Intercellular Calcium Signaling via Gap Junctions in Glioma Cells

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Abstract. Calcium signaling in C6 glioma cells in culture was examined with digital fluorescence video microscopy. C6 cells express low levels of the gap junction protein connexin43 and have correspondingly weak gap junctional communication as evidenced by dye coupling (Naus, C. C. G., J. F. Bechberger, S. Caveney, and J. X. Wilson. 1991. *Neurosci. Lett.* 126:33–36). Transfection of C6 cells with the cDNA encoding connexin43 resulted in clones with increased expression of connexin43 mRNA and protein and increased dye coupling, as well as markedly reduced rates of proliferation (Zhu, D., S. Caveney, G. M. Kidder, and C. C. Naus. 1991. Proc. Natl. Acad. Sci. USA. 88:1883–1887; Naus, C. C. G., D. Zhu, S. Todd, and G. M. Kidder. 1992. *Cell Mol. Neurobiol.* 12:163–175). Mechanical stimulation of a single cell in a culture of non-transfected C6 cells induced a wave of increased intracellular calcium concentration ($[Ca^{2+}]_i$) that showed little or no communication to adjacent cells. By contrast, mechanical stimulation of a single cell in cultures of C6 clones expressing transfected connexin43 cDNA induced a Ca^{2+} wave that was communicated to multiple surrounding cells, and the extent of communication was proportional to the level of expression of the connexin43 cDNA. These results provide direct evidence that intercellular Ca^{2+} signaling occurs via gap junctions. Ca^{2+} signaling through gap junctions may provide a means for the coordinated regulation of cellular function, including cell growth and differentiation.

INTERCELLULAR communication of changes in intracellular calcium concentration ($[Ca^{2+}]_i$) is a mechanism for direct signaling between adjacent cells (Sanderson et al., 1990; Charles et al., 1991). In the nervous system, Ca^{2+} signaling between glial cells may represent a system of widespread non-synaptic communication (Cornell-Bell et al., 1990; Charles et al., 1991). While it is generally believed that adjacent cells transmit signals through gap junctions, the nature of these signals is not well understood. Propagated changes in $[Ca^{2+}]_i$ which are suggestive of gap junctional communication have been observed in several types of cells including photocytes of Obelia (Dunlap et al., 1987; Brehm et al., 1989), hepatocytes (Saez et al., 1989), respiratory tract epithelial cells (Sanderson et al., 1990), Xenopus oocytes (Sandberg et al., 1990), astrocytes (Cornell-Bell et al., 1990), mixed glial cells (Charles et al., 1991), pancreatic cells (Gylfe et al., 1991), and endothelial cells (Demer, L., C. Wortham, E. Dirksen, and M. Sanderson. 1991. *Circulation.* 84(II):428). In each of these cell systems, an increase in $[Ca^{2+}]_i$ in a single cell leads to increases in $[Ca^{2+}]_i$ in surrounding cells. These increases in $[Ca^{2+}]_i$ are often observed to propagate as a wave across a cell and between cells, which we refer to as Ca^{2+} waves. The time course and spatial pattern of propagation of Ca^{2+} waves between cells suggests that these Ca^{2+} waves are communicated via gap junctions. However, none of the studies to date have definitively demonstrated that gap junctions are the route of communication of Ca^{2+} waves between cells. Inhibitors of gap junctional coupling such as octanol, heptanol, halothane, and low pH have been reported to inhibit intercellular Ca^{2+} signaling in Obelia cells (Dunlap et al., 1987), epithelial cells (Sanderson et al., 1990), and Xenopus oocytes (Sandberg et al., 1990). However, each of these inhibitors of gap junctional coupling may have non-specific effects on intracellular Ca^{2+} homeostasis (Haworth et al., 1989; Sanderson et al., 1990; Barnes and Bui, 1991; Chang et al., 1991; Mody et al., 1991; Still et al., 1991), raising the possibility that their inhibition of intercellular Ca^{2+} signaling may not be due to a direct effect on gap junctions.

To further investigate the role of gap junctions in intercellular Ca^{2+} signaling, we have examined the propagation of Ca^{2+} waves in C6 glioma cells and clones of C6 cells expressing varying levels of the gap junction protein connexin43. We have previously reported that in cultures of epithelial cells and glial cells, transient deformation of the membrane of a single cell with a micropipette induces a wave of increased $[Ca^{2+}]_i$ in the stimulated cell that is communicated to surrounding cells (Sanderson et al., 1990; Charles et al., 1991). We have used this technique of mechanical stimulation to induce Ca^{2+} waves in C6 glioma cells, and have found that the intercellular communication of Ca^{2+} waves in C6 cells is dependent upon the expression of connexin43.

Materials and Methods

Transfection of Connexin43 cDNA

Rat C6 glioma cells (American Type Culture Collection, Rockville, MD) were transfected with cDNA encoding connexin43 as previously described (Zhu et al., 1991). Briefly, the expression vector was derived by cloning the connexin43 cDNA clone G2 into the EcoR1 site of pLTR, a SV-40–based vector containing the *Escherichia coli* xanthine-guanine phosphoribosyltransferase (gpt)[1] as a dominant selectable marker. Cells were transfected using the Lipofectin reagent (Bethesda Research Laboratories, Gaithersburg, MD), and transfected clones were selected based on their expression of gpt. Transcription of the transfected plasmid resulted in a 2.25-kb connexin43 transcript (resulting from 1.4-kb G2 connexin43 cDNA + 0.85-kb SV-40 splice and polyadenylation region from pLTR).

