Human Connexin43 Gap Junction Channels
Regulation of Unitary Conductances by Phosphorylation


Abstract Connexin43 is the major gap protein in the heart and cardiovascular system. Single channel recordings of human connexin43 gap junction channels exogenously expressed in transfected SKHepl cells demonstrate two discrete classes of channel events, with unitary conductances of predominantly 60 to 70 and 90 to 100 pS when recorded with an internal solution containing CsCl as the major current-carrying ionic species and at moderate transjunctional voltages (<60 mV).

Human connexin43 expressed in SKHepl cells displays multiple electrophoretic mobilities (apparent Mr, ~41 to 45 kD) when resolved in Western blots. Treatment of connexin43 from these cells with alkaline phosphatase collapses the bands into a single 41-kD species; application of alkaline phosphatase to the cell interior through patch pipettes yields channels that are predominantly of the larger unitary conductance. The smaller 60- to 70-pS unitary conductance values correspond to the most common channel size seen in cultured rat cardiac myocytes; these channels were more frequently observed after treatment with the phosphatase inhibitor okadaic acid, which was shown to increase phosphorylation of human connexin43 in these cells under similar conditions. Exposure to the protein kinase inhibitor staurosporine shifted the proportion of events toward the largest unitary conductance and resulted in decreased phosphorylation of human connexin43 in seryl residues in these cells. Thus, the unitary conductance of human connexin43 gap junction channels covaries with the phosphorylation state of the protein. This change in unitary conductance appears to be a unique effect of phosphorylation on gap junction channels, since it has not been observed for other ion channels that have thus far been evaluated. (Circ Res. 1994;74:1050-1057.)

Key Words • heart connexin • single channel conductance • SKHepl cells • cDNA transfection

Gap junction proteins are encoded by the connexin multigene family. Based on both RNA hybridization analyses and immunodetection studies, connexin43 appears to be the most abundant and widespread of the connexins thus far characterized. Connexin43 is expressed throughout the cardiovascular system, most prominently in the heart and in vascular smooth muscle and endothelial cells (for reviews see References 3 through 5).

Recent studies using both nucleic acid and immunological probes demonstrate that individual tissues or even cell pairs from these tissues can express multiple connexin isoforms. Hence, the use of dissociated cells to characterize the physiological properties of channels composed of individual connexins is problematic. To evaluate properties of distinct connexin isoforms, including those that are ordinarily inaccessible to voltage-clamp analysis (such as human cardiac myocytes), exogenous expression systems have been developed.

A major advance in understanding gap junction channel function has been the expression of complementary RNAs in Xenopus oocytes. This system has clearly demonstrated that the expression of a single connexin type is sufficient to establish functional intercellular communication. However, because the large size of the oocytes prevents resolution of single channel currents (which for gap junction channels must be measured between voltage-clamped pairs of high-resistance cells), we have favored the use of communication-deficient mammalian cell lines stably transfected with connexin cDNAs. Furthermore, the expression of mammalian connexins within a similar cellular environment has the theoretical advantage of more faithfully reproducing channel behavior in situ.

Biophysical studies on cells stably transfected with cDNA encoding rat connexin32 demonstrated that this connexin formed a population of junctional channels that was homogeneous with regard to unitary conductance and voltage dependence. By contrast, detailed examination of the same cell line stably transfected with cDNA encoding human connexin43 revealed that the unitary junctional currents recorded at modest transjunctional potentials (<60 mV) were of two distinct conductances. We previously provided a brief report that treatments with phosphorylating and dephosphorylating agents lead to increased occurrence of the 60- to 70- and 90- to 100-picosiemens (pS) events, respectively, and that these treatments also produce major changes in the kinetics of voltage-dependent gating. In this report, we quantify changes in the electrophysiological behavior of exogenously expressed human connexin43 gap junction channels in response to intracellularly applied alkaline phosphatase and to the phosphatase inhibitor okadaic acid and the protein kinase inhibitor staurosporine. Even more importantly, we performed biochemical experiments under conditions similar to those used in the electrophysiological studies to evaluate changes in connexin43 phosphorylation occurring in...
concern with alterations in channel electrophysiology. These findings indicate that the unitary conductance of gap junction channels composed of human connexin43 exhibits distinct unitary conductance values that are correlated with the phosphorylation state of the protein, thereby demonstrating a unique type of channel modulation as a consequence of the action of second messenger molecules.

