$P2X_7$ receptor activates extracellular signal-regulated kinases ERK1 and ERK2 independently of Ca^{2+} influx

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$P2X_7$ nucleotide receptors modulate a spectrum of cellular events in various cells including epithelia, such as exocrine pancreas. Although the pharmacology and channel properties of the $P2X_7$ receptors have been studied intensively, signal transduction pathways are relatively unknown. In this study we applied a heterologous expression system of rat $P2X_7$ receptors in HEK-293 cells. We followed the receptor expression and function using the enhanced green fluorescent protein (EGFP) tag, activation of intracellular proteins and increases in cellular Ca^{2+}. EGFP-$P2X_7$ receptors localized to the plasma membrane, clusters within the membrane and intracellularly. Stimulation of $P2X_7$ receptors in HEK-293 cells led to an activation of extracellular signal-regulated kinases ERK1 and ERK2 and this activation was seen after just 1 min of stimulation with ATP. Using C- and N-terminal $P2X_7$-receptor mutants we show that the N-terminus is important in activation of ERKs, whereas deletion of the last 230 amino acids in the C-terminus did not effect ERK activation. On the other hand, Ca^{2+} entry was impaired in C-terminal but not in N-terminal mutants. In cell suspensions prepared from rat pancreas we show that $P2X_7$ receptors also activate ERK1 and ERK2, indicating that these signalling pathways are also turned on in native epithelium.

Key words: confocal microscopy, EGFP-$P2X_7$, HEK-293 cells, mitogen-activated protein kinase (MAPK), pancreas.

INTRODUCTION

During the last 10 years, many studies have shown that extracellular nucleotides can act as extracellular signalling molecules via specific receptors [1]. ATP-preferring purinergic P2 receptors are divided into two subfamilies according to their molecular structure: G-protein-coupled P2Y receptors and P2X ligand-gated ion channels [1]. The P2X receptor family comprises seven members that arise from distinct genes [2,3]. The P2X receptors have intracellular N- and C-termini and span the plasma membrane twice. Most P2X-receptor family members seem to function as multimeric complexes, i.e. they form either homo- or hetero-multimers [4,5]. Notably, P2X receptors affect or regulate a great variety of cellular functions including cell proliferation and growth, apoptosis, development of cancer, nociception, cell volume, cytokine release and epithelial transport [1].

In exocrine pancreas both families of P2 receptors are expressed [6]. Pancreatic acini have transcripts for $P2Y_2$, $P2Y_4$, $P2X_1$ and $P2X_4$ receptors, although functional receptors are difficult to detect in all cells [7]. Interestingly, acini release ATP, which can stimulate the downstream pancreatic ducts [8]. Pancreatic ducts, which secrete HCO_3^--rich fluid important for digestive processes, express four types of functional P2 receptors: $P2Y_2$, $P2Y_4$, $P2X_4$ and notably $P2X_7$ receptors [6]. Stimulation of $P2X_7$ (and possibly $P2X_4$) receptors leads to reversible Ca^{2+} and Na^+ influx and associated membrane voltage changes [6]. Although $P2X_7$ receptors would not elicit secretion alone, because they do not stimulate Cl^- or HCO_3^- transporters directly, they can up-regulate secretion stimulated by secretin [6,9].