Northern Blot RNA Analysis

Total cellular RNA from an equal number of C6 cells and transfected clones was extracted, electrophoretically separated in a 1.2% agarose–formaldehyde gel, capillary blotted onto nitrocellulose, and hybridized with connexin43 cDNA according to previously described methods (Zhu et al., 1991). Blots were examined with autoradiography and relative levels of connexin43 mRNA were measured by densitometric analysis.

Immunoblot Protein Analysis

An equal number (1×10^7) of dissociated cells of each type were lysed in 0.5% NP-40 and centrifuged at 10,000 r.p.m. for 3 min. The resulting pellets were added to 1 ml SDS loading buffer and resolved on 12% polyacrylamide gels followed by electrophoretic transfer to nitrocellulose. The nitrocellulose blot was incubated overnight at 4°C with a polyclonal antibody directed against the COOH-terminal domain (residues 302–319) of connexin43 (gift from Dr. B. J. Nicholson, affinity purified and diluted 1:1000). The blot was then washed five times with PBS containing 5% nonfat dry milk, incubated in ^{125}I-goat anti-rabbit IgG for 1 h, washed again, and examined with autoradiography.

Cell Culture

C6 cells and transfected clones were passaged and grown on glass coverslips in DME (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS, streptomycin, and penicillin (all from Sigma Chemical Co., St. Louis, MO) at 37°C in an atmosphere containing 5 or 10% CO_2. Cells of each type were grown to an equal confluence of ~90%. Cells were plated with an initial density of $\sim 10^4$ cells per each 15-mm diam coverslip and were grown for 2–6 d before experimentation. Since the rates of proliferation of the transfected clones were significantly less than the C6 cells, equal confluence was achieved either by a greater initial plating density of the transfected clones, or by a longer time in culture for the transfected clones. While all studies presented here were performed on cultures of similar confluence, other cultures grown to different levels of confluence gave similar results (data not shown) indicating that cell density was not a primary factor in the ability to communicate Ca^{2+} waves.

Mechanical Stimulation

Mechanical stimulation of a single cell was performed by briefly deforming the cell membrane with a glass micropipette as previously described (Sanderson et al., 1990; Charles et al., 1991). The distance and duration of the downward movement of the micropipette were controlled by a piezoelectric device. The duration of each stimulus was 150 ms. The total displacement of the micropipette was 10 μm, which included the distance between the pipette and the cell. The actual displacement of the cell membrane was estimated at 1–2 μm.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined by measurement of fura-2 fluorescence. Cells were loaded with fura-2 by incubation in HBSS with 25 mM Hepes buffer (HBSS/Hepes) (Sigma Chemical Co.) containing 5 μM fura-2-AM for 30 min at 37°C. After loading, cells were washed with HBSS/Hepes and left at room temperature for 30 min before experimentation. All experiments were performed at room temperature. The fluorescence imaging system and analysis methods were the same as those that have been previously described in detail (Sanderson et al., 1990; Charles et al., 1991).

1. *Abbreviation used in this paper*: gpt, guanine phosphoribosyltransferase.

Results

Expression of Transfected Connexin43

The level of connexin43 mRNA in cultures of C6 glioma cells is ~10% of that exhibited by primary cultures of glial cells (purified astrocytes, Naus et al., 1991). Transfection of C6 cells with the cDNA encoding connexin43 resulted in clones with varying levels of expression of connexin43 RNA and protein (Zhu et al., 1991; Naus et al., 1992). The level of connexin43 mRNA in the two clones which were used in this study, the Cx43-14 and Cx43-13 clones, was greater than that in nontransfected C6 cells by approximately 30- and 50-fold, respectively, as determined by densitometric analysis (Fig. 1 *a*). There was a corresponding increase in the level of the connexin43 protein in each clone (Fig. 1 *b*). The extent of dye coupling between cells was increased in proportion to the increased amount of connexin43 mRNA and protein in each clone. This correlation strongly suggests that there is increased functional gap junctional coupling in the transfected C6 clones (Zhu et al., 1991). Furthermore, ultrastructural studies using immunocytochemical (Naus et al., 1992) and freeze fracture EM techniques (Naus, C. C. G., S. Hearn, D. Zhu, R. R. Shivers. 1991. *J. Cell Biol.* 115:190*a*.) verified that increased numbers of gap junctions occurred in correlation with increased expression of connexin43 mRNA and protein in transfected C6 clones. Interestingly, clones expressing the transfected connexin43 cDNA had a significantly decreased rate of cellular proliferation (Zhu et al., 1991).

Intercellular Communication of Calcium Waves

In primary rat brain glial cultures, mechanical stimulation of a single cell induced a wave of increased $[Ca^{2+}]_i$ which spread from the point of micropipette contact throughout the stimulated cell to the cell boundaries. This wave of increased

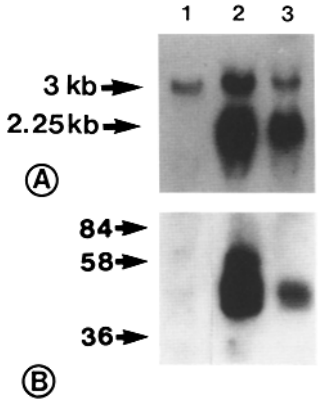

Figure 1. (*A*) Northern blot analysis of RNA from C6 cells (lane *1*) and transfected clones Cx43-13 (lane *2*) and Cx43-14 (lane *3*). All cells express some of the endogenous 3-kb connexin43 mRNA, but the transfected clones also express 2.25 kb mRNA resulting from expression of the transfected cDNA. The Cx43-13 clone shows heavy expression and the Cx43-14 shows moderate expression of the 2.25-kb connexin43 mRNA. (*B*) Immunoblot analysis of plasma membrane–enriched protein fractions from C6 cells (lane *1*) and transfected clones Cx43-13 (lane *2*) and Cx43-14 (lane *3*). Connexin43 was minimally detectable in the C6 sample, but was present in high levels in clone Cx43-13 and at moderate levels in clone Cx43-14. Numbers indicate positions of molecular weight standards in kilodaltons.