Materials and Methods

Cell Cultures

Cells used were a clone of the highly metastatic human hepatoma cell line SKHeP1.21 Hundreds of intracellular lucifer yellow injections have demonstrated that the parental cells never exhibit dye coupling. Unitary conductances of endogenous gap junction channels, which have been detected in only about 15% of the parental cell population or transfectants, are about 30 pS.17 Northern blot analysis of the parental cell line indicates that mRNA encoding connexin45 may be expressed at low levels22; weak expression of connexin45 presumably accounts for the low level of endogenous coupling observed in the parental population.

Transfection of SKHeP1 cells with vectors in which connexin cDNAs are driven by simian virus 40 and Rous sarcoma virus promoters has been described in detail.13,14 A key step in our selection procedure was identifying G418-resistant cells exhibiting dye coupling and then subcloning these populations for subsequent experimentation. Subclones obtained in this way were functionally coupled; for the cells used in this study, Northern blot analyses demonstrated the presence of human connexin434 but did not detect increased expression of the endogenous connexin (G.I.F., A.P.M., and D.C.S., unpublished observations).

Electrophysiology

Recordings of junctional conductance (gj) were obtained from cell pairs prepared by freshly dissociating pure populations of approximately 85% confluent cultures onto 1-mm-diameter glass coverslips. Experiments were performed at room temperature under conditions of constant superfusion with bathing solution (in mmol/L: NaCl 160, CsCl 7, CaCl2 0.1, MgCl2 0.6, HEPES 10; pH 7.4). Each cell of the pair was voltage clamped with heat-polished patch pipettes (3 to 7 MΩ) filled with internal solution (in mmol/L: CsCl 130, CaCl2 0.5, Na2ATP 2, MgATP 3, HEPES 10, EGTA 10; pH 7.2). Seals to cell surfaces were achieved with suction and were monitored by recording currents while applying simultaneously through both electrodes 20-millisecond, 4-mV pulses at 10 Hz. During suction, the current from the pipette was measured in the Track Mode of the voltage-clamp system (model 1-C, Axon Instruments, Inc.). Once a gigaohm seal was formed, the systems were switched to “hold,” and after access to the cell interior was obtained with strong suction, gj was determined by dividing the junctional current by the voltage step applied to the other cell.23-25

Single channel conductances were measured after conditions in which gj was reduced to low levels by halothane superfusion (2 mmol/L freshly diluted from saturated stock solution) or after phosphatase exposure. To obtain an all-points histogram, unitary currents of gap junction channels in one cell of a pair were filtered at 2 kHz, digitized (model DR-484, Neurodata Instruments Corp), and tape-recorded on a videocassette recorder (Fisher VSH-840). During playback, data were acquired through a Dell Computer 386 using PCLAMP software (Axon Instruments, Inc), filtering the original signal to 100 Hz (four-pole, Bessel low-pass variable frequency filter, AP Circuit Corp). For digitized traces, all points were grouped in 0.2-ps bins after the calibrated current values were divided by the driving force applied. For frequency histograms, unitary current events were measured from chart recordings (in which junctional channels were recog-
Normalized Values of $^{32}$P Incorporation Into Human Connexin43 Under Control Conditions and After Treatment With Staurosporine* or Okadaic Acid*

<table>
<thead>
<tr>
<th></th>
<th>Cx43-HP</th>
<th>Cx43-P2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.0</td>
<td>1.05±0.17</td>
</tr>
<tr>
<td>Okadaic acid</td>
<td>1.36±0.24</td>
<td>1.77±0.31</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>0.61±0.08</td>
<td>0.64±0.07</td>
</tr>
</tbody>
</table>

Cx indicates connexin. Values shown were normalized with respect to the counts obtained in the Cx43-HP band under control conditions in three separate experiments (actual values for $^{32}$P incorporation into Cx43-HP in these individual experiments were 4673, 852, and 1443 cpm).

