest that a persistence of coupling is needed [14,15]. In addition, while it is clear that coupling and uncoupling between follicular cells and the oocyte are developmentally regulated, the temporal sequence of these events has not been clearly defined [16,17]. The Atlantic croaker (*Micropogonias undulatus*) has recently emerged as an important in vitro model to study the mechanisms of oocyte differentiation and maturation under physiological conditions (reviewed in [18]). In this species, granulosa cell–oocyte gap junctions disappear after completion of follicular growth and reappear during the early stages of gonadotropin-induced oocyte maturation. Furthermore, the reappearance of these heterocellular gap junctions coincides with the acquisition of maturational competence [17].

Recently two connexin genes, connexin-32.2 (Cx32.2) and Cx32.7, have been cloned from Atlantic croaker ovary and shown to be developmentally regulated during the acquisition of oocyte maturational competence [19]. The purpose of this study was, therefore, to determine the functional characteristics of these two genes by expressing Cx32.2 and Cx32.7 in pairs of *Xenopus* oocytes. Our data report the first functional characterization of fish connexins and demonstrate striking differences in their functional competence.

2. Materials and methods

2.1. Molecular cloning, in vitro transcription and translation of fish connexins

Molecular biology protocols and reagent sources were essentially as described previously [20]. Cx32.2 (a 934 bp fragment obtained by *Bam*HI digestion of the 1.3 kb cDNA clone) and Cx32.7 (a *Not*I fragment spanning the entire 1.2 kb cDNA clone) were excised from pBluescript clones [19], blunted with DNA polymerase I (Klenow fragment), gel purified with Gelase (Epicentre Technologies, Madison, WI) and subcloned into the *Bgl*II site of the expression vector pSP64T [21]. Constructs were linearized with restriction endonucleases and capped cRNAs were produced in vitro with SP6 RNA polymerase, using the mMessage mMachine kit (Ambion Inc., Austin, TX) according to the manufacturer's instructions. The purity and yield of transcribed cRNAs were determined by comparison of the intensity of ethidium bromide staining to a known RNA standard ladder (Gibco, Grand

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Island, NY), following agarose gel electrophoresis. Aliquots (50–100 ng) of in vitro synthesized connexin cRNAs were translated (1 h at 37°C) in a rabbit reticulocyte lysate, following previously described protocols [20,22]. Radioactive products were separated by electrophoresis on a 13% SDS-polyacrylamide gel and visualized by fluorography.

2.2. Isolation of *Xenopus* oocytes and in vivo labelling of connexin proteins

Oocytes were prepared from *Xenopus laevis* females and processed for the paired oocyte expression assay as described [22,23]. After collagenase digestion and defolliculation, all subsequent steps were carried out at 18°C in modified Barth's (MB) medium [22,24]. For biochemical analysis, [³⁵S]methionine labelling of oocytes was carried out as previously described [22,24], except that each cell received 2 µCi of label together with water or cRNA. Approximately 1/20 of one oocyte was loaded onto each lane of a 13% SDS-polyacrylamide gel. Labelled proteins were visualized by fluorography. For physiological analysis, manually defolliculated oocytes were injected with an antisense oligonucleotide corresponding to a portion of the coding sequence of *Xenopus* Cx38 (3 ng/oocyte: 5'-CTGACTGCTCGTCTGTCCACACAG-3') [25], in order to eliminate the possible contribution of endogenous intercellular channels to the measured conductance [24]. Following an overnight incubation at 18°C, each antisense-treated oocyte was then injected with 40 nl of either water or cRNA (2–4 ng/cell).

2.3. Electrophysiological measurements of junctional currents

Micro-injected oocytes were immersed for a few minutes in hypertonic solution to strip the vitelline envelope [26], transferred to Petri dishes containing MB medium and manually paired with the vegetal poles apposed. Intercellular communication was quantitated by double voltage clamp 24–48 h after pairing [27]. Voltage clamping of oocyte pairs was performed using two GeneClamp 500 amplifiers (Axon Instruments, Foster City, CA) controlled by an IBM-PC compatible computer through a Digidata 1200 interface (Axon Instruments), as previously described [28]. Briefly, both cells of a pair were initially clamped at -40 mV to ensure zero transjunctional potential. Transjunctional potentials of increasing amplitude and opposite polarity were generated by hyperpolarizing or depolarizing one cell in 10–20 mV steps, while clamping the second cell at -40 mV. Current delivered to the cell clamped at -40 mV during the voltage pulse was equal in magnitude to the junctional current, and was divided by the voltage to yield the conductance. To ensure adequate control of voltage across the transjunctional membrane and avoid the risk of overestimating the actual junctional conductance at steady-state [29], oocyte pairs exhibiting conductance less than 5 µS were selected for analysis of voltage dependence. Following the imposition of voltage steps, initial currents were resolved at 5–10 ms, and steady state currents were measured at 30 s. Initial and steady state conductance values were normalized to their value at ± 10 mV, and plotted against the transjunctional potential. Data were fit to a Boltzmann equation of the form: $G_{jss} = (G_{jmax} - G_{jmin}) / \{1 + \exp(A[V - V_0])\} + G_{jmin}$, where G_{jss} is the steady state conductance, G_{jmax} is maximum conductance, G_{jmin} is minimum conductance, A is the cooperativity constant and V_0 is the voltage at which the decrease in G_{jss} is half-maximal [27].