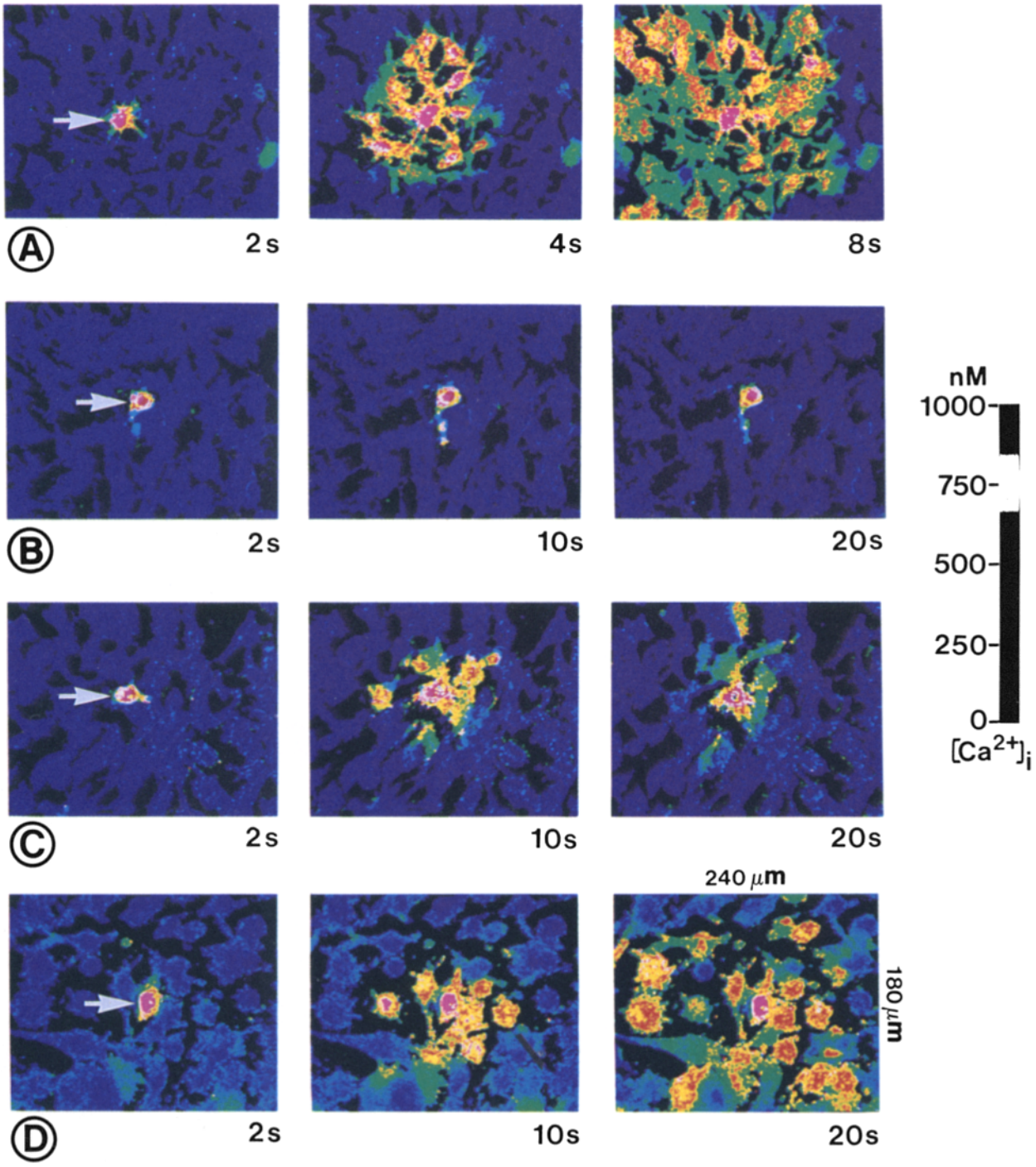

Figure 2. Calcium waves induced by mechanical stimulation in (*A*) a primary mixed glial culture, (*B*) a culture of C6 glioma cells, (*C*) a clone of transfected C6 cells showing moderate expression of connexin43 mRNA and protein (Cx43-14), and (*D*) a clone showing greater expression of connexin43 mRNA and protein (Cx43-13). Each panel shows the $[Ca^{2+}]_i$ in a field of cells at sequential times (indicated below each panel in seconds) following mechanical stimulation of a single cell. The stimulated cell is indicated by the white arrow in each sequence. (*A*) In a primary mixed glial culture, mechanical stimulation induces a wave of increased $[Ca^{2+}]_i$ in the stimulated cell that is communicated cell by cell to most surrounding cells in the microscopic field. (*B*) In C6 glioma cells, mechanical stimulation induces a Ca^{2+} wave in the stimulated cell that is not communicated to adjacent cells. (*C*) In clone Cx43-14, the Ca^{2+} wave induced by mechanical stimulation is communicated to eight adjacent cells in this example. (*D*) In clone Cx43-13 the Ca^{2+} wave induced by mechanical stimulation is communicated to 35 adjacent cells in this example (further communication occurred after the 20-s time point).