*300 nmol/L.

dimensional tryptic phosphopeptide mapping and phosphoamino acid analysis of the bands detected in control cells were performed as described.

The anti-connexin43 antibody used for immunoblotting and immunoprecipitation was previously characterized and provided by Dr E.L. Hertzberg, Department of Neuroscience, Albert Einstein College of Medicine, New York.

Results

Single channel recordings from SKHepl cells transfected with human connexin43 revealed distinct populations of single channel currents that were not present in the parental cells (a representative record and corresponding all-points histogram are shown in Fig 1; event histograms from numerous experiments are presented in the top panel of Figs 2 and 3). Currents corresponding to single channel conductances ($\gamma$) of 60 to 70 pS are similar in size to those channels previously reported between cardiac myocytes from various adult and embryonic mammalian species and in astrocytes... Events with the larger unitary conductance (about 90 to 110 pS) have not been reported to contribute appreciably to junctional conductance in cardiac myocytes or astrocytes, but they are prominent in other cell lines and primary cultures in which connexin43 is expressed. Also infrequently present in about 15% of the parental cell pairs and in some transfecants were events with $\gamma$=30 pS (see Figs 2 and 3), which we previously attributed to the occasional expression of endogenous channels. However, recent studies on the parental channel indicate that these endogenous channels are very voltage dependent and would thus be expected to exhibit few openings and closures at the potentials used in the present study; although abundant evidence now indicates that events of this size represent a substate of the connexin43 channel, the occurrence of these events was not affected by any of the treatments used in this study, and they are not considered further here.

The expression of multiple distinct unitary conductance values resulting from transfection with a single cDNA might be related to multiple forms of connexin43 produced by the transfected cells. An antibody to the carboxyl terminus of connexin43 (corresponding to amino acids 346 to 363 of the rat connexin43 sequence) was found to recognize several distinct bands on SDS gels of human connexin43, with mobilities corresponding to apparent molecular weights of 41 to 45 kD (Fig 1B, lane 2, and Fig 4A; see also Reference 14).

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Fig 1. Cells transfected with human connexin43 cDNA express gap junction channels with two distinct unitary conductances. A, Dwell histograms from junctional unitary currents. Inset shows digitized junctional currents after halothane uncoupling. The first peak in the histogram of digitized events represents the baseline or zero junctional conductance level. The second and third peaks represent unitary conductances of 56 and 100 pS, as determined by best fit to gaussian distributions (see “Materials and Methods”). No events corresponding to sizes of “endogenous” channels were observed during this recording epoch. B, Western blots from SKHepl cells transfected with human connexin43. Samples include, in lane 2, human connexin43 transfectants; in lane 1, human connexin43 transfectants, in which the pellet was treated with alkaline phosphatase after isolation.
Furthermore, phosphatase treatment of the protein sample collapsed these bands into the 41-kD form (Fig 1B, lane 1), as has been demonstrated for other connexin43 species in both primary cells and cell lines. Thus, the bands with lower electrophoretic mobilities are attributable to phosphorylated products of the 41-kD band.44-46

The above data indicate that human connexin43 expressed in the SKHep1 cell line forms channels with multiple unitary conductances and exhibits multiple posttranslationally modified products that differ in the state of phosphorylation of the protein. Moreover, in connexin43-expressing cells, the 60- to 70-pS \( \gamma \) value appears to be more frequently observed in cell types in which the phosphorylated forms of the protein are more abundant (see Fig 4 and “Discussion”). To test the hypothesis that the distinct unitary conductances might arise from differential phosphorylation of connexin43, we applied treatments favoring protein phosphorylation or dephosphorylation to cell pairs (see “Materials and Methods”).

