A Particle-Receptor Model for the Insulin-Induced Closure of Connexin43 Channels

Nobuo Homma, José Luis Alvarado, Wanda Coombs, Kathleen Stergiopoulos, Steven M. Taffet, Alan F. Lau, Mario Delmar

Abstract—Connexin43 (Cx43) channels can be regulated by a variety of factors, including low pH. Structure/function studies from this laboratory have demonstrated that pH gating follows a particle-receptor mechanism, similar to the “ball-and-chain” model of voltage-dependent inactivation of ion channels. The question whether the particle-receptor model is applicable only to pH gating or to other forms of Cx43 regulation as well remains. To address this question, we looked at the uncoupling effects of insulin and of insulin-like growth factor-1 (IGF) on Cx43 channels expressed in Xenopus oocytes. These agonists do not induce changes in pH, Junctional conductance (Gj) was measured by the dual 2-electrode voltage-clamp technique. Control studies showed that relative Gj did not change spontaneously as a function of time. Continuous exposure of Cx43-expressing oocytes to insulin (10 μL) led to a decrease in Gj. After 80 minutes, Gj was 54±5% from control (n=12). Exposure of oocytes to IGF (10 nmol/L) caused an even more pronounced change in Gj (37±4% of control, n=6). The time course of the IGF-induced uncoupling was similar to that observed after insulin exposure. The effect of insulin was abolished by truncation of the carboxyl-terminal domain of Cx43 at amino acid 257 (M257). Interestingly, as in the case of pH gating, coexpression of the carboxyl-terminal domain (amino acids 258 to 282) together with M257 rescued the ability of insulin to reduce coupling (Gj, 39±12% from control; n=6). Structure/function experiments using various deletion mutants of the carboxyl-terminal domain showed that insulin treatment does not modify Gj if amino acids 261 to 280 are missing from the Cx43 sequence. Our results suggest that a particle-receptor (or ball-and-chain) mechanism, similar to that described for pH gating, also applies to chemical regulation of Cx43 by other factors. (Circ Res. 1998;83:27-32.)

Key Words: connexin43 ▪ gap junction conductance ▪ insulin ▪ phosphorylation ▪ Xenopus oocyte

Gap junctions allow for the synchronization of electrical and biochemical activity between cells in a tissue. Studies on transgenic animals show that intercellular communication through gap junctions is critical for a variety of biological functions, such as female fertility,1 control of cell proliferation and growth,2 embryonic development,3 lens transparency,3,4 cardiogenesis,5,6 and normal cardiac electrophysiology.7 Moreover, medical genetic studies show that mutations of connexin genes are linked to hereditary diseases such as Charcot-Marie-Tooth,7,8 nonsyndromic sensorineural deafness,9 and some (though not all; see References 10 and 11) cases of a severe cardiac malformation called visceral atrial heterotaxia.12

In vertebrate systems, gap junctions are formed by oligomerization of an integral membrane protein called connexin. At least 13 different connexin genes have been identified. Our recent studies have focused on the regulation of Cx43. This 43-kD 382-amino acid connexin is expressed in a variety of tissues, including heart, ovary, and beta pancreatic cells (see References 8, 13, and 14 for review).

We have previously shown that truncation of the CT of Cx43 impaired pH gating.15 More recently, we showed that the CT of Cx43 can modulate acidification-induced uncoupling even if it is expressed separately from the rest of the channel.16 Our studies led to a particle-receptor model for pH gating, similar to the “ball-and-chain” model of voltage-dependent gating of nonjunctional channels.17 In its simplest version, we propose that the CT of Cx43 constitutes a gating particle and that a separate domain of Cx43, related to the pore-forming region, is a specific receptor for such a particle. On intracellular acidification, the particle would bind noncovalently to the receptor, thus causing channel closure.

Acidification of the intracellular space is only one of the several ways by which the intracellular and extracellular environments modulate cell-to-cell communication. Therefore, we asked whether the central premise of the particle-receptor model applies to the chemical regulation of connexins by factors other than pH. That is, we aimed to determine whether the regulatory domain, even if expressed separately from the rest of the protein, can recognize its receptor and interact with it to modify the channel function. To address this question, we developed an experimental model of insulin-induced uncoupling in Cx43-expressing oocytes. Our
results show that the insulin-induced regulation of Cx43 follows the particle-receptor paradigm. The data suggest that this mechanism is a common path for the chemical regulation of intercellular communication in Cx43-expressing cells.