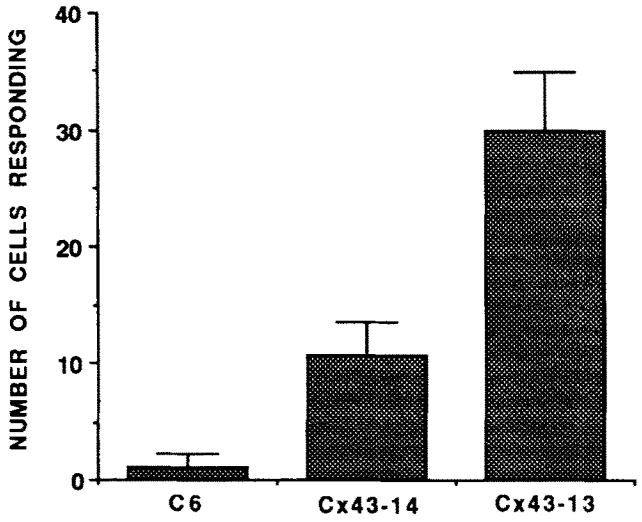

Figure 3. The average number of cells to which a Ca^{2+} wave was communicated in C6 glioma cells and two transfected clones showing moderate (Cx43-14) and greater (Cx43-13) expression of transfected connexin43 cDNA (n = 7 experiments for each cell type; error bars represent SD).

$[Ca^{2+}]_i$ was then communicated cell by cell in all directions to surrounding cells in the culture (Fig. 2 *A*) (Charles et al., 1991). In contrast to this extensively propagated Ca^{2+} response, mechanical stimulation of a single cell in cultures of non-transfected C6 glioma cells resulted in a Ca^{2+} wave in the stimulated cell which was not communicated to adjacent cells, or showed limited communication to at most one or two adjacent cells (Figs. 2 *B* and 3). However, in cultures of C6 clones expressing transfected connexin43 cDNA, Ca^{2+} waves induced by mechanical stimulation were communicated to multiple surrounding cells. The extent of this communication was proportional to the level of expression of the connexin43 cDNA. In the clone showing moderate expression of connexin43 (Cx43-14), the Ca^{2+} wave was communicated to 7–13 surrounding cells (Figs. 2 *C* and 3). In the clone showing greater expression of connexin43 (Cx43-13), the Ca^{2+} wave was communicated to a greater extent, to 20–38 adjacent cells (Figs. 2 *D* and 3). In the Cx43-14 and Cx43-13 clones, Ca^{2+} waves were propagated in a manner similar to that seen in primary glial cultures, frequently following a circuitous path throughout the culture (Figs. 2 and 4). Ca^{2+} waves within individual cells often originated from distinct sites of contact with other cells that had recently responded.

The magnitude of the increase in $[Ca^{2+}]_i$ associated with the Ca^{2+} wave in the stimulated cell in the C6 cells and Cx43-13 and Cx43-14 clones was similar to that observed in primary glial cultures (>1,000 nM, Fig. 2 and Table I). In the transfected clones, the magnitude of the Ca^{2+} wave generally decreased in proportion to the distance from the stimulated cell (Table I and Fig. 4), although the magnitude of the response varied widely in individual cells. The $[Ca^{2+}]_i$ of all cells, including the stimulated cell, eventually recovered to resting levels over 10 s to 5 min. The stimulated cell always showed the slowest recovery of $[Ca^{2+}]_i$ to resting levels (Fig. 4).

