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Modulation of Astrocyte P2Y₁ Receptors by the Carboxyl Terminal Domain of the Gap Junction Protein Cx43

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Abstract

Gap junction proteins, connexins, provide intercellular channels that allow ions and small signaling molecules to be transmitted to adjacent coupled cells. Besides this function, it is becoming apparent that connexins also exert channel-independent effects, which are likely mediated by processes involving protein–protein interactions. Although a number of connexin interacting proteins have been identified, only little is known about the functional consequences of such interactions. We have previously shown that deletion of the astrocytic gap junction protein, connexin43 (Cx43) causes a right-ward shift in the dose-response curve to P2Y₁R agonists and decreased P2Y₁R expression levels. To evaluate whether these changes were due to reduced gap junctional communication or to protein–protein interactions, Cx43-null astrocytes were transfected with full-length Cx43 and Cx43 domains, and P2Y₁R function and expression levels evaluated. Results indicate that restoration of P2Y₁R function is independent of gap junctional communication and that the Cx43 carboxyl terminus spanning the SH3 binding domain (260–280) participates in the rescue of P2Y₁R pharmacological behavior (shifting to the left the P2Y₁R dose-response curve) without affecting its expression levels. These results suggest that the Cx43 carboxyl-terminus domain provides a binding site for an intracellular molecule, most likely a member of the c-Src tyrosine kinase family, which affects P2Y₁R-induced calcium mobilization. It is here proposed that a nonchannel function of Cx43 is to serve as a decoy for such kinases. Such modulation of P2Y₁R is expected to influence several neural cell functions, especially under inflammation and neurodegenerative disorders where expression levels of Cx43 are decreased.

Keywords

glia; calcium signaling; connexin; purinergic receptor

INTRODUCTION

The gap junction protein connexin43 (Cx43) is widely expressed in most tissues (Beyer et al., 1987; Willecke et al., 2002), including the central nervous system (CNS). During early stages of CNS development, Cx43 expression is highly abundant in both neuronal and glial cell lineages (Duval et al., 2002; Rozental et al., 1998, 2000) but decreases as the cells differentiate and mature into neurons and oligodendrocytes, being restricted to the astrocytic glial cell population (Dermietzel et al., 1991; Rash et al., 2001a,b). Cx43 forms intercellular channels permeable to ions and molecules up to 900 Da. By providing continuity and exchange of signals between the interconnected intracellular compartments, Cx43 gap junction channels play important roles in astrocyte biology as well as during CNS development. Several factors can

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either decrease or increase intercellular communication between cells. For instance, closure of Cx43 gap junction channels in astrocytes and in other cell types may result from intracellular acidification (Dermietzel et al., 1991; Duffy et al., 2004; Ek-Vitorin et al., 1996; Liu et al., 1993; for review see Spray and Scemes, 1998), or as a consequence of changes in phosphorylation state following activation of protein kinases (Cooper et al., 2000; Kwak and Jongsma, 1996; Lampe et al., 2000; Li et al., 2005; Saez et al., 1998), whereas junctional conductance is increased by high extracellular K^+ (Enkvist and McCarthy, 1994; Pina-Benabou et al., 2001), and membrane permeant cAMP derivatives (Burghardt et al., 1995; Chanson et al., 1996; Darrow et al., 1996; Matesic et al., 1996; Paulson et al., 2000).

