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Gap junctions and tissue business: problems and strategies for developing specific functional reagents

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SUMMARY

The complex and overlapping tissue distribution of different members of the gap junctional connexin protein family is reviewed. Intermixing of different connexins in the building of intercellular channels and translational and posttranslational regulation of gap junctional channels add additional challenges to the interpretation of the possible functions played by gap junction-mediated intercellular communication in tissue business.

Key words: gap junctions, intercellular communication, phosphorylation

THE PROBLEMS: GAP-JUNCTIONAL CHANNELS ARE BIOCHEMICALLY AND PHYSIOLOGICALLY COMPLEX

Problem A: many proteins and overlapping expression

Gap junctions are membrane channels that directly join the cytoplasms of adjacent cells, forming the basis for synchronous electrical activity between excitable cells (Bennett and Goodenough, 1978; Bennett et al., 1991). Gap-junctional intercellular channels are almost universal between non-excitable cells as well, although their roles in diverse tissue functions, in cellular and tissue homeostasis and in differentiation, are largely unknown. A current challenge to research is to develop strategies for specifically interfering with junctional communication, such that the functional sequellae of junctional blockade can be directly observed. There are several experimental obstacles that make junctional blockade and interpretation of the data difficult.

The junctional intercellular channels are composed of paired connexons, oligomeric protein assemblies in each cell membrane (Caspar et al., 1977; Makowski et al., 1977; Unwin and Ennis, 1984). The connexins, a family of proteins that form the connexons (Beyer et al., 1988, 1990), have a complex, overlapping distribution in different cell types (Beyer et al., 1987, 1989; Bennett et al., 1991). The family can be divided into two general classes, called α and β (Kumar and Gilula, 1992) or I and II (Bennett et al., 1991), on the basis of sequence homology. Many (perhaps most) tissues express more than one type of connexin. In the lens there are three known connexins: connexin43 (Cx43), joining the epithelial cells (Beyer et al., 1989), and two connexins found in the junctions joining the lens fibers, Cx46 and Cx50 (also known as MP70) (Kistler et al., 1985; Paul et al., 1991; White et al., 1992; Tenbroek et al., 1992). Three connexins have been described in the chick heart (Veenstra et al., 1992). In some cases, individual cells express more than one connexin type. In stratified squamous epithelium, for example, there is a complex distribution of connexins (Hennemann et al., 1992a,b; Hoh et al., 1991) that varies as a function of the differentiated state of the cells and with the age of the animal (Risek et al., 1992).

In addition to overlap of expression, the levels of expression of connexins may vary as a function of physiological state. The explosive increase in expression of Cx43 in the myometrium immediately prior to parturition is a classic example, when smooth muscle cells acquire powerful synchronous contractility (MacKenzie and Garfield, 1985; Miller et al., 1989; Risek et al., 1990; Tabb et al., 1992). In a more functionally obscure example, Sertoli cells in the testis show a variation in the levels of Cx43 expression as a function of the cycle of the spermatogenic cells (Risley et al., 1992). The cellular location of Cx33, a testis-specific connexin (Haefliger et al., 1992), and its degree of overlap with Cx43 have not yet been studied.

Problem B: many channels, diverse physiological properties

Gap-junctional channels are generally thought of as weakly selective with respect to ions (Brink and Dewey, 1980; Brink, 1983; Brink et al., 1984; Baker et al., 1985; Verselis and Brink, 1986). Intercellular transfer of second messengers and metabolites has been demonstrated in a variety of cellular systems; for example, data are available that demonstrate a key role for gap junctions in the intercellular spread of calcium waves between glial cells, a long-range signaling phenomenon inducible with glutamate and blocked by conditions that activate protein kinase C (Cornell-Bell et al., 1990; Charles et al., 1991; Enkvist and McCarthy, 1992). A similar spread of calcium waves may be essential for ciliary metachronasy (Moss and Tamm, 1987; Sanderson et al., 1988). This intercellular synchro-

nization may result from passage of either Ca^{2+} or IP_3 between cells through gap junctions (Berridge and Irvine, 1989; Saez et al., 1989).

