Formation of Chimeric Gap Junction Channels in Mammalian Ovarian Follicle

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ABSTRACT

The oocyte and its surrounding granulosa cells co-exist in a closed compartment called a follicle, although they receive many signals from other parts of the body. It is well established that the intercellular communications between the oocyte and granulosa cells are required for normal oocyte development and ovulation during folliculogenesis. Gap junctions are intercellular channels allowing the direct transmission of ions and small molecules between coupled cells. Several lines of studies have shown that multiple connexins (Cx, subunits of gap junction) are expressed in mammalian ovarian follicles. Among them, two major connexins Cx37 and Cx43 are expressed in different manner. While the gap junction channels formed by Cx37 are localized between the oocyte and encompassing granulosa cells, the intercellular channels by Cx43 are located between granulosa cells. In this review, I will summarize the general properties of gap junction channels and discuss their possible formation (or compatibility) of intercellular channels formed by the oocyte and granulosa cells.

(Key words : Oocyte, Granulosa cell, Gap junction, Connexin, Intercellular channel)

INTRODUCTION

Gap junctions (or intercellular channels) are the conduits allowing the direct transmission of ions, second messengers, and cellular metabolites between coupled cells. Although they are originally observed and known as electrical synapses (Furshpan and Potter, 1959), they are now widely accepted as the structural basis for facilitating the direct intercellular communication between adjacent cells in whole body. Gap junctions are widely distributed in both excitable and most non- excitable tissues in all animals. These intercellular channels also have been found in mammalian ovarian follicles as early as primordial follicle stage. In follicle, the coordination between the oocyte and granulosa cells is bidirectional (reviewed in Eppig, 2001). The signals and nourishment from granulosa cells are required for oocyte development, whereas the factors from the oocyte are essential for granulosa cell development and function. Although the communications between the oocyte and granulosa cells are paracrine, intercellular communications mediated by gap junction channels are also required. So far, seven connexins (abbreviated as Cx, subunits of gap junction channel) have been identified in mammalian ovarian follicles. Cx37 and Cx43 proteins appear to be localized on the plaque (clusters of gap junction channels) between the oocyte and surrounding granulosa cells. Other connexins (e.g. Cx26, Cx30.3, Cx32, Cx40, and Cx45) also appear to be located on the sites between granulosa cells, though the expression levels of those connexins are relatively lower than those of Cx37 and Cx43.

Multiple connexins expressed in two types of cells (oocyte and granulosa cell) and the nature of gap junction channel formation would theoretically result in many different types of gap junction channels between the oocyte and granulosa cell, if the assembly of intercellular channels occurs among different connexins subunits. This hetero-oligomerization (referred as 'chimeric' in this review, however, most gap junction biophysicists favor the use of term 'heteromeric' and 'heterotypic') of subunits to form a complete gap junction is still possible in real system. The issue of 'Chimeric' gap junction channels will be discussed in later part of this review.

STRUCTURE OF GAP JUNCTION

The head to head union of two hemichannels (connex-

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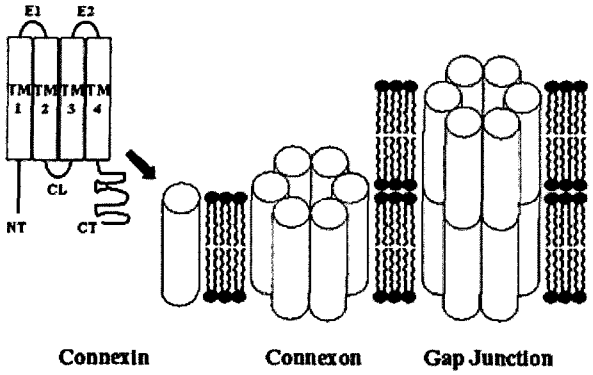

Fig. 1. A connexin is composed of four membrane-spanning segments (TM1, TM2, TM3, and TM4), two extracellular loops (E1 and E2), a cytolplasmic loop (CL), and intracellular amino and carboxyl termini (NT and CT respectively). A connexon (also called hemichannel) is comprised of six connexin subunits. The head to head union of two connexons in series forms a complete gap junction.

ons) in series, each of which is composed of six connexin subunits, forms a complete gap junction (Fig. 1). The currently accepted membrane topology of connexins is four transmembrane spanning segments (TM1, TM2, TM3, and TM4), two extracellular loops (E1 and E2), a cytoplasmic loop (CL), and intracellular amino and carboxyl termini (NT and CT respectively).

