Connexin-43 Hemichannels Opened by Metabolic Inhibition*

(Received for publication, August 28, 1998, and in revised form, October 13, 1998)

Scott A. John, Richard Kondo, Sheng-Yong Wang, Joshua I. Goldhaber, and James N. Weiss†

From the Cardiovascular Research Laboratory, Departments of Medicine (Cardiology) and Physiology, University of California at Los Angeles School of Medicine, Los Angeles, California 90095

The cause of altered ionic homeostasis leading to cell death during ischemia and metabolic inhibition is unclear. Hemichannels, which are precursors to gap junctions, are nonselective ion channels that are permeable to molecules of less than Mr 1000. We show that hemichannels open upon exposure to calcium-free solutions when they are either heterologously overexpressed in HEK293 cells or endogenously expressed in cardiac ventricular myocytes. In the presence of normal extracellular calcium, hemichannels open during metabolic inhibition. During ischemia and other forms of metabolic inhibition, activation of relatively few hemichannels will seriously compromise the cell's ability to maintain ionic homeostasis, which is an essential step promoting cell death.

Ischemia, hypoxia, and other forms of metabolic inhibition cause rapid disturbances in ionic homeostasis including intracellular Na and Ca gain and K loss that contribute to cellular injury and death (1); however, the mechanism is uncertain. Recent studies using dye uptake assays in immortalized cells have shown that some types of hemichannels open in low extracellular Ca (2). Hemichannels are the precursors to gap junctions (for a review, see Ref. 3); two hemichannels aligned end to end form an intercellular communicating channel. If they are activated in nonjunctional plasma membrane, hemichannels are nonselective and exchange intracellular K for extracellular Na or Ca due to the asymmetrical intracellular/extracellular distribution of these cations.

We have previously described a nonselective current that is activated by metabolic inhibition in isolated ventricular myocytes (4). The purpose of this study was to investigate whether activated nonjunctional hemichannels might be responsible for this current. The findings are positive, indicating that nonjunctional hemichannels may be involved in the pathogenesis of ionic disturbances during myocardial ischemia and hypoxia.

EXPERIMENTAL PROCEDURES

Molecular Biology—Plasmid encoding GFP (CLONTECH, Palo Alto, CA) was fused to the C terminus of Cx43 using the polymerase chain reaction overlap extension method (4, 5). The chimeric product was subcloned and sequenced. Cx43 provided by Dr. Bruce Nicholson (State University of New York, Buffalo, NY) was subcloned into pCDNA3. Both native and chimeric constructs used the cytomegalovirus promoter. Transfection was carried out using the calcium phosphate method (6).

Microscopy—The expression of Cx43-GFP was determined by examining cell monolayers grown on glass coverslips using a Nikon microscope fitted with a Xenon lamp and the appropriate filters (excitation bandpass, 450–480 nm; emission cutoff, <515 nm). Cardiac myocytes were examined for calcein and dextran-fluorescein loading using the same filters. Magnification was ×400 in the original slides, which were then scanned and assembled using an Adobe Photoshop/Macintosh G3 combination.

Electrophysiology—Whole cell and single channel currents were recorded with an Axopatch 200A clamp amplifier and a Digidata 1200 data acquisition system using pClamp software (Axon Instruments, Foster City, CA). For the whole cell clamp experiments, the patch pipette contained 140 mM KCl, 1 mM MgCl2, 5 mM NaCl, and 10 mM HEPES, pH 7.4, for HEK293 cells or 115 mM cesium glutamate, 30 mM TEA-Cl, 10 mM HEPES, 3 mM NaCl, 1 mM MgCl2, 1 mM NaH2PO4, 5 mM sodium pyruvate, and 1 mM Na-ADP for isolated ventricular myocytes. The bath solution contained 140 mM NaCl, 1 mM MgCl2, 5.4 mM KCl, 1.8 mM CaCl2, and 10 mM HEPES, pH 7.2, with or without 10 mM dextrose for HEK293 cells; CsCl was substituted for KCl for isolated ventricular myocytes. For the 0 Ca solution, CaCl2 was removed, and 2 mM EGTA was added (estimated free Ca, 1 nM). Metabolic inhibitors (which were dissolved in dimethyl sulfoxide as appropriate) or LaCl3 was added directly to the bath solution. For halothane, the appropriate bath solution was mixed with halothane and vigorously shaken. Excess halothane was suctioned off, and the halothane-saturated solution was covered with mineral oil throughout its use. Bath solutions were exchanged using a rapid solution exchanger with a 90% exchange time of <500 ms (7). For single channel recordings, the patch pipette contained the appropriate bath solution described above.

