Molecular Biology of the Cell
Vol. 18, 34–46, January 2007

Connexin Hemichannels and Gap Junction Channels Are Differentially Influenced by Lipopolysaccharide and Basic Fibroblast Growth Factor

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Submitted March 7, 2006; Revised September 29, 2006; Accepted October 19, 2006
Monitoring Editor: Asma Nusrat

Gap junction (GJ) channels are formed by two hemichannels (connexons), each contributed by the cells taking part in this direct cell–cell communication conduit. Hemichannels that do not interact with their counterparts on neighboring cells feature as a release pathway for small paracrine messengers such as nucleotides, glutamate, and prostaglandins. Connexins are phosphorylated by various kinases, and we compared the effect of various kinase-activating stimuli on GJ channels and hemichannels. Using peptides identical to a short connexin (Cx) amino acid sequence to specifically block hemichannels, we found that protein kinase C, Src, and lysophosphatidic acid (LPA) inhibited GJs and hemichannel-mediated ATP release in Cx43-expressing C6 glioma cells (C6-Cx43). Lipopolysaccharide (LPS) and basic fibroblast growth factor (bFGF) inhibited GJs, but they stimulated ATP release via hemichannels in C6-Cx43. LPS and bFGF inhibited hemichannel-mediated ATP release in HeLa-Cx43 cells, but they stimulated it in HeLa-Cx43 with a truncated carboxy-terminal (CT) domain or in HeLa-Cx26, which has a very short CT. Hemichannel potentiation by LPS was inhibited by blockers of the arachidonic acid metabolism, and arachidonic acid had a potentiating effect like LPS and bFGF. We conclude that GJ channels and hemichannels display similar or oppositely directed responses to modulatory influences, depending on the balance between kinase activity and the activity of the arachidonic acid pathway. Distinctive hemichannel responses to pathological stimulation with LPS or bFGF may serve to optimize the cell response, directed at strictly controlling cellular ATP release, switching from direct GJ communication to indirect paracrine signaling, or maximizing cell-protective strategies.

INTRODUCTION

Gap junctions (GJs) are specialized structures composed of a collection of channels that directly connect adjacent cells to allow the passage of small molecules such as amino acids, ions, and second messengers. Vertebrate GJ channels are composed of proteins encoded by the connexin gene family (Sohl and Willecke, 2004). Connexins span the membrane four times, are endowed with a single cytoplasmic loop and two extracellular loops, and have their amino and carboxy terminals (CTs) inside the cell. Most of the connexins are phosphorylated in vivo, primarily on serine residues and to a lesser extent on threonine and tyrosine residues located in the CT domain. Cx43 is a ubiquitously expressed connexin that has been well characterized in terms of phosphorylation sites and effects. A basal degree of phosphorylation on five serine residues seems to be required for the assembly and functioning of GJs composed of Cx43 (Musil and Goodenough, 1991; Laird *et al*., 1995; Cooper *et al*., 2000). Numerous studies have demonstrated the influence of growth factors, oncogene protein kinases, hormones, and inflammatory mediators on GJ communication via phosphorylations on the CT domain of the protein (amino acid 236–382) (Maldonado *et al*., 1988; Crow *et al*., 1990; Filson *et al*., 1990; Swenson *et al*., 1990; Kurata and Lau, 1994; Matesic *et al*., 1994). Several serine kinases have been identified, including protein kinase C (PKC) (Ser 368 and Ser 372), mitogen-activated protein kinases (MAPKs) (Ser 255, Ser 279, and Ser 282), cdc2/cyclinB (Ser 255), and casein kinase I (Ser 325, Ser 328, and Ser 330) (Lampe and Lau, 2004).

GJ channels are composed of two hemichannels, each contributed by the cells that share this junctional communication channel. Hemichannels are hexameric high-conductance plasma membrane channels that are normally closed and can act as a conduit to release paracrine signaling molecules such as ATP, NAD^+, glutamate, and prostaglandins when opened (Bruzzone *et al*., 2001b; Bennett *et al*., 2003; Ebihara, 2003; Goodenough and Paul, 2003; Ye *et al*., 2003; Cherian *et al*., 2005). Hemichannels are closed at physiological millimolar extracellular calcium concentrations, but they open in response to lowering extracellular calcium (alone or in combination with a lowering of other extracellular divalent cations) (Li *et al*., 1996; Pfahnl and Dahl, 1999; Quist *et al*., 2000; Kamermans *et al*., 2001; Muller *et al*., 2002; Stout and

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06–03–0182) on November 1, 2006.

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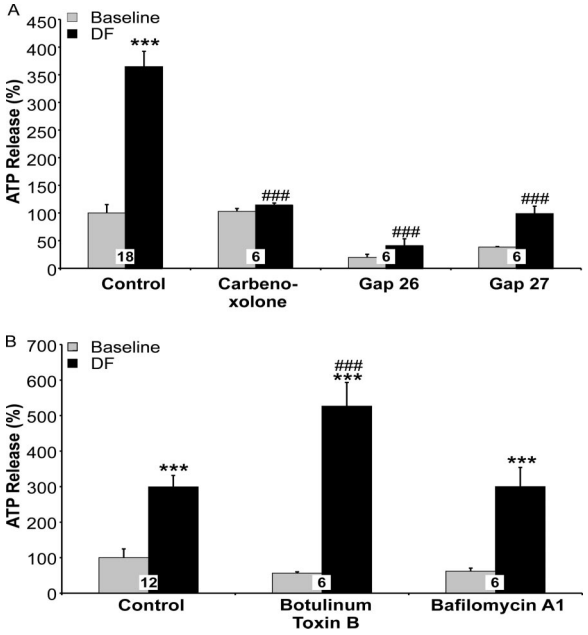

Figure 1. ATP release triggered in C6-Cx43 by DF exposure is mediated by hemichannels. (A) Exposure to DF conditions triggered significant ATP release that was blocked by carbenoxolone and the connexin mimetic peptides gap 26 and 27. (B) Botulinum toxin B and bafilomycin A1 had no inhibitory effects; botulinum toxin B even stimulated DF-triggered ATP release. Numbers on the bars indicate n. Star symbols indicate significant differences compared with baseline, whereas the number signs indicate significant differences compared with the corresponding control bar (single symbol, $p < 0.05$; double symbol, $p < 0.01$; and triple symbol, $p < 0.001$). Gap 26 and 27 also significantly depressed baseline ATP release ($p < 0.001$ and < 0.02 respectively; *t* test; not indicated on the graph).

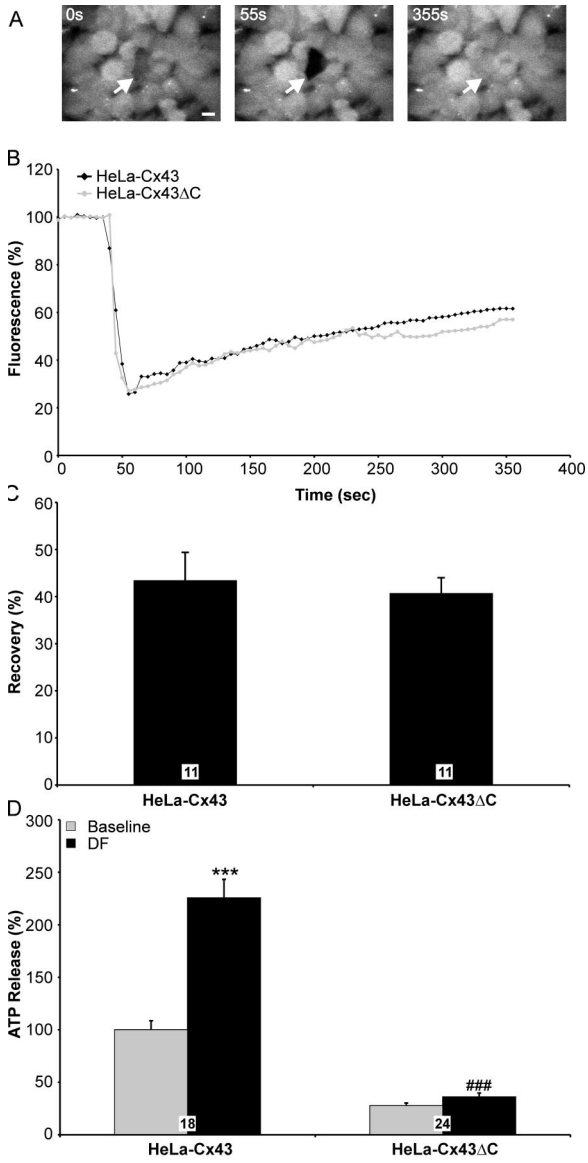

Figure 2. Effect of CT-truncation on GJ coupling and ATP release in HeLa-Cx43. (A) Example pictures of FRAP experiments used to investigate dye coupling. The white arrow points to the photobleached cell. (B) Average FRAP recovery traces of the experiments summarized in C. (C) Summary FRAP recovery data determined from data points over the last 25 s. CT truncation had no effect on GJ coupling (n is number of FRAP experiments on 2 different cultures). (D) CT-truncation largely depressed baseline and triggered ATP release. Stars indicate significant differences compared with baseline; number signs indicate significant differences compared with HeLa-Cx43. Baseline in HeLa-Cx43Δ C was significantly below baseline in HeLa-Cx43 ($p < 0.0001$; *t* test).