One well-described feature of the $P2X_7$ receptors is that they can form pores permeable to large molecules and cause lysis in some cells, e.g. in immunoreactive cells and in some expression systems [10–12]. It is not yet clear under what condition the channel-to-pore formation occurs and whether it depends on the receptor density; whether it is the pore-forming ability of only the multimeric receptor; whether it involves accessory signal proteins; or the association with other proteins such as porins [11,13–16]. In native exocrine glands, the $P2X_7$ receptor is probably equivalent to the earlier-described P2Z receptor [17]. In these tissues, the $P2X_7$ receptor's effects are reversible and in physiological contexts not associated with the pore formation, rather they regulate ion transporters compatible with secretion such as the Na^+/H^+ exchanger, Na^+–$2Cl^-$–K^+ co-transporter, K^+ channels and also protein secretion [6,17–20]. Therefore, signalling pathways other than those leading to the pore formation and lysis would be important in regulation of epithelial transport. However, cellular mechanisms underlying $P2X_7$-receptor-mediated effects, including intracellular signalling, are at present not clear and not widely studied. So far there are reports that the $P2X_7$ receptor is able to mediate an activation of phospholipase D, it interacts with cytoskeletal proteins and it activates stress-activated protein kinases ('SAPKs'). In view of the many various effects that $P2X_7$ receptors have on different cells, recently it has been considered whether the mitogen-activated protein (MAP) kinases are potential regulators. Thus it was shown that the $P2X_7$ receptor activates the p38 MAP kinase [21–24], and latest reports show that the $P2X_7$ receptor mediates activation of extracellular signal-regulated kinases (ERK1 and ERK2) in different cell lines. The signal transduction pathways leading to ERK1/ERK2 activation are beginning to be investigated, but dependence on Ca^{2+} is not resolved [25–27]. MAP kinases not only regulate a number of transcription factors, thereby controlling a large number of important genes, but also have non-genomic effects on various cell functions [28], and thus we consider whether they are also involved in regulation of epithelial transport [29,30].

The aim of the investigation was to study $P2X_7$-receptor signalling that may be relevant to epithelia, specifically for pancreatic duct cell function. To simplify our study, we expressed

Abbreviations used: BzATP, 2′-(or -3′)-*O*-4-benzoylbenzoyl-ATP; EGFP, enhanced green fluorescent protein; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; CLSM, confocal laser scanning microscopy; fura-2/AM, fura 2 acetoxymethyl ester.

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the tagged rat $P2X_7$ receptor and C- and N-terminal mutants in HEK-293 cells and began investigating signalling pathways. We followed $P2X_7$ expression using a enhanced green fluorescent protein (EGFP) tag and we monitored intracellular Ca^{2+} changes by confocal laser scanning microscopy (CLSM). Moreover, we looked at the $P2X_7$-chimera-mediated activation of one of the three MAP-kinase cascades involving ERK1/ERK2, also known as p44/42 MAP kinases. We find that the $P2X_7$ receptor and C-terminal, but not N-terminal, mutants activate ERK1/ERK2 in HEK-293 cells within 1 min. Results with both the C- and N-terminal mutants showed that an influx of Ca^{2+} through this receptor channel is most probably not involved in the activation of ERK1/ERK2. Similar activation of ERK1/ERK2 by $P2X_7$ receptor is seen in exocrine pancreas, indicating possible physiological relevance.

EXPERIMENTAL

Materials

The full-length rat $P2X_7$-receptor cDNA (pRK5-$P2X_7$) was provided kindly by Dr M. Voigt (St. Louis University School of Medicine, St. Louis, MO, U.S.A.). All nucleotides were from Sigma-Aldrich (Vallensbæk Strand, Denmark). Tissue-culture media were purchased from Life Technologies (Paisley, Scotland, U.K.). Primers for PCR were provided by MWG-Biotech AG (Ebersberg, Germany). Protease-inhibitor cocktail tablet was from Boehringer Mannheim (Hoersholm, Denmark). Anti-(phosphospecific MAP kinase) and anti-MAP kinase antibodies were purchased from New England Biolabs (Beverly, MA, U.S.A.). Anti-GFP, anti-FLAG (raised against the FLAG epitope, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) and horseradish peroxidase-linked (H + L) whole antibody were obtained from Sigma. pEGFP-C vectors were from Clontech (Palo Alto, CA, U.S.A.). Fura 2 acetoxymethyl ester (fura-2/AM) was purchased from Molecular Probes (Leiden, The Netherlands).