**Materials and Methods**

**Oocyte Preparation, Electrophysiological Recordings, and pH\textsubscript{I} Measurement**

Experiments were conducted in *Xenopus laevis* oocytes at stages V to VI of development. Procedures for oocytes preparation and injection have been previously described. Brieﬂy, cells were defolliculated and injected with 50 ng of antisense against the endogenous Cx38. After 2 to 5 days of incubation, the vitelline layer was mechanically removed, and the cells were injected with 8 to 20 ng of mRNA for rat Cx43 or its mutants. Cells were paired 24 hours after mRNA injection and tested 12 to 24 hours after pairing.

Oocytes were kept in culture medium (0.5× L-15) before electrophysiological analysis. Approximately 80 to 120 minutes before data collection, L-15 was substituted by a saline (Barth’s) solution. During that time, the cells were impaled, and the electrophysiological recordings were allowed to stabilize. Data acquisition started 10 minutes before insulin was added to the bath, but the cells had already been in Barth’s solution for 1.5 to 2 hours and impaled for at least 30 minutes.

Gj (ie, the electrical macroscopic conductance between the oocyte pair) was measured by conventional dual 2-electrode voltage clamp. The voltage-clamp protocol and the hardware for data acquisition were the same as previously described. Antisense-injected oocytes were not electrically coupled. Nevertheless, oocyte pairs showing Gj values <0.8 µS were not included in the study. This criterion was set to avoid recording from oocyte pairs that showed small levels of Cx43-induced endogenous coupling. Furthermore, to ensure good voltage control over the preparation, oocyte pairs expressing Gj values ≥10 µS were discarded. Experiments were conducted in a sodium acetate–buffered saline (Barth’s) solution (pH 7.4) of the following composition (mmol/L): sodium acetate 130, KCl 1, NaHCO\textsubscript{3} 2.4, MgSO\textsubscript{4} 0.82, CaCl\textsubscript{2} 0.74, HEPES 15, and NaCl 20. The pH was measured by detecting the light emission of the proton-sensitive fluorophore SNARF (dextran form), as previously described.

**cDNA and mRNA Preparation**

Rat cardiac Cx43 CDNA was subcloned into pBluescript IISK\textsuperscript{+} (Stratagene). A fragment encoding a large fraction of the CT domain of Cx43 (amino acids 259 to 382) was generated by conventional polymerase chain reaction of rat Cx43 and subcloned into pBluescript SK\textsuperscript{−} (Stratagene). cDNAs for all mutants were produced by oligonucleotide-directed mutagenesis, as previously described. Deletion mutants are identiﬁed by the symbol Δ, followed by 2 numbers, the ﬁrst and the last amino acid that is missing from the sequence (eg, A261–280 is a deletion mutant of Cx43 lacking amino acids 261 to 280). Truncation mutants are identiﬁed by the letter M and the number of the last amino acid in the sequence (eg, M361 is a truncation mutant of Cx43 at amino acid 361).

**Data Analysis**

Unless otherwise stated, Gj values were normalized to the Gj recorded immediately before the onset of agonist exposure (ie, Gj/Gj control). Results are reported as mean±SEM. Statistical comparisons by ANOVA, followed by a Bonferroni test, were conducted to establish whether the effect of insulin (or IGF) on the Gj of oocytes expressing a particular construct was different from that induced by insulin in Cx43-expressing oocytes. Statistical signiﬁcance set at P<0.05.

**Results**

**Insulin and IGF Induce a Reduction of Gj**

Our experiments required prolonged continuous recording of Gj. Thus, as a control, we determined whether spontaneous changes in the Gj of Cx43-expressing oocytes could be detected with time. Figure 1A shows average measurements of Gj (relative to the control Gj of each individual experiment) during continuous recording of Cx43-expressing oocyte pairs maintained in a normal saline solution. In this case, the value of Gj measured 10 minutes after the onset of recording was used as Gj control. Time zero corresponds to the beginning of data acquisition. The results show that no significant spontaneous changes in Gj were observed for up to 90 minutes of continuous recording. Gj/Gj control after 90 minutes was 0.99±0.05 (n=14).

Figure 1B shows the time course of changes in Gj that result from continuously exposing the oocytes to 10 µmol/L of insulin. After a delay of 10 to 15 minutes, the Gj of insulin-treated oocytes progressively decreased, until reaching an asymptotic value of 0.70 minutes after the onset of insulin exposure. The Gj/Gj control value at 80 minutes after the onset of insulin was 0.54±0.05 (n=12). These results show that insulin leads to a reduction of Gj in Cx43-expressing oocytes. In the present study, we use the term “insulin-induced uncoupling” to refer to this reduction on Gj caused by insulin exposure.

