Intracellular calcium changes trigger connexin 32 hemichannel opening

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Connexin hemichannels have been proposed as a diffusion pathway for the release of extracellular messengers like ATP and others, based on connexin expression models and inhibition by gap junction blockers. Hemichannels are opened by various experimental stimuli, but the physiological intracellular triggers are currently not known. We investigated the hypothesis that an increase of cytoplasmic calcium concentration ($[Ca^{2+}]_i$) triggers hemichannel opening, making use of peptides that are identical to a short amino-acid sequence on the connexin subunit to specifically block hemichannels, but not gap junction channels. Our work performed on connexin 32 (Cx32)-expressing cells showed that an increase in $[Ca^{2+}]_i$ triggers ATP release and dye uptake that is dependent on Cx32 expression, blocked by Cx32 (but not Cx43) mimetic peptides and a calmodulin antagonist, and critically dependent on $[Ca^{2+}]_i$ elevation within a window situated around 500 nM. Our results indicate that $[Ca^{2+}]_i$ elevation triggers hemichannel opening, and suggest that these channels are under physiological control.

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Introduction

The basic fuel molecule of the cell, ATP, has gained a lot of interest over the last decade as a paracrine messenger in various cell and tissue types (Novak, 2003). Besides being a remarkably versatile molecule, nonexcitable cells can be invoked to release ATP by widely differing stimuli, including mechanical cell stimulation (Stout *et al*, 2002), shear stress

(Cherian *et al*, 2005), hypotonic cell swelling (Boudreau and Grygorczyk, 2004), elevation of intracellular inositol trisphosphate ($InsP_3$) (Braet *et al*, 2003b) or exposure to low or zero extracellular Ca^{2+} conditions (Arcuino *et al*, 2002). The ATP release mechanisms involved appear to be equally diverse (reviewed in Lazarowski *et al*, 2003), including vesicular release, active transport via ABC transporters and diffusion via stretch-activated channels, voltage-dependent anion channels, pores opened by P_2X_7 receptors or connexin hemichannels. Connexin hemichannels are hexameric high-conductance plasma membrane channels (single-channel conductance ~ 90 and 220 pS for Cx32 and 43, respectively—Contreras *et al*, 2003; Gomez-Hernandez *et al*, 2003) that are normally closed and can act as a conduit for ATP, NAD^+ , glutamate and prostaglandins when opened (Bruzzone *et al*, 2001; Bennett *et al*, 2003; Ebihara, 2003; Goodenough and Paul, 2003; Ye *et al*, 2003; Cherian *et al*, 2005). Hemichannels are closed at normal millimolar extracellular $[Ca^{2+}]$, but open when Ca^{2+} is lowered (Li *et al*, 1996; Pfahnl and Dahl, 1999; Quist *et al*, 2000; Muller *et al*, 2002; Ye *et al*, 2003; Thimm *et al*, 2005). A Ca^{2+} -binding site composed of aspartate residues facing the external side has been reported for Cx32 hemichannels and is thought to translate changes of extracellular $[Ca^{2+}]$ to changes in channel gating (Gomez-Hernandez *et al*, 2003). Hemichannels also open in response to membrane depolarization and mechanical stimulation in a *Xenopus* oocyte expression system (Trexler *et al*, 1996; Bao *et al*, 2004), under conditions of metabolic inhibition in astrocytes, myocardial cells or renal epithelial cells (John *et al*, 1999; Kondo *et al*, 2000; Contreras *et al*, 2002; Vergara *et al*, 2003), after *Shigella* invasion of epithelial cells (Tran Van Nhieu *et al*, 2003) and in response to extracellular UTP in C6 glioma cells expressing Cx32 or 43 (Cotrina *et al*, 1998). The physiological intracellular signals controlling hemichannel opening are currently not known, but UTP-triggered ATP release via hemichannels was dependent on intracellular Ca^{2+} mobilization (Cotrina *et al*, 1998) and we demonstrated that photoactivation of $InsP_3$ in Cx43-expressing cells triggers Ca^{2+} -dependent ATP release that is blocked by gap junction blockers and peptides that mimic a short exposed sequence on the Cx43 subunit (Braet *et al*, 2003a,b), indicating that $InsP_3$ and downstream signals activate hemichannel opening (Leybaert *et al*, 2003). Recent work from the group of Mobbs and co-workers (Pearson *et al*, 2005) also points to intracellular Ca^{2+} changes triggering Cx43 hemichannel opening in native retinal pigment epithelium. The aim of the present work was to determine whether increases of cytoplasmic calcium concentration ($[Ca^{2+}]_i$) are sufficient to trigger hemichannel opening as probed with connexin mimetic peptides and connexin expression systems. Our results obtained in Cx32-expressing cells demonstrate that direct elevation of $[Ca^{2+}]_i$ by photoactivation of Ca^{2+} in the cytoplasm or stimulation of Ca^{2+} entry with a Ca^{2+} ionophore triggers ATP release and hemichannel-permeable dye uptake that was dependent on Cx32 expression and

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completely blocked by Cx32 mimetic peptides and calmodulin inhibition. Pores activated by P_2X_7 receptor activation were excluded and vesicular ATP release contributed to a limited extent, but hemichannel-mediated release was by far the predominant component. Our work shows that hemichannels can be activated by physiological $[Ca^{2+}]_i$, opening up a wide range of future investigations on hemichannel involvement in both physiology and pathophysiology.