Construction of $P2X_7$ mutants and truncations

pEGFP-$P2X_7$ was constructed from the plasmid pRK5-$P2X_7$. cDNA was amplified from the plasmid by PCR using forward and reverse primers containing restriction endonuclease sites at their 5′-ends. Both primers enabled us to ligate the endonuclease-digested fragment into pEGFP-C2. For all constructs the $P2X_7$ was ligated downstream of and in-frame with EGFP. For construction of the N-terminal deletion mutants, a forward primer annealing to the start of the first transmembrane domain (amino acid 24) was used. The same approach was used to construct the C-terminal deletion mutants. Together with the forward primer, primers containing a stop codon (denoted by *) at amino acids 365, 402 and 473 (Ser-365*, Ser-402* and Ser-473*) were used (see Figure 1).

Cell culture and transfection

HEK-293 cells (CRL 1573; ATCC) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Life Technologies), 10 mg/ml streptomycin and 100 units/ml penicillin at 37 °C in a 5%-CO_2-enriched humidified atmosphere. The cells were plated 24 h before transfection on plastic dishes (35 mm diameter), or in dishes containing glass coverslips (World Precision Instruments, Stevenage, Herts., U.K.) if they were to be used for fluorescence microscopy. Cells were transfected using ExGen 500 according to the manufacturer's instructions (Fermentas, Vilnius, Lithuania). The transfected cells were cultured for 48 h to allow full protein expression (see the Results section). Then, 24 h before stimulation with nucleotides, the medium was switched to Dulbecco's modified Eagle's medium with 0.5% fetal calf serum.

Figure 1 Schematic drawing of various $P2X_7$ constructs

The full-length $P2X_7$ receptor is shown at the top, followed by C-terminal and N-terminal mutants. The star shape shows EGFP, light-grey shading shows the intracellular domain (amino acids 1–25 and 356–595), mid-grey shading shows the transmembrane domains (amino acids 26–46 and 335–355) and dark-grey shading shows the extracellular domain (amino acids 47–334).

Preparation of pancreatic tissue

Pancreas was obtained from Wistar rats and cut into small pieces. Tissue preparation was based on our earlier method of collagenase digestion and trypsin inhibitor treatment, which yields viable acini and a small number of intra- and interlobular ducts [8]. The pancreatic cell suspension was incubated at 37 °C in a 1:1 mix of Dulbecco's modified Eagle's medium 1000/Ham's F12 gassed with 5% CO_2 in O_2, and stimulated with BzATP [2′-(or -3′)-*O*-4-benzoylbenzoyl-ATP]. After stimulation cells were homogenized and Western blot performed as outlined below.

Western immunoblotting

Following nucleotide stimulation, transiently transfected HEK-293 cells or pancreatic cells were homogenized in lysis buffer containing 20 mM Tris/acetate, pH 7.0, 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 50 mM NaF, 1% Triton X-100 and a protease-inhibitor cocktail tablet. After removal of cellular debris, the protein content in each sample was measured by using Bio-Rad Protein Assay Dye Reagent Concentrate according to the manufacturer's instructions (Bio-Rad, Hercules, CA, U.S.A.). Equal amounts of cell lysate were dissolved in 2 × Laemmli buffer and subjected to SDS/PAGE. Subsequently, the proteins were transferred to nitrocellulose membranes (Schleicher and Schüll, Dassel, Germany). Immunoreactive proteins were made visible using horseradish peroxidase-coupled secondary antibodies and chemiluminescence was detected using the ImageStation 440 CF (Eastman Kodak, Rochester, NY, U.S.A.). The band densities on immunoblots were measured using the ImageStation 440 CF. The activity of ERK1/ERK2 was determined by immunoblotting using the anti-(phosphospecific MAP kinase) antibody mentioned above.

CLSM

Fluorescence microscopy was performed on a confocal spectral laser scanning microscope (Leica TCS SP; Leica Microsystems

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Heidelberg GmbH, Germany) equipped with Ar/Kr and UV lasers, and 63 × 1.2 numerical aperture PL APO water immersion, 40 × 1.4 numerical aperture PL APO oil immersion and 20 × 0.7 numerical aperture HC PL APO objectives. HEK-293 cells were grown on coverslips mounted in a plastic dish. Cells were suspended in a medium or physiological saline containing 145 mM Na^+, 3.6 mM K^+, 1.5 mM Ca^{2+}, 1 mM Mg^{2+}, 140 mM Cl^-, 10 mM Hepes, 2 mM phosphate and 5 mM glucose, pH 7.4. Experiments were performed at 37 °C. EGFP was excited at 488 nm with the Ar/Kr laser and the emission intensity between 500 and 550 nm was selected with the CLSM spectrophotometer unit and collected with a photomultiplier.

