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Gap Junction Channels Coordinate the Propagation of Intercellular Ca^{2+} Signals Generated by P2Y Receptor Activation

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Abstract

Astrocytes express gap junction proteins and multiple types of P2Y receptors (P2YRs) that contribute to the propagation of intercellular Ca^{2+} waves (ICW). To gain access to the role played by gap junctional communication in ICW propagation generated by P2YR activation, we selectively expressed P2Y_{1,2,4}R subtypes and Cx43 in the human 1321N1 astrocytoma cell line, which lacks endogenous P2 receptors. Fluorescence recovery after photobleaching revealed that 1321N1 cells are poorly dyecoupled and do not propagate ICW. Forced expression of Cx43 in 1321N1 cells (which did not show functional hemichannels) increased dye coupling and allowed short-range ICW transmission that was mainly mediated by intercellular diffusion of Ca^{2+} generated in the stimulated cells. Astrocytoma clones expressing each of the P2YR subtypes were also able to propagate ICWs that were likely dependent on IP₃ generation. These waves exhibited properties particular to each P2YR subtype. Co-expression of eGFP-hCx43 and P2Y₁R modified the properties of P2Y₁R-generated ICW to those characteristics of P2Y₂R. Increased coupling in P2Y₄R clones induced by expression of eGFP-hCx43 abolished the ICWs observed in uncoupled P2Y₄R clones. No changes in the behavior of ICWs generated in P2Y₂R clones were observed after forced expression of Cx43. These data indicate that in 1321N1 cells gap junctional communication provides intercellular integration of Ca^{2+} signals generated by P2YR activation, thus coordinating the propagation of intercellular calcium waves.

Keywords

purinergic receptors; astrocytoma; calcium waves; connexin43; dye coupling

INTRODUCTION

In the central nervous system (CNS), spatial and temporal changes in intracellular Ca^{2+} levels control and modulate numerous cellular functions such as cell proliferation, neuronal migration, motility of dendritic growth cones, and clustering of postsynaptic receptors and channels (Kater et al., 1988; Ghosh and Greenberg, 1995; LoTurco et al., 1995; Komuro and Rakic, 1996; Gu and Spitzer, 1997). Intracellular Ca^{2+} transients in astrocytes can be transmitted to neighboring cells as intercellular Ca^{2+} waves (ICWs) (Cornell-Bell and Finkbeiner, 1991; Finkbeiner, 1992; Venance et al., 1995; Charles et al., 1996; Scemes et al., 1998). In these cells, IP₃ is the main second messenger mediating intracellular calcium rises that occur during the spread of ICW (Boitano et al., 1992; Venance et al., 1997; Leybaert et al., 1998). There are two routes by which ICWs travel between astrocytes; one involves the diffusion of IP₃ through gap junction channels and the other depends on the activation of surface

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membrane receptors due to the extracellular diffusion of molecules (ATP, glutamate) that are released from the stimulated cells (see Scemes, 2000).

Astrocytes in culture and *in situ* are coupled by gap junction channels mainly formed by Cx43 (Dermietzel et al., 2000; Nagy and Rash, 2000; Nagy et al., 2001) and express a multitude of purine and pyrimidine nucleotide receptors (Jimenez et al., 2000; Fumagalli et al., 2003; Suadicani et al., 2003). The relative contribution of gap junctions and P2 receptors for the propagation of ICW between astrocytes has been controversial. The first reports describing ICW propagation between astrocytes indicated that gap junction channels were the main, if not the only, route by which intercellular Ca^{2+} signals were transmitted (Charles et al., 1992; Finkbeiner, 1992; Nedergaard, 1994; Venance et al., 1995, 1997). Through the use of gap junction channel blockers (Finkbeiner, 1992; Nedergaard, 1994; Venance et al., 1995, 1997) or by overexpressing Cx43 in gap junction deficient cells (Charles et al., 1992), these studies showed that the extent of ICW spread was directly proportional to the degree of gap junctional communication between cells. However, evidence that an extracellular messenger (ATP) (Cotrina et al., 1998; Wang et al., 2000), hypothesized to be released from Cx43 hemichannels (Arcuino et al., 2002; Stout et al., 2002), was involved in ICW spread came from studies showing that Ca^{2+} waves could “jump” cell-free areas (Enkvist and McCarthy, 1992; Hassinger et al., 1996). Furthermore, the idea that the ATP pathway was the sole participant in ICW transmission gained support from studies showing that expression of P2Y₁R or P2Y₂R was sufficient to sustain the propagation of ICW between astrocytoma cells (Fam et al., 2000; Gallagher and Salter, 2003) and that in intact retina, ICW spread from astrocytes to Müller cells was entirely dependent on P2R activation (Newman, 2001).

Studies on Cx43-null astrocytes (Scemes et al., 1998,2000;Suadicani et al., 2003), in contrast, provided evidence for a more complex participation of Cx43 in ICW propagation than just being putative sites of ATP release. Through the use of gap junction channel blockers and P2R antagonists, these studies showed that there is a compensatory mechanism for ICW propagation such that in the absence of Cx43 gap junctional transmission there is increased participation of the purinergic pathway due to changes in the population of P2Y receptor subtypes that are expressed in Cx43-null compared with wild-type astrocytes (Scemes et al., 2000;Suadicani et al., 2003).

Although there is strong evidence supporting the participation of P2R in ICW propagation, very little is known about the contribution of gap junction channels in this process. One complicating factor for such an approach is the fact that astrocytes express several P2R receptor types and are coupled by more than one type of connexin. However, by selectively expressing P2YR subtypes and Cx43 in the human 1321N1 astrocytoma cell line that lacks P2 receptors (Parr et al., 1994) and are poorly dye-coupled (present work), we obtained evidence that Cx43 gap junction channels coordinate the ICWs generated by these purinergic receptor subtypes.

MATERIALS AND METHODS

Cell Cultures

The human astrocytoma 1321N1 cell line was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and maintained at 37°C in a humidified 5% CO₂ incubator. Astrocytoma cells were grown in Petri dishes containing Dulbecco's modified Eagle's medium (DMEM, Gibco Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Mediatech Cellgro, Herdon, VA). Stable clones of 1321N1 astrocytoma cells expressing P2YR receptor subtypes and rCx43 (see below) were maintained as described above in DMEM but containing G-418 (500 µg/mL; Gibco). One to 2 days prior to experimentation, parental cells and stable clones were trypsinized (0.05%

trypsin for 1 min at 37°C), centrifuged, resuspended in DMEM and seeded on glass-bottomed dishes (MatTek, MA).