Results

Divalent-free (DF) solutions trigger ATP release that is dependent on $[Ca^{2+}]_i$ in ECV304

Exposure of cells to solutions with lowered or zero Ca^{2+} and Mg^{2+} triggers ATP and glutamate release and has been applied in various cell types to investigate connexin hemichannel opening (Arcuino *et al*, 2002; Stout *et al*, 2002; Ye *et al*, 2003). We used ECV304 cells (bladder cancer epithelial cells) from which a subclone with prominent and spontaneous Cx32 expression (see Figure 3) was selected to study the role of $[Ca^{2+}]_i$ changes in triggering ATP release via hemichannels. Incubating ECV304 cells in zero Ca^{2+} and Mg^{2+} solutions (divalent-free [DF] solutions, applied 2.5 min) triggered ATP release that was significantly above baseline (Figure 1A) and was associated with transient and oscillatory $[Ca^{2+}]_i$ changes as observed in fluo-3 imaging experiments (Figure 1B). The ATP response to DF conditions varied between experiments and ranged from approximately 175 to 500% of baseline. Buffering $[Ca^{2+}]_i$ with BAPTA (5 μ M BAPTA-AM, 45 min loading time) or blocking the intracellular Ca^{2+} store SERCA pumps with thapsigargin (1 μ M, 10 min) significantly reduced the ATP response (Figure 1A). Blocking $InsP_3$ receptors with xestospongine-C (2–10 μ M, 10 min), ryanodine (Ry) receptors with dantrolene (25 μ M, 10 min) or pre-emptying Ry-sensitive Ca^{2+} stores with caffeine (10 mM, 30 min) all significantly inhibited DF-triggered ATP release (Figure 1C). $InsP_3$ - and Ry-sensitive Ca^{2+} stores are thus involved in the ATP response, and, in line with this, photoactivation of $InsP_3$ in the cytoplasm (Braet *et al*, 2003b) and exposure to the alkaloid Ry (10 μ M, 15 min) or caffeine (10 mM, 15 min) triggered significant ATP release (175 $\pm$ 38% for Ry and 400 $\pm$ 81% for caffeine, compared to 100 $\pm$ 12% baseline level, n = 15, P < 0.05 and < 0.0005, respectively).

Directly increasing $[Ca^{2+}]_i$ triggers ATP release in ECV304

Photoactivation of Ca^{2+} from ester-loaded NP-EGTA (5 μ M *o*-nitrophenyl EGTA acetoxymethyl ester (NP-EGTA-AM), 10 min; Ellis-Davies and Kaplan, 1994) triggered significant ATP release (Figure 1D). However, prolonged ester loading (1 h), applied to increase the amount of photoliberated Ca^{2+} , did not trigger significant ATP release. Stimulating Ca^{2+} entry with the Ca^{2+} ionophore A23187 (1 μ M, 4 min) triggered significant ATP release, but its application at higher concentrations (10 μ M) or longer incubations (10 min) failed to do so (Figure 1D). $[Ca^{2+}]_i$ changes thus trigger ATP release, but the response disappears with stronger stimulation.

DF- and Ca^{2+} -triggered ATP release are inhibited by gap junction blockers and a Cx32 mimetic peptide in ECV304

DF-triggered ATP release was significantly inhibited by the gap junction blockers 18 α -glycyrrhetinic acid (α -GA, 50 μ M,

Figure 1 Role of cytoplasmic Ca^{2+} in DF-triggered ATP release in ECV304 cells. (A) DF exposure triggered ATP accumulation in the medium that was assayed after the stimulation interval given in the text. Baseline and triggered ATP release were measured in different experimental groups. Buffering $[Ca^{2+}]_i$ with BAPTA or emptying the Ca^{2+} stores with thapsigargin reduced DF-triggered ATP release. Inhibitor concentrations and incubation times are given in the text; inhibitors were absent during stimulation. (B) $[Ca^{2+}]_i$ dynamics in response to DF exposure, demonstrating transient, steady and oscillatory changes. $[Ca^{2+}]_i$ changes are given as increases in fluo-3 fluorescence (ΔF , arbitrary units). (C) Xestospongine reduced DF-triggered ATP release at 2 μ M and completely blocked it at 10 μ M; dantrolene and pre-emptying Ca^{2+} stores with caffeine also reduced the response. (D) Photoactivation of Ca^{2+} from NP-EGTA or stimulation of Ca^{2+} entry with A23187 triggered ATP release. Prolonging NP-EGTA ester loading to 1 h or increasing the A23187 concentration or exposure time did not trigger ATP responses. *Significantly above the corresponding baseline; #significantly below the control response; a single symbol indicates P < 0.05, two symbols P < 0.01 and three symbols P < 0.001; numbers in the bars represent n .

30 min) and carbenoxolone (100 μ M, 30 min) (Figure 2A). In line with the connexin expression pattern of ECV304 (Figure 3), 32 gap 24 (0.25 mg/l, 30 min), a 13-amino-acid peptide mimicking a sequence on the intracellular loop of Cx32, completely blocked the ATP response, while 43 gap 27 (0.25 mg/l, 30 min), an 11-amino-acid peptide that mimicks a sequence on the second extracellular loop of Cx43, only showed a small, although significant, inhibition (Figure 2B). This last effect is probably related to a low Cx43 background expression (not detected on Western blots), because 43 gap 27 was previously found to have no effect on

Figure 2 Gap junction blockers and Cx32 mimetic peptides inhibit ATP release in ECV304. (A) α-GA and carbenoxolone completely blocked DF-triggered ATP release. (B) The Cx32 mimetic ³²gap 24 completely abolished DF-triggered ATP release, while the Cx43 mimetic ⁴³gap 27 only weakly inhibited it, in line with the connexin expression pattern in these cells (Figure 3). (C, D) Carbenoxolone and ³²gap 24 blocked ATP release triggered by Ca²⁺ photoactivation and 1 μM A23187. *Significantly above the corresponding baseline; #significantly below the corresponding control bar (baseline or trigger).