3. Results and discussion

The two croaker connexin cDNAs were used as templates in transcription reactions to produce cRNAs for the paired oocyte assay. The translational competence of transcripts was initially examined in a rabbit reticulocyte lysate system supplemented with [³⁵S]methionine. Labelled proteins were separated by SDS-gel electrophoresis. Translation reactions that received connexin cRNAs directed the synthesis of a major polypeptide band, whose electrophoretic mobility (30–35 kDa) was in agreement with the molecular weight deduced from the amino acid sequence (Fig. 1, lanes 2 and 3). In the case of Cx32.2, a second band of faster mobility, presumably a degradation product, was also observed. A translation reaction receiving only water synthesized no major protein products (Fig. 1, lane 1). Connexin

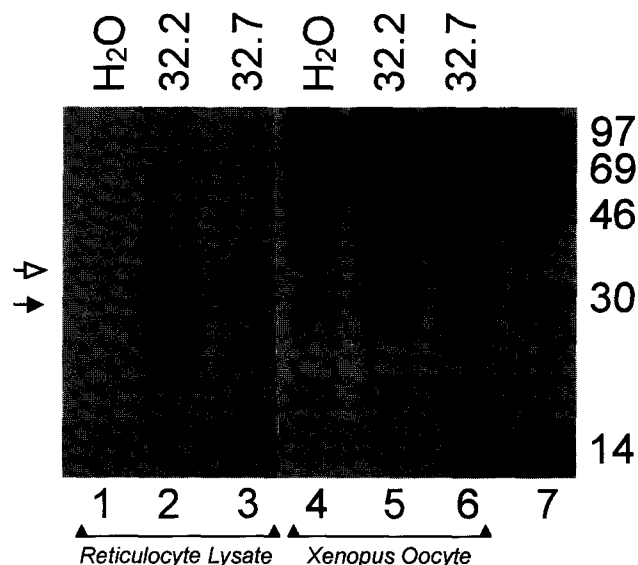


Fig. 1. Translation of Cx32.2 and Cx32.7 cRNAs in vitro and in micro-injected *Xenopus* oocytes. Experimental conditions are indicated on top of each lane. For in vitro translation (lanes 1–3), aliquots of cRNAs (50–100 ng) were incubated with a rabbit reticulocyte lysate in the presence of [³⁵S]methionine. For in vivo labelling (lanes 4–6), *Xenopus* oocytes were co-injected with 40 nl of either water or cRNAs (2–4 ng/cell) coding for Atlantic croaker connexins along with [³⁵S]methionine. Translated products and labelled proteins from oocyte homogenates were separated on a 13% SDS-polyacrylamide gel and detected by fluorography. The connexin-specific bands (open arrow for Cx32.2 and filled arrow for Cx32.7) observed in oocytes exhibited similar SDS-gel mobilities to the in vitro synthesized products. The migration of protein standards is shown in lane 7 with their molecular mass (in kDa) indicated on the right edge of the gel.

cRNAs were subsequently injected into single oocytes to determine whether similar products were synthesized in vivo. Lysates of metabolically labelled oocytes injected with water, that served as controls, produced a characteristic pattern of ³⁵S-labelled proteins (Fig. 1, lane 4) [22,24]. Injection of either Cx32.2 or Cx32.7 cRNA resulted in the appearance of a pronounced novel band with the expected electrophoretic mobility (Fig. 1, lanes 5 and 6). Together, these experiments demonstrate that oocytes can support the biosynthesis of croaker connexins and indicate that similar amounts of injected cRNAs produce similar amounts of labelled proteins.