Dye Uptake—HEK293 monolayers were washed twice with Ca-free Tyrodes containing 2 mM EGTA (EGTA-Tyrodes) and then incubated in EGTA-Tyrodes containing 1% Lucifer Yellow for 30 min at room temperature. Cells were extensively washed with normal Tyrodes containing 1.8 mM Ca before imaging. Isolated adult cardiac myocytes (8) were washed by resuspension and centrifugation in EGTA-Tyrodes and then incubated in normal Tyrodes or EGTA-Tyrodes containing 150 μM calcine or 1% dextran-fluorescein for 30 min at room temperature. They were washed in normal Tyrodes three times before imaging.

RESULTS

HEK293 cells were transfected with wild-type Cx43 or Cx43 linked in frame at the C terminus to the green fluorescent protein (Cx43-GFP). In monolayers transfected with Cx43-GFP, the pattern of expression showed long lines of fluorescence that were consistent with the formation of gap junctions in regions of contact between adjacent transfected cells, in addition to some perinuclear and plasma membrane fluorescence (Fig. 1, D and F). These long fluorescent lines were not observed between adjacent nontransfected and transfected cells (Fig. 1D), suggesting that the endogenous connexin-43 in the nontransfected HEK293 cells (9) either did not pair with the Cx43-GFP or was present at too low a level (9) to form detectable fluorescent structures with the exogenous Cx43-GFP. Cells transfected with GFP alone showed a homogenous pattern of fluorescence (Fig. 1B) that did not transfer to adja-
channel currents were detected in six of nine cells transfected with Cx43-GFP, but in none of the nontransfected cells. Furthermore, halothane inhibited the channels, with the NP decreasing from 0.28 ± 0.02 to 0.13 ± 0.03 (n = 3). The single channel conductance of the fully opened channel averaged 120 ± 25 pS. Several substrate conductance levels were also observed, although their dwell times were too inconsistent to analyze quantitatively.

Dye transfer experiments confirmed that the current observed in low extracellular Ca was due to functional hemichannels (Fig. 3). HEK293 cell monolayers containing either all nontransfected cells (Fig. 3, A and B) or a mixture of transfected and nontransfected cells (Fig. 3, C–F) were exposed to nominally Ca-free media containing 1% Lucifer Yellow (M, 522). After 30 min, Lucifer Yellow was washed out with Ca-containing media, and the cells were imaged. Cells expressing Cx43-GFP showed the brightest dye uptake (Fig. 3, C and D). Contacting nontransfected cells also showed a dimmer fluorescence, which is consistent with dye transfer from the adjacent nontransfected cells via gap junctions. No dye uptake occurred in nontransfected cells that were not close to transfected cells, suggesting that they did not take up Lucifer Yellow. The entry of Lucifer Yellow and the subsequent transfer to contacting cells indicate that the putative Cx43 hemichannels opened by low extracellular Ca are nonselective and are large enough to allow molecules of at least M, 522 to enter, which is consistent with the known properties of gap junctions (18). Comparable results were obtained when wild-type Cx43 was transfected alone (Fig. 3, E and F), although the successfully transfected cells could not be unequivocally identified.

These findings establish that Cx43 forms functional hemichannels with typical properties of gap junctions. Linkage to GFP did not fundamentally alter hemichannel properties. We therefore proceeded to test the effects of metabolic inhibition. HEK293 cells expressing Cx43-GFP were patch-clamped (whole cell mode) with 1.8 mM [Ca], and exposed to the metabolic inhibitors carbonyl cyanide-m-trifluoromethoxyphenylhydrazone (FCCP; 10 μM) and iodoacetate (IAA; 1 mM) to inhibit oxidative and glycolytic metabolism, respectively (Fig. 2, E and F). After ~15 min, a large linear current developed that was similar to that induced by low [Ca], but ~2-fold larger (1720 ± 282 pA at −80 mV; reversal potential, −7 ± 1.5 mV; n = 5). This current was reversibly inhibited by 73 ± 8% by halothane and irreversibly inhibited by 97 ± 1% by IAA (Fig. 2, E and F). In contrast, in nontransfected cells, only a small increase in current from 39 ± 15 to 132 ± 49 pA was observed during metabolic inhibition (Fig. 2H).

To rule out a nonspecific effect of the metabolic inhibitors, we tested alternative combinations of metabolic inhibitors: 5 μM rotenone (a mitochondrial inhibitor rather than an uncoupler like FCCP) + 1 mM IAA or 10 μM FCCP + 10 mM 2-deoxyglucose (a glycolytic inhibitor). Although the activation time course differed, a similar current developed in transfected cells (Fig. 2H). The combination of FCCP + IAA also activated the hemichannel current in cells co-transfected with wild-type Cx43 and βNaP-GFP (Fig. 2H). Halothane and La inhibited the putative hemichannel currents (Fig. 2H), although to a slightly lesser degree.