In some experiments transfected HEK-293 cells were loaded with fura-2/AM (5 μM) for 30–40 min, and subsequently Ca^{2+}-sensitive fura-2 signals were monitored in CLSM by ratiometric method, as implemented in our recent study [7]. Briefly, fura-2 was excited with 351- and 364-nm UV laser an using an acoustic-optical tunable filter ('AOTF') in the UV light path for fast switching of laser lines. The emission intensities were measured at 510 ± 20 nm with another photomultiplier. The real frame scanning time was usually set at 0.88 s for 512 × 512 pixels, and ratios were calculated from the 351- and 364-nm scans. Using consecutive scans of Ar/Kr and UV lasers it was possible to monitor both GFP and fura-2 fluorescence. Series of images were later analysed using the Physiology Package of the Leica CLSM software and MetaMorph 5.0 software (Universal Imaging Corporation, West Chester, PA, U.S.A.) and Microcal Origin 6.0 (Microcal Software, Northampton, MA, U.S.A.). The data obtained for fura-2 ratios are shown as means ± S.E.M. and ANOVA was used to determine the level of statistical significance.

RESULTS

Transfection of HEK-293 cells with EGFP-P2X$_7$

Although our main research interest lies in the investigation of ion transport and its regulation in pancreatic duct cells, we decided to investigate P2X$_7$-receptor-mediated signal transduction mechanisms by use of a heterologous expression system. This was done for the following reasons: pancreatic duct cells are difficult to isolate to purity in reasonable amounts, and they have a multitude of P2 receptors [6]. HEK-293 cells, the expression cell line of our choice, is a suitable model system for studying signal transduction mechanisms, and P2X$_7$ receptors have similar receptor kinetics/ion-channel properties to those seen in native cells [6,16,31].

In our experiments we fused the receptor to a fluorescent protein (EGFP), which enabled us to follow the receptor by fluorescence microscopy. We first investigated whether fusion of EGFP to the N-terminus of the P2X$_7$ receptor affected translocation of the receptor to the membrane. HEK-293 cells were transfected with either the full-length pEGFP-P2X$_7$ or pEGFP-C2 (empty vector) and viewed in CLSM. An example of fluorescent images is shown in Figure 2. Cells transfected with the empty vector, pEGFP-C2, showed a relatively even green fluorescence throughout the cell (Figures 2A and 2B). Cells transfected with the full-length pEGFP-P2X$_7$ showed a different pattern of fluorescence. After 24–48 h of transfection, the EGFP fluorescence became localized to the plasma membrane and in addition there were high-intensity spots; clusters within the membrane (Figures 2E and 2F). The clusters within the plasma membrane were 0.5–1 μm in diameter. The most intense fluorescence, and therefore highest receptor density, was detected at the sites of cell contact with the substratum and other cells and in the filopodia (Figures 2C