Recently, it has become apparent that connexins, specifically Cx43, may have other functions that are independent of intercellular channel formation. For instance it has been shown that forced expression of Cx43 in C6 glioma cells provides resistance to apoptotic signals (Lin et al., 2003a) and that over-expression or deletion of the Cx43 gene alters the expression of numerous unrelated gene products (Jacobas et al., 2003, 2004; Naus et al., 2000), including certain membrane receptors (Suadicani et al., 2003). One possible way by which connexins may exert channel independent effects may be through the action of Cx43 interacting proteins with either transcriptional activity, such as β -catenin (Ai et al., 2000; Xu et al., 2001) and members of src family of oncogenes (Kanemitsu et al., 1997; Loo et al., 1995), or with others with structural functions, such as the scaffolding protein ZO1 (Giepmans and Moolenaar, 1998; Toyofuku et al., 1998), and the microtubule protein tubulin (Giepmans et al., 2001), as well with the lipid raft protein caveolin-1 (Lin et al., 2003b; Schubert et al., 2002). Such functional diversity of molecules that have been shown to colocalize and to coimmunoprecipitate with Cx43, strongly suggest that Cx43 is a component of several supramolecular complexes (Duffy et al., 2002a; Spray et al., 1999). Among the cytoplasmic domains of Cx43, the carboxyl-terminus (CT) contains SH3, SH2 and PDZ binding sites that have been shown to be important for interaction with these proteins (for review see Delmar et al., 2004; Giepmans, 2004). Although the list of gap junction interacting proteins continues to grow, very little is known about the functional significance of such interactions. Our previous work on spinal cord astrocytes indicated that deletion of Cx43 alters the expression and function of purinergic (P2Y) receptors, thus affecting glial calcium signal transmission (Scemes et al., 2000b, Suadicani et al., 2003) and the rate of migration of neural progenitor cells (Scemes et al., 2003). Such effects of Cx43 on P2YR expression/function led us to hypothesize that these two proteins are part of a functional unit involving protein-protein interactions. To test this possibility, P2Y₁R function was evaluated in Cx43-null astrocytes transfected with full length Cx43, Cx43 truncated at amino acid residue 257, the Cx43 carboxyl terminus alone, and with a Cx43 construct lacking residues 260–280. Evidence is here provided for the functional interaction of Cx43 and P2Y₁R in astrocytes, involving the SH3 binding domain on the carboxyl terminus of Cx43. Such change in P2Y₁ receptor function is likely to have great impact in the CNS, especially under inflammatory conditions due to the decreased expression of Cx43.

MATERIALS AND METHODS

Astrocyte Cultures

Spinal cord astrocytes were obtained as previously described (Scemes et al., 2000b) from wild-type (WT) and Cx43-null neonatal mice (C57Bl/6J-Gja1 strain, heterozygotes were originally obtained from Jackson Laboratories and colonies maintained at the Albert Einstein College of Medicine; all experimental procedures were approved by the AECOM Animal Care and Use Committee). Briefly, cervical to lumbar vertebrae were dissected and spinal cord segments evicted from the vertebrae. After removal of the meninges, spinal cord tissues were digested in 0.025% collagenase (Sigma) for 10 min at 37°C. Cells were collected by centrifugation at

2,000 rpm for 2 min at room temperature. The final pellet was suspended in Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 5% fetal bovine serum and 1% antibiotics and cells seeded in 35 mm culture dishes. Studies were performed on spinal cord astrocytes maintained for 2–3 weeks in culture at which time about 95–98% of the cells in culture were immunopositive for glial fibrillary acidic protein.

Transient Transfection of Cx43-Null Astrocytes with Cx43 cDNAs

Confluent cultures of Cx43-null spinal cord astrocytes were transfected with 6–8 μ g of cDNA encoding either full length Cx43, Cx43 truncated at position 257 (M257), with Cx43 CT (255–382) or with Cx43 Δ 260–280 using lipofectamine 2000 (Invitrogen). Parallel experiments were performed on mock-treated (lipofectamine and/or empty vector) Cx43-null and WT spinal cord astrocytes. Cells were used 30–48 h after transfection. Cx43 constructs were originally obtained from Dr. Eric Beyer (full length Cx43) and Dr. Mario Delmar (Cx43M257, Cx43 Δ 260–280 and Cx43CT).

Western Blots

Samples of whole cell lysates of WT and of untransfected and transfected Cx43-null astrocytes were electrophoresed in 4–20% SDS-PAGE (Bio-Rad, Hercules, CA) and then transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Immunoblots were performed after overnight incubation of membranes with blocking solution (5% dry nonfat milk in 1X PBS) using primary antibodies. After several washes with 1X PBS containing 0.4% polyoxyethylenesorbitan monolaurate (Tween-20; Sigma), membranes were incubated with HRP-conjugated secondary antibodies (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA). Detection of bands was performed on X-ray films (Kodak, Rochester, NY) following incubation with enhanced chemiluminescence reagents (Amersham Pharmacia Biotechnology, Piscataway, NJ). Quantification of the expression level of Cx43 and P2Y₁R was performed by densitometric analysis (Scion-NIH Image software) using β -actin as an invariant control against which the protein of interest was normalized. The following primary antibodies were employed: polyclonal antiP2Y₁ (1:200; Alomone Labs), and two different polyclonal antiCx43 antibodies (gifts from Dr. E.L. Hertzberg, Albert Einstein College of Medicine), one recognizing Cx43 at position 241–261 (16A) and the other at position 345–360 (18A).