Cx32 hemichannels (Braet *et al*, 2003b). Carbenoxolone and ³²gap 24 also significantly inhibited ATP release triggered by Ca²⁺ photoactivation (Figure 2C) and A23187 (Figure 2D). In

Figure 3 Western blots for connexins and P₂X₇ receptors. (A, B) The ECV304 cells used showed clear Cx32 expression and no discernable expression of Cx43. (C) P₂X₇ receptor expression was absent in ECV304 and C6-Cx32. Ag + Ab = P₂X₇ antibody plus the corresponding antigenic peptide.

some of the experiments, inhibitor substances caused significant inhibition of the baseline signal (e.g. Figure 2A, B and D), indicating involvement of hemichannels under basal conditions. In all these cases, the net (trigger minus baseline) and relative (trigger over baseline ratio) responses were significantly below control ($P < 0.05$) except for the carbenoxolone experiment of Figure 2A, where the relative response appeared to be increased (an atypical response not observed in other experiments).

DF- and Ca²⁺-triggered ATP release and dye uptake are inhibited by Cx32 mimetic peptides and a calmodulin antagonist in C6-Cx32

The experiments on ECV304 cells strongly indicate a role for [Ca²⁺]_i in triggering ATP release via Cx32 hemichannels. We switched to another model system making use of C6 glioma cells to further establish hemichannel involvement based on both comparisons between wild-type (WT) and Cx32-expressing C6 (Bond *et al*, 1994) and connexin mimetic peptides as specific hemichannel blockers. DF conditions and A23187 (2 μM, 5 min) triggered significant ATP release in C6-Cx32 that was blocked with carbenoxolone (not shown) and ³²gap 24 (Figures 4A and 5A). ³²Gap 24 had no effect on DF-triggered ATP release in C6-Cx43 cells (data not shown). ³²Gap 27, a peptide that mimicks a sequence on the second extracellular loop of Cx32, blocked DF- and A23187-triggered ATP release to a similar extent as ³²gap 24 (Figures 4A and 5A). The two Cx32 mimetics (0.25 mg/l, 30 min) had no effect on gap junctional coupling as investigated with scrape loading and dye transfer (data not shown), similar to our observations with ⁴³gap 27 in Cx43-expressing cells (Braet *et al*, 2003a). Longer exposures to ⁴³gap 27 do however inhibit coupling (Braet *et al*, 2003a, b), presumably by preventing the formation of new gap junction channels or by the longer time needed to reach target interaction sites which are less accessible in the gap junction channel configuration. The Cx43 mimetic ⁴³gap 27 did not influence DF- or A23187-triggered ATP release in C6-Cx32 (data not shown). Both DF- and A23187-triggered ATP release were not significantly

Figure 4 DF-triggered ATP release in C6-Cx32. (A) 32 Gap 24 and 32 gap 27 drastically inhibited DF-triggered ATP release in C6-Cx32. DF-triggered ATP release was significantly lower in C6-WT as compared to C6-Cx32 (inset). (B, C) Bafilomycin A1 (Bafilo) had no effect, while botulinum toxin B (Botul) slightly but significantly inhibited ATP release. (D) Calmodulin inhibition with W7 drastically blocked ATP release. The P_2X_7 receptor antagonist oxidized ATP (Ox-ATP) had no effect, but KN62 displayed significant inhibition. *Significantly above the corresponding baseline; #significantly below control.

Figure 5 A23187-triggered ATP release in C6-Cx32. (A) 32 Gap 24 and 32 gap 27 blocked the ATP response. Bafilomycin A1 (Bafilo) had no effect, while botulinum toxin B (Botul) showed slight but nonsignificant inhibition (triggered ATP release was however not significantly above baseline). The ATP responses were completely blocked by adding 32 gap 24 together with either of the toxins, and was absent in C6-WT (inset). (B) W7 completely blocked the responses, but Ox-ATP and KN62 had no significant effects. (C, D) Recovery of ATP responses following washout (WO) of 32 gap 24 and W7, respectively. The effect of various washout periods was assessed by experiments on different cultures. *Significantly above the corresponding baseline; #significantly below the corresponding control bar.

above baseline in C6-WT and were significantly lower as compared to C6-Cx32 (insets to Figures 4A and 5A). In Figure 4A, DF exposure appears to trigger some ATP release in C6-WT (nonsignificant with ANOVA, but significantly above baseline with a *t*-test ($P < 0.05$)), which may be related to a low connexin background expression or the operation of other DF-responsive ATP release mechanisms. Cx32 contains two cytoplasmic calmodulin-binding domains (Torok *et al*, 1997) that may be involved in the Ca^{2+} -triggered ATP responses. The calmodulin antagonist W7 (20 μM , 1 h) was as potent as the Cx32 mimetics in blocking DF- and A23187-triggered ATP release (Figures 4D and 5B). The inhibitory effect of the peptides and W7 was reversible upon washout of

these substances: inhibition by W7 completely disappeared after 30 min washout (Figure 5D), inhibition by ^{32}gap 27 within 60 min (data not shown), and inhibition by ^{32}gap 24 took almost 120 min to disappear upon washout (Figure 5C).

DF conditions and A23187 (2 μM , trigger solutions applied for 5 min) triggered significant uptake of the hemichannel-permeable reporter dye propidium iodide (PI, MW 668.4 Da), but not of hemichannel-impermeable dyes (see Materials and methods) in C6-Cx32. Dye uptake was significantly lower in C6-WT as compared to C6-Cx32 with both DF and A23187 triggers (inset to Figure 6B and D). DF- and A23187-triggered dye uptake in C6-Cx32 were blocked by carbenoxolone, ^{32}gap 24 and W7 (Figure 6B and D).