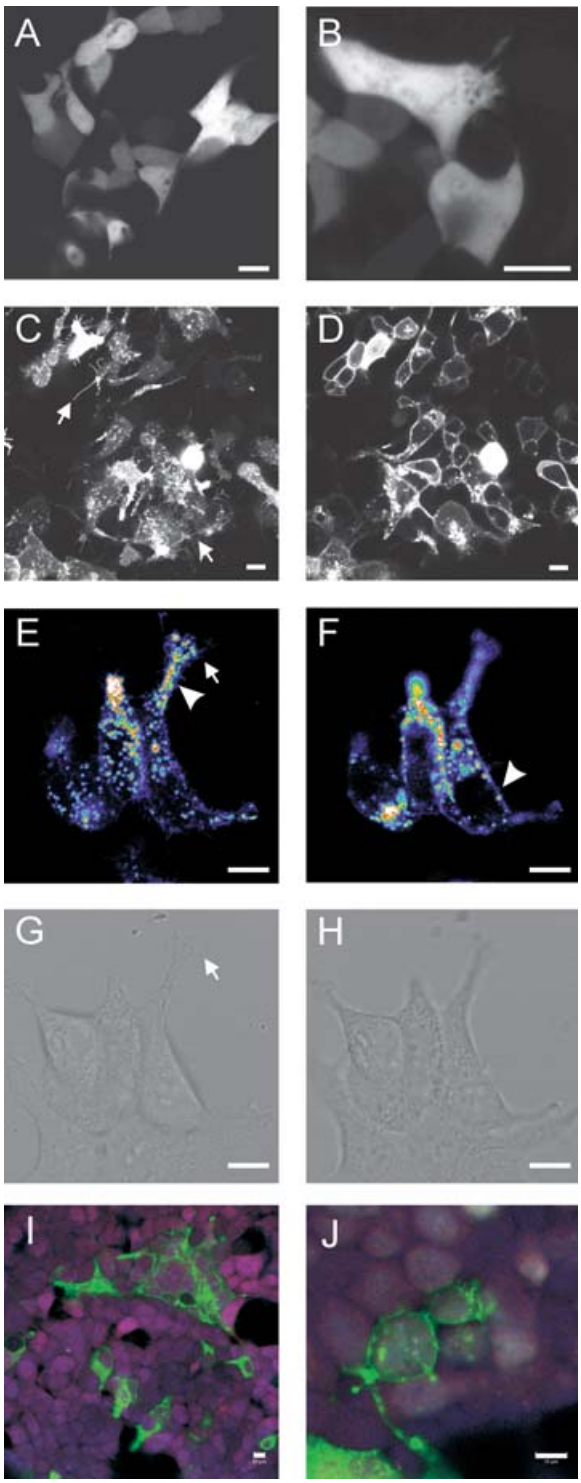

Figure 2 Localization of EGFP-P2X$_7$ receptors in transiently transfected HEK-293 cells

HEK-293 cells were transiently transfected with P2X$_7$ receptor tagged N-terminally with EGFP (see the Experimental section and Figure 1) and the EGFP fluorescence was visualized by CLSM. (**A**, **B**) Cells transfected with an empty vector. (**C**–**H**) Cells transfected with EGFP-P2X$_7$. (**C**, **D**) Images of the same cells taken at the level of their contact with the coverslip and about midway through most of the cells. (**E**, **F**) Two pseudo-colour images of three HEK-293 cells taken at two different levels showing fluorescent clusters at the membrane attachments to the glass and in filopodia (**E**) and in the plasma membrane 1.8 μm further through the cells (**F**). (**G**, **H**) Transmission images corresponding to (**E**) and (**F**). White arrows show examples of filopodia in (**C**), (**E**) and (**G**). White arrowheads show some EGFP-P2X$_7$ receptor clusters in the plasma membrane in (**E**) and (**F**). (**I**, **J**) Overlay images of EGFP-P2X$_7$ receptors (green) and fura-2 fluorescence (lilac). Scale bars, 10 μm.

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and 2E). These were also the sites of the clusters (also see Figure 4 below). In addition, there various amounts of intracellular fluorescence, some confined to small vesicles (1–2 μm) beneath the plasma membrane and occasionally to larger organelles. Almost all transfected cells retained their morphology, just like the proliferating untransfected HEK-293 cells. Only a very few cells were rounded and lost attachment to the substratum and neighbouring cells and the fluorescence increased, presumably as these cells were shrinking and dying, again similar to that seen in the untransfected cells. Transfected cells showing normal morphology were followed up to 5–7 days; when multinuclear cells became more abundant. The function of receptors was also monitored as Ca^{2+} signals in experiments where pEGFP-$P2X_7$-receptor fluorescence and fura-2 fluorescence could be visualized in transfected cells (Figures 2I and J; see also below).