Intracellular Calcium Transients

Agonist-induced intracellular Ca^{2+} transients were measured in WT and in mock-treated and transfected Cx43-null astrocytes loaded for 45 min at 37°C with Fura-2-AM (10 μ M; Molecular Probes, as previously described (Scemes et al., 2000b; Suadicani et al., 2003). Cells were then washed with Dulbecco's phosphate buffered saline (DPBS; pH 7.4; Cellgro, Herndon, VA) containing 5 mM glucose and imaged on an epifluorescence microscope (Eclipse TE2000-S; Nikon, Japan). Intracellular calcium measurements were performed on cells plated on Matek dishes and bathed in DPBS. Images were acquired with a CCD camera (Orca-ER; Hamamatsu, Japan). Ratio values of Fura-2 fluorescence intensities emitted at two excitation wavelengths (340 and 380 nm) were obtained using combined systems of filters and shutter (Lambda DG-4 Diaphot, Sutter Instruments Co., Burlingame, CA) driven by a computer through Metafluor software (Universal Imaging Systems, PA) and acquired continuously at a rate of 0.7 Hz. Measurements were obtained from regions of interest placed on cells, and ratio values were translated into intracellular Ca^{2+} concentrations according to an *in vitro* calibration curve of the form: $[\text{Ca}^{2+}]_i = K_D \{ (R - R_{\min}) / (R_{\max} - R) \} (F_{\min}^{380} / F_{\max}^{380})$, where $[\text{Ca}^{2+}]_i$ is the calculated intracellular calcium concentration, K_D is the dissociation constant of free- Ca^{2+} 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 with zero calcium at 380 nm, and $F_{\max}^{380}$ is the fluorescence

intensity measured with saturated calcium at 380 nm. Changes in intracellular calcium levels were monitored before and after bath application of the P2Y₁R agonist 2-methylthioadenosine 5'-triphosphate (2-MeS-ATP; Calbiochem, La Jolla, CA), as previously described (Scemes et al., 2000b; Suadicani et al., 2003).

For some experiments aimed to evaluate the responses of P2Y₁R to agonist, Cx43-null astrocytes were treated for 30 min with 10 μ M of a membrane permeant (TAT) peptide-FITC conjugated (GenScript) corresponding to amino acids 260–280 of Cx43CT and with the c-Src tyrosine kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2; 10 μ M; Tocris).

Fluorescence Recovery After Photobleach

Evaluation of dye-coupling in WT, Cx43-null and Cx43-null astrocytes transiently transfected with full length Cx43 and truncated Cx43 Δ 257 was performed using the fluorescence recovery after photobleaching (FRAP) technique (Lippincott-Schwartz et al., 2001; Wade et al., 1986), as previously described (Suadicani et al., 2004). Confluent cultures of astrocytes plated in Matek dishes were loaded with calcein-AM (1 μ M; Molecular Probes) for 30 min at 37°C. After several washes, cells were bathed in DPBS (Dulbecco's phosphate buffered salt solution; 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 $\times$ 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 $\times$ optical zoom (inserted between the light path and the objective). Immediately after the photobleach, changes in calcein fluorescence intensity were then monitored at low laser power and 1 $\times$ optical zoom. Images were acquired continuously at a rate of 1/60 s before, immediately after and for about 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 ($t = 1$ –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 Prism4.0 curve fitting software according to the following exponential equation: $Y = Y_{\max} [1 - \exp(-Kt)]$; 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 WT and Cx43-null astrocytes. At least three independent experiments were performed.

RESULTS

To evaluate whether changes in P2Y₁R expression and function observed in Cx43-null spinal cord astrocytes (Scemes et al., 2000b; Suadicani et al., 2003) were related to reduced coupling between Cx43-null cells or mediated through specific domains of the Cx43 protein, experiments described below were performed on Cx43-null spinal cord astrocytes transiently transfected with vectors encoding full length Cx43, Cx43 truncated at position 257 (M257), and the Cx43 carboxyl terminus (Cx43CT: amino acids 255–382). Parallel experiments were performed on WT astrocytes treated with the gap junction channel blocker carbenoxolone (CBX: 100 μ M).