Alkaline phosphatase, delivered through the patch electrodes, was tested for effects on \( \gamma \). In contrast to the other data presented in this paper, \( \gamma \) measurements in the presence of alkaline phosphatase could be obtained without the addition of halothane to the bath, because even unilateral exposure to this reagent resulted in a slow, progressive decline in \( g_0 \) to low levels (results not illustrated). Histograms in Fig 2A and 2B show the analyses of single channel current recordings in a cell pair under control conditions (Fig 2A, a recording made in the same dish as one of those recordings obtained after phosphate treatment) and in the analyses of three cell pairs obtained 45 minutes after dialysis with phosphatase (Fig 2B). Under control conditions (Fig 2A), events with unitary conductances of both 60 to 70 and 90 to 110 pS were readily detected (see also Fig 3). After

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**Fig 2.** Effect of intercellular addition of alkaline phosphatase on unitary conductance of gap junction channels. A. Frequency distribution of control recordings of unitary conductances in a cell pair in the same dish as one of the pairs analyzed in B. B. Frequency distribution of three cell pairs in which single channels were recorded in which both electrodes contained alkaline phosphatase.

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**Fig 3.** Frequency distributions of unitary junctional conductance (\( \gamma \)) events recorded under control conditions and after the application of the phosphatase inhibitor okadaic acid and the protein kinase inhibitor staurosporine. Top, Average of 14 experiments in which unitary conductances of junctional channels were measured after halothane application. The best fit to gaussian distributions was obtained with peaks at \( \gamma = 39 \pm 10 \) (SD, 9% of total events), 62 \( \pm 9 \) (black area, 25% of total events), and 89 \( \pm 18 \) (shaded area, 66% of total events) pS. Middle, Average of four experiments in which 300 nmol/L okadaic acid was added to the bathing solution for 30 minutes to 1 hour before recordings were begun. Best fits correspond to \( \gamma = 57 \pm 16 \) (71% of total events) and 103 \( \pm 7 \) (29% of total events) pS. Note shift in \( \gamma \) values to lower conductances. Bottom, Average of three experiments in which cells were treated with staurosporine (300 nmol/L) for 20 minutes; peaks occur at 30 \( \pm 6 \) (7% of total events), 61 \( \pm 7 \) (13% of total events), and 100 \( \pm 9 \) (60% of total events) pS. Note shift of the distribution of unitary conductances toward the highest \( \gamma \) values after treatment with staurosporine. All records are from cell pairs in which amplitudes of at least 100 unitary events were measured and are normalized with regard to the total number of events recorded in each experiment.
phosphatase dialysis, the channel population was marked-
edly uniform, with <5% of the total events being of
sizes other than the 90- to 110-pS population.

Representative histograms of $\gamma$ values after okadaic
acid (a phosphatase inhibitor) and staurosporine (a
protein kinase inhibitor) were applied are illustrated in
Fig 3. These experiments revealed that the frequency of
the 60- to 70-pS events relative to the frequency of the
90- to 110-pS events was increased more than sixfold
compared with control values after exposure to okadaic
acid. For other agents that would be expected to favor
the phosphorylated state (8-Br-cAMP, forskolin, tu-
mor-promoting phorbol ester), efficacy ranged from
about twofold in the case of forskolin to more than
sevenfold after exposure to phorbol ester (results not
illustrated, $n=3$ for each). The relative incidence of the
60- to 70-pS event was decreased about twofold after
treatment with the protein kinase inhibitor staurospo-
rine. The incidence of the 25- to 30-pS "endogenous"
channel was low in these experiments, which were
performed with transjunctional voltages $\leq 50$ mV, and
frequency was not systematically altered by the trea-
mens used in these experiments. To control against the
possibility that these treatments might induce the ex-
pression of 60- to 70- or 90- to 110-pS channels, single
cell conductances were measured in cell pairs of the
parental cell line in response to okadaic acid ($n=3$)
and staurosporine ($n=2$). No events other than the endog-
cuous 30-pS channels were seen, and treatment with
these agents did not detectably alter the occurrence of
these events (not illustrated). Thus, the shifts observed
in frequency histograms in transfected cells are unlikely
to be contributed by other channel types covertly pre-
sent in these cells.