Charles, 2003; Ye *et al*., 2003; Thimm *et al*., 2005; Srinivas *et al*., 2006), strong membrane depolarization (Trexler *et al*., 1996), mechanical stimulation (Bao *et al*., 2004a; Gomes *et al*., 2005), extracellular UTP (Cotrina *et al*., 1998), metabolic inhibition (John *et al*., 1999; Kondo *et al*., 2000; Contreras *et al*., 2002; Vergara *et al*., 2003), shigella infection (Tran Van Nhieu *et al*., 2003), and most recently also in response to an increase of cytoplasmic calcium (De Vuyst *et al*., 2006). Hemichannels have been reported to be inhibited by kinases such as PKC or Src (Li *et al*., 1996; Bruzzone *et al*., 2001a; Bao *et al*., 2004b). The purpose of the present study was to determine whether GJ channels and hemichannels are similarly or differentially influenced by various kinases or kinase-activating stimuli. Both channels and hemichannels are composed of the same connexin building blocks and would, at first glance, be expected to respond similarly to phosphorylations. Both channel types are, however, differentially regulated to start with, GJs being open under resting conditions and hemichannels being closed to prevent cell leakage.

Our work demonstrates that although channels and hemichannels are influenced in the same direction by certain stimuli, e.g., inhibition by PKC, Src, or LPA, both channels are differentially influenced by lipopolysaccharide (LPS) and basic fibroblast growth factor (bFGF), depending on the cell type and the presence or absence of the CT domain. In C6-Cx43 cells, LPS and bFGF potentiated ATP release via hemichannels, whereas in HeLa-Cx43 cells both substances inhibited these responses. Removal of the CT domain in HeLa-Cx43 cells reduced the ATP release, but LPS or bFGF treatment now potentiated the hemichannel-mediated ATP response. Likewise, HeLa cells expressing Cx26, which has a short CT domain, displayed hemichannel potentiation by LPS and bFGF. Antagonists of the arachidonic acid metabolism inhibited LPS enhancement of ATP release, and arachidonic acid itself mimicked the potentiation effect of LPS or bFGF. The cell specificity of hemichannel responses thus seemed to depend on the balance between phosphorylations (inhibition) and activation of the arachidonic acid signaling cascade (stimulation). LPS and bFGF came up as the most versatile modulators of connexin channels, because they

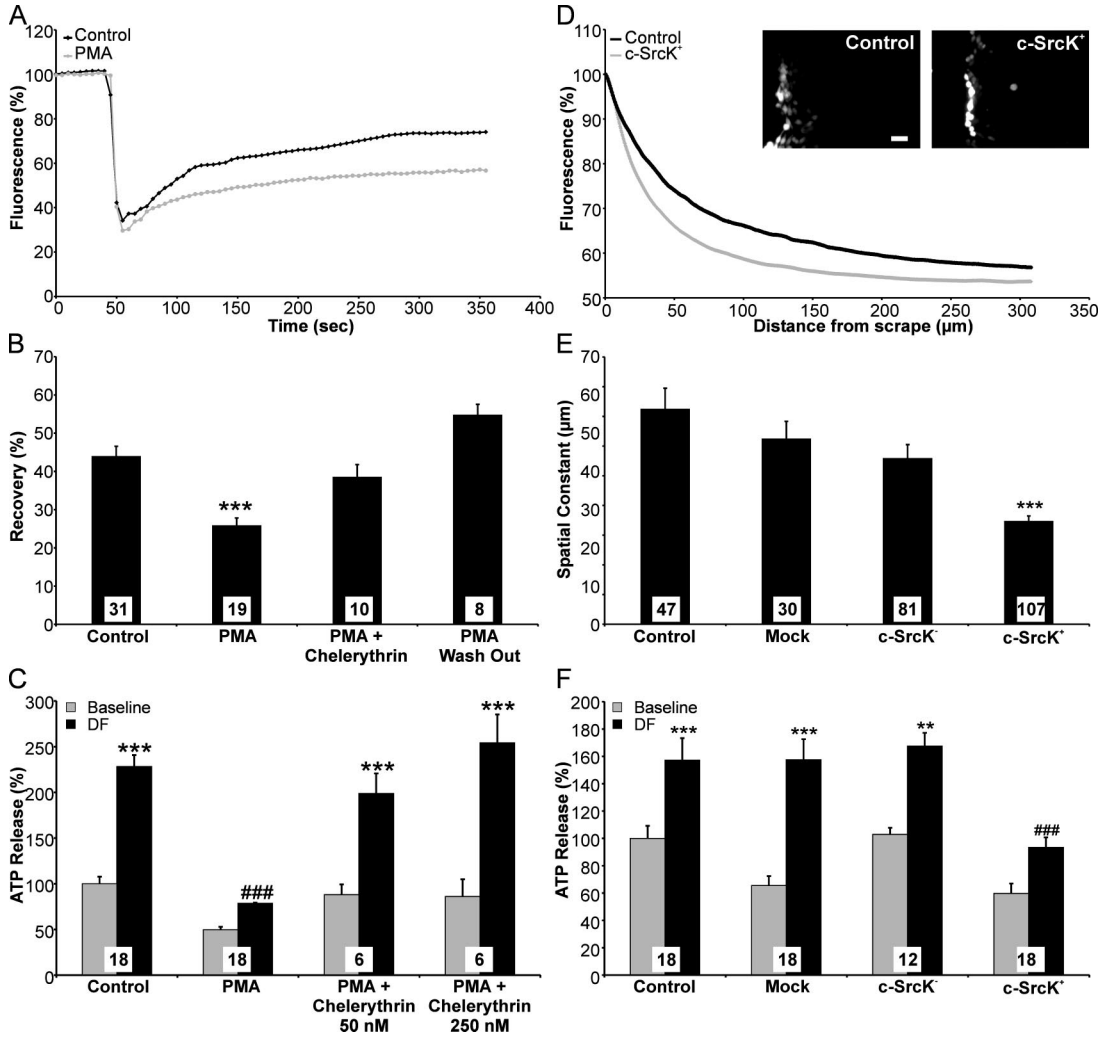

Figure 3. Effects of PKC and Src in C6-Cx43. (A) Average FRAP recovery traces (n = 31) in control and after PKC activation with PMA (n = 19). (B) FRAP summary data, showing inhibition of dye coupling by PMA and reversal by chelerythrin or PMA washout (30 min) (n is number of FRAP experiments on 3 different cultures for control and 2 different cultures for all other conditions). (C) Triggered ATP release was inhibited by PMA, an effect that was reversed by chelerythrin. (D) Example pictures illustrating SLDT in control and c-SrcK$^+$-transfected cells. The right border of the scrape is visible as the row of strongly 6-CF–stained cells, and dye spread proceeded from there to the right (bar, 100 μm). A fluorescence intensity profile was derived from each image, and a spatial constant of exponential decay was determined. (E) Summary data of SLDT experiments. Dye coupling was not significantly affected by mock or c-SrcK$^-$ transfection, but it was significantly inhibited in c-SrcK$^+$ (n is number of SLDT images obtained in 4 different cultures for control and 2 different cultures for all other conditions). (F) Triggered ATP release was not affected in mock or c-SrcK$^-$, but it was significantly depressed in c-SrcK$^+$. Stars indicate significant differences compared with control in B and E and compared with baseline in C and F. Number signs in C and F indicate significant differences compared with control. PMA significantly depressed baseline ATP release in C ($p < 0.001$; *t* test).

invariably inhibited GJs, but they inhibited or potentiated hemichannels depending on the dominance of kinase or arachidonic acid effects. The immunostimulant LPS and the growth factor bFGF thus exert powerful control over the release of ATP via hemichannels, a purine messenger that has both immune-modulatory and mitogenic actions (Fields and Burnstock, 2006). In addition, hemichannels play, like GJs, a role in cell death signaling, either as an antiapoptotic gate or a pathogenic pore (Plotkin *et al*., 2002; Hur *et al*., 2003; Kalvelyte *et al*., 2003; Krysko *et al*., 2005; Evans *et al*., 2006). Distinctive hemichannel responses may thus aim at optimizing cell–cell communication (stimulation of hemichannels compensating for the decreased GJ communication) or cell protection (inhibition of hemichannels), depending on the conditions and the cell type.