The most detailed three-dimensional density map was recently obtained from analyzing low-dose images of frozen-hydrated, tilted two-dimensional crystals of truncated Cx43 proteins using oleamide to induce closure of gap junctions (Unger et al., 1999). The data showed that gap junction had a thickness of about 150 Å. The outer diameter was about 70 Å within the membrane region, and then decreased to about 50 Å in the extracellular region. The pore of the channel was tapered. The channel narrowed from about 40 Å to about 15 Å in proceeding from the cytoplasmic to extracellular side. Within extracellular region, the pore then widened again to about 25 Å in diameter. In addition, 24 circular densities per hexameric connexon consistent with four a-helices per subunit were resolved. This finding confirmed the accepted topology of vertebrate gap junction channels. Furthermore, two transmembrane domains in each subunit were tilted, and thus twelve transmembrane helices contributed to the lining of the pore.

CONNEXIN GENE FAMILY

Since first connexin gene (connexin32, Cx32) has been identified from rat liver tissues (Paul, 1986), more than 20 members of the connexin gene family have been identified in vertebrates (reviewed in Sohl and Willecke, 2004). They include Cx23, Cx25 (in human), Cx26, Cx29 (Cx30.2 in human), Cx30, Cx30.2 (Cx31.9 in human), Cx30.3, Cx31, Cx31.1, Cx32, Cx33 (in mouse), Cx36, Cx37, Cx39 (Cx40.1 in human), Cx40, Cx43, Cx45, Cx46, Cx47, Cx50, Cx59 (in human), and Cx57 (Cx62 in human). Each connexin is referred to by its predicted molecular weight in kilodaltons (kDa) (Beyer et al., 1987) although an alternative nomenclature has been proposed (Kumar and Gilula, 1996). Except Cx31.1 (Hennemann et al., 1992) and Cx33 (Chang et al., 1996), all other connexins form functional intercellular channels when examined in *Xenopus* oocytes expression system.

Connexins are expressed in overlapping pattern, with most tissues expressing more than one connexin type. Tissue distribution profiles indicate that each connexin gene has its own unique expression pattern. For example, Cx43 is widely expressed in several organs by many cell types whereas Cx30.3, Cx31, Cx31.1 and Cx33 display a much more restricted distribution (reviewed in Bruzzone et al., 1996). Multiple connexins (e.g. Cx26, Cx30.3, Cx32, Cx37, Cx40, Cx43 and Cx45) have been identified in ovarian tissues obtained from different mammalian species (Grazul-Bilska, et al., 1997). Among them, Cx37 and Cx43 are the major subunits forming the intercellular channels in ovarian follicles.

MODULATION OF GAP JUNCTION

Gap junction channels can be modulated by various stimuli, which include pH, calcium and voltage and by chemical modification (phosphorylation). Several growth factors and hormones have also been reported to modulate intercellular coupling (Massa, et al., 1998; Hossain et al., 1998, 1999; Moorby and Gherardi, 1999; Patino and Kawaga, 1999). Modulation may be accomplished by rapid changes in conformation of intercellular gap junction channels (i.e. gating) or by changes in the expression of connexin proteins leading to changes in numbers of gap junctions. In this context, it should be noted that the normal turnover rate of gap junctions is relatively fast for a membrane protein. Half-lives of 1~2 hours have been reported (Laird et al., 1991).

Physphorylation of Cx43

The regulation of junctional communication by phosphorylation has been studied primarily in channels formed by Cx43 (reviewed in Hertzberg et al., 2000). Lau et al. (1992) showed that epidermal growth factor (EGF) not only inhibited gap junctional communication but also enhanced phosphorylation of Cx43. Warn-Cramer et al. (1996 and 1998) demonstrated that mitogen-activated protein (MAP) kinase phosphorylated Cx43 at three site (S255, S279 and S282) and that activation of MAP kinase by EGF stimulation was sufficient to inhibit junctional

communication. Moreno et al. (1994) reported that the unitary conductance of Cx43 channels expressed in SKHep1 cells depended upon the phosphorylation of the channel. Taken together, these studies suggest that phosphorylation of Cx43 channels implies the regulatory mechanism for the gating of the gap junction channels.