Expression of Cx43 Domains and Dye Coupling in Astrocytes

As illustrated in Western blots in Fig. 1A, transient transfection of Cx43-null astrocytes with full length Cx43, M257 and Cx43CT resulted in readily detectable expression of these three proteins. Functional studies performed using the FRAP assay (Fig. 1B) indicated that expression of both full length Cx43 and M257 provided dye coupling between Cx43-null astrocytes. The half-time of calcein recovery after photobleaching obtained for cells transfected with full length Cx43 (85.4 ± 2.91 s; $N = 15$ cells from three to four independent experiments) was not statistically different ($P > 0.05$, t -test) from that observed between WT cells (91.3 ± 0.72 s; $N = 15$ cells). Interestingly, dye coupling between cells expressing Cx43M257, which in oocytes produces pH-insensitive gap junction channels (Morley et al., 1996), was significantly lower (111.2 ± 0.28 s; $N = 15$ cells; $P < 0.001$, t -test) than WT cells. The possibility that such difference in dye coupling is related to distinct expression levels of each of the connexin constructs used cannot be ruled out. Because the two Cx43 antibodies used (18A and 16A) for western blot analyses are likely to have different affinities for the protein, comparison of band intensities generated by these two antibodies is unlikely to correlate with Cx43 expression levels.

Treatment of WT astrocytes with $100 \mu\text{M}$ CBX for 48 h (incubation time chosen to correspond to that evaluated after transfection) reduced dye coupling to levels similar to those seen in Cx43-null astrocytes (Fig. 1B); no changes in Cx43 expression levels were observed after CBX treatment (data not shown).

Expression of Specific Cx43 Domain But Not Blockade of Coupling Affects P2Y₁R Function

To evaluate whether the altered P2Y₁R function in Cx43-null astrocytes (Scemes et al., 2000b) was related to reduced gap junctional communication in these cells, experiments were performed by measuring the half-maximal increases in intracellular Ca^{2+} (EC_{50} values) induced by the P2Y₁R agonist 2-MeS-ATP in Fura-2-AM-loaded WT astrocytes untreated and treated with the gap junction channel blocker CBX. Figure 2A shows the dose-response curves obtained from untreated and CBX-treated WT and Cx43-null astrocytes. Exposure of WT spinal cord astrocytes to $100 \mu\text{M}$ CBX for 48 h did not significantly alter the EC_{50} value to 2-MeS-ATP ($\text{EC}_{50} = 11.8 \pm 0.7$ nM; $N = 8$ independent experiments) compared with those obtained for WT cells ($\text{EC}_{50} = 12.9 \pm 0.7$ nM; $N = 8$ experiments), which were significantly different from those obtained for Cx43-null astrocytes ($\text{EC}_{50} = 380.5 \pm 20.0$ nM; $N = 8$ experiments). Note that CBX treatment totally uncoupled astrocytes, as shown in Fig. 1. These data, therefore, indicate that altered P2Y₁R function in Cx43-null spinal cord astrocytes is unlikely to be due to reduced gap junctional communication between these cells and suggest that other mechanisms contribute to the change in agonist EC_{50} values.

That the effect of high concentrations of 2-MeSATP (10 and $100 \mu\text{M}$) used in the present study is not mediated by P2X receptors relies on the observation that calcium transients induced by 10 and $100 \mu\text{M}$ MeSATP were totally prevented by the specific P2Y₁R antagonist MRS2179 ($5 \mu\text{M}$) (see Fig. 4 in Scemes et al., 2003; Fig. S1 in supplemental material). Moreover, bath application of $300 \mu\text{M}$ α, β -meATP, an agonist of P2X₁ and P2X₃ receptors, which are sensitive to 2-MeSATP (Burnstock, 2007), failed to induce calcium elevation in astrocytes (Fig. S1 in supplemental material), as previously reported (Fumagalli et al., 2003; Scemes et al., 2000a).