MATERIALS AND METHODS

Cells and Reagents

We used the following cell lines: C6 rat glioma cells stably transfected with Cx43 (C6-Cx43) (Zhu *et al*., 1991), human epithelial HeLa cells stably transfected with Cx43 (HeLa-Cx43) or the truncated form of Cx43 (HeLa-Cx43ΔC) (Omori and Yamasaki, 1999), HeLa cells stably transfected with Cx26 (Mesnil *et al*., 1996), and human embryonic kidney (HEK)-293 stably transfected with P_2X_7 (HEK-P_2X_7) (Humphreys *et al*., 1998). C6-Cx43 was maintained in DMEM/Ham's F-12 (1:1), and the HeLa and HEK lines were maintained in DMEM (HEK cells received in addition Geniticin at 300 μg/ml). All media were supplemented with 10% fetal bovine serum and 2 mM glutamine. All cell culture reagents were obtained from Invitrogen (Merelbeke, Belgium).

Six-carboxyfluorescein, (6-CF), calcein-acetoxymethyl ester (AM) and 5-carboxyfluorescein diacetate (5-CFDA) were from Invitrogen. Phorbol 12-myristate 13-acetate (PMA), L-α-lysophosphatidic acid (LPA), LPS (*Escherichia coli* O111:B4), bafilomycin A1, botulinum toxin B, PD098.059, U0126, chelerythrine, genistein, arachidonic acid, indomethacin, and carbenoxolone were obtained

from Sigma (Bornem, Belgium). 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2) was from BIOMOL Research Laboratories (Plymouth Meeting, MA), bFGF was from Roche Diagnostics (Vilvoorde, Belgium), AACOCF3 and baicalein were from Tocris Cookson (Avonsmouth, United Kingdom), and ampicillin was from Invitrogen. The connexin mimetic peptides gap 26 (VCYDKSFPISHVR) and gap 27 (SRPTEKTIFII) were synthesized by Sigma-Genosys (Cambridge, United Kingdom).

Plasmid DNA Purification and Transfection

pcDNA constructs containing dominant-negative and dominant-positive mutants of Src kinase were kindly provided by Dr. W. H. Moolenaar (Division of Cellular Biochemistry and Center for Biomedical Genetics, The Netherlands Cancer Institute, Amsterdam, The Netherlands) (Giepmans *et al.*, 2001). Constructs (pcDNA3.1, pcDNA-SrcK$^+$, pcDNA-SrcK$^-$, and pDSRed, used as a marker for transfection efficiency) were transformed to DH5α heat-shock–competent bacteria and grown overnight on Luria broth (LB) agar plates. A single colony was picked up and grown in liquid LB media supplemented with ampicillin overnight. The next day, bacteria were harvested by centrifugation, and DNA was isolated using Midi kits (QIAGEN, Benelux, The Netherlands). Cells were seeded at a density of 25,000 cells/cm^2 and transfected 6 h after seeding with 0.45 ng of pcDNA plasmid and 0.05 ng of pDSRed plasmid/cm^2 by using Transfectin lipid reagent (Bio-Rad, Nazareth, Belgium), except for ATP measurements where pDSRed was excluded.

GJ Communication

GJ dye coupling was determined with fluorescence recovery after photobleaching (FRAP) or scrape loading and dye transfer (SLDT). For FRAP, confluent monolayer cultures grown on glass-bottomed microwell (MarTek, Ashland, MA) were loaded with 10 μM 5-CFDA or 5 μM calcein (used for HeLa-Cx26) in Hank's balanced salt solution (HBSS)-HEPES for 45 min at room temperature. Fluorescence within a single cell was photobleached by spot exposure to the 488-nm line of an Argon laser and imaging (again at 488-nm excitation) was done with a custom-made video-rate confocal laser scanning microscope (Leybaert *et al.*, 2005) with a 40× oil immersion objective (CFI Plan Fluor; Nikon, Tokyo, Japan). For SLDT, confluent monolayer cultures were washed three times with nominally calcium-free (CF)-HBSS-HEPES. Cells were incubated during 1 min in CF-HBSS-HEPES containing 0.4 mm 6-CF; a linear scratch (1 per culture) was made across the culture by using a syringe needle, and the cells were left for another minute in the same solution. Cultures were then washed four times with HBSS-HEPES, left for 15 min at room temperature, and images were taken with a Nikon TE300 inverted microscope in epifluorescence mode (tetramethylrhodamine B isothiocyanate excitation/emission) with a 10× objective and a Nikon DS-5M camera (Analis, Namur, Belgium). A fluorescence diffusion profile was derived from the images, fitted to a mono-exponential decaying function, and a spatial constant of intercellular dye spread was determined.

Cellular ATP Release

ATP release was measured with an ATP bioluminescent luciferin/luciferase assay kit (Sigma) in combination with a luminometer plate reader (Victor3 1420 multi label counter; Perkin-Elmer, Zaventem, Belgium) on subconfluent cultures grown on 24-well plates (BD Bioscience, Erembodegem, Belgium). Cells were seeded at a density of 25,000 cells/cm^2 and used the next day for experiments. ATP release was triggered with divalent-free (DF) HBSS-HEPES (Ca^{2+} and Mg^{2+} replaced with 4 mM EGTA), and ATP was accumulated over a 2.5-min stimulation period. Baseline measurements were done according to the same procedure but with standard HBSS-HEPES instead. ATP assay mix (75 μl), prepared in HBSS-HEPES (at 5-fold dilution), was added to 150 μl of solution bathing the cells, and the photon flux was counted over 10 s at the end of the 2.5-min stimulation period. Average ATP release in C6-Cx43 cells was 23.5 ± 2.4 pmol (n = 176) in baseline and 46.2 ± 3.4 pmol (n = 183) with DF stimulation. All pharmacological agents were preincubated for the times indicated, in HBSS-HEPES at room temperature or in culture medium at 37°C for incubations lasting 30 min or longer, and were not present during the 2.5-min stimulation.

Western Blotting

Cell protein lysates were extracted by treatment of confluent cultures with radioimmunoprecipitation assay buffer (25 mM Tris, 50 mM NaCl, 0.5% NP-40, 0.5% deoxycholate, 0.1% SDS, 0.055 g/ml β-glycerophosphate, 1 mM dithiothreitol, 20 μl/ml phosphatase inhibitor cocktail, and 20 μl/ml mini EDTA-free protease inhibitor cocktail) and sonicated by three 10-s pulses. Separation of Triton X-100–soluble and –insoluble material was done essentially according to the method of Cooper and Lampe (2002). After appropriate treatment, cells from 75-cm^2 culture flasks were washed two times with phosphate-buffered saline (PBS), pH 7.2. Cells were harvested in ice-cold 1% Triton X-100 in PBS supplemented with 50 mM NaF, 1 mM Na_3VO_4, 1% protease inhibitor cocktail, 1% phosphatase inhibitor cocktail 1 and 2 (Sigma), and 1X mini-EDTA-free protease inhibitor cocktail (Roche Diagnostics, Penzberg, Germany). These samples were separated into Triton-soluble and -insoluble fractions by centrifugation at 16,000 × g for 10 min. Triton-insoluble fractions (pellets) were resuspended in 1X Laemmli sample buffer and sonicated by five 10-s pulses. Protein concentration was determined with a Bio-Rad DC protein assay kit (Bio-Rad), and absorbance was measured on a plate reader with a 590-nm long-pass filter. Proteins were separated by electrophoresis on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Blots were probed with a rabbit polyclonal anti-rat Cx43 antibody (1/10,000; Sigma), a rabbit polyclonal anti-rat β-tubulin antibody (1/5000, loading control; Abcam, Cambridge, United Kingdom), a mouse monoclonal anti-rat Cx43 antibody (1/500, epitope located at the intracellular loop; Upstate Cell Signaling, Huissen, The Netherlands), a rabbit polyclonal anti-rat Cx26 (1/2000; Zymed, Invitrogen), or a polyclonal rabbit anti-rat P_2X_7 antibody (1/1000; Alomone Labs, Jerusalem, Israel) followed by alkaline phosphatase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1/8000-1/4000; Sigma), and detection was done with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate reagent (Zymed, Invitrogen). Specificity of the P_2X_7 receptor antibody was confirmed by competition experiments with an antigenic peptide corresponding to amino acids 576–595 of the rat P_2X_7 receptor. ImageJ (http://rsb.info.nih.gov/ij/) was used to quantify Western blot signals. A rectangular measurement window was drawn around the nonphosphorylated (P_0) and phosphorylated bands (P_{1+2}), and their respective intensities were determined. The same windows were used to measure the background signal in nitrocellulose membrane zones where no protein was present; this background was subtracted form the P_0 and P_{1+2} signals. The degree of phosphorylation was then calculated as the ratio between P_{1+2} and P_0, which was set to 100% for the control condition.