Stable and Transient Transfections

For stable transfection, parental 1321N1 cells plated on 35-mm dishes to confluence were transfected with 4–6 µg of the cDNA of interest (P2YR subtypes and rCx43) using 6 µl Lipofectamine 2000 reagent (Invitrogen Life Technologies, Carlsbad, CA). At 48 h after transfections, cells were transferred to 100-mm Petri dishes at low density (0.2–0.5 cell/mm²) and maintained in selection medium (DMEM supplemented with G-418) for 20–30 days. Clones were then isolated and tested for functional expression of the protein of interest (see below). For co-expression of Cx43 and one of the P2YR subtypes, the eGFP-hCx43 construct was used to transiently transfect clones expressing P2YR. Cells co-expressing eGFP-hCx43 and one P2YR subtype were used 48 h after transfection.

Mouse P2YR, Rat, and Human connexin43 Constructs

Genomic DNA isolated from mouse tails were purified using the DNeasy tissue kit (Qiagen, Valencia, CA). Oligonucleotide primers corresponding to regions flanking the start and stop codons of the published sequences of the mouse P2Y₁ (NCBI Accession No. NM-008772) and P2Y₄ (NCBI Accession No. NM-020621) receptors were designed with restriction sites added to the 5' ends of the primers and inserted in pcDNA3 vector. Primers sequences used were as follows: mP2Y₁R (pcDNA3) forward primer: 5'-AAAGGGATCCATGACCGAGGTGCCTTGG-3' and reverse primer: 5'-TCTCGAATTCTCACAACTCGTGTCTCC-3'; mP2Y₄R (pcDNA3) forward primer: 5'-CAGGGGATCCATGACCACTGCAGACTCCT-3' and reverse primer: 5'-CGTTCTCGAGTTATAATCGGTCACCCTCATAAGCAG-3'. The mouse P2Y₂R cDNA (a gift from Dr. Irene Yan, Columbia University, NY) was isolated from pP2R vector and cloned into a pcDNA3 at *Xba* I restriction site. The rat Cx43 cDNA (a gift from Dr. Eric C. Beyer, University of Chicago, IL) was isolated from the pSG5 vector and cloned into pIRES at an *Eco*RI restriction site. The human eGFP-Cx43 was obtained from Dr. Glen Fishman (Department of Cardiology, NYU, NY). Polymerase chain reaction (PCR) products and cloned fragments were tested for accurate sizes and sequences (DNA Sequencing Facility, Albert Einstein College of Medicine, NY).

Immunofluorescence

1321N1 parental cells were plated onto glass coverslips and fixed with 4% p-formaldehyde in 1× phosphate-buffered saline (PBS) for 5 min at room temperature (RT). After three washes in 1× PBS, cells were permeabilized with 1× PBS containing 0.4% Triton X-100 (Sigma, St. Louis, MO) for 30 min at RT. Following overnight incubation, at 4°C, in 1× PBS containing 10% goat serum and polyclonal anti-Cx43 antibody (1: 2,000; a gift from Dr. E.L. Hertzberg, Albert Einstein College of Medicine), cells were washed several times with 0.4% Triton X-100 in 1× PBS before incubation with Alexa Fluor 488 goat anti-rabbit IgG conjugate (1:2,000; Molecular Probes, Eugene, OR) for 1 h at RT. After several washes, coverslips were mounted on glass slides and cells photographed with a digital camera (Olympus U-CMDA-2) attached to an epifluorescence microscope (Olympus AX70, Olympus Optical, Tokyo, Japan) equipped with 488 nm filter sets, and a 40× objective (N.A. 0.85, WD 0.23 mm; Nikon, Tokyo, Japan).

Western Blotting

Whole cell lysates of 1321N1 parental cells grown to confluency in 35-mm culture dishes were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad, Hercules, CA) and proteins transferred onto nitrocellulose membranes (Schleider & Schuell, Keene, NH). After overnight incubation with blocking buffer (PBS with

5% nonfat dry milk) at 4°C, the membranes were incubated for 1 h at RT with polyclonal anti-Cx43 antibody (1:2,000) and monoclonal anti-GAPDH antibody (1:5,000; Research Diagnostics, Flanders, NJ). Following several washes in 1× PBS containing 0.5% Tween-20, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse IgG (1:3000, Vector Laboratories, Burlingame, CA) for 1 h at RT. Detection of bands was performed on x-ray film (Kodak) after incubation of the membranes with the enhanced chemiluminescence (ECL) reagents (Amersham, Buckinghamshire, UK).

Fluorescence Recovery after Photobleaching

Evaluation of dye coupling in 1321N1 parental cells, rCx43 clones and cells transiently transfected with eGFP-hCx43 was performed using the fluorescence recovery after photobleaching (FRAP) technique (Wade et al., 1986; Lippincott-Schwartz et al., 2001). Cells plated in MatTek dishes were loaded with calcein-AM (1 μM; Molecular Probes) for 30 min at 37°C. After several washes, cells were bathed in Dulbecco's phosphate-buffered salt solution (DPBS, pH 7.4, Cellgro) throughout the experiments performed at RT. Changes in calcein fluorescence intensity emitted at 510 nm when excited at 488 nm were monitored over time using a real time confocal microscope (Nikon RCM 8000) equipped with a 40× water immersion objective. Photobleach of calcein was attained within seconds by directing the laser beam, set at maximum power, to one to two cells using a 4× optical zoom (inserted between the light path and the objective). Immediately after the photobleach, changes in calcein fluorescent intensity were then monitored at low laser power and 1× optical zoom. Images were acquired continuously at a rate of 1/60 s before, immediately after and for 15 min following photobleach. OMDR stored images were played back and calcein fluorescence intensity values measured from regions of interest placed on the bleached cells using Polygon-Star software (Nikon, Japan). Evaluation of the degree of dye coupling was obtained by measuring the half times of fluorescence recovery, as follows. The absolute values of calcein fluorescence intensity obtained after photobleach (t = 1 to 15 min) were subtracted from the ones obtained immediately after photobleach (t = 0) and then normalized to the values obtained at the plateau phase of fluorescence recovery (t = 15 min). The half-time (t_{1/2}) of FRAP was obtained using GraphPad Prism3.0 curve fitting software according to the following exponential equation:

$$Y = Y_{\max} * [1 - \exp(-K * t)]$$

where Y is the change in fluorescence intensity over time, Y_{max} is the maximum fluorescence intensity attained at t = 15 min after photobleach and K is the rate constant (t_{1/2} = 0.69/K). The t_{1/2} values were then used as parameters to evaluate the degree of dye coupling in 1321N1 parental and Cx43 transfected cells. At least four independent experiments were performed.