IONIC SELECTIVITY AND MOLECULAR PERMEABILITY

Although the pore sizes of the gap junction channels are relatively larger than those of other cellular channels (e.g. potassium and sodium channels), gap junction channels have their own properties of selectivity and permeability. Both electrical (measuring the ionic currents) and molecular devices (spreading the fluorescent dyes) are widely used to approach these issues. Simpson et al. (1977) reported that an insect gap junction was permeable to molecules up to 1.8 kDa. Subsequently, Flagg-Newton et al. (1979) reported that the upper limit of permeability of vertebrate gap junctions expressed in "rat fibroblast line B" was about 0.9 kDa using a series of fluorescent amino acid and peptide molecules differing in size and charge. They also demonstrated that insect gap junction channels allow the passage of substantially larger molecules than their vertebrate counterparts. Despite having similar morphology and functional properties, vertebrate and invertebrate gap junction proteins are encoded by two unrelated gene families, termed 'connexins' and 'innexins' respectively. Generalizations based on comparative properties of gap junctions expressed in cell lines and tissues are perhaps less meaningful now, as there are more than 20 members of the vertebrate connexin gene family. Channels comprised of different members of the connexin gene family are known to have different permeabilities. For example, Bevans et al. (1998) reported that Cx26 channels have a smaller exclusion limit than those formed with Cx32 in a liposome reconstitution system. Brink and Dewey (1980) showed that fixed charge, rather than just the size of the probe could influence the measured permeability of gap junctions. The properties of the gap junction channels formed by Cx37 and Cx43 are briefly summarized as below.

Cx37 Gap Junction Channels

Cx37 channel forms a channel which is strongly cation-selective and permits passage of cations of different size with differing mobilities. Cx37 channel fails to spread Lucifer Yellow (LY, an anionic dye) from cell to cell (Waltzman, 1996). Cx37 channel has a smallest pore diameter with a largest ionic conductance. The gap junction channels formed by Cx37 are required for the oocyte development and timely ovulation (Simon et al., 1997). Mice lacking Cx37 gene show multiple defects in ovarian follicles, including loss of coupling between the oocyte and cumulus cells, arrest of oocyte growth, absence of mature Graafian follicles and ovaries resembling corpora lutea.

Cx43 Gap Junction Channels

Cx43 channels are non-selective. Most charged and uncharged fluorescent dyes can pass through the gap junction channels formed by Cx43. These biophysical properties are relevant to the pore dimension of Cx43 channel which has a largest pore diameter. Several reports have supported that Cx43 channel is required for granulosa cells to proliferate. Homozygous mutant mice lacking Cx43 are neonatal lethal with a several heart defect (Reaume et al., 1995). Neonatal ovaries cultured *in vitro* showed that postnatal folliculogenesis were retarded (Juneja et al., 1999). In graft experiments, Cx43-deficient ovaries failed to be developed while wild-type ovaries were developed a full range of follicles from primordial to antral follicles (Ackert et al., 2001).

INTERCELLULAR CHANNEL FORMATION

Connexons (hemichannels) can be composed of either the same connexin subunits (homomeric) or different connexin subunits (heteromeric). Intercellular channels formed by identical connexons are termed 'homotypic' channels, whereas intercellular channels formed by two different connexons are termed 'heterotypic' channels (Fig. 2). Since many cell types express more than one type of connexins, many combinations of heteromeric connexons and heterotypic intercellular channels are theoretically possible.

Heterotypic Channels

The ability of homomeric connexons to form heterotypic channels has been studied in some detail in exogenous expression systems (*Xenopus* oocytes and/or transfected cell lines) (Verselis and Veenstra, 2000). In some cases the ability of connexons to pair appears to be regulated in some unknown manner. For example, *Xenopus* oocytes form Cx43/Cx38 heterotypic junctions but not form Cx38/Cx38 homotypic junctions in some culture conditions. Heterotypic compatibilities among connexins expressed in ovarian follicles are summarized in Table 1.