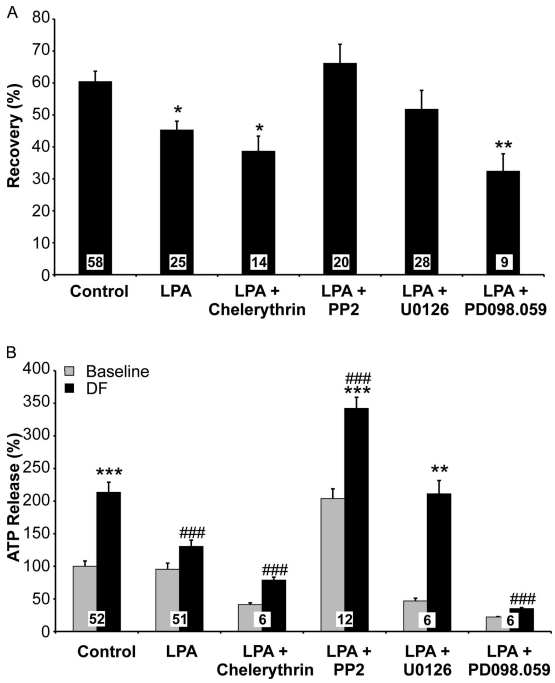

Figure 4. Effects of LPA treatment in C6-Cx43. (A) GJ coupling (FRAP) was significantly inhibited by LPA, and this was reversed by PP2 and U0126 but not by chelerythrin or PD098.059 (n is number of FRAP experiments on 5 different cultures for control and 2 different cultures for all other conditions). (B) Triggered ATP release was significantly inhibited by LPA, an effect that was reversed by PP2 and U0126, but not by chelerythrin and PD098.059. Stars indicate significant differences compared with control in A and compared with baseline in B. The number signs in B indicate significant differences compared with control.

Data Analysis and Statistics

The data are expressed as mean ± SEM with n (indicated on the bar graphs) denoting the number of experiments. Comparison of two groups was done with a one-tailed unpaired t test with a p value below 0.05 indicating significance. Comparison of more than two groups was done with one-way analysis of variance (ANOVA) and a Bonferroni posttest. Statistical significance is indicated in the graphs with a single symbol (* or #) for $p < 0.05$, two symbols for $p < 0.01$, and three symbols for $p < 0.001$. Some substances or treatments also influenced the baseline signal, but these effects most often did not reach

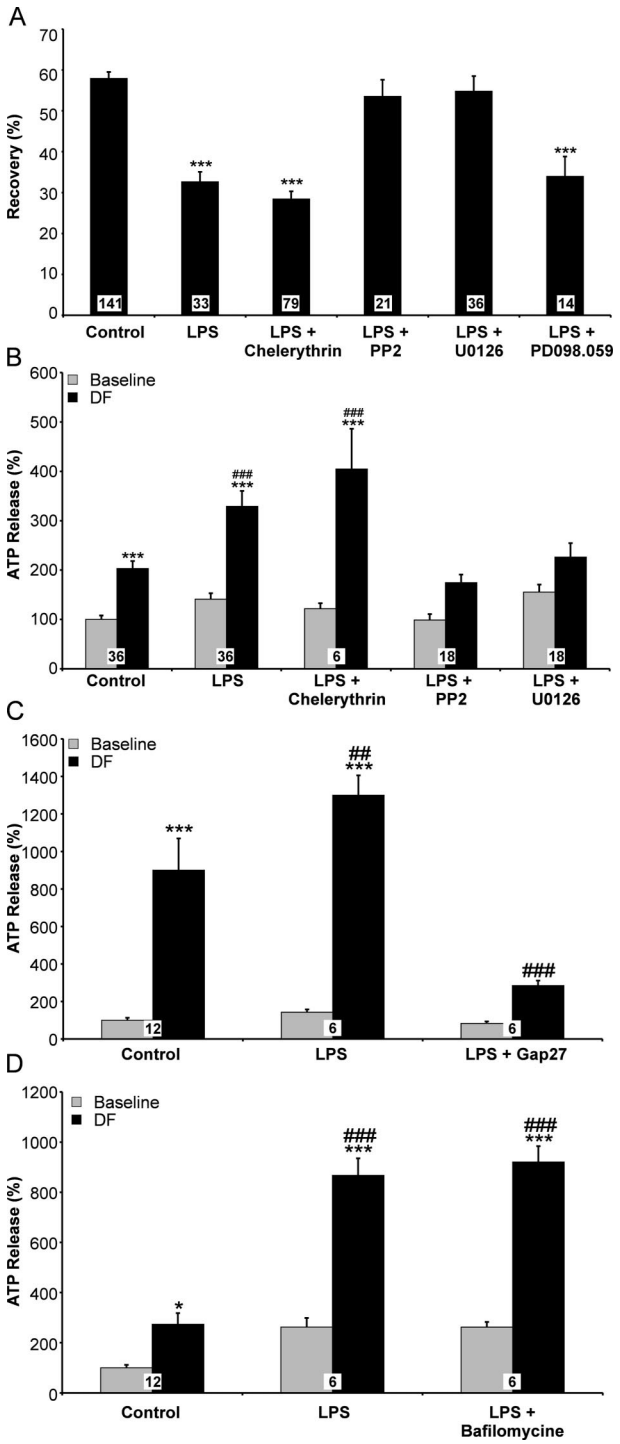

Figure 5. Effects of LPS treatment in C6-Cx43. (A) GJ coupling (FRAP) was inhibited by LPS, and this was reversed by PP2 and U0126, not by chelerythrin or PD098.059 (n is number of FRAP experiments on 5 different cultures for control and 2 different cultures for all other conditions). (B) Triggered ATP release was significantly enhanced by LPS, an effect that was reversed by PP2 and U0126, but not by chelerythrin. (C) LPS enhancement of ATP release was strongly inhibited by gap 27, to an extent comparable to that in Figure 1A. (D) Bafilomycin A1 had no effect. Stars indicate significant differences compared with control in A and compared with baseline in B–D; number signs indicate significant differences compared with control. LPS also significantly stimulated baseline ATP release ($p < 0.001$ in B, <0.05 in C, and <0.001 in D; *t* test).

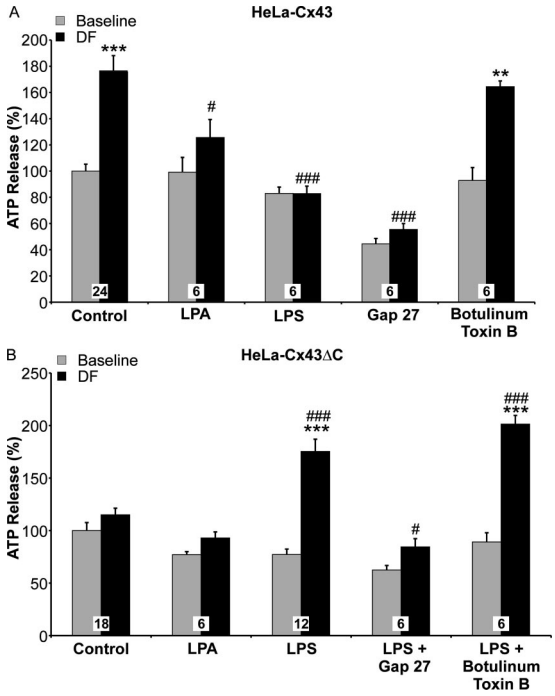

Figure 6. Effect of CT-truncation on LPA and LPS modulation of ATP release in HeLa-Cx43. (A) Experiments on HeLa-Cx43 illustrating inhibition of triggered ATP release by LPA and LPS. Gap 27 blocked the triggered ATP release, whereas botulinum toxin B had no effect (gap 27 also inhibited baseline ATP release, as noted in Figure 1A, $p < 0.0001$). (B) Experiments on CT-truncated HeLa-Cx43 illustrating absence of triggered ATP release in these cells, no effect of LPA, but significant stimulation of triggered ATP release by LPS. Gap 27 blocked the LPS-enhanced ATP response, but botulinum toxin B had no effect, analogous to the experiments on C6-Cx43 illustrated in Figure 5, C and D. Stars indicate significant differences compared with baseline, whereas the number signs indicate significant differences compared with control.

significancy with ANOVA. Selected comparisons for baseline effects were redone with a *t* test, and significant differences, if relevant, are given in the figure legend.