Semi-quantitative RT-PCR

Total RNA extracted from 1321N1 parental cells and P2YR clones using Trizol reagent (Gibco) was treated with RNase-free DNaseI (Roche Diagnostic GmbH, Penzberg, Germany) to eliminate contaminating genomic DNA. Reverse transcription (RT) was performed using random hexamer primers. PCR reactions were performed within the linear range of amplification previously determined for each P2YR subtype (27 cycles for P2Y₁R, 29 cycles for P2Y₂R and 38 cycles for P2Y₄R) and the hCx43 (27 cycles) using a PTC-100 Thermocycler (M.J. Research, Watertown, MA). PCR amplification consisted of initial denaturation at 94°C for 1 min followed by 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The last cycle was followed by a final extension cycle at 72°C for 8 min. Reaction products were analyzed by electrophoresis on 2.5% agarose gels. Primers for ribosomal 18S and competitor at 1:9 ratio (Ambion, Austin, TX) were added to the PCR reaction and used as an invariant endogenous control against which the products from the gene of interest were normalized. Primers used were: mP2Y₁R sense 5'-CTGTGTCTTATATCCCTTTCC-3' and

antisense 5'-CTCCATTCTGCTTGAATC-3'; mP2Y₂R sense 5'-AATAGCACCATCAATGGCAC-3' and antisense 5'-TAATAAACCAACAGCGGCAG-3'; mP2Y₄R sense 5'-GCCCTCAATGCCCCAACC and antisense 5'-ATGCCCATGTAGCGGTGC-3'; hCx43 sense 5'-GGATTGTCCTTAAGTCCCTG-3' and antisense 5'-CACAAGTCCATTGACACCTG-3'.

Intracellular Ca²⁺ Levels

Analysis of functional expression of P2YR subtypes was performed by measuring changes in cytosolic calcium levels induced by P2 agonists, as previously described (Scemes et al., 2000). Briefly, 1321N1 parental cells and P2Y_{1,2,4}R clones plated on MatTek dishes were loaded with Fura-2-AM (10 μM; Molecular Probes), for 45 min, at 37°C. After several washes with DPBS (pH 7.4), cells were imaged on an epifluorescence microscope (Eclipse TE2000-S; Nikon, Tokyo, Japan) equipped with a CCD digital camera (Orca-ER, Hamamatsu Photonics, Hamamatsu, Japan) and a 20× objective (N.A. 0.45; Nikon). Ratio values of Fura-2 fluorescence intensities emitted at two excitation wavelengths (340 nm and 380 nm) were obtained using the Lambda DG-4 (Sutter Instruments, Burlingame, CA) driven by a computer through Metafluor software (Universal Imaging, West Chester, PA). Changes in Fura-2 fluorescence ratio were acquired continuously at 0.7 Hz before and after the addition of P2R agonists. Fluorescence ratio values obtained online from regions of interest placed on cells were translated into intracellular Ca²⁺ concentrations according to an in vitro calibration curve of the form:

$$[Ca^{2+}]_i = K_D \{ (R - R_{min}) / (R_{max} - R) \} (F_{min}^{380} / F_{max}^{380})$$

where $[Ca^{2+}]_i$ is the calculated intracellular calcium concentration, K_D is the dissociation constant of free Ca²⁺ for Fura-2 ($K_D = 224$ nM) (Grynkiewicz et al., 1985), R is the ratio intensity, R_{min} is the ratio of the intensity obtained at zero calcium, R_{max} is the ratio of the intensity at saturated calcium, F_{min}^{380} is the fluorescence intensity measured at zero calcium at 380 nm, and F_{max}^{380} is the fluorescence intensity measured with saturated calcium at 380 nm. Noncumulative dose-response curves were obtained for 2-methylthioadenosine 5'-triphosphate (2-MeS-ATP; Calbiochem, San Diego, CA), uridine 5'-triphosphate (UTP; Sigma) and adenosine 5'-triphosphate (ATP; Sigma). An interval of 5 min after several washes with DPBS was maintained between the additions of increasing doses (10 nM to 1 mM) of the same agonist. The EC₅₀ values (effective concentration at which half-maximal increase in intracellular Ca²⁺ levels is reached) for each agonist were calculated from the sigmoidal fittings of the dose-response curves using GraphPad Prism 3.0 software.

Interacellular Calcium Waves

The properties of ICWs were determined as previously described (Scemes et al., 1998). Briefly, 1321N1 parental cells, rCx43 and P2Y_{1,2,4}R clones, and P2YR-subtype expressing cells transiently transfected with eGFP-hCx43 plated on MatTek dishes were incubated for 45 min, at 37°C, with Indo-1 AM (10 μM; Molecular Probes). Indo-1 was excited at 351 nm with a UV laser (Nikon RCM 8000, large pinhole, 40× water immersion objective, N.A. 1.15, W.D. 0.2 mm) and the ratio of the fluorescence intensity emitted at two wavelengths (390–440 nm and >400 nm) was acquired at 1 Hz after background and shading correction. The properties of mechanically induced ICW were analyzed in relation to its amplitude (peak of response/basal Indo-1 fluorescence ratio), velocity [distance (μm) between the stimulated and the nonstimulated cells/time interval (s) between the half-maximal Ca²⁺ increase within the stimulated and responding cells] and efficacy (number of responding cells/total number of cells in field of view). The overall properties of the Ca²⁺ signal transmission were evaluated by the E.V.A. factor (product of the relative values, test/control, obtained for the efficacy, velocity, and amplitude). Furthermore, to determine the range of ICW propagation, the distances

between the stimulated and foremost responding cells were measured; the ICW profile was estimated graphically after plotting the number of responding cells (efficacy) per tier (15 μm radius, centered on the stimulated cell) and by a 3D graph of the time, distance and Ca^{2+} transient amplitude of the ICW.

RESULTS

Gap Junctional Communication in 1321N1 Parental and Cx43 Transfected Cells

RT-PCR and Western blot analysis revealed that 1321N1 human astrocytoma cells express Cx43 (Fig. 1A,B). Although abundant, Cx43 immunoreactivity was diffuse and mainly found in intracellular compartments, with little expression at cell-cell contacts (Fig. 1C). As expected from the distribution of Cx43 immunoreactivity, 1321N1 parental cells were found to be poorly dye-coupled, as indicated by the half-time ($t_{1/2}$) values of dye transfer obtained from FRAP experiments (Fig. 1D). Compared with the half-time values of dye transfer obtained for cultured astrocytes ($t_{1/2} = 107.1 \pm 11.4$ s; $N = 18$ cells from six independent experiments; Fig. 1D), the higher half-time values recorded between parental 1321N1 cells ($t_{1/2} = 693.1 \pm 71.6$ s; $N = 19$ cells from 4 independent experiments; Fig. 1D) indicates that the degree of coupling between 1321N1 cells is about 6–7 times lower than that of astrocytes.