Heteromeric Channels

Several lines of studies have supported the possibility that heteromeric channels can form both in native tissues and in exogenous expression systems. Kuraoka et al. (1993) showed intermingled expression domains of

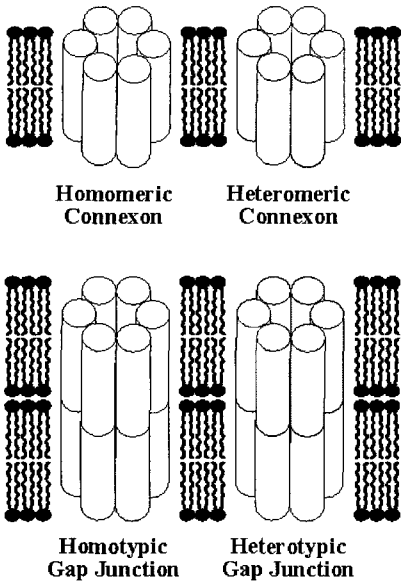

Fig. 2. Connexon can be composed of either the same connexin subunits (homomeric) or different connexin subunits (heteromeric). A complete gap junction channel formed by identical connexons is termed 'homotypic' channel, whereas an intercellular channel formed by two different connexons is termed 'heterotypic' channel.

Table 1. Heterotypic compatibilities among connexins expressed in ovarian follicles

	Cx26	Cx30.3	Cx32	Cx37	Cx40	Cx43	Cx45
Cx26	o						
Cx30.3	-	o					
Cx32	+	-	o				
Cx37	-	+	-	o			
Cx40	-	+	-	+	o		
Cx43	-	+	-	+	+	o	
Cx45	-	-	-	+	+	+	o

Formation of homotypic channels (o), formation of heterotypic channels (+), no formation of heterotypic channels (-).

Cx26 and Cx32 in same plaque of hepatic gap junctions of rat and guinea pig by double-labeling immunoelectron microscopy. They suggested that this expression pattern reflected the presence of heteromeric connexons although other interpretations are equally feasible. Stauffer (1995) reported that connexons purified by gel filtration from insect cells co-transfected with Cx26 and Cx32 were labeled with both Cx32 and Cx26 antibodies. Heteromeric connexons composed of Cx46 and Cx50 were also found in ovine lens (Jiang and Goodenough, 1996). In this case, immunoprecipitation of ovine lens connexons with antibodies against Cx46 were demonstrated to contain Cx50 by Western blots (and vice versa). Elenes et al. (1999) provided the evidence that connexons expressed in canine right atria contained Cx40 and Cx43 subunits. Reconstitution of isolated liver gap junctions into liposomes showed that heteromeric Cx26/Cx32 hemichannels had different permeability properties from those of homomeric forms (Bevans et al., 1998).

Heteromeric assembly of connexins is likely to be an interesting problem in that it may be regulated at several levels. These may include differences in the sites and timing of connexon assembly and specific protein interactions among different connexin subunits. If the assembly of connexins into connexons takes place in the endoplasmic reticulum(as the assembly of most membrane proteins does), then it is most likely that the heteromeric channels would form according to the ratio of available subunits. However, it is possible that different connexins may be assembled in different cellular compartments. Musil and Goodenough (1993) showed that the assembly of Cx43 into hexamers occurs in the trans-Golgi network in NRK cells, CHO mutant cells, and *Xenopus* oocytes. Cx32 has been reported to oligomerize in the endoplasmic reticulum of BHK cells (Kumar et al., 1995). Thus, it would be unlikely that heteromeric connexons containing Cx43 and Cx32 subunits would be found if these sites of assembly for Cx43 and Cx32 were conserved across all cell lines.

Heteromeric Channels between Cx37 and Cx43 in Exogenous System

The single channel properties of transfected N2A cell lines co-expressing both Cx37 and Cx43 were examined (Brink et al., 1997). In this system, it was possible to compare single channel properties (i.e. conductance and gating) obtained from those channels (both homotypic and heterotypic channels) when both connexins were co-expressed. Multiple types of channels that could not be explained by the formation of homotypic and heterotypic channels were indicative of the existence of the heteromeric channels.