RESULTS

ATP Release Triggered in Cx43-transfected Cells by Divalent-free Extracellular Conditions Is Related to Hemichannel Opening

Incubating C6 glioma cells stably transfected with Cx43 (C6-Cx43) (Zhu *et al*., 1991) in divalent cation-free (DF) solutions triggered ATP release that was significantly above baseline (Figure 1A), as reported previously (Arcuino *et al*., 2002; Stout and Charles, 2003; Ye *et al*., 2003). DF-triggered ATP release in C6-Cx43 was significantly suppressed by the GJ blocker carbenoxolone (25 μM; 15 min), the connexin mimetic peptide gap 26 (amino acids 64–76 on the first extracellular loop of Cx43), and gap 27 (amino acids 201–211 on the second extracellular loop) (Figure 1A). These peptides specifically block Cx43 hemichannels and not GJ channels when applied 30 min at 0.25 mg/ml (Braet *et al*., 2003a,b; Leybaert *et al*., 2003; Evans *et al*., 2006). Gap 26 and 27 also significantly reduced baseline ATP release (Figure 1A), indicating the presence of open hemichannels without DF stimulation. Botulinum toxin B, a blocker of vesicular release that cleaves synaptobrevin (1.5 nM; 24 h) (Schiavo *et al*., 1992) or bafilomycin A1 (100 μM; 1 h), a v-ATPase inhibitor known to inhibit ATP storage (Coco *et al*.,

2003), did not diminish triggered ATP release (Figure 1B). Activation of the P_2X_7 receptors with subsequent opening of a large ATP-permeable pore is another pathway for ATP release (Chessell *et al*., 1997; Duan *et al*., 2003), but Western blots did not indicate P_2X_7 receptor expression in C6-Cx43 (Figure 10A), and exposure to the P_2X_7 receptor pore inhibitors Ox-ATP (100 μM; 2 h) and KN62 (1 μM; 1 h) did not significantly affect DF-triggered ATP release in C6-Cx43 cells (data not shown). DF conditions also triggered ATP release in HeLa cells stably transfected with Cx43 (HeLa-Cx43) (Omori and Yamasaki, 1999), and this response was, like in C6-Cx43, blocked by gap 27 and not influenced by botulinum toxin B (Figure 6A); HeLa-Cx43 cells, like C6-Cx43, did not express P_2X_7 receptors (Figure 10A).

CT Truncation of Cx43 Does Not Affect GJ Communication but Abolishes ATP Responses in HeLa-Cx43

The CT of Cx43 is known to contain multiple consensus phosphorylation sites for PKC, Src, and MAPK (Warn-Cramer and Lau, 2004). To determine the gross effect of phosphorylations at these sites, we performed experiments on HeLa-Cx43 with the CT-truncated at position 239 (HeLa-Cx43ΔC) (Omori and Yamasaki, 1999). HeLa-Cx43 cells were well dye-coupled in FRAP experiments, and the level of coupling was not different in HeLa-Cx43ΔC (Figure 2, A–C). By contrast, DF-triggered ATP release was reduced to baseline level in HeLa-Cx43ΔC, and the baseline level itself was also significantly lower as in HeLa-Cx43 (Figure 2D). Subsequent experiments on these cells confirmed the absence of triggered ATP release in HeLa-Cx43ΔC (Figures 6B, 7D, and 9E), but in one series (Figure 9B) a small significant release component was apparent. In sum, removal of the Cx43 CT inhibits hemichannel opening and does not affect GJs. The intact GJ communication as shown in our experiments and by Omori and Yamasaki (1999) indicates the presence of Cx43ΔC at the plasma membrane and the formation of functional GJs.

Activation of PKC and c-Src Reduces GJ Communication and ATP Responses in C6-Cx43

PMA (10 nM; 2 h), a known PKC activator (Lampe, 1994; Malfait *et al*., 2001), significantly reduced dye coupling (FRAP) in C6-Cx43 (Figure 3, A and B). Washing out PMA during 30 min or coincubating the cells with the PKC inhibitor chelerythrin (250 nM; 30-min preincubation followed by 2-h coincubation with PMA) (Herbert *et al*., 1990) abolished the PMA effect. PMA also significantly reduced DF-triggered ATP release, and this was reversed by chelerythrin (Figure 3C). The effect of c-Src was investigated by transient transfection of C6-Cx43 with a vector containing constitutively active or inactive c-Src. Mock transfection or transfection with the inactive c-Src mutant (c-SrcK^-; K295M) had no significant effect on GJ communication, but transfection with the constitutively active mutant (c-SrcK^+; Y527A) significantly inhibited dye transfer between the cells (SLDT) (Figure 3, D and E). Triggered ATP release was affected in a similar manner, with a significantly decreased ATP response in c-SrcK^+ (Figure 3F). Figure 10B illustrates Cx43 phosphorylation in response to PMA and in c-SrcK^+.

LPA Inhibits GJ Coupling and ATP Responses in C6-Cx43, whereas LPS Inhibits Coupling but Stimulates ATP Release

LPA activates various kinases, including PKC (Takeda *et al*., 1999; Kelley *et al*., 2006), Src kinase (Takeda *et al*., 1998; Kranenburg and Moolenaar, 2001), and the MAPK family (Takeda *et al*., 1999; Kranenburg and Moolenaar, 2001). In C6-Cx43, LPA (10 μM; 1 h) significantly inhibited GJ cou-

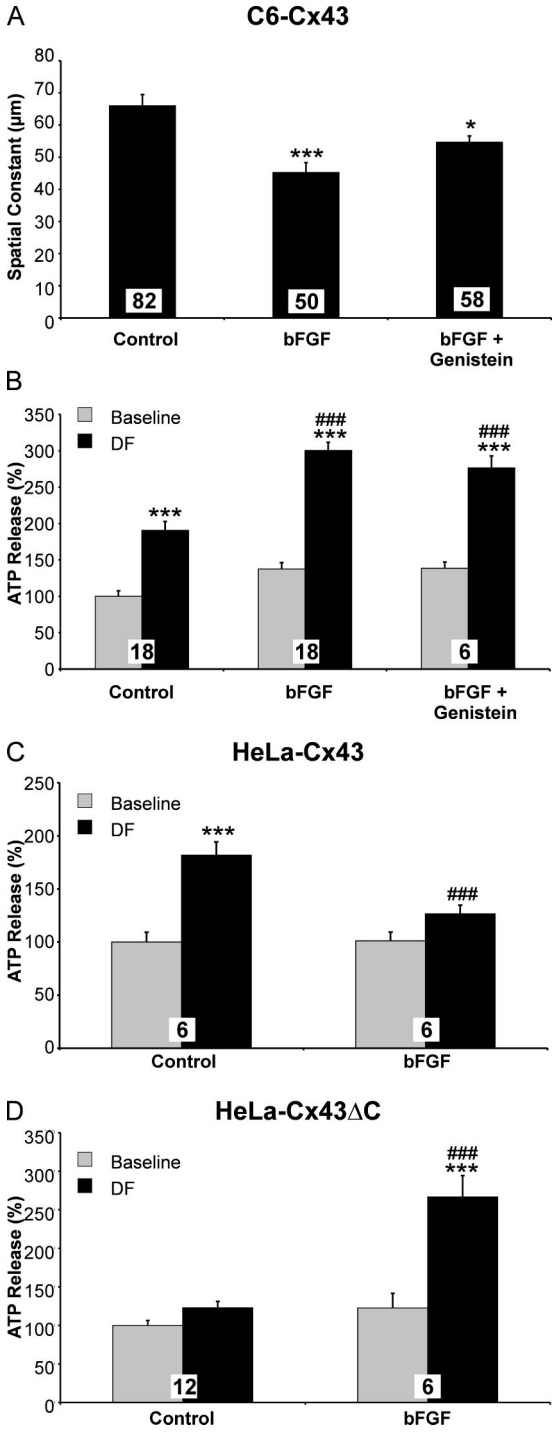

Figure 7. Effects of bFGF are analogous to the effects observed with LPS. (A) bFGF significantly inhibited GJ coupling (SLDT) in C6-Cx43, and this effect was partly reversed by genistein (n is number of SLDT images in 4 different cultures). (B) bFGF significantly stimulated the triggered ATP release in C6-Cx43, but this could not be reversed by genistein. (C) bFGF significantly inhibited the triggered ATP release in HeLa-Cx43. (D) bFGF significantly stimulated the triggered ATP release in CT-truncated HeLa-Cx43. Stars indicate significant differences compared with control in A and compared with baseline in B–D. The number signs in B–D indicate significant differences compared with control.