Figure 6 Dye uptake in C6-Cx32 and HEK293-P₂X₇. (A) Example images illustrating baseline and DF-triggered dye uptake in C6-Cx32 cells. The white calibration bar measures 20 μm . (B) Summary data obtained in C6-Cx32. DF-triggered PI uptake was blocked by carbenoxolone, ^{32}gap 24 and W7, and was significantly lower in C6-WT (inset). The ordinate expresses the percentage of PI-positive cells relative to the total number of cells in the field. (C) Example images of baseline and A23187-triggered dye uptake in C6-Cx32 cells. (D) A23187-triggered PI uptake in C6-Cx32 was inhibited by carbenoxolone, ^{32}gap 24 and W7, and absent in C6-WT (inset). (E) Dye uptake in HEK293-P₂X₇ in response to various trigger conditions. DF and A23187 (2 μM) did not stimulate significant dye uptake, while benzoyl-ATP (Bz-ATP, 2 μM , 30 min) was an effective stimulus. ^{32}Gap 24 did not significantly inhibit the Bz-ATP-triggered response. *Significantly above baseline; [#]significantly below control.

P₂X₇ receptors are not involved in ATP release

Activation of P₂X₇ receptors also triggers a pore-like ATP release pathway (Duan *et al*, 2003) that should be distinguished from hemichannels (Papura *et al*, 2004). ECV304 and C6-Cx32 cells did not express P₂X₇ protein (Figure 3C). Oxidized ATP (100 μ M, 1 h), an irreversible P₂X₇ receptor antagonist that also blocks pore-forming P₂X₂ receptors (North, 2002), did not inhibit DF- and A23187-triggered ATP release in C6-Cx32 (Figures 4D and 5B). KN62, another P₂X₇ antagonist (North, 2002), significantly inhibited DF-triggered ATP release (Figure 4D), but was without effect on ATP release triggered by A23187 (Figure 5B). Experiments on HEK293-P₂X₇ cells showed that DF conditions did not trigger significant ATP release (not shown) or PI uptake (Figure 6E). KN62 inhibition of DF-triggered ATP release is thus not related to P₂X₇ receptor antagonism, but is probably the result of inhibition of calmodulin-dependent kinases acting on the connexins (Hidaka and Yokokura, 1996). HEK293-P₂X₇ cells did neither show PI uptake in response to A23187; benzoyl-ATP triggered significant PI uptake in these cells, but this response was not significantly affected by ³²gap 24 (Figure 6E).

Vesicular release slightly contributes to Ca²⁺-triggered ATP release in C6-Cx32

Botulinum toxin B, a protease that cleaves the v-SNARE protein synaptobrevin (Schiavo *et al*, 1992) (1.5 nM, applied 24 h in the culture medium), significantly reduced DF-triggered ATP release in C6-Cx32, but the v-ATPase inhibitor bafilomycin A1 (100 μ M, 1 h), known to inhibit ATP storage (Coco *et al*, 2003), had no effect (Figure 4B and C). Both toxins displayed partial but nonsignificant inhibition of A23187-triggered ATP release in C6-Cx32 (Figure 5A). Addition of ³²gap 24 together with botulinum toxin B or bafilomycin A1 (the last 30 min) depressed the ATP response to below baseline levels, indicating predominance of the release component inhibited by the Cx32 mimetics (Figure 5A).

Relation between [Ca²⁺]_i and ATP release or dye uptake in C6-Cx32

We tested various A23187 concentrations to construct a dose-response curve for Ca²⁺-triggered ATP release. These experiments confirmed the observations in ECV304 cells (Figure 2) and demonstrated a response curve with activation of ATP release within a very narrow range of A23187 concentrations (only 1.5 and 2 μ M were effective—Figure 7A); a similar sharp response pattern was found for A23187-triggered dye uptake (Figure 7B). Expression of ATP release or dye uptake as a function of the [Ca²⁺]_i (measured with fura-2) attained with various A23187 concentrations (Figure 7C and D) demonstrated a bell-shaped response curve with maximal response at ~500 nM (Figure 7E and F).

We further investigated the relation between cytoplasmic Ca²⁺ changes and dye uptake by simultaneous imaging of [Ca²⁺]_i and the PI fluorescence in individual cells. Under baseline conditions, sparse PI-positive cells could be distinguished ($\times 40$ objective). Closer inspection of these cells demonstrated that the dye was located at small discrete spots most often at the periphery of the cell (Figure 8A–F). This staining pattern is different from the nuclear and cytoplasmic staining shown in Figure 6A and C, because the PI

concentration was 100 times lower and the exposure time shorter in these experiments. Resting [Ca²⁺]_i in baseline PI-positive cells averaged 96.5 ± 12.4 nM ($n = 20$), which is slightly but significantly ($P < 0.02$) above the 62.5 ± 8.7 nM ($n = 20$) in PI-negative cells in the same cultures or 61.0 ± 13.7 nM from the experiments used for Figure 7D. Upon exposure of the cultures to 2 μ M A23187, additional cells with PI-positive spots appeared (Figure 8I and J). The intensity of these PI spots increased with time and [Ca²⁺]_i (Figure 8K), and attained half-maximal intensity at 463 ± 102 nM [Ca²⁺]_i (12 cells from 12 experiments), which is in the range of the ~340 nM half-maximum concentration for activation of the ATP and dye uptake responses derived from the graphs in Figure 7E and F. The peak [Ca²⁺]_i increase triggered by 2 μ M A23187 was slightly but not significantly higher in cells that subsequently became PI-positive as compared to cells that remained PI-negative (622 ± 107 versus 515 ± 100 nM, respectively; $n = 12$). In another series of experiments, [Ca²⁺]_i changes were triggered with 4 μ M A23187 (increasing [Ca²⁺]_i to 3300 ± 530 nM; $n = 21$) and PI was introduced here just after the peak (Figure 8L). A limited number of experiments (three cells from three experiments) showed the appearance of PI-positivities during [Ca²⁺]_i recovery in previously PI-negative cells, indicative of hemichannel opening when [Ca²⁺]_i decreases, and falls into the concentration range of the right flank of the dose-response curves of Figure 7E and F.