Insulin is known to activate the IGF-R in oocytes. As a first approach to determine whether the effect of insulin could be mediated by IGF-R activation, Cx43-expressing oocyte pairs were exposed to 10 nmol/L of IGF. As shown in Figure 1C, IGF led to a reduction of Gj similar to that observed when a 1000-fold greater concentration of insulin was used (Gj/Gj control, 0.57±0.04; n=6).

**The Insulin Effect Is Not Mediated by a Reduction in pH\textsubscript{I}**

Because acidification-induced closure is a common property of gap junctions, we conducted control experiments to eliminate the possibility that the observed insulin-induced uncoupling resulted indirectly from intracellular acidification. Figure 2 shows the magnitude of pH\textsubscript{I} (measured by the
fluorescence emission of the pH-sensitive fluorophore SNARF; see Reference 16) recorded from a Xenopus oocyte as a function of time. Switching the bathing solution from L-15 to modified sodium acetate–buffered Barth’s solution led to a drop in pHi from 7.86 to 7.78. pH i slowly recovered to more alkaline levels, reaching 7.85 after 130 minutes. Insulin exposure (10 μmol/L) was initiated at that time and maintained for the rest of the recording period. Clearly, addition of insulin to the bath did not reduce pHi over the ensuing 80 minutes. Similar results were obtained in 3 additional experiments. As noted in Materials and Methods, data acquisition for the electrophysiological experiments presented in the present study started 1.5 to 2 hours after the culture medium (L-15) was substituted for saline (Barth’s) solution. Thus, relative to the onset of Barth’s superfusion, the timing of insulin addition in the experiment shown in Figure 2 is similar to that used for the electrophysiological experiments. The results show that (1) pHi had reached a steady state by the time data acquisition started and (2) insulin addition did not modify pHi. Thus, the effects of insulin on Gj cannot be ascribed to changes in pH.

Insulin-Induced Uncoupling of Cx43 Channels Requires the CT Domain of Cx43

Structure-function studies have shown that most of the regulatory functions of Cx43 involve the CT domain. 13,25 In particular, truncation of the CT domain at amino acid 257 of Cx43 (mutant M257) prevented pH gating.15,16 Therefore, we tested the effect of insulin exposure on the Gj of M257-expressing oocytes. As shown in Figure 3 (open circles)

Figure 1. Insulin and IGF induce uncoupling of Cx43 gap junctions. A, Stability of Gj with time. Gj was continuously recorded from a pair of Cx43-expressing oocytes maintained in normal saline. Gj control (Gj cont) was defined as the Gj value recorded 10 minutes after the onset of data acquisition. All measurements were standardized relative to Gj cont. Bars indicate SEM. The value of Gj/Gj cont after 90 minutes of recording was 0.99±0.05 (mean±SEM, n=14). B, Insulin-induced uncoupling in Cx43-expressing oocytes. The horizontal dotted line and the arrow indicate the onset of 10 μmol/L insulin exposure. Gj cont was defined as the Gj value recorded immediately before the addition of insulin to the superfusate. For each experiment, Gj measurements were normalized to their own Gj cont. Values of Gj/Gj cont at each point in time from several experiments were then averaged. The plot shows the averaged values, and the bars represent the SEM for the indicated data point. For the sake of clarity in the diagram, only 1 of every 10 error bars is displayed. Insulin caused a reduction in Gj. After 80 minutes of insulin exposure, Gj/Gj cont was 0.54±0.05 (n=12). C, IGF (10 nmol/L) induces partial uncoupling of Cx43-expressing Xenopus oocytes. IGF was applied at the time indicated by the broken line and maintained throughout. Gj/Gj cont dropped to 0.37±0.04 after 70 minutes of IGF exposure. Bars indicate SEM (n=6).

Figure 2. Insulin does not induce intracellular acidification in Xenopus oocytes. The plot shows the magnitude of pH i as a function of time after the solution is switched from the culture medium (L15) to the acetate-containing saline solution. Addition of insulin to the bath is noted by the vertical broken line.

Figure 3. The CT fragment of Cx43 rescues the insulin sensitivity of M257 channels. Data from 3 different groups of experiments are depicted: wild-type Cx43 (solid circles, same data as in Figure 1B), M257 (open circles), and a third group (stippled circles) in which oocytes were injected with 2 separate mRNAs: M257 and another mRNA coding exclusively for the CT fragment (amino acids 258 to 382 of Cx43). In contrast to the effect of insulin on the Gj/Gj control (Gj/Gj cont) values recorded from M257-expressing oocytes 80 minutes after the onset of insulin exposure were not different from control (Gj/Gj cont =1.06±0.12, n=8). Coexpression of the CT fragment provided the M257 channels with insulin sensitivity (Gj/Gj cont for M257+CT=0.39±0.12, n=6).
truncation of the Cx43 CT domain caused the loss of insulin sensitivity. The effect of insulin on wild-type Cx43 channels is displayed for comparison (solid circles). The Gj recorded from M257-expressing oocytes after 80 minutes of insulin exposure was not significantly different from the untreated control (Gj/Gj control, 1.06±0.12; n=8).