We also recorded single channel currents from cell-attached patches on Cx43-GFP-transfected cells during metabolic inhibition with FCCP + IAA (Fig. 2G) with normal extracellular Ca (1.8 mM) in the patch pipette and bath. After a delay of 8–20 min, noisy-appearing currents were observed. Single channel amplitude was similar to that induced by low [Ca], 5 ± 1.2 versus 4.5 ± 0.9 pA at −40 mV. However, the kinetics differed, due to a greater substate occupancy during metabolic inhibi-
Connexin-43 Hemichannels Opened by Metabolic Inhibition

Fig. 2. Activation of hemichannel currents by low extracellular Ca (A–D) and by metabolic inhibition (E–H) in HEK293 cells expressing Cx43-GFP or wild-type Cx43. A, time course activation of whole cell hemichannel currents by low extracellular Ca (0Ca) in a patch-clamped HEK293 cell transfected with Cx43-GFP. Current amplitude was measured at −80 mV. The current was reversibly blocked by the application of halothane (H). B, current-voltage relationships at the selected time points (a–d) indicated in A. Currents were recorded during a voltage ramp from −100 to +60 mV at 0.1 mV/ms. Selected time points were as follows: a, control with 1.8 mM [Ca]; b, after the removal of extracellular Ca (0Ca); c, after the reappearance of 1.8 mM [Ca]; d, in the presence of halothane (H) after removing extracellular Ca again. C, single channel currents from a cell-attached patch with 0Ca in the patch electrode (NP, versus time before and during exposure to halothane); representative single channel recordings are shown below. D, summary of the effects of extracellular Ca removal on whole cell currents in HEK293 cells. Nontransfected cells (NT) showed a modest increase in inward current at −80 mV upon Ca removal (0Ca) compared with a much larger increase in cells transfected with either Cx43-GFP (n = 4) or wild-type Cx43 (WT; n = 3). The latter currents were partly blocked by halothane (H). E, time course activation of whole cell hemichannel currents during MI in a patch-clamped HEK293 cell transfected with Cx43-GFP. Current amplitude was measured at −80 mV. Approximately 10 min after exposure to MI (with FCCP + IAA), a large current activated that was reversibly blocked by halothane (H) or lanthanum (La). F, current-voltage relationships at the selected time points (a–d) indicated in E. The selected time points were as follows: a, control; b, 12 min after the onset of MI in the presence of 1.8 mM [Ca]; c, in the presence of halothane (H); d, in the presence of 1 mM La. Currents were recorded during a voltage ramp from −100 to +60 mV at 0.1 mV/ms. G, single channel currents from a cell-attached patch during MI. After 6 min of MI, NP; increased progressively with time and was decreased by halothane (H); representative single channel recordings are shown below. H, summary of the effects of MI on whole cell currents in HEK293 cells. Compared with nontransfected cells (NT; n = 3), cells transfected with Cx43-GFP or wild-type Cx43 (WT) and exposed to the metabolic inhibitors FCCP + IAA developed a large current at −80 mV that was partially blocked by halothane (H) and completely blocked by La (n = 5). Similar results were obtained in cells transfected with Cx43-GFP subjected to different combinations of metabolic inhibitors, either rotenone (Rot) + 2-deoxyglucose (DOG; n = 2) or FCCP + DOG (n = 2).
Connexin-43 Hemichannels Opened by Metabolic Inhibition

Cx43 dye transfer characteristics ($M_r$ cutoff, ~1000). Rounded dead myocytes, however, readily took up this dye. Electrophysiological evidence also supported the existence of functional hemichannels in isolated myocytes. After the calcein dye uptake protocol, brightly fluorescent rod-shaped myocytes were patch-clamped (whole cell mode). The removal of extracellular Ca (with or without EGTA present) rapidly induced a current with a linear voltage relationship that reversed near 0 ($n = 6$; Fig. 5, A–C). Current amplitude at ~80 mV averaged 7 ± 1.8 pA/pF ($n = 6$). The current was reversibly blocked by