The phosphorylation state of connexin43 was evalu-
ated in cells treated for 20 minutes with either stauro-
sporine or okadaic acid, which were chosen to com-
pare with the electrophysiological findings because these
agents are membrane permeant and were found to
cause extreme effects on the distribution of $\gamma$ under
dual voltage clamp conditions. Immunoblots of rat heart
homogenates (Fig 4A) showed a single band corre-
sponding to a phosphorylated form of rat connexin43,
and immunoblots of transfected SKHep1 cells showed
at least five distinct bands in the human connexin43
protein (Fig 4A): Cx43-NP corresponded to the de-
phosphorylated form, and Cx43-P', Cx43-P1, Cx43-P2,
and Cx43-HP corresponded to phosphory-
lated forms of connexin43 as shown in other systems (see text). B, Radiolabeled cells (labeled $^{32}$P) showed bands that comigrated with the bands labeled Cx43-P2 and Cx43-HP under control conditions. Incorporation of label was decreased after 20 minutes of exposure to staurosporine and increased after 20 minutes of exposure to okadaic acid. The lane marked P.I. corresponds to the control sample after treatment with alkaline phosphatase (see "Materials and Methods"). C and D, Two-dimensional tryptic phosphopeptide mapping and phosphoamino acid analysis of the bands Cx43-P2 (D) and Cx43-HP (C) detected in control cells. Asterisks indicate the origin, and numbers 1 through 5 indicate common phosphopeptides displaying different relative incorporation of $^{32}$P. Phosphoamino acid analysis of these samples indicated that both forms of connexin43 were phosphorylated $>95\%$ in seryl residues and $<5\%$ in threonyl residues (see Fig 5).
indicated that neither treatment caused a major change in the amount of immunoprecipitated connexin43 (Fig 4A, lanes C, S, and O.A.; densitometric data given in legend). However, small changes in relative abundance of specific bands were detected (eg, Cx43-NP was about 9% to 10% lower and Cx43-P’ about 5% higher in okadaic acid–treated cells compared with controls or cells treated with staurosporine). The minor effects of these drugs on electrophoretic mobility of connexin43 contrast with the striking changes in P incorporation into the two forms with lowest electrophoretic mobility (bands labeled Cx43-HP and Cx43-P2 in Fig 4A and 4B). P incorporation into both bands was reduced by staurosporine and was increased by okadaic acid (lanes C, S, and O.A. in Fig 4B); the similarity of the two-dimensional tryptic phosphopeptide maps of these Cx43-P2 and Cx43-HP bands (Fig 4C and 4D) indicates that both bands corresponded to the same protein that was phosphorylated to different extents in the same sites. Phosphoamino acid analysis of a mixture of P-labeled forms of Cx43 immunoprecipitated under each condition showed that serine was the main amino acid phosphorylated (Fig 5). A minor amount of phosphothreonine was detected when the acid hydrolysis was carried out for only 45 minutes but not when it was performed for 2 hours (not shown).

### Discussion

Gap junctions were originally described on the basis of their tissue source. The initial molecular cloning studies demonstrated a “liver” isoform and a second, distinct “cardiac” isoform, encoded by a separate gene. More recently, about a dozen distinct genes have been identified in the mouse genome, and expression at the level of RNA and/or protein has been described for many of these. Studies of both tissues and cultured cells have now confirmed that multiple connexins may be expressed in single cells. Thus, the initial classification based on source is less tenable. We initially developed an exogenous expression system using communication-deficient cells stably transfected with connexin cDNA sequences to provide a permanent source for biophysical studies. In particular, we generated the pGF1 cell line expressing human connexin43 to model cellular coupling in human cardiac myocytes. Because recent studies demonstrated the expression of several connexin isoforms in cardiac myocytes, the transfected cells have become especially useful for the study of channels composed of connexin43, unencumbered by the coexpression of other gap junction proteins.