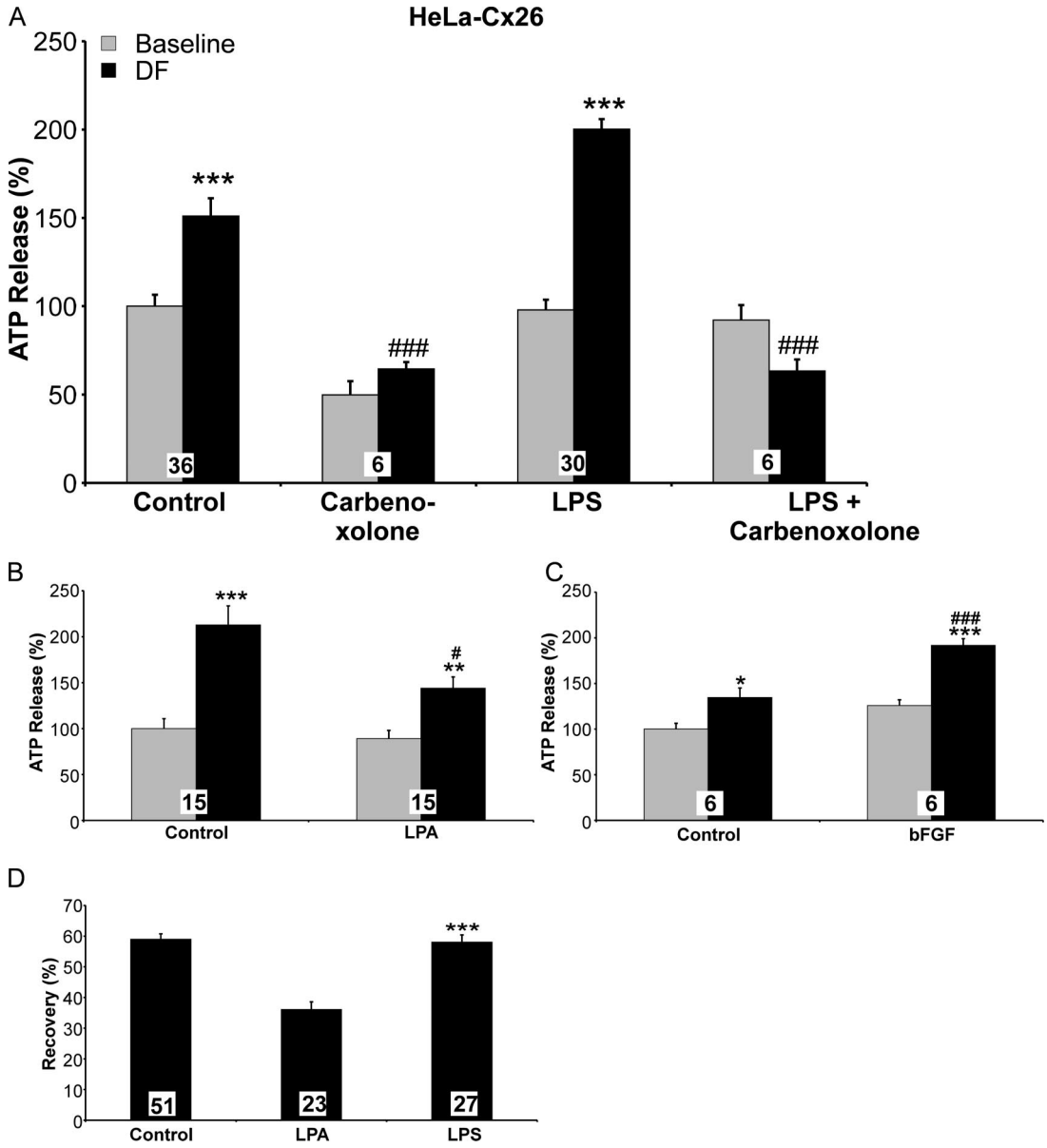

Figure 8. Modulation of ATP release and GJ coupling by LPS, LPA, and bFGF in HeLa-Cx26 cells. (A) DF conditions triggered significant ATP release that was stimulated by LPS and inhibited by carbenoxolone. (B) LPA inhibited triggered ATP release C. bFGF slightly but significantly stimulated ATP release. (D) Effect of LPA and LPS on GJ coupling in HeLa-Cx26 cells as measured via FRAP, using calcein-AM as a gap junctional-permeable dye (n is number of FRAP experiments in 2 different cultures). Stars indicate significant differences compared with baseline in A–C and compared with control in D; number signs indicate significant differences compared with control. Carbenoxolone also significantly depressed the baseline signal in A ($p < 0.05$).

pling (FRAP) and DF-triggered ATP release (Figure 4). Both LPA effects were abolished with the Src inhibitor PP2 (10 μM; 15-min preincubation followed by 1-h coincubation with LPA) or with the MEK1/2 inhibitor U0126 (20 μM; 1-h preincubation followed by 1-h coincubation with LPA) (Figure 4). The MEK1/2 inhibitor PD098.059 (50 μM; same protocol as for U0126) had no significant effect, nor did the PKC inhibitor chelerythrin (Figure 4).

LPS is another broad-spectrum kinase activator, which binds to the Toll-like receptor and the coreceptor CD14 (Ulevitch, 1993) and thereby activates PKC, c-Src, and the MAPK signaling pathway (Lidington *et al*., 2000; Lidington *et al*., 2002; Schorey and Cooper, 2003). LPS (100 ng/ml; 1 h) inhibited dye coupling (like LPA), but (unlike LPA) stimulated DF-triggered ATP release (Figure 5, A and B). Both LPS effects were reversed by PP2 and U0126 but not by chelerythrine or PD098.059. Gap 27 blocked the LPS-enhanced ATP release (Figure 5C), to a similar extent as observed without LPS stimulation (Figure 1A), whereas bafilomycin A1 had no effect on LPS-enhanced DF-triggered ATP release (Figure 5D). LPS can up-regulate P_2X_7 receptor expression in macrophages (Le Feuvre *et al*., 2002), but Western blot experiments did not show any evidence for such effect in C6-Cx43 (Figure 10A). Together, LPA and LPS have opposing effects on ATP release and similar effects on GJ coupling. These influences involve c-Src and MEK1/2 and are exerted at the level of hemichannels. Figure 10B illustrates Cx43 phosphorylation in response to LPA and LPS.

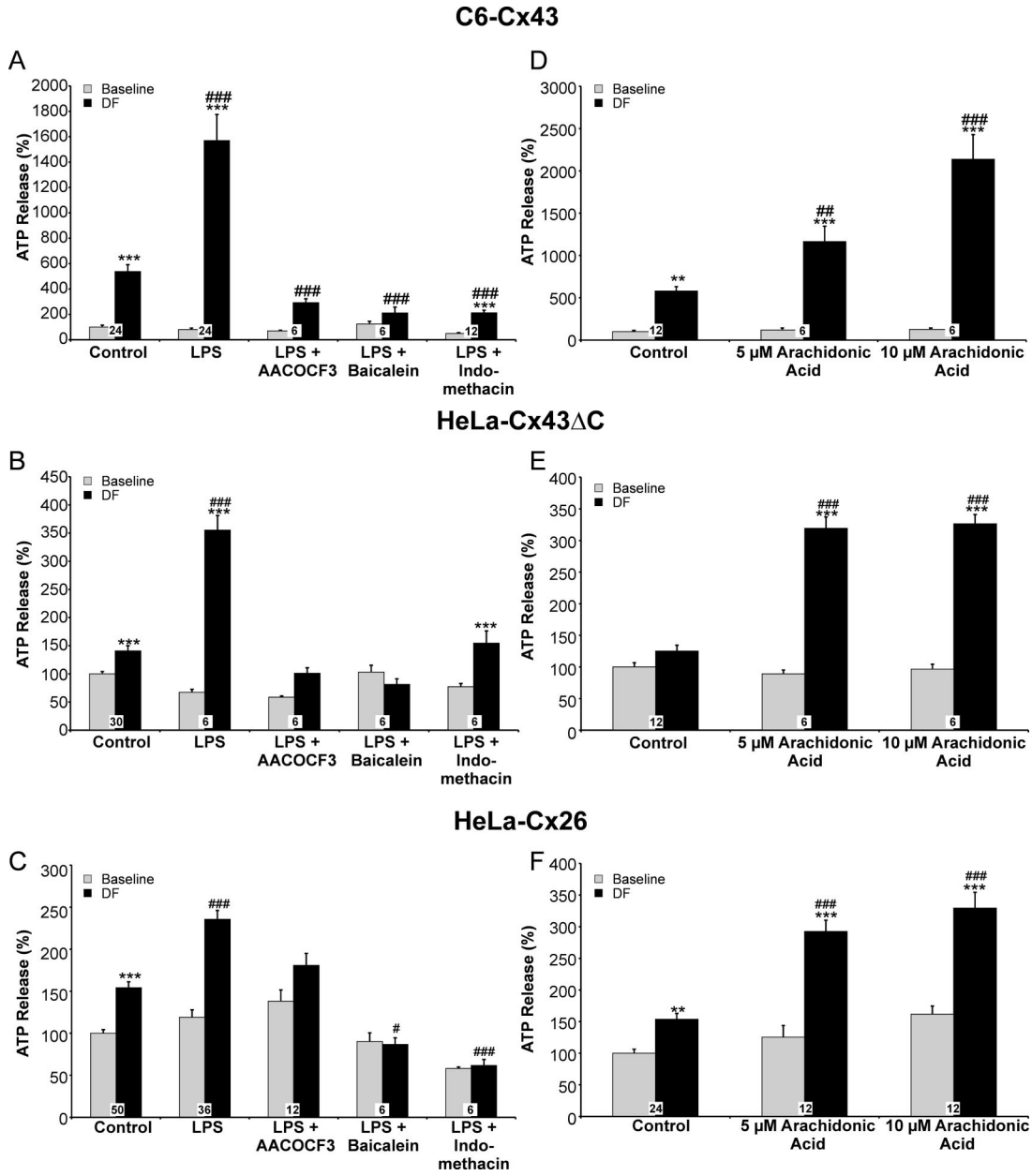

Figure 9. Arachidonic acid metabolism and LPS enhancement of ATP release. (A) Inhibition of arachidonic acid production with AACOCF3, of lipo-oxygenase with baicalein, or cyclo-oxygenase with indomethacin all drastically suppressed LPS enhancement of triggered ATP release in C6-Cx43. Similar results were observed in HeLa-Cx43Δ C (B) and HeLa-Cx26 (C). (D–F) Arachidonic acid potentiated DF-triggered ATP release in C6-Cx43, HeLa-Cx43Δ C, and HeLa-Cx26. Stars indicate significant differences compared with baseline; number signs indicate significant differences compared with control.