**FIG. 3.** Dye uptake and transfer in HEK293 cells. A and B, double exposure of bright-field and fluorescence images (A) or the fluorescence image alone (B) of a monolayer of nontransfected HEK293 cells after exposure to 0Ca solution containing 1% Lucifer Yellow for 30 min. Occasionally, dead cells took up Lucifer Yellow (inset) but did not transfer the dye to adjacent cells. C and D, double exposure of bright-field and fluorescence images (E) and the fluorescence image alone (F) of a monolayer of HEK293 cells transfected with Cx43-GFP after exposure to 0Ca solution containing Lucifer Yellow for 30 min. The fluorescence from Cx43-GFP is very bright and localized at discrete spots, as seen in Fig. 1, whereas Lucifer Yellow fluorescence is homogeneous throughout the cell. Lucifer Yellow fluorescence is most intense in the Cx43-GFP-labeled cells and becomes dimmer in the adjacent nontransfected cells, which is consistent with dye transfer. In contrast, the more distant nontransfected cells did not show any Lucifer Yellow fluorescence. E and F, double exposure of bright-field and fluorescence images (C) and the fluorescence image alone (D) of a monolayer of HEK293 cells transfected with wild-type Cx43 after exposure to 0Ca solution containing Lucifer Yellow for 30 min. Note the bright fluorescence of the (presumably) transfected cell in the upper left corner, with dimmer fluorescence consistent with dye transfer to surrounding cells. In contrast, distant cells show no fluorescence.

**FIG. 4.** Size-selective dye uptake by isolated rabbit ventricular myocytes exposed to Ca-free media. A and B, representative paired bright-field and fluorescence images of isolated myocytes after a 30-min incubation with either (A) 150 μM calcein ($M_r$ 660) or (B) 1% dextran conjugated to fluorescein ($M_r$ 1500–3000) in the presence (+Ca) or absence (0Ca) of 1.8 mM Ca. Note that live rod-shaped cells take up calcein only in the absence of Ca, whereas rounded dead cells take up both dyes irrespective of Ca. C and D, summary of the number of rod-shaped cells taking up the dye. The ratio of fluorescent rod-shaped cells to the total number of rod-shaped cells is indicated above each column.

**FIG. 5.** Activation of putative hemichannel currents in isolated rabbit ventricular myocytes by either low extracellular Ca (A–C) or metabolic inhibition (D–F). A, time course of whole cell current at ~80 mV holding potential after the removal of extracellular Ca (0Ca) in a patch-clamped myocyte preloaded with calcein. The current was blocked with 2 mM lanthanum (La). B, current-voltage relationships at the selected time points (a–c) as indicated in A. Currents were recorded during a voltage ramp from −100 to +80 mV at 0.18 mV/ms. C, summary of the effects of extracellular Ca removal on whole cell currents in isolated cardiac myocytes. D, time course of whole cell current at ~80 mV in a patch-clamped myocyte during MI with rotenone in the presence of 1.8 mM [Ca]. The current was blocked by 2 mM lanthanum (La). E, current-voltage relationships at the selected time points (a–c) indicated in D, F, summary of the effects of metabolic inhibition.
1.8 mM Ca or 2 mM La.

To determine whether the endogenous hemichannels in myocytes could be activated by metabolic inhibition with normal extracellular Ca, calcine-labeled myocytes were exposed to rotenone (5 μM) in dextrose-free Tyrode’s solution containing 1.8 mM Ca. After 8–10 min, a linear nonrectifying current developed that was fully blocked by 2 mM La (Fig. 5, D–F). The reversal potential of the La-sensitive component was 5.6 ± 2.6 mV compared with 3.9 ± 2.5 mV for the current induced by Ca removal. Myocytes underwent a rapid and progressive contraction as the current activated, indicating a severe disturbance of ionic homeostasis and intracellular Ca overload. We have previously shown (4) that this current is nonselective to molecules as large as N-methyl-D-glucamine (Mᵣ 195), which is consistent with hemichannel properties in HEK293 cells (Fig. 2).

DISCUSSION

The first major finding of this study is that both wild-type Cx43 and the Cx43-GFP assemble into functional hemichannels when heterologously overexpressed in HEK293 cells. The hemichannels open upon the removal of extracellular Ca, are nonselective and permeable to large molecules such as Lucifer Yellow (Mᵣ 450), and are reversibly blocked by halothane and irreversibly blocked by La. To our knowledge, this study is the first detailed electrophysiological characterization of hemichannels formed from heterologously expressed Cx43 and defines a convenient, useful assay system for future structure-function studies. The findings are generally consistent with previously described studies of heterologously expressed Cx46 (13, 15, 16) and Cx56 (12) and studies of native hemichannels in skate (20) and catfish retina (11). Whether other connexins (17, 18) and Cx56 (12) and studies of native hemichannels (24).