To test the hypothesis that P2Y₁R function is dependent on a mechanism that requires the expression of Cx43, Cx43-null spinal cord astrocytes were transiently transfected with full length Cx43 and the half maximal responses to 2-MeS-ATP were measured in Fura-2-AM loaded cells, as described above. As shown in Fig. 2B, expression of full length Cx43 in Cx43-null astrocytes rescued P2Y₁R behavior; the half-maximal value of intracellular Ca^{2+} increase induced by the P2Y₁R agonist observed in mock-treated Cx43-null astrocytes ($\text{EC}_{50} = 321.0$

± 24.6 nM; $N = 12$ experiments) shifted to 42.1 ± 6.6 nM ($N = 9$ experiments) in full length Cx43 transfectants; the EC_{50} values obtained for transfected Cx43-null cells were not different ($P > 0.05$; ANOVA) from those obtained for WT cells ($EC_{50} = 38.5 \pm 4.9$ nM; $N = 10$ experiments). Thus, these data strongly suggest that expression of Cx43 protein is important for the proper function of P2Y₁R.

To evaluate whether specific Cx43 domains mediated the rescue of P2Y₁R function, dose-response curves to 2-MeS-ATP were performed in Cx43-null astrocytes transfected with Cx43CT and with M257. As shown in Fig. 2B, forced expression of Cx43CT in Cx43-null astrocytes, but not of M257, shifted the dose-response curve obtained for 2-MeS-ATP toward that obtained for WT cells. The EC_{50} values obtained for 2-MeS-ATP in Cx43CT transfectants (52.7 ± 1.6 nM; $N = 10$ experiments) were similar to those obtained for WT cells and full length Cx43 transfectants ($P > 0.05$; ANOVA). No significant difference ($P > 0.05$; ANOVA) was observed between M257 transfectants ($EC_{50} = 254.1 \pm 30.2$ nM; $N = 10$ experiments) and mock-treated Cx43-null cells ($EC_{50} = 321.0 \pm 24.6$ nM; $N = 12$ experiments).

The domain of Cx43CT spanning 264–287 comprises an SH3 binding site, which has been shown to play an important role in the competition between c-Src and ZO1 (Sorgen et al., 2004). Therefore, we hypothesized that the effect of Cx43CT on P2Y₁R pharmacology could be mediated by a member of the Src tyrosine kinase family. To test this hypothesis, Cx43-null astrocytes were transfected with a Cx43 mutant construct missing the SH3 domain (deletion of amino acids 260–280). As shown in Fig. 3A, transfection of Cx43-null astrocytes with Cx43 Δ 260–280 did not rescue the EC_{50} value of 2-MeS-ATP observed in WT cells ($EC_{50} = 849.6$ nM for Cx43 Δ 260–280; $EC_{50} = 40.0$ nM for WT). Conversely, treatment of Cx43-null astrocytes with a membrane permeant peptide corresponding to amino acids 260–280 of Cx43CT shifted the dose-response curve to the left (Fig. 3B; $EC_{50} = 49.4$ nM and 57.2 nM for peptide and $EC_{50} = 16.4$ nM for WT).

These results suggest that altered P2Y₁R function in Cx43-null astrocytes is dependent on the expression of Cx43, specifically the Cx43 carboxyl terminal spanning the SH3 domain of the c-Src binding site. To further test this hypothesis, WT, untransfected and transfected Cx43-null astrocytes were treated with the c-Src kinase inhibitor PP2 (10 μ M) prior to stimulation with 2-MeS-ATP (100 nM). As shown in Fig. 3C, intracellular calcium mobilization induced by 2-MeS-ATP was enhanced in Cx43-null astrocytes treated with PP2 to levels similar to those recorded from WT and from Cx43-null astrocytes transfected with Cx43CT; intracellular calcium mobilization was not altered in astrocytes expressing the truncation mutant Cx43M257 compared with untransfected cells.

P2Y₁R Expression Levels in Cx43-Null Transfectants

To evaluate whether the rescue of P2Y₁R function in Cx43-null transfectants was related to increased expression levels of P2Y₁R in these cells, western blot analyses were performed in untransfected and transfected Cx43-null astrocytes with the three different Cx43 constructs. As shown in Fig. 4, the reduced expression level of P2Y₁R in Cx43-null astrocytes was not restored to that measured from WT cells following forced expression of either Cx43CT or M257 ($P < 0.001$; ANOVA followed by Newman-Keuls Multiple Comparison Test). Interestingly, although the levels of P2Y₁R in full length Cx43 transfectants were not statistically different from that of WT, they also did not significantly differ from that seen in Cx43-null cells ($P > 0.05$; ANOVA followed by Newman-Keuls Multiple Comparison Test).