Discussion

Exposure to lowered extracellular divalent ion conditions is a well-known procedure to potentiate or trigger the opening of hemichannels (Stout *et al*, 2002; Ye *et al*, 2003). In Cx32-expressing ECV304 cells, DF-triggered ATP release was reduced by buffering [Ca²⁺]_i and inhibiting the SERCA pump, InsP₃ receptors or Ry receptors. Directly increasing [Ca²⁺]_i by stimulating Ca²⁺ entry with A23187 or photoliberating Ca²⁺ in the cytoplasm also triggered ATP release. A23187 gave the largest responses and was therefore used in the remainder of the study. A23187 also triggered PI reporter dye uptake (but not of larger reporter molecules), indicating activation of a bidirectionally permeable pathway. ATP release triggered by A23187 or Ca²⁺ photoactivation and dye uptake triggered with A23187 were all blocked (inhibited to the baseline or below) by gap junction blockers and the Cx32 mimetics ³²gap 24 and ³²gap 27, as was DF-triggered ATP release or dye uptake. Similar drastic blocking effects were previously reported for Cx43 mimetics (⁴³gap 26 and ⁴³gap 27) on DF-, InsP₃- and mechanical stimulation-triggered ATP release in Cx43-expressing cells (Braet *et al*, 2003a,b; Gomes *et al*, 2005). ³²Gap 24 is a tridecapeptide composed of residue numbers 110–122 of Cx32. Unlike ³²gap 27 that mimicks a sequence on the second extracellular Cx32 loop, the ³²gap 24 sequence is located on the intracellular Cx32 loop. Peptides mimicking a sequence on the intracellular loop of Cx43 have previously been demonstrated to influence the gating of gap junction channels (residue numbers 119–144; Seki *et al*, 2004), but ³²gap 24 was not found to have any effect on dye coupling between the cells in the present work. The details of peptide–connexin interactions leading to hemichannel block are currently unknown, but the effects of ³²gap 24 are connexin-specific as this peptide did not influence

Figure 7 Dose-response curve for A23187-triggered ATP release in C6-Cx32. (A) A23187 only triggered significant ATP responses at 1.5 and 2 μM , while responses were absent at lower or higher concentrations. (B) Dose-response curve for Ca^{2+} -triggered dye uptake, illustrating the same narrow concentration dependence as observed for ATP release. *Significantly above the corresponding baseline. (C) Time course of $[\text{Ca}^{2+}]_i$ responses to increasing A23187 concentrations as determined in Ca^{2+} imaging experiments. (D) Average peak $[\text{Ca}^{2+}]_i$ response to various A23187 concentrations ($n=4$). (E) ATP release as a function of $[\text{Ca}^{2+}]_i$ (graph constructed from data presented in (A) and (D)). (F) Dye uptake as a function of $[\text{Ca}^{2+}]_i$ (constructed from the data shown in (B) and (D)).

DF-triggered ATP release in C6-Cx43 cells and ^{43}gap 27 had no effect in C6-Cx32- and other Cx32-expressing cells (Braet *et al*, 2003a). If ^{32}gap 24 interacts with the intracellular loop, then either this loop must be accessible from the outside via the pore or the peptide must get access to the cytoplasm (^{32}gap 24 does not, however, contain any currently known cell-penetrating peptide sequence—sequences reviewed in Zorko and Langel, 2005). The fact that washout of the ^{32}gap 24 effect took twice as long as for ^{32}gap 27 favors the hypothesis of interaction with an intracellular target.

The v-ATPase inhibitor bafilomycin A1, reported to inhibit vesicular ATP storage and Ca^{2+} -dependent ATP release in astrocytes (Coco *et al*, 2003), had no significant effect on DF- and A23187-triggered ATP release in C6-Cx32. Botulinum toxin B selectively cleaves the v-SNARE synaptobrevin (Schiavo *et al*, 1992) and similar toxins acting at the same target have been demonstrated to inhibit osmotic swelling-induced ATP release in epithelial cells (van der Wijk *et al*, 2003). The toxin inhibited DF-triggered ATP release and also slightly (but nonsignificantly) affected A23187-triggered ATP

release. ATP release in C6-Cx32 thus appears to involve a small vesicular component. Exposing the cells to either of the toxins combined with a Cx32 mimetic peptide completely suppressed A23187-triggered ATP release. The inhibition by the peptide, added alone or in combination, was so nearly complete that the pathway blocked must be the predominant ATP release pathway or the one located most upstream in a cascade of ATP release signals or events.

An alternative ATP release pathway involves P_2X_7 receptors (Duan *et al*, 2003) that, upon exposure to ATP in the 100 μM concentration range applied over several minutes, opens a pore that is permeable to below 1 kDa substances (North, 2002). ATP released by the cell, by whatever pathway, may thus activate these receptors and amplify the ATP responses. DF conditions potentiate P_2X_7 pore opening (North, 2002), but these conditions are by themselves not sufficient to initiate pore opening, as illustrated in the experiments now reported with HEK293- P_2X_7 cells. The absence of P_2X_7 receptor expression in ECV304 and C6-Cx32, together with the evidence obtained with P_2X_7 receptor antagonists,