Data are accumulating from studies with transfected cells and other cell lines that show that the different connexin types have different single channel conductances, generally in the range of 50-150 pS (Jaslove and Brink, 1989; Brink and Fan, 1989; Dermietzel et al., 1991; Eghbali et al., 1990; Moreno et al., 1991a,b; Donaldson and Kistler 1992; Fishman et al., 1990; Moore et al., 1991; Salomon et al., 1992; Rup et al., 1993; Bennett and Verselis, 1992). Larger and smaller conductances are also observed (Spray et al., 1991; Perez-Armendiriz et al., 1991), as well as complex intermediate states, which may result from different conductance states of a single channel (Chen and DeHaan, 1992), simultaneous opening of more than one channel, or from channels consisting of more than one connexin type. This exquisite fine tuning of channel conductance between the different connexin types adds another level of complexity to the possible functions, and additional complexity to the interpretation of specific connexin ablation.

Problem C: selectivity and mixing in connexin interactions

Dahl and Werner (Dahl et al., 1987, 1988) have developed a *Xenopus* oöcyte-pair assay, in which individual oöcytes can be directed to synthesize specific connexins by intracellular injection of the appropriate cRNAs. Pairs of injected oöcytes that have had their vitelline envelopes removed can be manipulated into contact such that they form gap junctions. The functional participation of an endogenous *Xenopus* connexin can be substantively controlled by preinjecting oöcytes with antisense oligonucleotides specific for *Xenopus* Cx38 (Barrio et al., 1991; Willecke et al., 1991; White et al., 1992). Using this assay, it has been possible to demonstrate that heterotypic intercellular channels form between oöcytes expressing different connexins (Swenson et al., 1989; Werner et al., 1989; see also Rook et al., 1992). In some cases, the formation of heterotypic intercellular channels by programming each oöcyte with a different cRNA results in a channel with hybrid electrical characteristics. This has been demonstrated by pairing oöcytes injected with rat connexin32 and -26 cRNAs (Barrio et al., 1991). Cx32 and Cx26 are found naturally intermixed in gap junctions joining rat hepatocytes in situ, albeit with functionally obscure individual gradients across the hepatic lobule. Barrio et al. (1991) have shown that connexins26 and -32 are differently affected by applied voltage when assembled in heterotypic 26/32 pairs as compared to when they are assembled in homotypic 32/32 and 26/26 channels. These data suggest a *trans* effect on electrical properties exerted by one connexin on another in an adjacent cell. The observation that Cx43 injected into one oöcyte is able to recruit endogenous *Xenopus* Cx38 in the other oöcyte of the pair demonstrates that individual connexins may also exert complex *trans* effects on gap junction assembly (Swenson et al., 1989; Werner et al., 1989).

While it is possible for heterotypic channels to form using some connexins, it has become equally clear that other connexins are unable to pair with each other in the *Xenopus* oöcyte assay. This selectivity has been studied in detail in the case of rat connexins 43, 40 and 37 (Bruzzone et al., 1993). The 43/37 and 37/40 heterotypic intercellular channels readily assemble, each showing asymmetric voltage profiles characteristic of each connexin. In contrast, 43/40 heterotypic channels do not form, even though the proteins are expressed at high levels. While the meaning of this selectivity is not clear, it may certainly permit the formation of communication compartments within a given organ where adjacent cells are functionally required to maintain separate domains of homeostasis.