Previous studies from our laboratory have shown that acidification-induced uncoupling follows a particle-receptor model of gating. Therefore, we tested whether the CT fragment could also act as an independent domain during insulin-induced uncoupling. Each oocyte was injected with 2 separate mRNAs: one coding for the insulin-insensitive Cx43 channel (M257) and the other one coding exclusively for the CT region of Cx43 (amino acids 258 to 382). Oocyte pairs coexpressing these constructs were exposed to insulin following the protocol described above. Figure 3 shows that coexpression of the separate Cx43 CT fragment rescued the insulin sensitivity of the otherwise insulin-insensitive (M257) channel (stippled circles). The results demonstrate that the regulatory domain (the CT fragment) can specifically recognize and noncovalently interact with the rest of the channel protein to switch the channel from a conductive to a nonconductive state. In this regard, the particle-receptor mechanism is not unique to acidification-induced uncoupling but common to other forms of chemical regulation of Cx43.

Structure-Function Relation of Insulin-Induced Uncoupling
Insulin and IGF are known to activate the IGF-R in oocytes, thus triggering a cascade that involves activation of MAPK.26 Separate in vitro studies have shown that MAPK can phosphorylate Cx43 at serines 255, 279, and 282.27 As a first approach to characterize the structural bases for insulin-induced uncoupling, we tested the effect of insulin on the Gj of oocyte pairs expressing a variety of deletion and truncation mutants of the Cx43 CT domain. Figures 4 and 5 show the results. In all panels, the open circles show the data obtained from a mutant channel. Data obtained from wild-type Cx43 (same data as in Figures 1B and 3, solid circles) are shown for comparison.

Figure 4 shows results obtained from deleting 20 amino acid segments that included the known MAPK consensus sites. The results indicated that deletion of amino acids 261 to 280 was sufficient to prevent the insulin effect (Figure 4A). Indeed, after 80 minutes of insulin exposure, Gj between oocyte pairs expressing mutant Δ261-280 was not different from its own Gj control. Moreover, the Gj/Gj control value recorded from mutant Δ261-280 at the end of insulin exposure was statistically different from that recorded from Cx43-expressing channels. On the other hand, the Δ241-260 mutant was virtually indistinguishable from the wild-type channel (Figure 4B). The Δ281-300 mutant showed a slightly increased sensitivity to insulin-induced changes (Figure 4C). These results showed that preservation of region 261 to 280 in Cx43 is essential for the insulin-induced uncoupling.

Figure 5 shows results obtained from oocytes expressing mutants Δ301-320, Δ321-340, Δ341-360, and M361 (ie, truncation of Cx43 at amino acid 361). Clearly, all of these mutants were insulin sensitive. In fact, ANOVA tests show that insulin-induced uncoupling effect of insulin was observed for all mutants, only deletion 261-280 eliminated the sensitivity of Cx43 to insulin (statistical analysis by ANOVA corrected by Bonferroni test, P<0.01). Values from the other 2 mutants were not statistically different from those of the wild-type channel. Gj/Gj cont values were 0.54±0.05, 0.63±0.04, 0.98±0.03, and 0.30±0.09 for Cx43, Δ241-260, Δ261-280, and Δ281-300, respectively.

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induced uncoupling was facilitated by deletion of amino acids 301 to 320 (Figure 5A). The reason for the facilitated effect of the 301-320 deletion is unclear, but it may be related to modifications in the secondary structure of the CT domain consequent to deletion. Comparison of the insulin sensitivity of the other deletion mutants with that of the wild-type channel revealed no statistically significant difference (although they all showed a similar trend of enhanced sensitivity).

Discussion

The experiments presented here show the following: (1) Exposure of Cx43-expressing oocytes to insulin, or to IGF, leads to a reduction in Gj. (2) The effect of insulin on Gj requires preservation of the CT region, particularly at amino acids 261 to 280. (3) Insulin-induced gating of Cx43 follows a particle-receptor (or ball-and-chain) model.