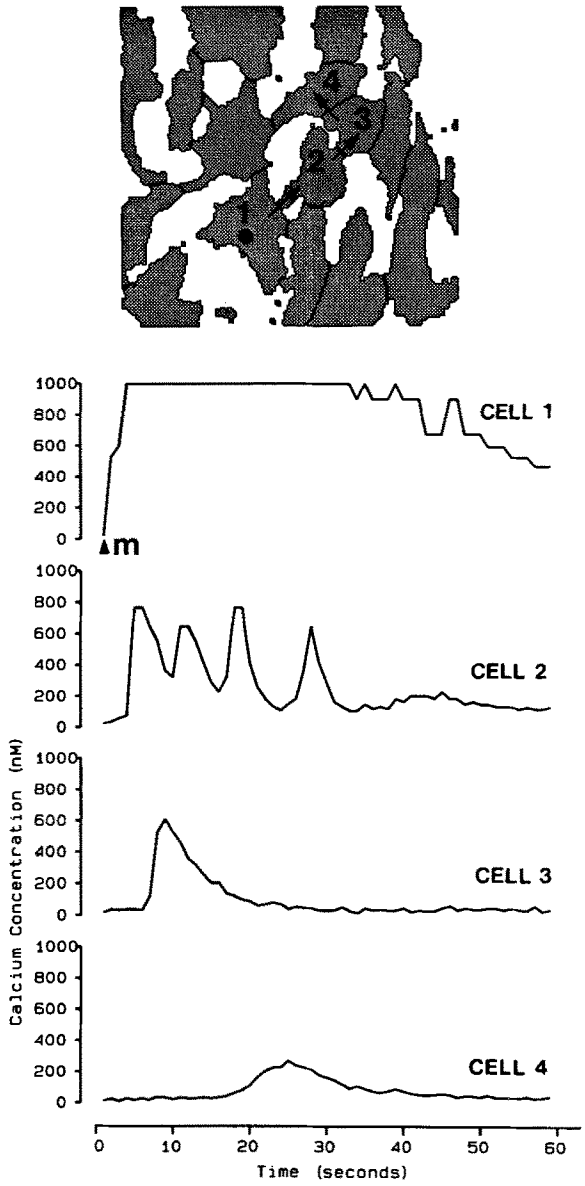

Figure 4. The propagation of a Ca^{2+} wave from a mechanically stimulated cell through three adjacent cells in a culture of the Cx43-13 clone. The diagram at the top of the figure demonstrates the position of the stimulated cell (*CELL 1*) and three adjacent cells. The arrows on the image indicate the direction of intercellular communication of the Ca^{2+} wave in these four cells. CELL 1 was mechanically stimulated at the point indicated by the closed circle at the time indicated on the graph by the arrow labeled (*M*). The stimulated cell showed a sustained increase in $[Ca^{2+}]_i$ to at least 1,000 nM (the maximum $[Ca^{2+}]_i$ level accurately measurable with our system) which began to recover toward resting levels after 40 s. A Ca^{2+} wave appeared in an adjacent cell (*CELL 2*) after an intercellular lag period of ~3 s. The Ca^{2+} wave was then propagated sequentially to CELL 3 and CELL 4, with an increasing intercellular lag period. The amplitude of the Ca^{2+} wave decreased with increasing distance from the stimulated cell. CELL 2 showed oscillations in $[Ca^{2+}]_i$ which were not communicated to adjacent cells. Although their responses are not shown, most other cells in this field of cells showed a similar outward propagation of the Ca^{2+} wave.

Table I. Maximum $[Ca^{2+}]_i$ in Cells Propagating Ca^{2+} Wave

	Cell position					
	1 (stim)	2	3	4	5	6
C6	>1,000 nM	NR	NR	NR	NR	NR
Cx43-14	>1,000 nM	713 nM (±262)	437 nM (±282)	400 nM (±316)	NR	NR
Cx43-13	>1,000 nM	715 nM (±209)	584 nM (±199)	382 nM (±185)	349 nM (±172)	228 nM (±87)

Each column represents the average maximum $[Ca^{2+}]_i$ (±SD) in cells showing a propagated Ca^{2+} wave in response to mechanical stimulation (average from six different cultures of each cell type). Cell position refers to the position of the cell relative to the stimulated cell in the path of Ca^{2+} wave propagation (for example, see Fig. 4). NR indicates that there was no response in cells in this position. All stimulated cells reached a level of at least 1,000 nM (the highest $[Ca^{2+}]_i$ accurately measurable with our system).

Intercellular Lag Period

There was a lag period between the arrival of a Ca^{2+} wave at the boundary of a cell and the subsequent appearance of a Ca^{2+} wave in an adjacent cell. As a result, the Ca^{2+} wave was propagated throughout a culture by a series of intracellular Ca^{2+} waves interrupted by intercellular delays. The duration of this intercellular delay increased in proportion to the distance from the stimulated cell, varying from 1 to 30 s (Fig. 4 and Table II). The average intercellular lag period was greater in the Cx43-14 clone than the Cx43-13 clone for all corresponding cell positions relative to the stimulated cell (Table II). While the pattern of Ca^{2+} wave propagation in transfected C6 cells was similar to that observed in primary mixed glial cultures (Charles et al., 1991), the rate of intercellular spread of Ca^{2+} waves was considerably slower in the transfected C6 cells (2–10 μm/s in both Cx43-13 and Cx43-14 cells) than in primary glial cultures (15–27 μm/s) (Fig. 2).

Oscillations in $[Ca^{2+}]_i$

Individual resting C6 cells and transfected cells showed occasional spontaneous, transient, repetitive increases in $[Ca^{2+}]_i$, or Ca^{2+} oscillations. In addition, some cells (5–40%) in each of the Cx43-13 and Cx43-14 cultures showed asynchronous oscillations in $[Ca^{2+}]_i$ after propagation of the Ca^{2+} wave (Fig. 4, cell *2*). These Ca^{2+} oscillations were similar to those that have been previously described in primary glial cultures. The periodicity of the Ca^{2+} oscillations after Ca^{2+} wave propagation varied from 5–40 s. Neither the spontaneous Ca^{2+} oscillations nor those induced by propagation of a Ca^{2+} wave were communicated to adjacent cells, even when the magnitude of the Ca^{2+} oscillation was similar to that of the communicated Ca^{2+} wave (Fig. 4). A Ca^{2+} wave induced by mechanical stimulation could be propagated across Cx43-13 or Cx43-14 cells undergoing spontaneous asynchronous oscillations in $[Ca^{2+}]_i$.

Table II. Intercellular Lag Period in Ca^{2+} Wave Communication

	Cell position				
	1-2	2-3	3-4	4-5	5-6
Cx43-14	2.4 s (±1.2)	5.0 s (±1.9)	9.2 s (±4.5)	NR	NR
Cx43-13	1.7 s* (±0.8)	3.9 s* (±1.1)	5.3 s* (±1.1)	7.9 s (±1.7)	19 s (±3.9)

Each column represents the average lag period (±SD) between arrival of a Ca^{2+} wave at the border of a cell and the subsequent appearance of a Ca^{2+} wave in an adjacent cell (average from six different cultures for each cell type). Cell position indicates position relative to the stimulated cell (position 1) in the path of Ca^{2+} wave propagation. NR indicates cells which did not show a response.
* Statistically significant difference ($P < .05$) from values for Cx43-14 clone for corresponding cell positions.

Discussion

Deformation of the membrane of a single cell with a mechanical stimulus can initiate a cellular response which is communicated from the stimulated cell to many surrounding cells (Sanderson and Dirksen, 1986; Sanderson et al., 1990; Charles et al., 1991). Mechanical stimulation of a single cell in primary mixed glial cultures induced a wave of increased $[Ca^{2+}]_i$ in the stimulated cell which was communicated extensively to many surrounding cells. By contrast, C6 glioma cells, which express low levels of connexin43 and show minimal gap junctional coupling, showed little or no intercellular communication of Ca^{2+} waves in response to mechanical stimulation. In clones of C6 cells expressing transfected connexin43 cDNA, the ability to communicate Ca^{2+} waves was restored, and the extent of this communication was correlated with the level of expression of the connexin43.