These results showing that expression of Cx43 domains in Cx43-null astrocytes do not restore P2Y₁R expression levels suggest that the increased agonist sensitivity of P2Y₁R induced by forced expression of full length Cx43 and Cx43CT (Fig. 2B) is independent of either direct or indirect dependence of transcriptional activity of Cx43 on P2Y₁R expression levels. These

data, however, raise the possibility that the binding of Cx43 to a tyrosine kinase of the Src family facilitates calcium mobilization induced by P2Y₁R activation.

Altered P2Y₁ Receptor Function Following Cytokine-Induced Decrease in Cx43

Given that Cx43 expression levels in astrocytes can be greatly reduced under inflammatory conditions (Brand-Schieber et al., 2005; Hinkerohe et al., 2005; John et al., 1999; Meme et al., 2006), we evaluated whether such decrease could also affect P2Y₁R function. For that, we used confluent cultures of WT spinal cord astrocytes treated with the proinflammatory cytokine interleukin (IL)-1 β to evaluate Cx43 expression levels and P2Y₁R induced calcium mobilization. Western blot analysis of whole cell homogenates of confluent cultures of untreated and IL1- β treated (20 ng/mL; Cedarlane) WT mouse spinal cord astrocytes showed, as expected, a significant decrease of Cx43 expression levels 6 h after treatment (Fig. 5) that was further decreased after 12 and 24 h. The dose-response curves obtained for the P2Y₁R agonist 2-MeS-ATP performed on WT treated for 24 h with IL-1 β yield EC₅₀ values of 291.5 ± 1.3 nM (see Fig. 5), which are about 10–20 times higher than those observed for untreated WT astrocytes (see Fig. 2 and Fig. 3). Because no significant changes in the P2Y₁R agonist EC₅₀ values were observed between untreated and IL-1 β treated Cx43-null astrocytes (Fig. 2, Fig. 3, and Fig. 5), it is likely that the altered P2Y₁R behavior observed under these conditions is related to the reduced expression of Cx43 rather than to downstream effects of IL-1 β on P2Y₁R. Moreover, no significant changes in P2Y₁R expression levels were observed in astrocytes treated for 24 h with the cytokine (Fig. S2 in supplemental material). Similarly to what was here observed for IL-1 β , activation of Toll-like receptor 3 in human fetal astrocytes, which also leads to decreased Cx43 expression level, does not alter P2Y₁R receptor expression levels (Zhao et al., 2006).

DISCUSSION

Astrocytes “*in vivo*” and “*in vitro*” are equipped with distinct purinergic P2 receptors (Ballerini et al., 1996; Fumagalli et al., 2003; Jimenez et al., 2000; Neary et al., 1988; Pearce and Langley, 1994; Suadicani et al., 2003; Zhu and Kimelberg, 2001) and gap junction proteins (Dermietzel et al., 2000; Nagy et al., 2003; Scemes et al., 2000b; Spray et al., 1998). Although these protein families are genetically unrelated, they share at least the common function of regulating calcium levels in astrocytes. Activation of P2 receptors by exogenous purine and pyrimidine nucleotides leads to elevation of intracellular calcium levels that can be constrained within a single cell or transmitted through gap junction channels to adjacent coupled astrocytes (for reviews see Scemes, 2000; Scemes and Giaume, 2006). Changes in cytosolic calcium levels are not only important for several aspects of astrocyte biology but can also impact on neuronal activity due to the exocytotic release of ATP and glutamate from astrocytes (Bezzi et al., 2004; Montana et al., 2004; Striedinger et al., 2007; Zhang et al., 2004). Because the main gap junction protein in astrocytes (Cx43) forms intercellular channels permeable to Ca²⁺ mobilizing second messengers, such as IP₃, cADP-ribose, Ca²⁺ (Christ et al., 1992; Churchill and Louis, 1998; Saez et al., 1989), Cx43 gap junctional channels are expected to modulate P2R mediated Ca²⁺ signals within the astrocytic compartment. Indeed, we have recently shown that gap junctional communication between glial cells in culture can either increase or decrease the number of cells responding to P2R activation depending on the levels of second messenger generated by each P2Y receptor subtype (Suadicani et al., 2004).