Possible Mechanisms of Insulin-Induced Uncoupling

Previous studies have shown that insulin and IGF, both at the same concentrations used in the present study, activate IGF-R in Xenopus oocytes and trigger a complex intracellular signaling cascade. The ball-and-chain model involves a separate protein, can identify and react with a receptor, thus eliciting a change in channel function. We recently demonstrated that this condition applies to the acidification-induced uncoupling of Cx43. Insulin-induced uncoupling differs from the particle-receptor (or ball-and-chain) model, based on the biochemical effects known for this hormone in Xenopus oocytes. These results, by themselves, should not be interpreted as demonstrative of an insulin-mediated modulation of gap junctions in mammalian systems, nor do we ascribe at the moment a role to insulin on Xenopus develop-

ment. However, it is tempting to speculate that changes in intercellular communication may be part of the cellular processes related to the effect of IGF on cardiac and vascular tissue. Although perhaps such modulatory mechanisms exist, their demonstration and analysis go beyond the goals of the present project.

A delay was observed between the onset of insulin exposure and the development of uncoupling. It is unlikely that such a delay resulted simply from the time required for the solution exchange in the recording chamber. Indeed, the delay was not observed in some of the mutants (see Figure 5). Furthermore, the time course of insulin-induced uncoupling of Cx43 was similar to that observed for insulin-induced phosphorylation of S6 kinase (one of the target proteins of MAPK) in Xenopus oocytes. The MAPK-mediated effect of epidermal growth factor receptor activation on Cx43 was also observed 5 to 10 minutes after epidermal growth factor exposure. Although these observations are consistent with our results, the nature of the rate-limiting step between insulin exposure and Cx43 uncoupling remains to be determined.

Insulin-Induced Uncoupling and the Particle-Receptor Hypothesis

The ball-and-chain model has been applied to the fast inactivation of Shaker B potassium channels. A key element of the ball-and-chain model is that the gating particle acts as an independent domain that, even when expressed as a separate protein, can identify and react with a receptor, thus eliciting a change in channel function. We recently demonstrated that this condition applies to the acidification-induced uncoupling of Cx43. A remaining question, though, was whether the ball-and-chain model was unique to pH gating or common to other forms of connexin regulation. The present results show that, as in the case of pH gating, insulin-induced closure follows the ball-and-chain model. The data thus indicate that both manners of chemical regulation converge into a common mechanism. Results from the laboratory of Bruce Nicholson (Zhou and Nicholson, presented in abstract form) suggest that a ball-and-chain model also applies to the src-induced closure of gap junctions. The term “chemical gating” is therefore loosely justified from these results. However, it should not be interpreted to indicate that the effect of insulin is necessarily (or exclusively) mediated by a conformational change in a preexisting channel that switches the oligomer from an open to a closed state. Given the time course of insulin-induced uncoupling, it may also be possible that insulin induces a reduction in cell-cell communication by, for example, the removal and/or degradation of connexins from the membrane. The latter does not modify the central premise of the present study, ie, that noncovalent intramolecular interactions between the CT domain and its receptor are essential for the regulation of intercellular communication by either pH or other membrane agonists.

Structure-function studies show that regions of the CT domain necessary for pH gating are not required for insulin-induced uncoupling. In particular, our previous studies have shown that deletion 281–300, as well as truncation at amino acid 361, interferes with acidification-induced closure. Those same deletions do not affect the sensitivity of Cx43 to
insulin. This difference suggests that these 2 chemically induced mechanisms of gating are not mediated by a common intermediary. More likely, intracellular acidification and insulin exposure trigger 2 different intermediary cascades that have different structural requirements of the CT region and different time course, both leading to uncoupling by a particle-receptor type of mechanism.

The concept of particle-receptor interaction opens the possibility that the regulation of gap junctions can be modified by small analogous molecules that compete for the receptor without modifying its function (ie, competitive inhibition). This strategy has been successfully implemented by our laboratory to prevent pH gating of Cx43 in Xenopus oocytes.33 The latter approach could be used as a tool to study the role of Cx43 in the cellular changes induced by IGF and, perhaps, other cytokines in the cardiovascular system.34

Spray and Burt35 originally proposed that pH gating of Cx43 may involve protonation of histidine residues in the cytoplasmic loop. Our studies18 have supported this hypothesis; we have further suggested that such histidines may be part of the receptor for the gating particle. Future studies will address the question whether the histidines in the cytoplasmic loop are also a possible part of the receptor for insulin-induced gating.

In conclusion, the present study shows that the particle-receptor (or ball-and-chain) model is a common mechanism of chemical gating of Cx43. Understanding the molecular intricacies of chemical gating may allow for the development of new approaches to interfere with gap junction channel closure in native systems. The latter would have implications in the study of gap junction function both in health and disease.

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