Problem D: regulation of gap junctions at multiple levels

There is a substantial amount of literature that has reported studies of the regulation of gap-junctional intercellular communication. It is almost axiomatic that cells that are joined by low resistance channels must have fail-safe systems to close the channels, should either of the cells undergo injury or apoptosis, since a catastrophic loss of membrane potential of one cell would rapidly depolarize an adjacent cell to which it was junctionally joined. Perhaps related to this fail-safe system are the data that demonstrate that gap-junctional channels between cells are sensitive to increases in intracellular calcium and/or decreases in intracellular pH (Rose and Loewenstein, 1975; Turin and Warner, 1980: Obaid et al., 1983). These injury-related responses are of particular interest to the student of myocardial cells, since ischemic injury may trigger these uncoupling mechanisms by Ca^{2+}, pH or lipid metabolites (Burt, 1987, 1989), and their rapid reversal may represent a critical survival mechanism (Burt et al., 1991; Moore et al., 1991). There are documented experimental and developmental situations when groups of cells establish or terminate intercellular communication in the absence of tissue injury (Potter et al., 1966; Dixon and Cronly-Dillon, 1974; Nagajski et al., 1989; Olson et al., 1991; Olson and Moon, 1992; Warner, 1992). Given the relatively short half-life of certain connexins both in vivo and in vitro (Fallon and Goodenough, 1981; Yancey et al., 1981; Musil et al., 1990a), it is possible that these changes in gap-junctional communication levels are regulated at the level of either transcription or translation. The very close temporal correspondence between the metabolic turnover of Cx32 and the loss of morphological and physiological gap-junctional channels in regenerating liver provides a compelling example (Yee and Revel, 1978; Meyer et al., 1981; Dermietzel et al., 1987; Traub et al., 1989). In some systems, the regulation of connexin gene expression has been studied, and this promises to be an interesting area of investigation in the future (reviewed by Musil and Goodenough, 1990).

Gap-junctional channels can also be regulated post-translationally by a variety of effectors (reviewed by Bennett et al., 1991). In many instances, this regulation is thought to involve phosphorylation of connexins, several of which have been shown to be phosphoproteins (e.g. connexins 32, 43, 46 and 50 (MP70) Swenson et al., 1990; Crow et al., 1990; Musil et al., 1990a,b; Musil and Goodenough, 1991; Filson et al., 1990; Saez et al., 1986; Tenbroek et al., 1992). In non-transformed cells, connexin phosphorylation is largely confined to serine residues (Saez et al., 1986, 1990; Swenson et al., 1990; Musil et al., 1990b). Expression of the transforming tyrosine protein kinase $pp60^{v\text{-}src}$ results in tyrosine phos-

phorylation of Cx43 (but not Cx32) and selective inhibition of Cx43-mediated gap-junctional conductance between *Xenopus* oöcytes (Swenson et al., 1990). Mutation of tyrosine 265 to phenylalanine prevents both tyrosine phosphorylation of Cx43 and loss of gap-junctional conductance in the presence of $pp60^{v\text{-}src}$, demonstrating that Cx43 is a functional substrate for this transforming tyrosine kinase in the oöcyte-pair system. The tyrosine phosphorylation of Cx43 and concomitant loss of cell-cell communication has also been observed in tissue culture cells infected with Rous sarcoma virus (Crow et al., 1990; Filson et al., 1990).

In contrast to tyrosine phosphorylation, the functions subserved by phosphorylation of Cx43 on serine residues are not yet understood. It has been shown that serine phosphorylation to the mature Cx43-P_2 form, which results in a shift in the electrophoretic mobility of Cx43 to a more slowly migrating species, does not occur unless gap junctions are assembled (Musil et al., 1990b). Non-phosphorylated Cx43 can be solubilized with Triton X-100 buffers, leaving Cx43-P_2 in the insoluble pellet (Musil and Goodenough, 1991). Using this fractionation method in combination with pulse-chase and cell surface biotinylation studies, it is clear that nonphosphorylated Cx43 can be exported to the cell surface in a Triton-soluble form prior to assembly into Triton-insoluble gap-junctional maculae. Phosphorylation of Cx43 in NRK cells therefore does not serve as an export signal to target transport of Cx43 through intracellular compartments to the cell surface. This conclusion is indirectly supported by the demonstration that a mutant of Cx43, which had much of its cytoplasmic tail truncated, was able to assemble into functional channels in transfected SKHep cells (Fishman et al., 1991).