The technique of mechanical stimulation is particularly useful in the study of intercellular calcium signaling because it reproducibly induces a wave of increased $[Ca^{2+}]_i$ in a single cell which may be propagated from cell to cell (Sanderson et al., 1990; Charles et al., 1991). Several lines of evidence indicate that the Ca^{2+} wave induced by mechanical stimulation is not a result of a propagated mechanical wave. First, a mechanical stimulus lasting 150 ms initiates a spreading increase in $[Ca^{2+}]_i$ that occurs over the following 30–60 s; a mechanical wave would be expected to propagate much more rapidly. Second, there is a lag period of up to 30 s between the arrival of a Ca^{2+} wave at the boundaries of a cell and the subsequent communication of the Ca^{2+} wave to an adjacent cell: a mechanical wave would be expected to proceed without interruption from cell to cell. Finally, there is little or no communication of Ca^{2+} waves induced by mechanical stimulation in cells which have little gap junctional coupling: propagation of a mechanical wave should not require gap junctional coupling. It is possible that a mechanical stimulus injures the stimulated cell. However, in these experiments, the $[Ca^{2+}]_i$ of the stimulated cell always recovered to resting levels following a mechanical stimulus, and in most cases there was no decrease in fura-2 fluorescence intensity suggesting that there was no leakage of the indicator

from the cell. In addition, after recovery of $[Ca^{2+}]_i$ to resting levels a cell could be repeatedly stimulated, each time inducing an identical Ca^{2+} response.

The pattern of intercellular propagation of Ca^{2+} waves in glial cells and other cell types suggests that increases in $[Ca^{2+}]_i$ are communicated at sites of specific intercellular connections. The results of the present study demonstrate a direct correlation between the expression of connexin43 and the intercellular communication of increases in $[Ca^{2+}]_i$. In light of the increased dye coupling and immunohistochemical and electron microscopic demonstration of increased gap junctions associated with the increased expression of connexin43 mRNA (Zhu et al., 1991; Naus, C. C. G., S. Hearn, D. Zhu, R. R. Shivers. 1991. *J. Cell Biol.* 115:190*a*; Naus et al., 1992), the most direct explanation for this correlation is that Ca^{2+} waves are propagated via gap junctions. It is possible that the overexpression of connexin43 somehow alters the mechanisms of intracellular messenger production or results in channels in intracellular membranes which might result in different patterns of Ca^{2+} signaling. However, if expression of transfected connexin43 cDNA had some effect on intracellular Ca^{2+} release, it might be expected to result in other changes in Ca^{2+} homeostasis, such as a change in resting $[Ca^{2+}]_i$, a difference in the peak $[Ca^{2+}]_i$ associated with a Ca^{2+} wave, or different patterns of Ca^{2+} oscillations—no such differences were observed. In the C6 cells and transfected clones, the only difference in Ca^{2+} signaling associated with increased expression of connexin43 was a greater extent of intercellular communication and a decreased lag period in the communication of the response from cell to cell. The response to mechanical stimulation in the transfected C6 cells was very similar to that observed in primary glial cultures, except for a faster rate of intercellular propagation of Ca^{2+} waves in the primary cultures. The similarity of these intracellular Ca^{2+} responses, in contrast to the marked difference in the intercellular communication of Ca^{2+} waves, argues against a nonspecific effect of expression of transfected connexin43.

Not all increases in $[Ca^{2+}]_i$ were communicated: Ca^{2+} oscillations occurred independently in individual cells, even in adjacent cells which showed communication of a Ca^{2+} wave in response to mechanical stimulation. The coexistence of communicated Ca^{2+} waves and asynchronous Ca^{2+} oscillations has also been observed in cultures of mixed glial cells (Charles et al., 1991), purified astrocytes (Cornell-Bell et al., 1990), and epithelial cells (Sanderson, M. J., A. C. Charles, and E. R. Dirksen. 1990. *J. Cell Biol.* 111:389*a*). The differential communication of these otherwise similar increases in $[Ca^{2+}]_i$ could be explained by distinct mechanisms of intracellular Ca^{2+} release. We have previously reported that microinjection of IP_3 induces a communicated Ca^{2+} wave which is similar to that which is seen in response to mechanical stimulation (Sanderson et al., 1990). Thapsigargin, an inhibitor of endoplasmic reticulum Ca^{2+}-ATPases which depletes IP_3-sensitive intracellular Ca^{2+} stores, has been found to block intercellular propagation of Ca^{2+} waves (Charles et al., 1992). Dantrolene, an inhibitor of Ca^{2+}-induced Ca^{2+} release, has been observed to reduce the magnitude of Ca^{2+} release and abolish Ca^{2+} oscillations, without affecting the rate of intercellular propagation of Ca^{2+} waves. Based on these results, we have proposed that intercellular communication of Ca^{2+} waves is mediated by IP_3, whereas intracellular Ca^{2+} waves are amplified and subsequent Ca^{2+} oscillations are generated primarily by Ca^{2+}-induced Ca^{2+} release (Sanderson et al., 1990; Charles et al., 1991, 1992).

An alternative explanation for the difference in communication of Ca^{2+} waves and Ca^{2+} oscillations is that gap junctions are opened under specific conditions which allow propagation of Ca^{2+} waves, but remain closed during Ca^{2+} oscillations. However, Ca^{2+} waves induced by mechanical stimulation could be propagated through Cx43-13 and Cx43-14 cells undergoing spontaneous asynchronous oscillations in $[Ca^{2+}]_i$. In addition, in primary glial cell cultures, a Ca^{2+} wave could be propagated across cells undergoing asynchronous Ca^{2+} oscillations induced by exposure to glutamate. These observations suggest that increases in $[Ca^{2+}]_i$ can be communicated via gap junctions even in cells undergoing asynchronous oscillations in $[Ca^{2+}]_i$.