We used paired oocytes to investigate whether croaker connexins could form functional intercellular channels and to analyze their voltage gating behavior. In order to eliminate the contribution of endogenous intercellular channels to the recorded junctional conductance, all cells were pre-treated with oligonucleotides antisense to *Xenopus* Cx38. The amounts of connexin cRNAs injected were identical to those used for biochemical measurements. As previously reported [20,24,30,31], water-injected oocyte pairs were not coupled (Table 1). In contrast, pairing oocytes that received cRNA encoding Cx32.2 resulted in the formation of intercellular channels. The levels of macroscopic conductance (Table 1) were of the same order of magnitude as those obtained in pairs injected with similar amounts of cRNA encoding mammalian connexins [20,22–24,28,30–33]. The physiological characteristics of channels composed of Cx32.2 was further analyzed in a series of pairs that were injected with more diluted cRNA (40–80 pg/cell) and

displayed conductances not exceeding $5 \mu\text{S}$. This choice was made necessary to avoid overestimation of the actual transjunctional potential due to differences in access resistance [29]. Fig. 2 shows a typical family of junctional currents evoked by voltage steps of opposite polarity and increasing amplitude (A) and plots of normalized conductance (G_j) versus transjunctional voltage (V_j) (B) for Cx32.2 channels. Junctional currents decreased in a time- and voltage-dependent manner (Fig. 2A). While the rate of channel closure increased with increasing V_j , currents decayed slowly in comparison with other well-characterized voltage-gated ion channels [34]. This decay was slightly asymmetric, with positive V_j inducing a greater decline over the duration of the voltage step. A certain degree of asymmetry has also been reported for rat Cx26 [32] and Cx43 [20,31] and attributed to a dependence on the potential difference between the cell cytoplasm and the outside bath. Voltage dependence was quantitated by plotting G_j as a function of V_j . G_j values for both initial (G_{j0}) and steady-state (G_{jss}) were normalized to the maximal conductance measured at the lowest V_j ($\pm 10 \text{ mV}$). G_{j0} was ohmic, showing independence from both size and polarity over the range of V_j tested (Fig. 2B, open circles). In contrast, G_{jss} decreased with increasing V_j of either polarity at relatively large potentials ($>40 \text{ mV}$; Fig. 2B, filled circles). Plots of G_{jss} were fitted to a Boltzmann equation of the form given in section 2. For positive V_j , Cx32.2 pairs had the following parameters: $V_0 = 62 \text{ mV}$; $G_{jmin} = 0.13$; $A = 0.08$. For negative V_j , the following parameters were obtained: $V_0 = -64 \text{ mV}$; $G_{jmin} = 0.24$; $A = 0.11$. Although some of the Boltzmann assumptions may not be met by all intercellular channels [35,36], this analysis still provides useful parameters for comparing the responses of different connexins when expressed in the same experimental system. We have suggested that functional criteria can aid in the determination of homologous gap junction proteins between species [4]. On the basis of its voltage-dependent behavior, Cx32.2 appears to be the functional homologue of rat Cx43, which is also present in the ovary and undergoes dramatic changes of expression during pregnancy [37].

Surprisingly, pairs of oocytes expressing Cx32.7 failed to develop measurable junctional currents. Because levels of protein expression were similar to those of Cx32.2 (Fig. 1), other mechanisms must account for the functional incompetence of Cx32.7. One possibility is that connexons composed of Cx32.7 cannot interact with homologous connexons in the adjacent cell. In fact, the failure of homotypic intercellular channel formation (both connexons composed of the same connexin) has been previously reported [20,38]. However, in one instance, the channel forming ability was restored when heterotypic channels (each connexon composed of a different connexin) were tested

Table 1
Conductances developed by paired *Xenopus* oocytes injected with Atlantic croaker connexin cRNA

Cell injection (cell1/cell2)	Conductance (μS ; mean \pm S.E.M.)	Number of pairs
Cx32.2/Cx32.2	30.21 ± 3.68	28
Cx32.7/Cx32.7	0.09 ± 0.08	17
Cx32.2/Cx32.7	0.02 ± 0.01	12
H ₂ O/H ₂ O	0.01 ± 0.01	14

Antisense-treated oocytes were stripped of their vitelline envelope and manually paired 24–48 h before performing measurements of junctional conductance by dual voltage clamp.