Transfected 1321N1 cells overexpressing either the eGFP-hCx43 or the rCx43 were found to be more strongly dye-coupled than nontransfected parental cells; a significant decrease ($P < 0.001$; ANOVA) in the half-time values of dye transfer was observed in rCx43 clones ($t_{1/2} = 287.8 \pm 52.8$ s, $N = 30$ cells, 4 independent experiments) and in cells transfected with eGFP-hCx43 ($t_{1/2} = 310.5 \pm 32.4$ s, $N = 51$ cells, 8 independent experiments) (Fig. 1D); no significant difference in dye coupling was observed between cells transiently transfected with eGFP-hCx43 (Fig. 1D) and the rCx43 clones ($P > 0.05$; Tukey's multiple comparison test). These results show that stable and transient transfections of Cx43 increased by twofold the degree of coupling between 1321N1 cells. [Transfection of parental 1321N1 cells with an irrelevant (non-connexin) cDNA construct did not alter the degree of coupling measured by FRAP ($t_{1/2} = 742.0 \pm 128.5$ s; $N = 11$ cells from four independent experiments).]

Characterization of P2Y Receptor Expressing Clones

Analysis of functional expression of P2YR subtypes was performed in Fura-2 loaded cells exposed to three P2 agonists (2-MeS-ATP, ATP, and UTP). The P2Y₁R clones displayed intracellular Ca^{2+} transients in response to bath application of ATP and 2-MeS-ATP, but not to UTP. The maximal amplitudes of Ca^{2+} transients induced by 2-MeS-ATP varied among the P2Y₁R clones, ranging from 50 nM to 2 μM with 2-MeS-ATP EC₅₀ values from 2.0 nM to 0.1 μM . Similar variability was observed among the P2Y₂R expressing clones in response to UTP application, with maximal Ca^{2+} transients ranging from 75 nM to 1.5 μM and UTP EC₅₀ values from 1.0 μM to 21 μM . None of the P2Y₂R clones responded with Ca^{2+} elevations to the selective P2Y₁R agonist 2-MeS-ATP. Only two of the 10 selected P2Y₄R clones displayed functional receptors, as evidenced by bath application of ATP and UTP; in these cases, the maximal amplitudes of Ca^{2+} transients induced by ATP (from 600 nM to 1.3 μM) were higher than those induced by UTP (52–720 nM). As expected from RTPCR assays showing lack of P2YR mRNA in 1321N1 parental cells (Fig. 2A), these astrocytoma cells were completely unresponsive to any of the P2R agonists employed even at a concentration of 1 mM.

For experiments aimed at evaluating the contribution of P2YR subtypes to ICW propagation (see below), we used P2YR clones that displayed the lowest EC₅₀ values for their selective agonists and highest Ca^{2+} amplitudes. Thus, the P2Y₁R clone selected for these studies displayed an averaged maximal response to 2-MeS-ATP of 1.3 ± 0.3 μM and an EC₅₀ value

of 2.3 nM (IC95%: 0.4–13.8 nM, N = 40 cells); the selected P2Y₂R clone showed an averaged maximal response to UTP of 1.4 ± 0.2 μ M and EC₅₀ of 15.7 μ M (IC95%: 7.7–32.2 μ M, N = 50 cells); the selected P2Y₄R clone responded to ATP application with averaged maximal Ca²⁺ amplitude of 0.8 ± 0.2 μ M and EC₅₀ value of 2.1 μ M (IC95%: 1.08–4.04, N = 32 cells). The dose response curves obtained for these selected P2YR clones as well as their respective P2YR mRNA expression levels are shown in Figure 2.

Intercellular Ca²⁺ Waves Propagated by Cx43 Gap Junction Channels in the Absence of Purinergic Receptors

As expected from the low degree of coupling between 1321N1 parental cells (Fig. 1D), only 3% of the cells within the confocal field of view displayed Ca²⁺ transients following focal mechanical stimulation of a single cell (Table 1; see also Fig. 3A). These responding cells were located not farther than 60 μ m from the source of stimulation. In view of the low degree of dye coupling, it is likely that the small range of ICW spread is related to the presence of few gap junction channels between these cells.

In cells expressing rCx43, ICW spread with higher velocities (10.00 ± 3.70 μ m/s; N = 236 cells) and traveled farther distances (90 μ m; N = 13 fields) than waves spreading between parental cells (velocity = 6.90 ± 2.38 μ m/s, N = 216 cells; range = 60 μ m, N = 12 fields). However, the mean amplitude of Ca²⁺ transients in the rCx43 clone was smaller (1.19 ± 0.12 -fold; N = 13 cells) while the efficacy (0.3 ± 0.01 ; N = 13 fields) was similar to that of parental cells (amplitude = 1.47 ± 0.20 -fold, N = 12 cells; efficacy = 0.3 ± 0.01 , N = 12 fields). Thus, these data indicate that Cx43 gap junction channels can enhance by 17% their ability to transmit Ca²⁺ signals, as indicated by the E.V.A. factor ($E.V.A._{rCx43/parental} = 1.17$; Table 1).

Intercellular Ca²⁺ Waves Propagated by P2Y_{1,2,4} Receptor Activation

Focal mechanical stimulation of 1321N1 cells stably expressing P2YR subtypes induced increases in the [Ca²⁺]_i in the stimulated cells that were not different from those measured in parental cells (Table 1). The number of cells recruited in the ICW propagation between P2YR expressing cells was significantly increased compared with parental and Cx43 expressing cells (Table 1). Further confirmation that the propagation of ICW was mediated through P2YR activation was obtained by exposing P2Y₁R expressing cells to 5 μ M MRS-2179, a specific P2Y₁R antagonist, which reduced the efficacy of the ICW from 0.23 ± 0.03 to 0.03 ± 0.01 (N = 7 fields), a value similar to that obtained in parental cells (see Table 1).

The overall properties of intercellular Ca²⁺ signal transmission between P2YR expressing cells were markedly enhanced by individual expression of P2Y₁R, P2Y₂R and P2Y₄R, when compared with parental cells ($E.V.A_{P2Y1R/parental} = 18.21$; $E.V.A_{P2Y2R/parental} = 17.98$; $E.V.A_{P2Y4R/parental} = 4.04$; Table 1). Interestingly, however, ICW propagation had distinct characteristics when spreading between the different P2YR subtype clones (Table 1).

The efficacy of the ICW propagation in the P2Y₁R clone was 52% lower than that of waves traveling between P2Y₂R cells, but 22% higher than the efficacy of waves propagating between P2Y₄R clones (Table 1). The velocity (12.13 ± 0.94 μ m/s), amplitude (1.57 ± 0.04 -fold) and range (100 μ m) of ICW propagating between P2Y₁R cells were higher than those measured in P2Y₂R cells (velocity = 8.91 ± 0.59 μ m/s; amplitude = 1.40 ± 0.03 -fold; range = 75 μ m; Table 1). With the exception of the range to which ICW spread, all other ICW parameters measured in P2Y₁R cells were also higher than those of P2Y₄R cells, which were smaller than those of the P2Y₂R clone (Table 1).