Activation of ERK1/ERK2 by ATP in HEK-293 cells

The $P2X_7$ receptor does not contain any intrinsic tyrosine kinase activity, nor does it have any known binding sites for G-proteins, or other conserved binding sites for protein kinases. Therefore, we decided first to look at the downstream regulation of one of the MAP-kinase cascades, ERK1 and ERK2 activation. Figure 3 shows the Western blot of $P2X_7$ receptors and ERK1/ERK2 expression in HEK-293, and of phosphorylated ERK1/ERK2 by the use of phosphospecific antibodies against ERK1 and ERK2. In cells transiently transfected with an empty vector, incubation of cells with ATP (300 μM) for 5 min did not activate ERK1/ERK2 (Figure 3). When HEK-293 cells were transiently transfected with the full-length $P2X_7$ receptor, ATP activated the ERK1/ERK2. After 5 min of stimulation with ATP, phosphorylated ERK1/ERK2 increased 4-fold above the basal level, and ERK2 appeared to be slightly more phosphorylated than ERK1. Since there is a clear difference in ERK activation between the $P2X_7$-expressing and the non-expressing HEK-293 cells, it seems most likely that endogenous P2 receptors were not involved in the activation. Notably, reverse transcription PCR on HEK-293 cells revealed that they do not have transcripts for $P2X_7$ (J. Amstrup, M. R. Hansen and I. Novak, unpublished work). The following studies were designed to investigate which part of the receptor was involved in ERK1/ERK2 activation.

$P2X_7$-receptor mutants and effects on ERK1/ERK2

Despite the great interest in the $P2X_7$ receptor's pharmacology and structural motifs related to channel/pore formation, there is little information available regarding the receptor structural motifs related to signalling [1]. All P2X receptors contain two homologous transmembrane loops and rather variable intracellular N- and C-terminal tails [3]. The $P2X_7$ receptor is distinct from the other P2X receptors in that it contains a rather long C-terminal tail, thought to be involved in pore formation [12,32]. There are 240 amino acids in the rat $P2X_7$-receptor C-terminal tail, while there are only 29–125 amino acids in the C-termini of the $P2X_1$–$P2X_6$ receptors [3]. In the present study we decided to investigate the involvement of the C-terminal tail in the activation of ERK1/ERK2, and several C-terminal truncations were made (Figure 1). These constructs were made such that the C-terminal part from residue Ser-365 was missing (the last 230 amino acids), or such that two-thirds of the terminus (Ser-402*) or half of the terminus (Ser-473*) was missing. These constructs were made with the EGFP fused to their N-terminus, such that we could also determine whether the truncated receptors were able to integrate into the plasma membrane.