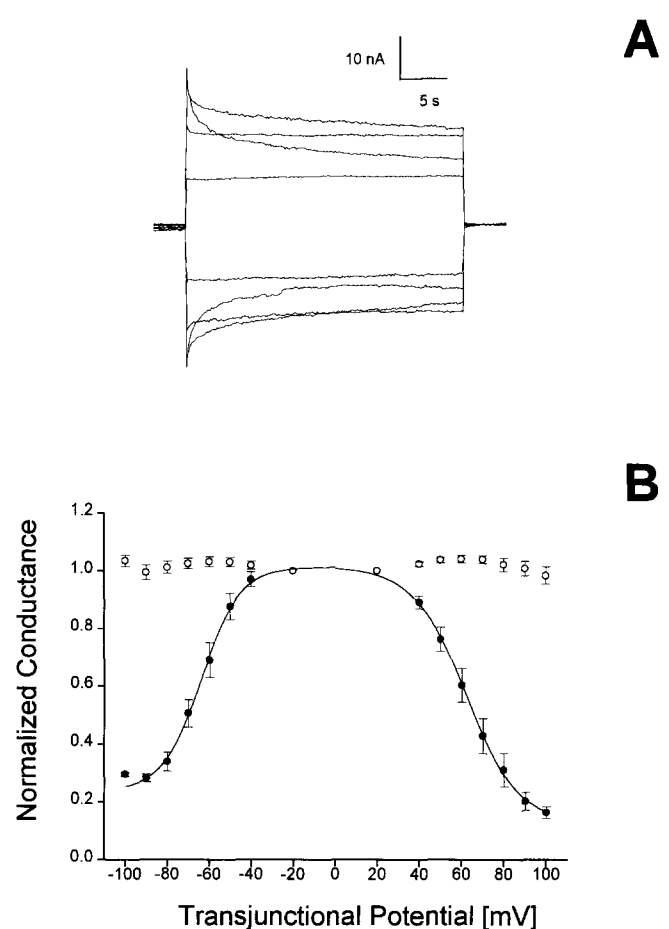


Fig. 2. Voltage-dependent gating of intercellular channels composed of Cx32.2. Pairs of *Xenopus* oocytes were studied by dual voltage clamp 24–48 h after pairing, as detailed in section 2. The two paired oocytes were initially clamped at -40 mV to ensure zero transjunctional voltage (V_j). While one cell was held at a constant potential, V_j steps of increasing amplitude and opposite polarity were sequentially imposed to the other cell and the resulting junctional currents (I_j) and conductance (G_j) were recorded. (A) Time-dependent decay of I_j induced by V_j steps of the duration of 30 s, applied in 20 mV increments. (B) Plots describe the relationship of V_j to initial (G_{j0} , open circles) and steady-state (G_{jss} , filled circles) junctional conductance. Both G_{j0} and G_{jss} were normalized to the values obtained at $\pm 10 \text{ mV}$. Results are shown as mean \pm S.E.M. of 5 oocyte pairs, whose conductance was $2.5 \pm 1.1 \mu\text{S}$ (mean \pm S.E.M.). Smooth curves represent the best fit to Boltzmann equations, whose parameters are given in the text.

[20]. Therefore, we paired oocytes expressing Cx32.2 with those expressing Cx32.7 but found that these pairs were also devoid of intercellular channels. Thus, Cx32.2 was unable to functionally 'rescue' Cx32.7. If only heterotypic interactions are allowed to Cx32.7, then Cx32.2 is not a compatible partner. Another possible explanation is that Cx32.7 does not form functional channels but regulates communication by interfering with other connexins in a dominant inhibitory fashion. In support of this hypothesis, a preliminary report suggests that rat Cx33, which does not form functional channels in paired oocytes [20], may act as a dominant negative inhibitor of intercellular communication [39]. Therefore, we paired oocytes co-expressing Cx32.2 and Cx32.7 but found that Cx32.7 does not interfere with the functional competence of Cx32.2 ($n = 5$). Finally, the possibility remains that cell-specific post-translational modifications, not

carried out in *Xenopus* oocytes, are needed to confer Cx32.7 functional competence. For example, it is clear that oocytes do not phosphorylate some connexins as extensively as observed in vivo (reviewed in [40]). However, those phosphorylations are not required for the formation of functional intercellular channels [20,24,30,31,41,42].

In conclusion, we have used the paired oocyte assay to present the first functional characterization of two teleost connexins, Cx32.2 and Cx32.7, that are expressed in the ovary of Atlantic croaker. We have demonstrated that Cx32.2 was competent to form functional intercellular channels whereas Cx32.7 was not. While the precise role of intercellular channels during the course of follicular growth and oocyte maturation is still unclear, the ovarian follicle contains homologous (between granulosa cells) and heterologous (oocyte-granulosa cells) gap junctions that undergo dramatic changes following specific hormonal stimuli [8–10,15–17,37]. In the Atlantic croaker it has been found that the acquisition of maturational competence correlates with the induction of Cx32.2 mRNA and with the re-establishment of heterologous gap junctions [17,19]. It is noteworthy that this connexin exhibited functional competence in the paired oocyte assay. This finding is consistent with a critical role for gap junctions during the activation of the ovarian follicle in response to gonadotropin induction. At present the role of Cx32.7 remains puzzling. More studies are needed to determine whether the ovary expresses other connexins that could either function as compatible partners for heterotypic interactions or could be inhibited in a dominant fashion.

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