A striking difference between ICW propagating between cells expressing each of these P2YR subtypes was observed when analyzing the graphic representation of ICW profiles (Fig. 3). In

P2Y₁R-expressing cells, the profiles of ICW transmission had a “saltatory” nature with Ca²⁺ transients of uniform amplitudes occurring in about 20% of cells/tier at different time points (Fig. 3A,B). Regardless of the variability in agonist EC₅₀ values (29–2.3 nM) obtained for the different P2Y₁R clones, similar “saltatory” behavior of ICW was observed in all the clones tested. In the P2Y₂R clone, ICW profile had a “decremented” nature with Ca²⁺ signal amplitudes and efficacies decaying with increased distance from the stimulated cell (Fig. 3C). In the P2Y₄R clone, the ICW profile had a “restricted” nature with larger Ca²⁺ transients occurring near the source of stimulation (Fig. 3D).

Contribution of Cx43 Gap Junction Channels to ICW propagation in P2YR Expressing Cells

To evaluate the role of Cx43 gap junction channels in Ca²⁺ signal propagation, the properties of ICW transmission were analyzed in P2YR clones transiently transfected with eGFP-hCx43. For these experiments we selected confocal fields that contained a large number of P2YR clonal cells exhibiting eGFP fluorescence at cell-cell contacts. Focal mechanical stimulation of single 1321N1 cells co-expressing P2YR and eGFP-hCx43 increased [Ca²⁺]_i in the stimulated cell to levels that were not different from those measured in parental or in cells stably expressing the P2YR subtypes (Table 1). When compared with the ICW spreading between P2Y₁R clones, the amplitude and efficacy of ICW traveling between cells co-expressing eGFP-hCx43 and P2Y₁R were significantly increased by 17% and 69%, respectively, while the velocity was reduced by 42% (Table 1). More dramatically, expression of eGFP-hCx43 in P2Y₁R clones changed the P2Y₁R ICW profile from a “saltatory” to a “decremented” form of wave propagation, in which larger Ca²⁺ transients occurred in cells located close to the stimulus and decayed along the cell tiers (Fig. 3B); thus, the “saltatory” profile of these waves become similar to the ICW profile observed in the P2Y₂R clones (Fig. 3C).

Although not altering the overall properties (E.V.A. factor; Table 1) or the “decremented” nature of ICW observed in P2Y₂R clones (Fig. 3C), eGFP-hCx43 expression in these cells caused a 42% decrease in the velocity and a 30% increase in amplitude of the waves (Table 1).

In contrast to what was observed for Cx43-P2Y₁R and Cx43-P2Y₂R cells, expression of eGFP-hCx43 in a P2Y₄R clone caused a striking reduction in the efficacy (43%) and E.V.A. values (42%) of ICW when compared with those observed in P2Y₄R clones (Table 1). Such attenuation in the transmission of Ca²⁺ signals in cells co-expressing eGFP-hCx43 and P2Y₄R can be visualized in Figure 3D displaying the wave profile, which changed from a “restricted” to a “nonexistent” ICW.

Contribution of Extracellular and Intracellular Ca²⁺ Compartments to the Propagation of Intercellular Calcium Waves

As showed above, ICWs traveling between rCx43 were significantly improved when compared with nontransfected parental 1321N1 cells (Table 1). However, this ICW transmission is quite poor when considering the relatively high permeability of these channels to second messengers (Leybaert et al., 1998; Paemeleire et al., 2000; Fry et al., 2001). To gain insight regarding whether such constrained spread of ICW between rCx43 cells was related to the type of second messenger (IP₃, Ca²⁺) generated by the mechanical stimulation, cells loaded with Fura-2-AM were bathed in Ca²⁺-free DPBS containing 1.0 mM EDTA and imaged using an epifluorescence microscope. The amplitudes of Ca²⁺ transients induced in the mechanically stimulated cell and the efficacy of ICW were then measured and compared with those values obtained from cells exposed to Ca²⁺ containing solution. As shown in Figure 4, in the absence of extracellular Ca²⁺ the rise in intracellular Ca²⁺ levels of the mechanically stimulated rCx43-expressing cells was greatly reduced compared with control values (from 1722.0 ± 145.7 nM to 69.4 ± 21.51 nM; N = 10; Fig. 4A) and the efficacy of ICW propagation was reduced by

50% (Fig. 4B). These data thus strongly suggest that the increases in $[Ca^{2+}]_i$ induced by mechanical stimulation of cells lacking P2 receptors is due to the influx of Ca^{2+} from the extracellular compartment, with minor contribution of intracellular Ca^{2+} stores. Furthermore, these data suggest that the constraint imposed to ICW propagation between 1321N1 cells expressing rCx43 channels is likely related to the high Ca^{2+} buffering power of these cells, thus limiting the diffusion range of this second messenger through the coupled cells (Allbritton et al., 1992).

In contrast to what was observed for cells lacking P2 receptors, when 1321N1 cells expressing P2Y₁R were bathed in Ca^{2+} -free solution, Ca^{2+} transients recorded from the mechanically stimulated cells were not abolished (Fig. 4A). No significant changes in the amplitudes of Ca^{2+} transients recorded from mechanically stimulated P2Y₁R expressing cells were observed after removal of extracellular Ca^{2+} (from 1902.0 ± 11.1 nM to 1708.0 ± 224.0 nM; $N = 5$, $P > 0.05$; Fig. 4A). Moreover, no significant change in the efficacy of ICW propagating between P2Y₁R cells was recorded in Ca^{2+} -free solution compared with that obtained in the presence of extracellular Ca^{2+} (Fig. 4B). These results, therefore, indicate that, in this case, mobilization of Ca^{2+} from intracellular stores is sufficient to sustain ICW propagation between P2Y₁R cells.

Interestingly, although the amplitudes of Ca^{2+} transients of mechanically stimulated cells expressing P2Y₂R were substantially decreased by removing Ca^{2+} from the extracellular solution (from 1908.0 ± 10.5 nM to 390.5 ± 159.6 nM; $N = 11$; Fig. 4A), no change in the efficacy of ICW was observed (Fig. 4B). In contrast, however, the attenuation of Ca^{2+} transient amplitudes observed in mechanically stimulated P2Y₄R cells bathed in Ca^{2+} -free solution (from 1922.0 ± 6.77 nM to 110.6 ± 25.2 nM; $N = 8$; Fig. 4A) greatly affected the efficacy of ICW, which was reduced by 77% (Fig. 4B).