The most significant finding is the demonstration that endogenous and heterologously expressed Cx43 or Cx43-GFP hemichannels open when subjected to metabolic inhibition, even when extracellular Ca remains normal. In a beating cardiac myocyte, 10 open hemichannels producing a 100 pA (0.7 pA/pF) current at −80 mV would increase the Na influx by 75% (22), and the fully developed current (5 pA/pF or about 70 open hemichannels) would increase the Na influx 5-fold. This represents a tiny fraction of the estimated 2.6 × 10⁶ junctional connexins in a typical cardiac myocyte (23). To the extent that the Na-K pump is unable to compensate for this additional Na load, intracellular Na accumulation and K loss result (22), causing membrane depolarization, intracellular Ca overload by reverse Na-Ca exchange, and possibly direct Ca entry through hemichannels. We cannot absolutely exclude that hemichannel density in isolated myocytes is artifically increased by the isolation procedure. However, existing evidence suggests that gap junction plaques are pulled off intact with either one myocyte or its partner, rather than separating into plaques of hemichannels (24).

The gap junction blocker halothane has been shown to have cardioprotective effects during myocardial ischemia (25), which could be mediated in part by inhibition of the Cx43 hemichannel. Also, activators of protein kinase C have been shown to prevent Cx43 hemichannels from opening during the removal of extracellular Ca (5), raising the possibility that the role of protein kinase C in ischemic preconditioning may be linked in part to the suppression of hemichannel activation.

Acknowledgment—We thank Dr. Yujuan Lu for technical assistance.

REFERENCES

1. Wilde, A. A., and Aksnes, G. (1995) Cardiovasc. Res. 29, 1–15
2. Li, H., Liu, T. P., Lazrak, A., Perecchia, C., Goldberg, G. S., Lampé, P. D., and Johnson, R. G. (1996) J. Cell Biol. 134, 1019–1030
3. Laird, D. W. (1996) J. Biomed. Biolumin. 26, 311–318
4. Kondo, R. P., Weiss, J. N., and Goldhaber, J. I. (1997) Circulation 96, 1359
5. Ho, S., Hont, H. D., Pullen, J. K., and Pease, L. R. (1989) Gene (Amst.) 77, 51–59
6. Chen, C. A., and Okayama, H. (1988) Biotechniques 6, 632–638
7. Goldhaber, J. I., Parker, J. M., and Weiss, J. N. (1991) J. Physiol. 433, 371–386
8. Mitra, R., and Morad, M. (1986) Am. J. Physiol. 249, H1056–H1060
9. Toyofuku, T., Yabuki, M., Otsu, K., Kuzuya, T., Hori, M., and Tada, M. (1998) J. Biol. Chem. 273, 1519–1526
10. Flagg-Newton, J., Simpson, I., and Loewenstein, W. R. (1979) Science 205, 404–407
11. DeVries, S. H., and Schwartz, E. A. (1992) J. Physiol. 445, 201–230
12. Ebihara, L., Berthoud, V. M., and Beyer, E. C. (1995) Biophys. J. 68, 1796–1803
13. Ebihara, L., and Steiner, E. (1995) J. Gen. Physiol. 102, 59–74
14. Ebihara, L. (1996) Biophys. J. 71, 742–748
15. Paul, D. L., Ebihara, L., Takamoto, L. J., Swenson, K. I., and Goodenough, D. A. (1991) J. Cell Biol. 115, 1077–1089
16. Tresli, E. B., Bennett, M. V., Bargiello, T. A., and Verselis, V. K. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5836–5841
17. Lazrak, A., Peres, A., Giovannardi, S., and Perecchia, C. (1994) Biophys. J. 67, 1052–1059
18. Kumar, N. M., and Gilula, N. B. (1996) Cell 84, 381–388
19. Manjunath, C. K., Goings, G. E., and Page, E. (1985) J. Membr. Biol. 85, 159–168
20. Malchow, R. P., Qian, H., and Ripps, H. (1995) J. Neurosci. Res. 39, 237–245
21. Rodrigo, G. C., and Chapman, B. A. (1991) J. Physiol. 434, 627–645
22. Shivkumar, K., Deutsch, N. A., lamp, S. T., Khu, K., Goldhaber, J. I., and Weiss, J. N. (1997) J. Clin. Invest. 100, 1782–1788
23. Manjunath, C. K., and Page, E. (1985) Am. J. Physiol. 248, H783–H791
24. Severs, N. J., Slade, A. M., Powell, T., Twa, V. W., and Green, C. R. (1990) J. Muscle Res. Cell Motil. 11, 154–166
25. Sieglund, B., Schlaack, W., Ladilov, Y. V., Balser, C., and Piper, H. M. (1997) Circulation 96, 4372–4379