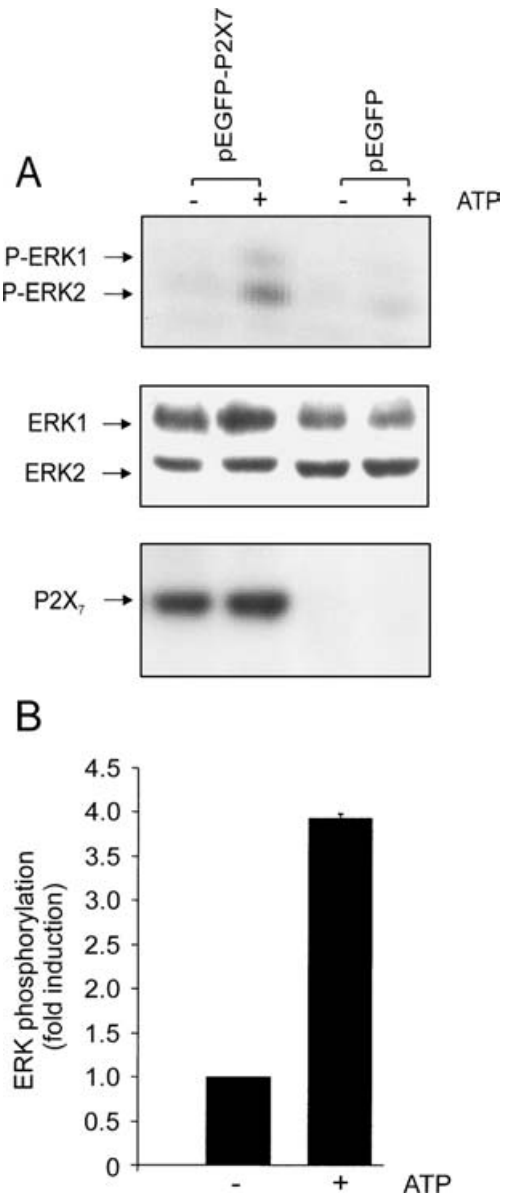

Figure 3 $P2X_7$ receptor activates ERK1/ERK2 in HEK-293 cells

HEK-293 cells were transiently transfected with empty vector, or with the rat $P2X_7$ receptor. After transfection (48 h) the HEK-293 cells were stimulated for 5 min with 300 μM ATP and then lysed according to the Experimental section. (**A**) Western blots show the control for the $P2X_7$ receptor, ERK1/ERK2 and the active phosphorylated ERK1/ERK2, with and without ATP. One of at least three independent experiments is shown. (**B**) Total ERK activation of EGFP-$P2X_7$-transfected cells in three experiments was quantified on the ImageStation as described in the Experimental section. Bars show means ± S.E.M.

Figure 4 shows the EGFP fluorescence in HEK-293 48 h after transfection with various $P2X_7$ mutants. The partial C-terminal mutants Ser-473* and Ser-402*, shown respectively in Figures 4(A)–4(C) and Figures 4(D)–4(F), had a similar fluorescence distribution as the full-length tagged receptors (Figure 2), including targeting of the fluorescent receptors to the plasma membrane, cluster formation and some intracellular distribution. Notably, HEK-293 cells with the whole C-terminal part of the $P2X_7$ receptor missing, Ser-365*, also showed a similar distribution of green fluorescence (Figures 4G–4I). Overall, the transfected cells had very similar morphology to the cells expressing

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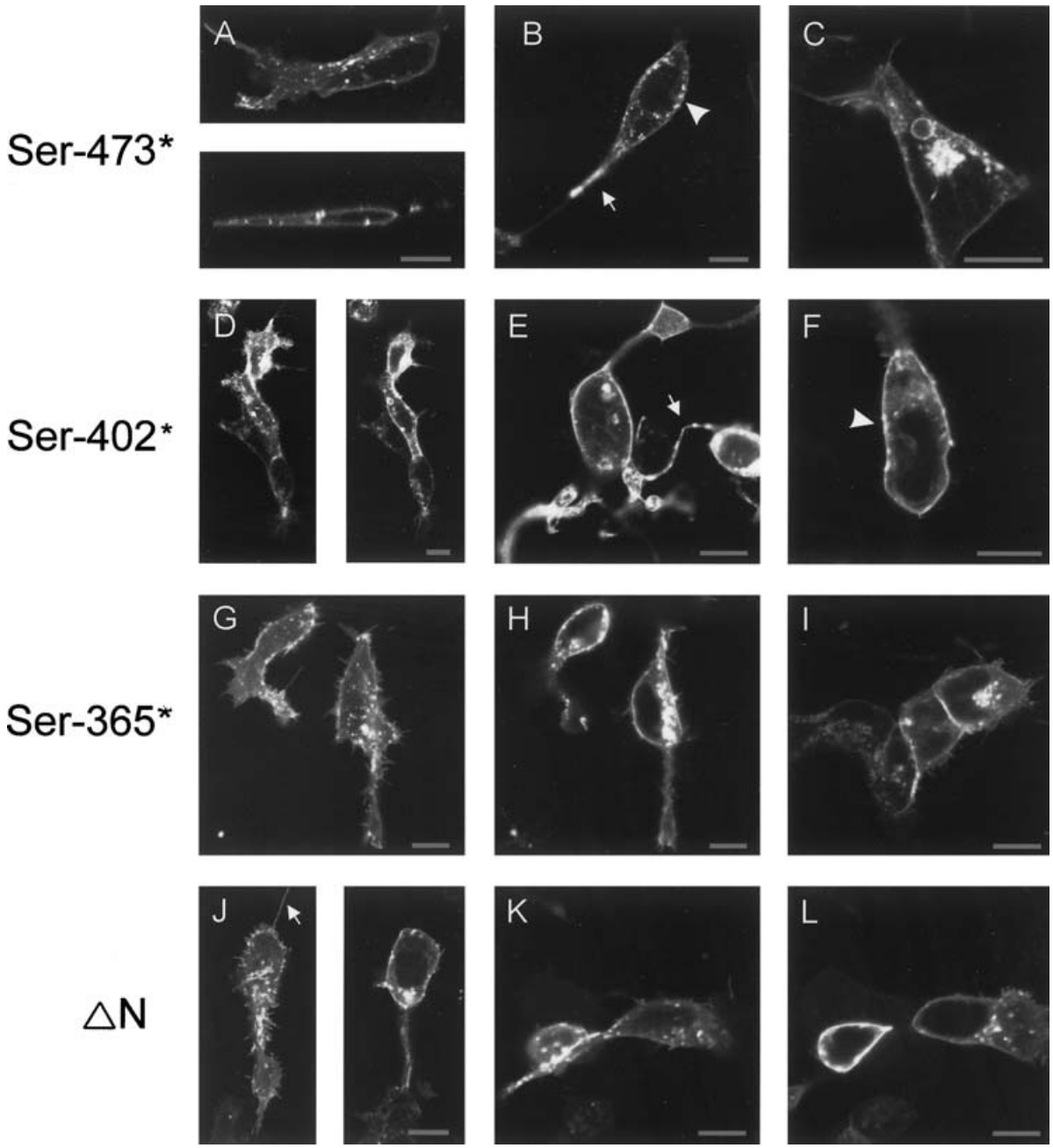