Together, these data suggest that mechanical stimulation of cells expressing P2YR can lead to Ca^{2+} mobilization from intracellular stores. Such increases in cytosolic Ca^{2+} are likely induced by IP₃ generation due to autocrine activation of these P2 metabotropic receptors in response to ATP/UTP released from 1321N1 cells during the mechanical stimulation (Lazarowski et al., 1997,2000,2003;Lazarowski and Harden, 1999;Lazarowski and Boucher, 2001). However, because 1321N1 cells display intense ectonucleotidase activity that rapidly degrades ATP and UTP into ADP and UDP (see Zimmermann, 2000;Lazarowski et al., 2003), it is expected that, due to differences in agonist sensitivity, autocrine activation of these three P2YR subtypes would generate different levels of IP₃. Thus, it is not surprising that mechanical stimulation of cells expressing P2Y₁R, which are activated by ATP and ADP, mobilizes higher levels of intracellular Ca^{2+} than do cells expressing the ATP- and UTP-sensitive P2Y₂R and P2Y₄R. Therefore, it is likely that the observed differences in Ca^{2+} levels that were mobilized from intracellular stores on the mechanically stimulated cells bathed in Ca^{2+} -free solution (Fig. 4A) are related to distinct levels of IP₃ generated by each of these P2YR subtypes. In addition, because the efficacy of ICW traveling between cells exposed to Ca^{2+} -free solution differed among the P2YR subtype clones (Fig. 4B), it is likely that the IP₃ generated by activation of P2Y₁R or P2Y₂R, but not of P2Y₄R, is sufficient to sustain long range Ca^{2+} signal transmission between coupled cells.

Do connexin43 hemichannels contribute to ICW propagation?

Recently, Cx43 hemichannels have been proposed to be sites of ATP release (Stout et al., 2002; Braet et al., 2003a, b; Stout and Charles, 2003). Following mechanical stimulation of a single astrocyte in culture, Stout and Charles (2003) reported that ICW propagated to larger areas and to a higher number of cells when bathed in a solution deprived of Ca^{2+} . Such potentiation of ICW propagation was attributed to the increased release of ATP through Lucifer Yellow (LY) permeable Cx43 hemichannels (Stout et al., 2002; Stout and Charles, 2003) whose

openings have been reported to be favored by low divalent conditions (Ebihara and Steiner, 1993; Li et al., 1996; Stout et al., 2002; Ye et al., 2003).

Therefore, to evaluate the contribution of Cx43 hemichannels to ICW propagation between 1321N1 astrocytoma cells, the efficacy of ICW between cells co-expressing eGFP-hCx43 and P2Y₁R were measured in Ca²⁺-free solution containing 1.0 mM EDTA. It should be noted that tagging eGFP to Cx43 does not interfere either with gap junction or hemichannel formation/function (Bukauskas et al., 2001; Contreras et al., 2003; see also results of FRAP above).

As shown in Figure 4B, when bathed in Ca²⁺-free solution, no significant alteration in the efficacy of ICW traveling between cells co-expressing P2Y₁R and eGFP-hCx43 was observed. Furthermore, exposure of Cx43 transfectants to Ca²⁺-free solution did not lead to increased permeability to LY (LY fluorescence intensity in DPBS = 43.9 ± 3.1; LY fluorescence intensity in Ca²⁺-free-DPBS = 32.2.5 ± 2.5; N = 150 cells from 3 independent experiments). These results showing lack of potentiation of ICW propagation between 1321N1 cells bathed in low divalent solution, together with data showing lack of LY uptake, do not support the hypothesis that opening of Cx43 hemichannels favors the release of ATP from these cells. Although the reasons for the difference between the results obtained in astrocytes (Stout and Charles, 2003) and those reported here are not clear, it is possible that other pathways present in astrocytes and absent in 1321N1 cells would provide the sites of ATP release (e.g., ABC transporters, pl-VDAC, stretch-activated channels, P2X₇ receptors: Lazarowski et al., 2003; Anderson et al., 2004).

DISCUSSION

A growing body of evidence indicating that astrocytes not only respond to neuronal activity but also modulate synaptic transmission led to the proposal that astrocytes are active participant in neuronal information processing (for reviews, see Araque et al., 1999; Haydon, 2001). Intra- and intercellular calcium waves in astrocytes have been shown to mediate such bi-directional neuro-glial interaction (Dani et al., 1992; Porter and McCarthy, 1996; Araque et al., 1998; Newman and Zahs, 1998). Intracellular Ca²⁺ elevations in response to electrical, chemical or mechanical stimulation can be transmitted to neighboring cells as propagating intercellular Ca²⁺ waves (ICWs). Diffusion of intracellular second messengers (e.g., IP₃, Ca²⁺) through gap junction channels and activation of surface membrane receptors by extracellular messengers (e.g., ATP, glutamate) provide the two pathways for intercellular Ca²⁺ Signal propagation (see Scemes, 2000).

Although this form of intercellular communication has been extensively demonstrated between glial cells, both in culture and *in situ*, little is actually known about the relative contribution of these two pathways to ICW propagation. One complicating factor for such studies is that astrocytes are coupled by more than one type of gap junction protein (Dermietzel et al., 2000; Nagy and Rash, 2000; Nagy et al., 2001) and express a multitude of purinergic receptor subtypes (Jimenez et al., 2000; Suadicani Fumagalli et al., 2003; et al., 2003). The lack of selectivity of gap junction channel blockers (Rozental et al., 2001) and of P2 receptor antagonists (Ralevic and Burnstock, 1998), together with the observation that deletion of the gap junction protein, Cx43, affects expression of other genes (Iacobas et al., 2003), including P2Y receptors (Suadicani et al., 2003) add to the complexity when trying to evaluate the contribution of pathways to glial Ca²⁺ transmission.

Using the 1321N1 human astrocytoma cell line, which does not express P2 receptors (Parr et al., 1994) and is poorly coupled (this work), we here report on the properties of the intercellular Ca²⁺ wave (ICW) propagation mediated by the individual and combined forced expression of P2YR and Cx43 gap junction proteins. However, it should be kept in mind that although this

simplified system certainly provides advantages for studying the contribution of specific targets to ICW propagation, other components (i.e., ionotropic P2Rs and functional connexin hemichannels; see Parpura et al., 2004) absent in 1321N1 cells may also contribute to Ca^{2+} signal transmission, as has been proposed in other cells types including astrocytes.

In the present study, we show that expression of either Cx43 gap junction channels or one of the three P2YR subtypes (P2Y₁R, P2Y₂R, and P2Y₄R) is sufficient to sustain ICW propagation between 1321N1. However, the properties of ICW conferred by the expression of each of these proteins are quite distinct. In terms of the overall ICW property, measured by the E.V.A. factor, expression of P2Y₁R and P2Y₂R improved ICW by 18-fold compared with nontransfected cells, while expression of Cx43 or P2Y₄R lead to a much smaller increment in the E.V.A. values (1.17 and 4.0, respectively). Such differences in the overall properties of ICW generated by each protein are most likely related to the type and amount of second messenger generated during the mechanical stimulation used to induce ICWs. As evidenced from experiments performed in the absence of extracellular Ca^{2+} , in cells lacking metabotropic P2 receptors, Ca^{2+} is the main second messenger that diffuses between Cx43 coupled cells. In contrast, in cells expressing P2YR subtypes, the amount of IP₃ generated by autocrine activation of P2YR due to ATP and UTP release (Lazarowski et al., 1997,2000,2003;Lazarowski and Harden, 1999;Lazarowski and Boucher, 2001) is likely to govern the overall property of ICW. Moreover, because of the short range of diffusion of Ca^{2+} compared with IP₃ (diffusion distances of 0.08 μm and 13 μm , respectively; Kasai and Petersen, 1994), it is not surprising that ICW mediated by diffusion of Ca^{2+} through gap junction channels (in the case of cells lacking P2 receptors) would rapidly attenuate and therefore be expected to produce less competent ICWs than coupled cells expressing IP₃ generating P2YR.