Evidence supporting a role for Cx43 phosphorylation in gap junction formation and/or function came from studies with the communication-deficient S180 and L929 cell lines, which lack morphologically or physiologically recognizable gap junctions. In these cells, Cx43 is not phosphorylated to the P_2 form despite being synthesized and degraded with kinetics equivalent to that seen in NRK cells, which both assemble gap junctions and phosphorylate Cx43. Gap-junctional assembly and communication in S180 cells can be 'rescued' by stable transfection with a cDNA encoding the cell-cell adhesion molecule L-CAM (Mege et al., 1988), which results in the phosphorylation of Cx43 to the P_2 form (Musil et al., 1990b). Acute uncoupling of NRK or other communication-competent cells by cytoplasmic acidification or with 1-heptanol does not result in measurable Cx43 dephosphorylation (Musil et al., 1990), suggesting that the experimentally induced fail-safe gating response occurs via mechanisms other than gross changes in Cx43 phosphorylation.

Cx43 cDNA-transfected SKHep cells, which have had intracellular protein kinases stimulated, show differences in single channel conductance and in the kinetics of voltage dependence, suggesting that phosphorylation may alter these electrophysiological parameters (Moreno et al., 1992). Changes in single channel conductance have also been seen in cardiac myocytes expressing endogenous Cx43 in response to stimulation of cyclic GMP protein kinase (Takens-Kwak and Jongsma, 1992). Direct demonstration that a change in gap-junctional single channel conductance is due to Cx43 phosphorylation is experimentally difficult, however, and it is also clear that Cx43 experiences multiple phosphorylations, leaving open the possibility that several functions may be involved. Given the meager knowledge of the function of gap junctions as outlined above, a detailed dissection of the function of connexin phosphorylation is likely to involve students of intercellular communication for some time.

STRATEGIES FOR SPECIFIC ABLATION OF GAP-JUNCTIONAL INTERCELLULAR COMMUNICATION

In addition to changes in intracellular pH and/or calcium levels, gap-junctional intercellular communication is sensitive to exogenous compounds normally not present in a cell's life history, such as alkanols (Johnston et al., 1980) and local anesthetics (Hauswirth, 1968; Burt and Spray, 1989). However, the mechanisms of action of any of these uncoupling agents are largely unknown, and each agent has additional effects on the biology of the cell. It is not possible to use these uncouplers to experimentally determine the roles of gap-junctional communication in tissue biology.

Strategy A: use of specific antisera and peptides

In theory, the intracellular injection of anti-connexin sera would be an effective way to specifically block gap-junctional communication. A few polyclonal antisera directed against one or more connexins have been reported in the literature as being effective in inhibiting gap-junctional communication (Hertzberg et al., 1985; Traub et al., 1989; Yancey et al., 1989), but these have not been used for further functional studies. In one notable exception, Warner et al. (1984) microinjected an active antiserum into embryonic cells in the mouse and the frog and reduced ionic conductance or transfer of the tracer dye Lucifer Yellow (Warner et al., 1984; Lee et al., 1987). When the injected cells included precursors of the anterior central nervous system, abnormalities in the development of the brain, eye and other anterior structures result (Warner et al., 1984). Developmental effects following injection of other lineages have also been reported (Warner, 1985). These results might indicate the involvement of junctional communication in any of a series of tissue interactions, since a block to coupling is present throughout a prolonged and ill-defined period. It is possible, for instance, that gap junctions could play a role in a progression of cell-cell signaling and interactional events that are essential for proper specification of mesoderm, for neural plate induction by the underlying dorsal mesoderm, for neural plate folding, or simply for maintenance of the ectoderm and later neural epithelium. Although Warner and Gurdon (1987) showed in recombination experiments that induction of muscle differentiation in ectoderm is unaffected by inhibiting junctional permeability among cells of the inductively active blastula endoderm, they did not investigate the role of coupling within the responding tissue, nor could they determine whether the pattern of mesodermal induction was perturbed. Preliminary studies have shown the feasibility of using anti-Cx43 antisera directed against putative extracellular domains as a method for blocking gap junction assembly in cultured cells (Meyer et al., 1992). Similarly, channel assembly between *Xenopus*