Figure 8 Simultaneous imaging of $[Ca^{2+}]_i$ and dye uptake in C6-Cx32. (A–F) Examples illustrating resting $[Ca^{2+}]_i$ in baseline PI-positive cells. (A, D) Fura-2-loaded cells at 380 nm excitation. (B, E) Corresponding $[Ca^{2+}]_i$ maps indicating 153 nM $[Ca^{2+}]_i$ in (B) and 54 nM in (E). The PI spots are indicated with small circles on the fura-2 and $[Ca^{2+}]_i$ images. (D–F) Two PI spots in the same cell. (G–J) Image series illustrating PI spots appearing in response to A23187. (G) Fura-2 image at 380 nm excitation. (H) Time series of $[Ca^{2+}]_i$ maps (times as indicated in (J)). The first image is before and subsequent images after exposure to 2 μ M A23187. (I) 380 nm image with indication of the PI spot (circle) observed in the next sequence. (J) Time series of PI images acquired simultaneously with the $[Ca^{2+}]_i$ maps. A small spot at the periphery of a cell becomes PI positive when switching to 2 μ M A23187. The position of the PI spot is also indicated in (H). The intensity of the PI spot increased with time and $[Ca^{2+}]_i$. The white calibration bars measure 20 μ m. (K) Time course of $[Ca^{2+}]_i$ and PI intensity (open circles) in the point indicated in (H) and (I). The $[Ca^{2+}]_i$ increase was associated with an increasing intensity of the PI spot (expressed in arbitrary units (AU)). Half-maximal intensity was reached at $\sim$ 800 nM in this experiment. (L) Experiment where PI was introduced after induction of a large $[Ca^{2+}]_i$ increase with 4 μ M A23187. In this case, a PI spot appeared during the $[Ca^{2+}]_i$ recovery phase.

the ineffectiveness of DF conditions as well as A23187 to trigger dye uptake in HEK293-P₂X₇ and the absence of any effect of Cx32 mimetics on dye uptake triggered in these cells by benzoyl-ATP, excludes involvement of P₂X₇ receptors. Faria *et al* (2005) recently reported that A23187 triggers opening of a P₂X₇-related pore, presumably a pore activated by maitotoxin, that was furthermore inhibited by W7 (Faria *et al*, 2005). The identity of the maitotoxin receptor is currently not known, but the pores activated by this toxin are blocked by DF conditions rather than opened (Lundy *et al*, 2004) and are virtually inactive at room temperature (Schilling *et al*, 1999).

The present studies combine to show that increasing $[Ca^{2+}]_i$ triggers hemichannel-permeable dye uptake and ATP release that is inhibited by gap junction blockers and Cx32 mimetics, and is absent in cells not expressing connexins. Vesicular ATP release contributes to a limited extent to the responses, but the release pathway blocked by the peptides is the most prominent or upstream one. The magnitude of the Ca^{2+} stimulus is critical in order to trigger hemichannel opening: both small and large stimuli were ineffective and only $[Ca^{2+}]_i$ changes in the range of above 200 nM and below

1000 nM were successful. $[Ca^{2+}]_i$ changes passing through the optimum concentration range towards a higher peak level are ineffective, presumably because the time spent within the trigger window is too short. The narrow bell-shaped response curve probably explains some controversy in the literature whether hemichannel-mediated ATP release is or is not dependent on $[Ca^{2+}]_i$. Our work also indicates that baseline hemichannel activity allows PI to enter the cell, while having only small (although significant) effects on the resting $[Ca^{2+}]_i$. In line with this, A23187-triggered $[Ca^{2+}]_i$ changes were slightly (but not significantly) larger in cells that experienced PI uptake and hemichannel opening as compared to those that remained PI-negative. Presumably, PI influx through hemichannels is quite limited in the conditions used for these experiments and only stains RNA in close proximity to the channel. Ca^{2+} influx through hemichannels is probably equally limited and/or effectively removed by Ca^{2+} pumps or buffers.

The finding that $[Ca^{2+}]_i$ increases trigger hemichannel opening is not in contradiction with the widespread notion that $[Ca^{2+}]_i$ elevation closes gap junction channels. Recent work from the group of Li and co-workers (Dakin *et al*, 2005)

has elegantly demonstrated that only capacitative Ca^{2+} entry via store-operated channels is effective in blocking gap junctional communication, while Ca^{2+} ionophores were without effect. How $[\text{Ca}^{2+}]_i$ changes are linked to hemichannel opening is currently unknown. Direct interactions of Ca^{2+} at the cytoplasmic side of the connexin subunit are unlikely (Peracchia, 2004). Cx32 has two calmodulin interaction sites, one in the N-terminal tail and the other close to the C-terminal tail (Torok *et al*, 1997), while Cx43 has only one on its N-terminal (reviewed in Peracchia, 2004). The calmodulin inhibitor W7 blocked Ca^{2+} -triggered ATP release and dye uptake as efficiently as the Cx32 mimetic, and the $[\text{Ca}^{2+}]_i$ successfully activating ATP release (500 nM) were in the K_d range for Ca^{2+} -calmodulin interactions (500 nM–5 μM) (Chin and Means, 2000), strongly pointing to the involvement of calmodulin in the signaling cascade. Calmodulin may act either directly on calmodulin interaction sites on the connexin subunit, or indirectly via calmodulin-dependent kinases. Further work is under way to characterize the Ca^{2+} dependency of Cx43 hemichannels, which contain a single calmodulin interaction site. Preliminary work shows that the Ca^{2+} dependency is much more smeared out over a much broader range of Ca^{2+} concentrations.