Furthermore, it is also likely that differences observed in each of the parameters (amplitude, range, profile, and efficacy) of ICWs propagating between poorly coupled P2YR expressing cells are related to the individual characteristics of the P2YR subtypes. Included here are the differential sensitivity of P2YR subtypes to ATP and UTP and their products such as ADP and UDP that are generated by ectonucleotidase activities (see Zimmermann, 2000). For instance, it is possible that the difference in ICW profile observed between uncoupled cells expressing P2Y₁R (“saltatory” wave), P2Y₂R (“decremented” wave), and P2Y₄R (“restricted” wave) is related to the higher sensitivity of P2Y₁R to ADP than are the other two receptors (Ralevic and Burnstock, 1998). In this case, the amplitude, efficacy and range of calcium signal transmission in cells expressing P2Y₁R should not follow the attenuation of extracellular ATP concentration gradient that occur with increased distance from the point of release. In fact, it is here shown that amplitude and efficacy of ICWs traveling between P2Y₁R cells do not vary along the tiers of cells (see Fig. 3B). Conversely, waves spreading between P2Y₂R and P2Y₄R expressing cells would be expected to follow the extracellular ATP/UTP concentration gradient, as evidenced by the “decremented” and “restricted” behavior of these ICW (see Fig. 3C,D).

The broad variability in properties of Ca^{2+} signal transmission observed in different cell types and in astrocytes from different regions of the nervous system can be in part attributed to the differences in the composition of the P2 receptors expressed in these cells. For instance, in rat cortical astrocytes, six out of the seven cloned ligand-gated P2X receptors (P2X₁₋₅ and P2X₇), all G-protein-coupled P2Y receptors (P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₂) and the P2Y-like UDP-glucose receptor recently recognized as the P2Y₁₄ receptor (Abbracchio et al., 2003) are functionally expressed (Fumagalli et al., 2003). With the exception of P2X₁, P2X₃, and P2Y₆ receptors, all other P2 receptors were shown to be capable to induce intracellular Ca^{2+} transients in response to ATP (Fumagalli et al., 2003). Therefore, in cells expressing mixed populations of P2 receptors, the ATP/UTP-mediated transmission of intercellular Ca^{2+} signals should recruit the participation of each purinoceptor coupled to Ca^{2+} mobilization,

which will presumably be different, as shown to be the case for mouse P2Y₁, P2Y₂, and P2Y₄ receptors (this study) and also for rat P2Y₁ and P2Y₂ receptors (Gallagher and Salter, 2003).

Especially important when analyzing the contribution of P2YR to ICW spread between coupled cells is the possibility that the higher levels of IP₃ generated by activation of P2Y₁R than by P2Y₂ or P2Y₄ receptor subtypes (evidenced by the levels of Ca²⁺ mobilized from intracellular stores; Fig. 4A), contribute to differences observed between ICW properties, as discussed below.

Analysis of the properties of ICW spreading between 1321N1 cells co-expressing purinergic receptors and Cx43 revealed that gap junction channels coordinate the propagation of ICW generated by P2YR stimulation. Such coordination conferred by Cx43 gap junction channels to ICW propagation is best exemplified following P2Y₁R expression; in this case, ICW changed from a “saltatory,” noncoordinated wave to a coordinated, “decremented” wave-like form of ICW propagation in cells co-expressing Cx43 and P2Y₁R. Thus, it is likely that such “decremented” ICW profile reflects the intercellular concentration gradient of IP₃ generated by P2Y₁R activation in the vicinities of the point of stimulation that declines with distance as it diffuses through the coupled cells.

Using similar reasoning, it is not surprising that the “decremented” nature of ICW spreading between P2Y₂R cells was not altered when eGFP-hCx43 was co-expressed. Because the mobilization of intracellular Ca²⁺ stores resultant from P2Y₂R activation was smaller than that observed following P2Y₁R activation, it is likely that the 35% attenuation on the overall property of ICW (E.V.A. value) is related to the dilution of IP₃ among the coupled cells. The more dramatic reduction (76%) in the overall properties of ICWs observed in cells co-expressing P2Y₄R and eGFP-hCx43 could be related to a steeper decrease in IP₃ concentration as it diffuses through the coupled cells. As mentioned before, it is likely that activation of P2Y₄R generates less IP₃ than the other two P2 receptor types, and thus the levels of IP₃ could be below the threshold necessary to induce Ca²⁺ release.

In conclusion, based on the results presented here, it is proposed that Cx43 gap junction channels, besides providing coordination for Ca²⁺ signal transmission by expanding the volume of the intracellular compartment, can either amplify or minimize the distribution of signaling molecules between coupled cells. Whether or not gap junction channels limit the extent of ICW propagation seems to depend on the amount and nature of the second messenger generated by receptor activation.

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Fig. 1.

Gap junctional communication in 1321N1 cells. **A:** Semiquantitative RT-PCR indicating the expression level of Cx43 mRNA endogenously expressed in 1321N1 parental cells. The 324bp band corresponds to 18S used as an invariable internal control. **B:** Representative Western blot showing the endogenous expression levels of Cx43 in parental 1321N1 astrocytoma cells. Note the presence of the nonphosphorylated (NP) and one of the phosphorylated (P1) forms of Cx43 in these cells. GAPDH was used as an invariable internal control. **C:** Cx43 immunofluorescence images obtained from 1321N1 parental cells indicating that Cx43 immunoreactivity (arrows) is mainly localized in intracellular compartments. **D:** Bar histogram showing the mean half-time values of dye transfer obtained from 4–8 independent fluorescence

recovery after photobleaching (FRAP) experiments performed in primary cultures of astrocytes, 1321N1 parental, and in 1321N1 transfected with rCx43 and eGFP-hCx43. Inset: representative time course curves of FRAP obtained for astrocytes, 1321N1 parental cells and Cx43 transfectants. Astrocyte cultures were obtained as previously described (Scemes et al., 2000). (***) $P < 0.001$, ANOVA, Tukey's multiple comparison test).