The decrease in the peak $[Ca^{2+}]_i$ observed with increasing distance from the stimulated cell suggests that the Ca^{2+} wave is mediated by a diffusible messenger which is depleted as the Ca^{2+} wave is propagated. However, the lag period of 1 to 30 s between the arrival of a Ca^{2+} wave at the border of a cell and the subsequent appearance of a Ca^{2+} wave in an adjacent cell indicates that a linear response to a diffusible intercellular messenger cannot fully explain the intercellular communication of the Ca^{2+} wave. This intercellular lag period could be explained by the existence of a threshold concentration of a messenger required to initiate a Ca^{2+} wave: the lag period may represent the time taken for the concentration of messenger to reach that threshold. The observation that the intercellular lag period is decreased with increased expression of connexin43 is consistent with this hypothesis, since a greater number of gap junctions may allow a messenger to reach a threshold concentration more quickly in the adjacent cell. Alternatively, the lag period could be due to a delay in the opening of gap junctions which delays the movement of a messenger from one cell to the next.

Increases in $[Ca^{2+}]_i$ have been observed to decrease gap junctional coupling in a number of different cell types, although it is not known whether physiologic increases in $[Ca^{2+}]_i$ are involved in gating of gap junctions (reviewed by Bennett et al., 1988). Our results suggest that increases in $[Ca^{2+}]_i$ in the physiologic range do not inhibit communication via gap junctions. If the increase in $[Ca^{2+}]_i$ associated with the Ca^{2+} wave resulted in a closure of gap junctions, this should interrupt the intercellular propagation of the messenger which mediates the Ca^{2+} wave. Assuming that the adjacent cell responded directly to a threshold concentration of a messenger such as IP_3, a Ca^{2+} response to this messenger in the adjacent cell should not occur after the peak increase in $[Ca^{2+}]_i$ in the proximal cell. However, we observed that the $[Ca^{2+}]_i$ of the proximal cell often reached its peak during the lag period of intercellular communication, before the initiation of a response in an adjacent cell (Fig. 4 cells *2*, *3*, and *4*). This observation suggests that increases in $[Ca^{2+}]_i$ of up to 1 μM did not inhibit gap junctional communication. However, we cannot exclude the possibility that an increase in $[Ca^{2+}]_i$ in the proximal cell did in fact close the gap junctions, but the adjacent cell nonetheless showed a delayed Ca^{2+} response to messenger which was transmitted before the closure of gap junctions. Additional evidence against the closure of gap junctions by physiologic increases

in $[Ca^{2+}]_i$ has been provided by studies using mixed glial cultures, in which Ca^{2+} waves induced by mechanical stimulation are propagated with normal velocity across cells whose $[Ca^{2+}]_i$ has been increased to up to 500 nM in response to glutamate (Charles et al., 1991). The intercellular propagation of Ca^{2+} waves may represent a functional measure of gap junctional communication which may provide further insight into the gating of gap junctions.

There is evidence for widespread gap junctional coupling throughout the central nervous system (Gutnick et al., 1981; Kettenmann et al., 1983, Bennett et al., 1991; Micevich and Abelson, 1991). Intercellular Ca^{2+} signaling between glial cells could coordinate such functions as the regulation of extracellular potassium, the generation of slow electrical fields, or the response of glial cells to a localized injury (Charles et al., 1991; Dermietzel et al., 1991). In addition, communication of Ca^{2+} waves through gap junctions may be involved in the phenomenon of spreading depression in the nervous system (Charles et al., 1991; Cornell-Bell and Finkbeiner, 1991). Changes in $[Ca^{2+}]_i$ are also known to be important in the control of the cell cycle (Means and Rasmussen, 1988; Johnson et al., 1990; Whitaker and Patel, 1990). Thapsigargin, a substance which alters IP_3-mediated Ca^{2+} release and inhibits intercellular Ca^{2+} waves (Charles et al., 1992), has tumor-promoting effects (Hakii et al., 1986; Thastrup et al., 1990). The decreased rate of cell proliferation which results from the expression of transfected connexin43 cDNA in C6 cells suggests that increased gap junctional communication inhibits cellular proliferation (Zhu et al., 1991). Intercellular Ca^{2+} signaling through gap junctions may therefore be a mechanism for the coordinated control of cellular growth.

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References

Barnes, S., and Q. Bui. 1991. Modulation of calcium-activated chloride current via pH-induced changes of calcium channel properties in cone photoreceptors. *J. Neurosci.* 11:4015–4023.

Bennett, M. V. L., V. Verselis, R. L. White, and D. C. Spray. 1988. Gap junctional conductance: gating. *In* Gap Junctions. E. L. Hertzberg and R. G. Johnson, editors. Alan R. Liss. New York. 167–175.

Bennett, M. V. L., L. C. Barrio, T. A. Bargiello, D. C. Spray, E. Hertzberg, and J. C. Saez. 1991. Gap junctions: new tools, new answers, new questions. *Neuron.* 6:305–320.

Brehm, P., J. Lechleiter, S. Smith, and K. Dunlap. 1989. Intracellular signaling as visualized by endogenous calcium-dependent bioluminescence. *Neuron.* 3:191–198.

Chang, D., N. L. Kushman, and D. C. Dawson. 1991. Intracellular pH regulates basolateral K+ and Cl- conductances in colonic epithelial cells by modulating Ca^{2+} activation. *J. Gen. Physiol.* 98:183–196.

Charles, A. C., J. E. Merrill, E. R. Dirksen, and M. J. Sanderson. 1991. Intercellular signaling in glial cells: calcium waves and oscillations in response to mechanical stimulation and glutamate. *Neuron.* 6:938–992.