LPA and LPS Inhibit ATP Responses in HeLa-Cx43, but LPS Potentiates These Responses in CT-truncated HeLa-Cx43

To determine whether the opposing effects of LPA and LPS on ATP release were perhaps specific for C6-Cx43, we repeated these experiments in HeLa-Cx43. LPA and LPS inhibited DF-triggered ATP release in HeLa-Cx43 (Figure 6A) in contrast to C6-Cx43 where LPA inhibited and LPS potentiated these responses. No effect of LPA was found in HeLa-Cx43ΔC, but quite remarkably, LPS restored the DF-triggered ATP response in these cells (while giving inhibition in nontruncated HeLa-Cx43) (Figure 6). LPS-enhanced ATP release in HeLa-Cx43ΔC was blocked by gap 27 and not affected by botulinum toxin B (Figure 6B). The potentiation of ATP release by LPS is thus, as in C6-Cx43, the consequence of hemichannel stimulation.

bFGF Has Similar Effects as LPS

Basic FGF is another broad-spectrum kinase activator (Shiokawa-Sawada *et al*., 1997; Mergler *et al*., 2003); this substance (10 ng/ml in serum-free medium for 6 h) significantly inhibited dye coupling (SLDT) in C6-Cx43, like LPS and LPA, an effect that was partly reversed by the tyrosine kinase blocker genistein (Figure 7A). The bFGF effects on triggered ATP release were similar to those of LPS, that is, stimulation of ATP release in C6-Cx43 and HeLa-Cx43ΔC and inhibition in HeLa-Cx43 (Figure 7,

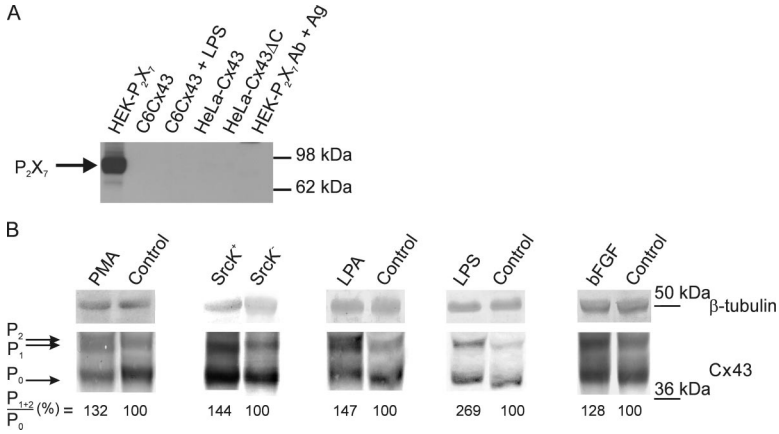

Figure 10. (A) Western blots illustrating the absence of P_2X_7 receptor expression in the various cell lines and conditions used in this study. (B) The influence of different kinases/kinase activators on the phosphorylation status of Cx43 in C6-Cx43 cells. The ratio of nonphosphorylated versus phosphorylated Cx43 is given under each lane, and the ratio for control conditions is converted to 100%. The data are representative for three different experiments. The effect on the phosphorylation status can also be appreciated from the blots shown in Figure 11 (the effect of bFGF is more clear there).

B–D). Stimulation of ATP release by bFGF in C6-Cx43 was not reduced by genistein, indicating involvement of other pathways. Figure 10B illustrates Cx43 phosphorylation after bFGF exposure.

bFGF and LPS Potentiate ATP Release Triggered in HeLa-Cx26

We next performed experiments on HeLa cells expressing Cx26, which is a connexin with a very short CT domain of only 16 amino acids, and the only connexin known not to be phosphorylated. GJ communication was inhibited by LPA and not affected by LPS in HeLa-Cx26 (Figure 8D). DF conditions triggered ATP release significantly above baseline, and this was potentiated by LPS and bFGF (Figure 8, A and C), as observed in HeLa-Cx43ΔC. No connexin mimetic peptides are currently available to block Cx26 hemichannels, so we applied carbenoxolone, which inhibited the triggered ATP release, both in control and after potentiation with LPS (Figure 8A). LPA inhibited the DF-triggered ATP release (Figure 8B). LPS and bFGF thus potentiate ATP release via hemichannels in C6-Cx43, CT-truncated HeLa-Cx43, and the short CT connexin in HeLa-Cx26.

LPS Potentiation of ATP Responses Involves Arachidonic Acid Signaling

The kinases activated by LPS, Src, and MEK1/2 can lead to phosphorylation and activation of cytosolic phospholipase A_2 ($cPLA_2$) and calcium-independent phospholipase A_2 ($iPLA_2$) (Luo *et al*., 2005) with subsequent production of arachidonic acid, a proposed candidate for hemichannel activation (Contreras *et al*., 2002). We investigated the involvement of arachidonic acid and its downstream products with the $cPLA_2$ inhibitor AACOCF3 (5 μg/ml; 1 h) (Riendeau *et al*., 1994; Chakraborti *et al*., 2004), the lipoxygenase inhibitor baicalein (30 μM; 1 h) (Vivancos and Moreno, 2002), and the cyclooxygenase inhibitor indomethacin (50 μM; 1 h) (Fujiwara *et al*., 2006). All these substances significantly suppressed LPS potentiation of ATP release in C6-Cx43, HeLa-Cx43ΔC, and HeLa-Cx26 (Figure 9, A–C). In addition, exposure to arachidonic acid significantly enhanced ATP release in these cell lines (Figure 9, D–F).

Influence of LPA, LPS, and bFGF on the Cellular Distribution of Connexins

We used Triton X-100 extraction to separate soluble and insoluble connexin fractions. The insoluble fraction most likely represents connexins in GJs and not in hemichannels, because Triton X-100 insolubility is only observed after assembly into GJ plaques (Musil and Goodenough, 1991, 1993; Govindarajan *et al*., 2002). Treatment with LPA, LPS and bFGF did not induce systematic and consistent changes in the distribution of soluble and insoluble fractions in the various cell types used (Figure 11).

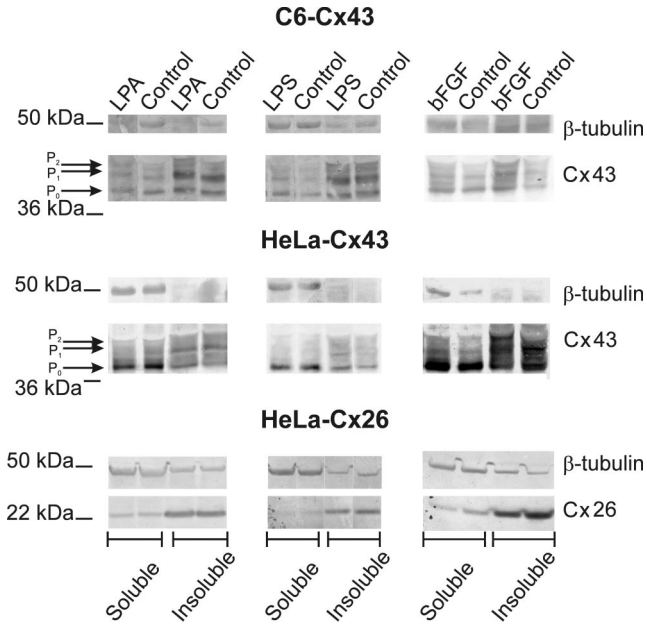

Figure 11. Western blots illustrating the separation of soluble and insoluble connexin fractions in C6-Cx43, HeLa-Cx43, and HeLa-Cx26 by using Triton X-100 extraction. In HeLa-Cx43, bFGF slightly increased the soluble fraction (at the cost of the insoluble fraction), and a similar tendency was observed for LPS in C6-Cx43. No gross and consistent shifts were however observed over three different experiments.

DISCUSSION

Exposure to DF conditions is a well-known trigger for hemichannel opening, and ATP release provoked in this way proceeded through hemichannels, because it was strongly inhibited by the hemichannel blockers gap 26 and 27 (Braet *et al*., 2003a,b; Leybaert *et al*., 2003; Gomes *et al*., 2005; Pearson *et al*., 2005; Evans *et al*., 2006). A substantial contribution of other ATP release mechanisms is unlikely

because of the potency of the suppressive effect of the mimetic peptides, the absence of inhibition by the vesicular release inhibitors botulinum toxin B (which unexpectedly potentiated the release for unknown reasons) and bafilomycin A1, the lack of P_2X_7 receptor expression, and the absence of effects of P_2X_7 receptor antagonists.