Materials and methods

Cell cultures

We used ECV304 (bladder cancer epithelial cells—ECACC, Salisbury, UK), C6 glioma wild type (C6-WT), C6 stably transfected with Cx32 (C6-Cx32) or 43 (C6-Cx43) (Zhu *et al*, 1991; Bond *et al*, 1994) and HEK293 cells stably transfected with P_2X_7 receptors (HEK293- P_2X_7) (Humphreys *et al*, 1998). ECV304 was maintained in Medium-199 (Gibco, Merelbeke, Belgium), C6 in DMEM-Ham's F12 (1:1) and HEK293- P_2X_7 in DMEM, all supplemented with 10% fetal bovine serum and 2 mM glutamine. Cells were seeded at a density of 25 000 or 50 000 cells/cm² (specified further) on either glass bottom microwells (MatTek Corporation, Ashwood, MA), Nunclon four-well plates (NUNC Brand Products, Denmark) or 24-well plates (Falcon3047, Becton Dickinson, Erembodegem, Belgium) and used for experiments the next day (nonconfluent cultures). The experiments were carried out in Hanks' balanced salt solution buffered with 25 mM HEPES (HBSS-HEPES, pH 7.4).

Agents

Fluo-3 acetoxymethyl ester (fluo-3-AM), fura-2-AM, NP-EGTA-AM, ethylenedioxymethyl(o-phenylenetriol)tetraacetic acid acetoxymethyl ester (BAPTA-AM), 4-bromo-A23187 (A23187), 6-carboxyfluorescein (6-CF), dextran fluorescein conjugate (MW 10 kDa) and PI were obtained from Molecular Probes (Leiden, The Netherlands). Thapsigargin, W7, KN62, oxidized ATP, 2'-3'-O-(4-benzoylbenzoyl) ATP (benzoyl-ATP), bafilomycin A1, botulinum toxin B, carbenoxolone and α -GA were from Sigma (Bornem, Belgium), dantrolene sodium salt and xestospongine-C from Calbiochem (Darmstadt, Germany) and ryanodine and caffeine from Alomone Labs (Jerusalem, Israel). The connexin mimetic peptides ³²gap 24 (GHGDPLHLEEVKC, intracellular loop, position 110–122), ³²gap 27 (SRPTEKTVFT, extracellular loop 2, position 182–191) and ⁴³gap 27 (SRPTEKTIFII, extracellular loop 2, position 201–210) were synthesized by solid-phase chemistry and purified by HPLC to 95% purity. Monoclonal mouse anti-rat Cx43 antibody was obtained from Transduction Laboratories (Becton Dickinson, Erembodegem, Belgium; 1/500), polyclonal rabbit anti-rat Cx32 antibody from Sigma (Bornem, Belgium; 1/1000) and polyclonal rabbit anti-rat P_2X_7 antibody plus the corresponding antigenic peptide (residue 576–595) from Alomone Labs (Jerusalem, Israel; 1/1000).

Extracellular ATP measurements

Cellular ATP release was determined with a luciferin/luciferase assay kit (product no. FL-AA, Sigma, Bornem, Belgium) and was measured either in a sample collected from the medium bathing the

cells (sample procedure) or directly in the medium above the cells (plate reader procedure). In the sample procedure, 100 μl of 200 μl bathing medium was transferred to 100 μl ATP assay mix solution used at five-fold dilution, and the photon flux was counted with a photomultiplier luminometer (type 9924B, Thorn-Emi Electron Tubes, Middlesex, UK; 10 s counting time). In the plate reader procedure, 75 μl ATP assay mix prepared in HBSS-HEPES (at five-fold dilution) was added to 150 μl medium above the cells and photon flux was counted (Victor-3, type 1420 multilabel counter, Perkin-Elmer, Brussels, Belgium). ATP release was triggered with a DF HBSS-HEPES (Ca^{2+} and Mg^{2+} replaced with 4 mM EGTA), by photoactivation of Ca^{2+} inside the cells (described below) or by applying A23187 or other agonists mentioned in the text. Standard cell seeding density was 25 000 cells/cm², but 50 000 cells/cm² for DF stimulation and simultaneous Ca^{2+} /PI imaging. Cellular ATP release was accumulated over the period of trigger exposure specified in the text; for Ca^{2+} photoactivation, a 2.5-min collection period was included after the short photo-stimulus. Baseline measurements were carried out on separate cultures according to the same procedure, but with standard HBSS-HEPES vehicle instead. The ATP assay was calibrated in the range of 5–100 pmol, with baseline corresponding to 23.8 ± 1.88 pmol ($n = 152$) in ECV304 and 10.1 ± 1.63 pmol ($n = 155$) in C6-Cx32. The DF stimulus increased ATP release to $351 \pm 17.9\%$ ($n = 122$), while 2 μM A23187 increased it to $230 \pm 12.4\%$ ($n = 186$) in C6-Cx32. Taking into account the number of cells that display dye uptake (see further) and 100×10^{-15} mol intracellular ATP contents per cell, both stimuli were calculated to trigger the release of approximately 2% of the cellular ATP contents (in line with previous estimates—Braet *et al*, 2004). All pharmacological or inhibitory 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 stimulation. The same protocol applies for dye uptake experiments described further.

Photoactivation of Ca^{2+}

Ester loading with NP-EGTA was carried out with 5 μM NP-EGTA-AM in 1 ml HBSS-HEPES for the times indicated, followed by 30 min de-esterification, all performed at room temperature. UV field illumination during 2 s was used to photoliberate Ca^{2+} in a large zone of NP-EGTA-loaded cells on glass bottom microwells, as described in detail in Braet *et al* (2004). Baseline measurements were carried out in cultures that received the UV light, but were not loaded with NP-EGTA.