Charles, A. C., E. R. Dirksen, J. E. Merrill, and M. J. Sanderson. 1992. Mechanisms of intercellular calcium signaling in glial cells studied with dantrolene and thapsigargin. *Glia.* In press.

Cornell-Bell, A. H., and S. M. Finkbeiner. 1991. Calcium waves in astrocytes. *Cell Calcium.* 12:185–204.

Cornell-Bell, A. H., S. M. Finkbeiner, M. S. Cooper, and S. J. Smith. 1990. Glutamate induces calcium waves in cultured astrocytes: long range glial signaling. *Science (Wash. DC).* 247:470–474.

Dermietzel, R., E. L. Hertzberg, J. A. Kessler, and D. C. Spray. 1991. Gap junctions between cultured astrocytes: immunocytochemical, molecular, and electrophysiological analysis. *J. Neurosci.* 11:1421–1432.

Dunlap, K., K. Takeda, and P. Brehm. 1987. Activation of a calcium-dependent photoprotein by chemical signalling through gap junctions. *Nature (Lond.).* 325:60–62.

Gutnick, M. J., B. W. Connors, and B. R. Ransom. 1981. Dye coupling between glial cells in the pig neocortical slice. *Brain Res.* 213:486–492.

Gylfe, E., E. Grapengiesser, and B. Hellman. 1991. Propagation of cytoplasmic Ca^{2+} oscillations in clusters of pancreatic B-cells exposed to glucose. *Cell Calcium.* 12:229–240.

Hakii, H., H. Fujiki, M. Suganuma, M. Nakayasu, T. Tahira, T. Sugimura, P. J. Scheuer, and S. B. Christensen. 1986. Thapsigargin, a histamine secretagogue, is a non-12-O-tetradecanoylphorbol-13-acetate (TPA) type tumor promoter in two-stage mouse skin carcinogenesis. *J. Canc. Res. Clin. Oncol.* 111:177–181.

Haworth, R. A., A. B. Goknur, and H. A. Berkoff. 1989. Inhibition of Na-Ca exchange by general anesthetics. *Circ. Res.* 65:1021–1028.

Johnson, C. L., C. G. Johnson, E. Bazan, D. Garver, E. Gruenstein, and M. Ahluwalia. 1990. Histamine receptors in human fibroblasts: inositol phosphates, Ca2+, and cell growth. *Am J. Physiol.* 90:PC533–543.

Kettenmann, H., R. K. Orkand, and M. Schachner. 1983. Coupling among identified cells in the mammalian nervous system. *J. Neurosci.* 3:506–516.

Means, A. R., and C. D. Rasmussen. 1988. Calcium, calmodulin, and cell proliferation. *Cell Calcium.* 9:313–319.

Micevich, P. E., and L. Abelson. 1991. Distribution of mRNAs coding for liver and heart gap junction proteins in the rat central nervous system. *J. Comp. Neurol.* 305:96–118.

Mody, I., D. L. Tanelian, and M. P. MacIver. 1991. Halothane enhances tonic neuronal inhibition by elevating intracellular calcium. *Brain Res.* 538: 319–323.

Naus, C. C. G., J. F. Bechberger, S. Caveney, and J. X. Wilson. 1991. Expression of gap junction genes in astrocytes and C6 glioma cells. *Neurosci. Lett.* 126:33–36.

Naus, C. C. G., D. Zhu, S. Todd, and G. M. Kidder. 1992. Characteristics of C6 glioma cells overexpressing a gap junction protein. *Cell Mol. Neurobiol.* In press.

Sáez, J. C., J. A. Connor, D. C. Spray, and M. V. Bennett. 1989. Hepatocyte gap junctions are permeable to the second messenger inositol 1,4,5-trisphosphate, and to calcium ions. *Proc. Natl. Acad. Sci. USA.* 86:2708–2712.

Sandberg, K., M. Bor, H. Ji, A. Marwick, M. A. Millan, and K. J. Catt. 1990. Angiotensin II-induced calcium mobilization in oocytes by signal transfer through gap junctions. *Science (Wash. DC).* 249:298–301.

Sanderson, M. J., and E. R. Dirksen. 1986. Mechanosensitivity of cultured ciliated cells from the mammalian respiratory tract: implications for the regulation of mucociliary transport. *Proc. Natl. Acad. Sci. USA.* 83:7302–7306.

Sanderson, M. J., A. C. Charles, and E. R. Dirksen. 1990. Mechanical stimulation and intercellular communication increases intracellular Ca^{2+} in epithelial cells. *Cell Regulation.* 1:585–596.

Still, J. C., C. Uhl, S. Eskuri, R. Van Dyke, and J. Tarara. 1991. Halothane inhibits agonist-induced inositol phosphate and Ca^{2+} signaling in A7r5 cultured vascular smooth muscle cells. *Mol. Pharm.* 40:1006–1013.

Thastrup, O., P. J. Cullen, B. K. Drobak, M. R. Hanley, and A. P. Dawson. 1990. Thapsigargin, a tumor promoter, discharges intracellular Ca2+ stores by specific inhibition of the endoplasmic reticulum Ca2+-ATPase. *Proc. Natl. Acad. Sci. USA.* 87:2466–2470.

Whitaker, M., and R. Patel. 1990. Calcium and cell cycle control. *Development.* 108:525–542.

Zhu, D., S. Caveney, G. M. Kidder, and C. C. Naus. 1991. Transfection of C6 glioma cells with connexin43 cDNA: analysis of expression, intercellular coupling, and cell proliferation. *Proc. Natl. Acad. Sci. USA.* 88:1883–1887.