oöcytes can be blocked using synthetic peptides corresponding to the extracellular loop sequences of Cx32 (Dahl et al., 1992). To date none of these extracellularly targeted reagents have been used for functional studies.

While these studies provide clear information, the methodology of injecting antisera is experimentally difficult with some cell types. In addition, it is difficult to inject whole populations of cells for study. This problem was circumvented by Fraser et al. (1987), who were able to disrupt patterning during regeneration in *Hydra* by loading all the cells with an active anti-gap-junction antiserum using a DMSO procedure.

Strategy B: development of dominant negative inhibitors and transgenic animals

A relatively unexplored possibility for the development of specific inhibitors of gap-junctional function would be a study that focuses on the coöligomerization of different connexins into heteromeric connexons and intercellular channels. *cis* interactions between connexins in the formation of intercellular channels, if they exist, offer the possibility of developing dominant negative constructs that would be of great value in specific knockout of gap junction function. Indeed, it might be feasible to design a dominant negative specific for each connexin, such that the meaning of the overlapping distribution of different connexins could be functionally understood. Design strategies would be to combine sequences between two connexins that are unable to pair in *trans*, such as Cx43 and Cx40, to create a single chimeric molecule capable of physically, but not functionally, interacting with one or both of the parental connexins. An interacting but non-functional connexin might also be designed by selectively deleting or mutating highly conserved regions common to all connexins (Dahl et al., 1992). Once developed, these dominant-negative connexins, under the control of tissue-specific promoters, could be used to construct transgenic animals for functional studies, where a given connexin is ablated in a specific tissue at a defined developmental time. Alternatively, the single-copy connexin genes (Willecke et al., 1990; Hsieh et al., 1991; Haefliger et al., 1992; Hennemann et al., 1992a,b) could be ablated by homologous recombination, denying expressing of a specific connexin in all its developmental and tissue locations.

CONCLUSION

The application of molecular biological methods to the study of intercellular communication via gap junctions has revealed a number of single-copy genes that code for the connexins, a family of oligomeric integral membrane proteins. The cloning of a large number of these genes has opened up the possibility of using genetic strategies to specifically interfere in gap-junctional communication, including development of transgenic animals with individual connexins knocked out by homologous recombination, or of transgenics engineered with connexin-specific dominant negative constructs driven by tissue-specific promoters. The design and interpretation of such experiments will, however, have to take into account several of the complexities of gap junction structure and functional regulation discussed above. First, connexins show a complicated, overlapping tissue and cellular distribution, such that knocking out a specific connexin may well leave cells functionally coupled by other members of the connexin family. Second, knockout of a widely distributed connexin such as Cx43 is both a potentially lethal event and experimentally complex, since this connexin is found not only very early in development (Barron et al., 1989; Nishi et al., 1991; Yancey et al., 1992) but also in a myriad of different tissue locations (Kadle et al., 1991; Ruangvoravat and Lo, 1992), under different regulatory control in different cell types (Risek et al., 1990). As a further complication to the understanding of gap junction function, the degrees to which different connexins assemble into heteromeric connexons, and different connexons into heterotypic intercellular channels, are not yet fully known. Furthermore, genetic approaches do not address the fact that gap junction function can be regulated post-translationally. Despite these complexities, it is anticipated that genetic manipulation of the expression of specific connexins will serve as a powerful tool to elucidate the functions of individual connexins in tissue business.

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