Ca^{2+} imaging

Cell cultures were loaded with fluo-3 or fura-2 by ester loading for 1 h as described for NP-EGTA-AM. Imaging was performed on an inverted epifluorescence microscope (Nikon Eclipse TE 300, Analis, Ghent, Belgium) with an $\times 40$ oil immersion objective and a filter switch (Cairn, Kent, UK) providing 490 nm excitation for fluo-3 and excitation alternating between 340 and 380 nm for fura-2 (each applied over 1 s, resulting in one Ca^{2+} image every 2 s). Fluo-3 measurements were carried out with a standard FITC dichroic mirror and emission filter; for fura-2 the dichroic was a 430 nm long-pass with emission bandpass filtering at 510 nm (40 nm bandwidth). Images were acquired with an intensified CCD (Extended Isis camera, Photonic Science, East Sussex, UK) and stored in a PC equipped with a frame grabber (Data Translation, DT 3152, Marlboro, MA). Ratio images were generated with software written in Microsoft Visual C++ 6.0, after standard background and shade correction procedures. Fura-2 *in situ* calibrations were carried out with Ca^{2+} -free and fura-2 saturating solutions containing 10 μM A23187; a K_d of 224 nM was used to convert ratios to Ca^{2+} concentrations.

Simultaneous Ca^{2+} and PI imaging was performed with triple excitation wavelength switching (340, 380 and 560 nm, each applied over 1 s, resulting in a Ca^{2+} /PI image pair every 3 s) in combination with a triple band dichroic mirror and emission bandpass filter (XF2050 and XF3063, respectively, Omega Optical, Brattleboro, VT). Separation of the three emission light channels was excellent and the PI fluorescence had no discernable influence on the fura-2 images (see Figure 8A and D). Cells on glass bottom microwells were superfused on the stage of the microscope at a rate of 1 ml/min. Each experiment where PI-positive cells appeared in response to the Ca^{2+} trigger was concluded with an inspection of the PI channel at different Z objective positions to verify that the PI

positivity was not located in a cell occasionally overlaying the cell originally in focus. The $[Ca^{2+}]_i$ time courses presented are measurements at the center of the PI-positive spot and are representative for the time course at other locations within the same cell.

Dye uptake

Dye uptake was determined with the hemichannel permeable reporter dye PI (1 mM). In the combined Ca^{2+} /PI imaging experiments, the PI concentration was lowered to 10 μ M to minimize fluorescence from the PI-containing superfusate during recording and to avoid saturation of the ICCD camera at PI positivities. In all dye uptake experiments, except the combined Ca^{2+} /PI imaging experiments described before, the protocol was such that the cells were exposed for 5 min to the trigger solution and were then washed four times with HBSS-HEPES. Images, nine for each culture, were acquired on a Nikon TE300 inverted microscope in epifluorescence mode (TRITC excitation/emission) with an $\times 10$ objective and a Nikon DS-5M camera (Analisis, Namur, Belgium). The number of PI-positive cells was counted in each image using ImageJ software after application of a threshold corresponding to the upper level of the background signal. Cell counts were expressed in the graphs as a percentage relative to the total number of cells in view counted after DAPI staining (65 ± 4.5 cells per $\times 10$ objective camera field for 25 000 cells/cm² seeding density and 127 ± 7.8 for 50 000 cells/cm²; $n = 36$). Overall, the number of PI-positive cells was 1.2 ± 0.1 (747 images from 83 experiments) in baseline, 16.6 ± 0.7 (333 images from 37 experiments) with DF and 7.6 ± 0.4 (414 images from 46 experiments) with 2 μ M A23187, corresponding to a procentual increase of 1383% for DF and 633% for A23187. In the combined Ca^{2+} /PI experiments where imaging was carried out with an $\times 40$ objective (Figure 8), the chance of finding a cell responding to A23187 was in the order of one every two $\times 40$ camera fields. A very large number of experiments on different cultures ($n = 120$) was therefore necessary to obtain meaningful data.

The selectivity of dye uptake was verified with the fluorescent reporters 6-CF (MW 376 Da) and dextran fluorescein (MW 10 kDa) ($\times 40$ objective, FITC excitation/emission). DF-triggered dye uptake was significant for the hemichannel-permeable dye 6-CF (baseline $1.04 \pm 0.496\%$ of the cells, trigger $10.6 \pm 1.99\%$, $n = 9$, $P < 0.0003$) and nonsignificant for the hemichannel-impermeable 10 kDa dextran (baseline $0.80 \pm 0.56\%$, trigger $2.39 \pm 1.02\%$, $n = 9$). Experiments with the A23187 trigger (2 μ M) gave similar results (6-CF: baseline $3.75 \pm 1.75\%$ of the cells, trigger $20.9 \pm 6.07\%$,

$n = 9$, $P < 0.008$; 10 kDa dextran: baseline $1.03 \pm 1.03\%$, trigger $3.75 \pm 2.01\%$, $n = 9$).

Western blotting

Cell protein lysates were extracted with RIPA buffer (25 mM Tris, 50 mM NaCl, 0.5% NP40, 0.5% deoxycholate, 0.1% SDS, 0.055 g/ml β -glycerophosphate, 1 mM DTT, 20 μ l/ml phosphatase inhibitor cocktail, 20 μ l/ml mini EDTA-free protease inhibitor cocktail) and sonicated by three 10-s pulses. Total protein was determined with a BioRad (Nazareth, Belgium) DC protein assay kit and a plate reader. Proteins were separated on a 10% Bis-Tris gel (Invitrogen, Merelbeke, Belgium) and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). Blots were probed with antibodies, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse (Cell Signalling, Beverly) and detection was carried out with the ECL chemiluminescent reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Data analysis and statistics

The data are expressed as mean $\pm$ s.e.m., with 'n' denoting the number of independent experiments on different cultures. In dye uptake experiments, n reflects the number of images. The variations in baseline and triggered signals observed in some figures represent normal variability between different experimental groups; in some cases it may be related to different experimental conditions (e.g. the presence of DMSO). Comparison of two groups was carried out using a one-tailed unpaired t -test, with a P -value below 0.05 indicating significance. Comparison of more than two groups was carried out with one-way ANOVA and a Bonferroni post test. 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 in case $P < 0.001$.

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