'Chimeric' Channel Formation in Ovarian Follicles

The observations of immunoelectron microscopy have shown that Cx37, Cx45 and Cx43 subunits formed both homotypic and heterotypic gap junction channels in the mouse cumulus-oocyte complex (Kidder and Mhawi, 2002). Homotypic Cx37 channels (Cx37/Cx37) and heterotypic channels comprised of Cx37 and Cx43 (Cx37/Cx43) appeared on the surface of oocyte, while homotypic Cx43 channels (Cx43/Cx43) and heterotypic channels with Cx43 and Cx45 (Cx43/Cx45) appeared between cumulus cells. Additional connexins (e.g. Cx26, Cx32, Cx30.3, and Cx43) are known to be present in cumulus cells, but their distributions have not been determined at the ultrastructural level.

Confocal immunofluorescence microscopy and oocyte preloading functional assay using mouse cumulus-oocyte complex have shown that homotypic gap junction cha-

nnels formed by Cx37 are required for the intercellular communications between the oocyte and cumulus cells (Veitch et al., 2004). Lucifer yellow (fluorescent dye commonly used for studying the intercellular coupling) was transferred from the oocyte (preloaded with dye) to the cumulus cell layers, whereas dye was moved to only the first layer of cumulus cells obtained from Cx43-deficient mice. Wild-type oocytes (preloaded with calcein, another fluorescent dye) transferred dye to wild-type granulosa cells whereas no dye transfer occurred when Cx37 was missed from either the granulosa cells or the oocyte.

Lucifer yellow is a negatively charged molecule (charge of -2) and calcein is more strongly charged (net charge of -4). Because homotypic gap junction channels formed by Cx37 are markedly cation-selective (Waltzman, 1996), it is unlikely that both dyes (Lucifer yellow and calcein) can easily pass through Cx37 channels. Instead, dye coupling between the oocyte and cumulus cells in wild-type follicles can be mediated by gap junction channels formed by either Cx43 and/or undetermined connexins. The studies showing both Cx26 and Cx43 express in the oocyte of different species (Ithana et al., 1996; Grazul-Bilska et al., 1998; Johnson et al., 1999; Granot et al., 2002) support this idea.

Alternative mechanism for dye transfer could be explained if heteromeric gap junction channels formed by Cx37 and other connexins are located on the site between the oocyte and cumulus cells. Although the expression of Cx37 is predominated in the oocyte, small portion of other connexin subunits can form hetermeric channels with Cx37 subunits. A hetermeric connexon comprised of 5 of Cx37 subunits and 1 of unknown connexin subunits is theoretically predicted if the expression of Cx37 is twenty times more than that of other connexin and the assembly of connexon randomly occurs (binomial distribution). The biophysical behavior of the resulting 'chimeric' channel containing 5 of Cx37 subunits and 1 of other connexin subunits can be changed (from strong cation- selective to mild cation- or non-selective). This prediction is supported by a recent report characterizing the gating polarity of heteromeric channel comprised of 5 of wild- type Cx32 subunits and 1 of mutant subunits (Oh et al., 2000). While homomeric Cx32 channels were closed by negative potential, heteromeric Cx32 channels were closed by both negative and positive potentials. The data imply that a single subunit is sufficient for changing the behavior of entire channel.

CONCLUSIONS

The intercellular communications between the oocyte and granulosa cells are essential for normal oocyte development and timely ovulation during mammalian ovarian folliculogenesis. These intercommunications are mediated by gap junction channels. Although both Cx37 and Cx43 are abundant in ovarian follicles, other connexins are certainly existed in. The expression of multiple connexins in two types of cells suggests that heterooligomerization of connexin subunits to form a complete gap junction channel might be occurred. These 'chimeric' gap junction channels formed by different types of connexin subunits are hard to be defined using the conventional biochemical and molecular approaches due to the limitation of resolution. The identification of these chimeric' channels is feasible if aided by electrophysiological measurements. The biophysical characterizations of chimeric' gap junction channels obtained from the exogenous expression systems (e.g. transfected cell lines) will give a clear evidence for the existence of functional chimeric' channels. It will be interesting to examine how connexins are targeted to same and/or different assembly sites and if this process occurs in a cell-specific manner and if it can be regulated. Also the distribution of other connexins should be determined at the ultrastructural level. These efforts for defining the types of gap junction channels will provide a more detailed picture for the intercommunications between the oocyte and granulosa cells in ovarian follicles.

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