It is becoming apparent that gap junction proteins have roles other than providing direct ionic and metabolic continuity between the cytosol of coupled cells. Previous studies performed on Cx43-null astrocytes indicated that numerous unrelated genes are altered, such as those involved in cell cycle, motility and metabolism (Iacobas et al., 2003, 2004; Naus et al., 2000). The mechanisms by which connexins exert channel- independent effects are not clear.

It is possible that they are mediated by connexin interacting proteins (for reviews see Delmar et al., 2004; Giepmans, 2004), implying that connexins are part of a supra-molecular complex (Duffy et al., 2002a; Spray et al., 1999).

In this regard, evidence is here provided suggesting that the major gap junction protein in astrocytes, Cx43, modulates purinergic receptor activity, altering P2Y₁R agonist concentration necessary to attain half maximal intracellular calcium elevation. In accordance with our previous work showing that deletion of Cx43 by homologous recombination or by the use of anti-sense oligonucleotide altered the behavior of P2Y₁R (Scemes et al., 2000b; Suadicani et al., 2003) are the results obtained here showing that IL-1 β -induced decrease in Cx43 expression levels causes a similar shift in 2-MeS-ATP EC₅₀ value as that seen in Cx43 KO astrocytes.

Moreover, it is shown here that this modulation is independent of gap junctional communication but is dependent on the presence of the Cx43 carboxyl-terminal domain. Evidence in support for a gap junction-independent modulation of P2Y₁R activity includes the findings that the dose-response curves obtained for P2Y₁R agonist were not affected by (1) blockade of gap junctional communication in WT astrocytes and by (2) coupling Cx43 KO astrocytes with Cx43M257 gap junction channels. In favor of P2Y₁R modulation through Cx43 specific domains are the results showing that P2Y₁R function is rescued when Cx43-null astrocytes were transfected with either full length Cx43 or with its CT domain (Cx43CT).

A possible mechanism by which such interplay between connexins and purinergic receptors occurs may include protein-protein interactions. For instance, it has been shown that specific sequences of the C-termini of P2Y₁ and P2Y₂ receptors, distinct from their PDZ-binding motif, modulate the voltage dependence of the endogenous *Xenopus* oocyte transient inward (Tin) channels (Lee et al., 2003). Moreover, the binding of the SH3 domain of c-Src to P2Y₂R was shown to lead to the recruitment of the epidermal growth factor receptor (EGFR) into the P2Y₂R complex, thus facilitating EGFR phosphorylation by c-Src (Liu et al., 2004). Although the number of Cx43 binding partners has been increasing (see Giepmans, 2004; Jacobas et al., 2007), little is known about their functional consequences. Binding of the Cx43 C-terminus to its cytoplasmic loop has been proposed as a mechanism for the pH gating of Cx43 gap junction channels (Duffy et al., 2002b; Ek-Vitorin et al., 1996; Morley et al., 1996), while the binding of c-Src SH3 domain to Cx43CT, following prolonged intracellular acidification was shown to lead to the unbinding of the PDZ domain of Cx43 from the scaffolding protein ZO-1 and to Cx43 internalization (Duffy et al., 2004).

We here show that the amino acid sequence 260–280 of Cx43 CT that contains an SH3 sequence motif (Sorgen et al., 2004) is necessary for proper function of P2Y₁R. This is based on the observation that a peptide spanning the amino acids 260–280 of Cx43, but not expression of Cx43 lacking this motif, was able to restore P2Y₁R function. A possible mechanism by which Cx43 modulates P2Y₁R is by providing a binding site for c-Src, i.e., by serving as a decoy. In such a model, reduction of a c-Src binding site (i.e., Cx43) could lead to increased binding and phosphorylation of other proteins (P2Y₁R), thus altering their function. Support for a role of c-Src on P2Y₁R is our observation that the Src tyrosine kinase inhibitor PP2 restored P2Y₁R-induced calcium mobilization in Cx43-null astrocytes.