Fig. 2. Characterization of P2YR expressing 1321N1 cells. **A:** Semi-quantitative RT-PCR showing the expression levels of P2Y₁R, P2Y₂R and P2Y₄R obtained from parental (1321N1) and from astrocytoma clones expressing each of these P2YR subtypes. **B:** Representative dose-response curves to P2YR agonists (2-MeS-ATP, UTP, and ATP) obtained for P2Y₁R, P2Y₂R and P2Y₄R clones showing proper functional expression of these purinergic receptors as indicated by the EC₅₀ values.

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Fig. 3.

Characteristics of intercellular calcium wave propagation mediated by P2Y_R activation in the presence and absence of gap junctional communication. **A:** Time-lapse images showing changes in Indo-1 fluorescence ratio (pseudo-colored scale) following mechanical stimulation (arrow) of a single parental cell (1321N1) and of cells expressing P2Y₁R and P2Y₁R plus eGFP-hCx43 (Cx43+P2Y₁R). Note the low number of 1321N1 parental cells participating in ICW propagation compared with that between P2Y₁R cells and also the change in the behavior of ICW transmission between P2Y₁R when Cx43 was co-expressed in these cells. **B—D:** Graphic illustration of the changes in the profile of ICW propagation induced by forced expression of Cx43 in cells expressing P2Y₁R (B), P2Y₂R (C) and P2Y₄R (D). The ICW profiles are displayed in two forms, as 3D graphs (Ca²⁺ amplitude × time × distance from stimulated cells; left and middle graphs) and as bar histograms of the number of cells responding with increase in cytosolic calcium (efficacy) per tiers (right graphs). Note in part B that forced expression of Cx43 in P2Y₁R clones altered the ICW profile from a “saltatory”

to a decremented” wave and greatly increased the efficacy of ICW transmission per tiers of cells. Forced expression of Cx43 in P2Y₂R clones (part C), however, did not greatly alter the profile of ICW transmission, while in cells expressing P2Y₄R (D), it reduced the efficacy of ICW per tiers of cells and changed ICW profile from a “restricted” to a “nonexistent” wave.

Fig. 4. Contribution of the extracellular and intracellular Ca^{2+} compartments to intercellular calcium wave propagation. Bar histograms showing the mean values of intracellular Ca^{2+} transients recorded in the mechanically stimulated cells (**A**) and of the efficacy of ICW propagation (**B**) between parental and cells expressing rCx43, P2Y₁R, P2Y₂R, P2Y₄R, and cells co-expressing rCx43 and P2Y₁R (Cx43+P2Y₁R) when bathed in DPBS containing 1.0 mM Ca^{2+} (black bars) and in Ca^{2+} -free DPBS with 1 mM EDTA (gray bars). (* $P < 0.05$; *** $P < 0.001$; unpaired t -test).

TABLE 1

Properties (Efficacy, Velocity, and Amplitude) of Intercellular Ca^{2+} Wave (ICW) Propagation in 1321N1 Parental Cells, 1321N1 Cells Expressing the rCx43, P2Y_{1,2,4}R, and 1321N1 Cells Co-expressing P2YR and Cx43 (Cx43+P2Y_{1,2,4}R)[†]

	Ca ²⁺ amplitude of stimulated cells (fold basal)	ICW efficacy (No. resp cells/total No. cells)	ICW velocity (μm/s)	ICW amplitude (fold basal)	EVA
Parental	2.38 ± 0.16 (N = 12 cells)	0.03 ± 0.01 (N = 12 fields)	6.90 ± 2.38 (N = 8/216 cells)	1.47 ± 0.20 (N = 8/216 cells)	-
rCx43	2.18 ± 0.04 (N = 13 cells)	0.03 ± 0.01 ^{e,i} (N = 13 fields)	10.00 ± 3.70 (N = 9/236 cells)	1.19 ± 0.12 ^d (N = 8/236 cells)	1.17
rCx43/Parental P2Y ₁ R	2.26 ± 0.15 (N = 13 cells)	1.0 (N = 13 fields)	1.45 (N = 102/415 cells)	0.81 (N = 102/415 cells)	18.21
P2Y ₁ R/Parental P2Y ₂ R	2.35 ± 0.09 (N = 15 cells)	0.29 ± 0.04 ^b (N = 13 fields)	12.13 ± 0.94 (N = 149/375 cells)	1.57 ± 0.04 (N = 149/375 cells)	17.98
P2Y ₂ R/Parental P2Y ₄ R-1321N1	2.32 ± 0.13 (N = 8 cells)	0.44 ± 0.05 ^{b,c,g} (N = 15 fields)	8.91 ± 0.59 ^c (N = 27/212 cells)	1.40 ± 0.03 ^c (N = 27/212 cells)	4.04
P2Y ₄ R/Parental Cx43+P2Y ₁ R	2.26 ± 0.10 (N = 9 cells)	0.13 ± 0.02 ⁱ (N = 8 fields)	7.66 ± 0.87 (N = 126/226 cells)	1.24 ± 0.06 ^c (N = 126/226 cells)	1.16
Cx43+P2Y ₁ R/P2Y ₁ R	2.57 ± 0.09 (N = 8 cells)	4.33 (N = 9 fields)	1.11 (N = 117/309 cells)	0.84 (N = 117/309 cells)	0.64
Cx43+P2Y ₂ R/P2Y ₂ R	2.39 ± 0.06 (N = 19 cells)	0.49 ± 0.06 ^{b,d,g,k} (N = 8 fields)	7.02 ± 0.46 ^e (N = 20/570 cells)	1.84 ± 0.04 ^{e,g,i,k} (N = 20/570 cells)	0.27
Cx43+P2Y ₄ R/P2Y ₄ R		1.69 (N = 19 fields)	0.58 (N = 20/570 cells)	1.18 (N = 20/570 cells)	
		0.38 ± 0.05 ^{b,g,j} (N = 8 fields)	5.17 ± 0.25 ⁱ (N = 117/309 cells)	1.82 ± 0.04 ^{e,g,i,k} (N = 117/309 cells)	
		0.86 (N = 19 fields)	0.58 (N = 20/570 cells)	1.3 (N = 20/570 cells)	
		0.03 ± 0.06 ^{e,i} (N = 19 fields)	7.81 ± 1.35 (N = 20/570 cells)	1.25 ± 0.07 (N = 20/570 cells)	
		0.26 (N = 20/570 cells)	1.02 (N = 20/570 cells)	1.0 (N = 20/570 cells)	

[†] Compared with parental

^a $P < 0.001$; compared to P2Y₁R-1321N1

^b $P < 0.05$

^c $P < 0.01$

^d $P < 0.001$; compared with rCx43-1321N1

^e $P < 0.01$

^f $P < 0.001$; compared with P2Y₂R-1321N1

^g $P < 0.01$

^h $P < 0.001$; compared with P2Y₄R-1321N1

$i_P < 0.01$
 $j_P < 0.001$