The truncation of the CT tail of Cx43 at position 239 did not influence GJ coupling, indicating that the CT is not essential for the assembly and membrane insertion of GJ channels. This confirms previous work on the same cell line (Omori and Yamasaki, 1999) and is in line with observations on CT truncations at slightly different positions: position 244 (Fishman *et al*., 1991) or 257 (Liu *et al*., 1993; Hur *et al*., 2003). PKC activation depressed GJ coupling and ATP release via hemichannels in C6-Cx43, in line with other studies reporting inhibition of dye uptake (Li *et al*., 1996) and NAD^+ release (Bruzzone *et al*., 2001a) via hemichannels. Recent work shows that this occurs via phosphorylations on Ser 262 and Ser 368 of Cx43 (Bao *et al*., 2004c). c-Src kinase inhibits GJs via phosphorylations at Tyr 265 and Tyr 247 (Goldberg and Lau, 1993; Kanemitsu *et al*., 1997; Giepmans *et al*., 2001; Lin *et al*., 2001), and hemichannel inhibition has also been reported (Li *et al*., 1996). In line with this, v-Src inhibited both GJs and hemichannels in C6-Cx43. The phospholipid mediator LPA activates various protein kinases, including PKC, Src, and the MAPK family (Takeda *et al*., 1998, 1999; van Leeuwen *et al*., 2003; Kelley *et al*., 2006). LPA inhibited GJ channels and hemichannels, and experiments with the inhibitors PP2 and U0126 indicated involvement of Src and MEK1/2, respectively (absence of effect of the MEK1/2 inhibitor PD098.059 is probably related to its poor solubility; Davies *et al*., 2000; Ahn *et al*., 2001). MAPKs are known to inhibit GJs (Kim *et al*., 1999), and our work indicates that MEK1/2 (an MAPK family member) has a similar action on hemichannels.

LPS, a glycolipid immunostimulant from Gram-negative bacteria, is another activator of PKC, c-Src, and the MAPKs (Lidington *et al*., 2000, 2002; Schorey and Cooper, 2003). LPS may also stimulate NO production, but this needs exposures longer than the 1 h used here (Shin *et al*., 2001). LPS reduced GJ coupling but stimulated triggered ATP release in C6-Cx43, and these opposite actions involved Src and MEK1/2 (reversed by PP2 and U0126, respectively). An enhancement of ATP release may result from the stimulation of hemichannels or the recruitment of other release mechanisms. LPS-enhanced ATP release was suppressed by gap 27, to an equal and drastic extent as observed under control conditions. The strong inhibition by the mimetic peptides together with the lack of any effect of bafilomycin A1 or botulinum toxin B, and the absence of P_2X_7 receptor up-regulation in response to LPS, indicate that the enhancement of triggered ATP release is most likely due to stimulation of hemichannels.

LPS inhibited ATP release via hemichannels in HeLa-Cx43, and surprisingly, inhibition was turned into stimulation in CT-truncated HeLa-Cx43. bFGF, acting on membrane-bound tyrosine kinase receptors (Shiokawa-Sawada *et al*., 1997), had similar effects, i.e., depression of GJ coupling in C6-Cx43, stimulation of ATP release in C6-Cx43 and CT-truncated HeLa-Cx43, and inhibition of ATP release in HeLa-Cx43. LPS and bFGF also potentiated ATP release in HeLa-Cx26, a connexin with a very short CT domain. In sum, the CT domain seems to be necessary for hemichannel opening, whereas its absence is required for hemichannel stimulation by LPS or bFGF in HeLa cells.

The different effects of LPS and bFGF on different connexins implicate the involvement of other signaling pathways.

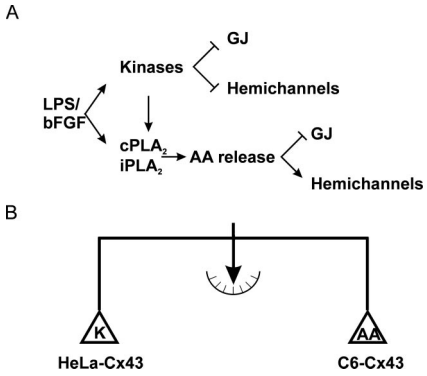

Figure 12. Scheme summarizing the major findings of this study. (A) LPS and bFGF activate various kinases that suppress GJs and hemichannel-mediated ATP release. In addition, both substances also trigger the production of arachidonic acid (AA) via activation of $cPLA_2$ and $iPLA_2$. Arachidonic acid is known to inhibit GJs, and the present study demonstrates that it stimulates hemichannel-mediated ATP release. (B) We conclude that the net effect of LPS and bFGF depends on the balance between activation of kinases, inhibition of hemichannels, and activation of the arachidonic acid metabolic pathway, stimulating hemichannel-mediated ATP release. In HeLa-Cx43 cells the kinase component predominates, whereas in C6-Cx43 it is the arachidonic acid pathway that adds most weight. Truncation of the CT in HeLa-Cx43 removes inhibition by kinases and thereby reveals the arachidonic acid component.

LPS and bFGF can, either directly or indirectly, via intermediate kinases (Luo *et al*., 2005), lead to activation of $cPLA_2$ and/or $iPLA_2$ (Vivancos and Moreno, 2002; Antoniotti *et al*., 2003) with subsequent production of arachidonic acid (Figure 12A). Treatment with arachidonic acid indeed mimicked the effects of LPS and bFGF in C6-Cx43, HeLa-Cx43ΔC, and HeLa-Cx26, and inhibitors of arachidonic acid production or metabolism furthermore reduced the potentiating effect of LPS. Stimulation of hemichannels by LPS and bFGF is thus related to the activation of the arachidonic-acid signaling pathway. In C6-Cx43, this pathway is presumably more active and overrides the inhibition of hemichannels by phosphorylations at the CT. At the level of GJs, the two pathways lead to inhibition, as reported here for LPS and by others for arachidonic acid (Giaume *et al*., 1989; Criswell and Loch-Caruso, 1995; Velasco *et al*., 2000). The soluble and insoluble fractions were not grossly or systematically affected by LPS or bFGF treatments. Hemichannel modulation by LPS and bFGF is therefore likely to be mediated by effects at the level of hemichannel functioning (such as its gating) rather than by introducing shifts in the distribution of GJ channels and hemichannels.

Our work demonstrates that GJ channels and hemichannels, although composed of the same connexins, can be differentially modulated. The factors determining the hemichannel responses are related to the applied stimulus, the cell type, and the CT domain containing the phosphorylation consensus sites, and they depend on the balance between connexin phosphorylations and activation of the arachidonic acid pathway (Figure 12B).

The role of oppositely directed responses of GJs and hemichannels is not known, but they may serve cell-protective and restorative purposes. GJs close under pathological conditions, for example, in response to bFGF released after brain trauma and ischemia (Logan *et al*., 1992) or in response to LPS present during bacterial infection (Campos de Carvalho *et al*., 1998), and this may help to prevent the spread of cell death-promoting factors to neighboring cells (Krysko

et al., 2005). In this case, paracrine ATP signaling via hemichannels may be solicited to compensate for the lost GJ communication. Moreover, ATP release via hemichannels may stimulate the recovery of injured tissues (e.g., brain and liver) by its mitogenic actions (Thevananther *et al*., 2004; Pearson *et al*., 2005) and its vasodilatory and neuroprotective degradation product adenosine (Erlinge, 1998; Burnstock, 2002; Stone, 2002). Hemichannels can, however, also be considered as pathogenic pores promoting cell death (Evans *et al*., 2006), and closure of both GJs and hemichannels may thus be preferred when cell protection is the primary target. We conclude that the immunostimulant LPS and the growth factor bFGF exert a powerful control over hemichannel ATP release, with inhibition or stimulation being determined by the cell type, the intracellular signaling machinery, and the connexin type present. Given the pleiotropic effects of extracellular ATP, these results suggest that the final effect of LPS and bFGF may, in addition to the various intracellular cascades activated by these agents, also depend on hemichannel modulation.

ACKNOWLEDGMENTS

We thank Dirk De Gruytere, Cyriel Mabilde, and Eric Tack for excellent technical support and expertise. In addition, we thank Drs. B. N. Giepmans and W. H. Moolenaar for providing the pcDNA Src kinase constructs and Drs. G. Tran Van Nhieu and M. Mesnil for the HeLa-Cx26 cells. We appreciate the helpful discussions with Dr. B. Vanheel. This work was supported by the Fund for Scientific Research Flanders, Belgium (FWO Grants 3G023599, 3G001201, G0335.03, and G.0354.07 to L.L.), the Belgian Society for Scientific Research in Multiple Sclerosis (WOMS Grant 51F06700 to L.L.), Ghent University (BOF Grants 01115099, 01107101, and 01113403 to L.L.), and the Queen Elisabeth Medical Foundation Grant 365B5602 (to L.L.).

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