Given that P2Y₁R plays important roles in multiple biological functions (production and release of NO from astrocytes: Horiuchi et al., 2003; astrogliosis: Franke et al., 2001; progenitor cell migration: Scemes et al., 2003; Weisman et al., 2004), the modulation of P2Y₁R activity by Cx43 is likely to impact on CNS physiology and pathology, especially under conditions of CNS inflammation and in neuro-degenerative disorders, where Cx43 has been shown to be down-regulated (Brand-Schieber et al., 2005; Hinkerohe et al., 2005; John et al., 1999; Meme et al., 2006).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Gap junctional communication

(A) Western blot showing expression of Cx43 in WT (lane 1) and in Cx43-null astrocytes transfected with full length Cx43 (lane 3), with Cx43 carboxyl terminus (lane 4) and with Cx43M257 (lane 5). Lane 2 corresponds to untransfected Cx43-null cells. Lanes 1–4 correspond to immunoblots performed with Cx43-18A antibody and lane 5 with Cx43-16A. (B) Time courses of calcein FRAP obtained from untreated (black squares) and 48 h carbenoxolone-treated (open triangles) WT astrocytes, and from untransfected (open circles) and transfected Cx43-null astrocytes with full length Cx43 (open squares) and Cx43M257 (black circles). Data were obtained from astrocytes cultured from a minimum of 3 litters of mice.

Fig. 2. Modulation of P2Y₁R activity by Cx43 domains

(A) Dose-response curves obtained for untreated (black squares) and 48 h carbenoxolone-treated (open triangles) WT and untransfected Cx43-null spinal cord astrocytes exposed to the P2Y₁R agonist 2-MeS-ATP. Note that blockade of gap junctional communication in WT astrocytes does not alter the half-maximal response (EC_{50} value) induced by the P2Y₁R agonist. Mean values were obtained from four to seven independent experiments. (B) Dose-response curves obtained for WT (black squares), untransfected (open circles) and transfected Cx43-null astrocytes with full length Cx43 (open squares), Cx43M257 (black circles) and Cx43CT (black triangles). Note that both full length Cx43 and Cx43CT but not Cx43M257

shifted the EC_{50} values of 2-MeS-ATP obtained for Cx43-null astrocytes to those obtained in WT cells. Data were obtained from 5 to 8 litters.

Fig. 3. Functional interaction between P2Y₁R and the SH3 domain of Cx43

(A) Dose-response curves obtained for WT (black squares) and untransfected (open circles) and Cx43 $\Delta 260-280$ transfected (black triangles) Cx43-null astrocytes exposed to 2-MeS-ATP, showing that deletion of the Cx43 SH3 domain does not rescue P2Y₁ receptor function. Mean $\pm$ SE values are from 100 to 200 cells obtained from three litters. (B) Dose-response curves obtained from WT (black squares), and Cx43-null astrocytes untreated (open circles) and treated (black triangles) with a membrane permeant peptide corresponding to amino acids 260–280 of Cx43CT. (C) Bar histograms of the mean $\pm$ SE values of intracellular calcium mobilization induced by 100 nM 2-MeS-ATP recorded from WT, Cx43-null and Cx43-null transfected with Cx43CT and Cx43M257 in the absence and presence of 5 μ M PP2. Note that

only untransfected and Cx43M257 transfected Cx43-null astrocytes that were not exposed to PP2 did not respond to agonist with intracellular calcium levels similar to WT astrocytes. Mean $\pm$ SE values are from 4 litters (** $P < 0.001$; ANOVA followed by Newman-Keuls' Multiple Comparison Test).

Fig. 4. P2Y₁R expression levels in Cx43-null transfectants

Bar histograms showing the mean values of P2Y₁R expression levels in WT, untransfected Cx43-null (KO), and in Cx43-null transfected with Cx43 CT, Cx43 truncated at position 257 (M257) and with full length Cx43. An example of western blots for Cx43 and β -actin is shown above.

Fig. 5. Effect of IL-1 β on Cx43 and P2Y₁R

(A) Western blot showing decreased expression levels of Cx43 following exposure of spinal cord astrocytes to IL-1 β (B) Dose-response curves obtained for 2-MeS-ATP performed on Fura-2 loaded WT and Cx43 KO astrocytes treated for 24 h with IL-1 β (20 ng/mL). Note that exposure to the cytokine altered the agonist EC₅₀ values in WT astrocytes. About 180 cells from three independent experiments were used in each condition.