Studies of SKHeP1 cells transfected with connexin32 revealed relatively homogeneous channel behavior. Distinct unitary conductances of approximately 120 to 140 pS were observed that were relatively voltage dependent, in agreement with gap junction channel recordings from primary hepatocytes, which express abundant connexin32. Initial studies of connexin43 transfectants demonstrated channel events that corresponded well to gap junction activity recorded in primary cultures of cardiac myocytes, with unitary conductances of approximately 60 to 70 pS that were relatively voltage insensitive. More detailed studies, including recordings from more than 100 cell pairs, have now demonstrated complex channel activity that is regulated at multiple levels. In addition to the predicted 60- to 70-pS unitary event, a unitary conductance class of 90- to 110-pS events has now been observed, a finding not unique to the SKHeP1 line. The larger unitary conductance has been observed in cultures of WB (possible liver stem) cells, leptomeningeal cells, smooth muscle from human corpus cavernosum, and mouse Leydig cells, all of which express predominantly connexin43.

Posttranslational modification of connexin43 by phosphorylation has been well documented, and phosphorylation sites on the connexin43 molecule have been identified. Although previous studies have stressed the changes in electrophoretic mobility that accompany long-term changes in phosphorylation state, we have found that short-term treatment with staurosporine and okadaic acid results in altered P incorporation without grossly affecting the abundance of connexin43 forms with various electrophoretic mobilities. This finding indicates rapid phosphate turnover within these specific species, which may correspond to topologically distinct pools of protein within the cell. A similar result was obtained from studies on neonatal rat cardiocytes in which 30 minutes of treatment with staurosporine did not significantly affect the pattern of connexin43 detected by immunoblot but profoundly decreased the incorporation of P into the Cx43-P2 form. In most endogenous systems analyzed, connexin43 can be phosphorylated on seryl and threonyl residues. Similarly, in the exogenous expression system used in these studies, human connexin43 was found to be phosphorylated predominantly on seryl and to a minor extent on threonyl residues. This result is consistent with our interpretation that the unitary conductance of connexin43 forms channels that can be affected by the phosphorylation mediated by second messengers that activate...
cAMP-dependent protein kinase or protein kinase C, both of which are ser&from=copyothyrosine kinases.55 Although the data presented in this article do not specifically identify which of the phosphorylated forms of connexin43 corresponds to the 60- to 70-pS channel population, the fraction of low-conductance connexin43 channels is shown to correlate directly with the extent to which the junctional protein is phosphorylated after exposure to phosphatase or kinase inhibitors for comparable periods of time.

Changes in γ have been detected after phosphorylating treatments in other systems in which endogenous connexin43 expression is prominent.42,56 although in those studies it remained possible that phosphorylating treatments were altering the frequency distributions of channels formed by different connexins. The present demonstration on transfected cells indicates that the same connexin can form channels with different unitary conductances, as opposed to the possibility that expression of another channel type is altered rapidly by treatments that affect protein phosphorylation.

Junctional conductance (gJ) is the product of γJ and the number of conducting channels. After treatment with okadaic acid, gJ is decreased by about 10% to 20% (not illustrated), which is in reasonable agreement with the effect on γJ illustrated here. After staurosporine, gJ also declines by about 20%, and conductance decreases toward zero after phosphatase injection (not illustrated); since γJ is increased by these treatments, either the total number of junctional channels or their open probability must be markedly reduced.

Modulation of unitary conductance of human connexin43 gap junction channels by phosphorylation is a unique effect of second messenger molecules on ion channels. For the ACh receptor and voltage-dependent Na+ channels and Ca2+-channels of snail ganglia and heart, phosphorylation has been associated with increased desensitization, reduced ion flux, and increased ionic current, respectively.57-60 For the delayed rectifier K+ channel in squid axon, phosphorylating treatment shifts voltage dependence of activation and inactivation without affecting mean open times or unitary conductance.61 If changes in γJ result in alterations of gap junction channel permeability or selectivity (which have not yet been demonstrated), connexin phosphorylation could restrict intercellular diffusion of ions of different charge or molecules of different size. Alternatively, the larger unitary conductance that correlates with decreased connexin43 phosphorylation might be viewed as a compensatory mechanism in systems such as the heart, in which the major function of gap junctions is in mediating intercellular current flow. Thus, dephosphorylation of the human cardiac gap junction channel, which might occur in metabolically compromised tissues, including the ischemic heart, would be predicted to increase the current-carrying capacity of those channels that remain open.

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