Figure 4 Localization of the $P2X_7$ C- and N-terminally truncated $P2X_7$ receptors

HEK-293 cells were transiently transfected with C- and N-terminally truncated versions of the $P2X_7$ receptor (see Figure 1). (**A**–**C**) Cells expressing the C-terminal mutant Ser-473*. Images in (**A**) were of the same cell taken in the *x*–*y* and *x*–*z* planes. (**D**–**F**) Cells expressing the C-terminal mutant Ser-402*. Images in (**D**) were of the same cells taken in different *z* planes. (**G**–**I**) Cells expressing the C-terminal mutant Ser-365*. Two cells in (**G**) and (**H**) are images taken in different *z* planes. (**J**–**L**) Cells expressing the N-terminal truncation mutant ΔN-EGFP-$P2X_7$. The pair of images in (**J**) and those in (**K**) and (**L**) are of the same cells, again in different *z* planes. Scale bars, 10 μm. White arrows point to examples of filopodia in (**B**), (**E**) and (**J**); white arrowheads point to receptor clusters in the plasma membrane in (**B**) and (**F**).

the full-length $P2X_7$ receptor (Figure 2) and the non-transfected cells.

Figure 5 shows the effect of ATP on ERK1/ERK2 in HEK-293 cells expressing one of the three $P2X_7$-receptor C-terminal mutants. Within 5 min stimulation with ATP (300 μM) mediated an activation of ERK1/ERK2 in all mutants and the stimulation pattern was similar to the full-length receptor (Figures 3 and 5). Figure 5(B) shows that there was a 4.5-fold increase in phosphorylation of the fully truncated mutant Ser-365*, and the Ser-402* and Ser-473* truncations both gave a 3–4-fold increase in ERK phosphorylation. Together, these observations indicate that the $P2X_7$-receptor C-terminally truncated mutants could activate ERK1/ERK2.

The importance of the N-terminal part of the receptor in ERK1/ERK2 phosphorylation was tested in the next series of experiments. The N-terminal part of the P2X receptors is a very short region consisting of between 22 and 34 amino acids and there is a small amount of similarity in the N-termini of different P2X receptors [3]. HEK-293 cells were transiently transfected with the N-terminally truncated version of the receptor chimera

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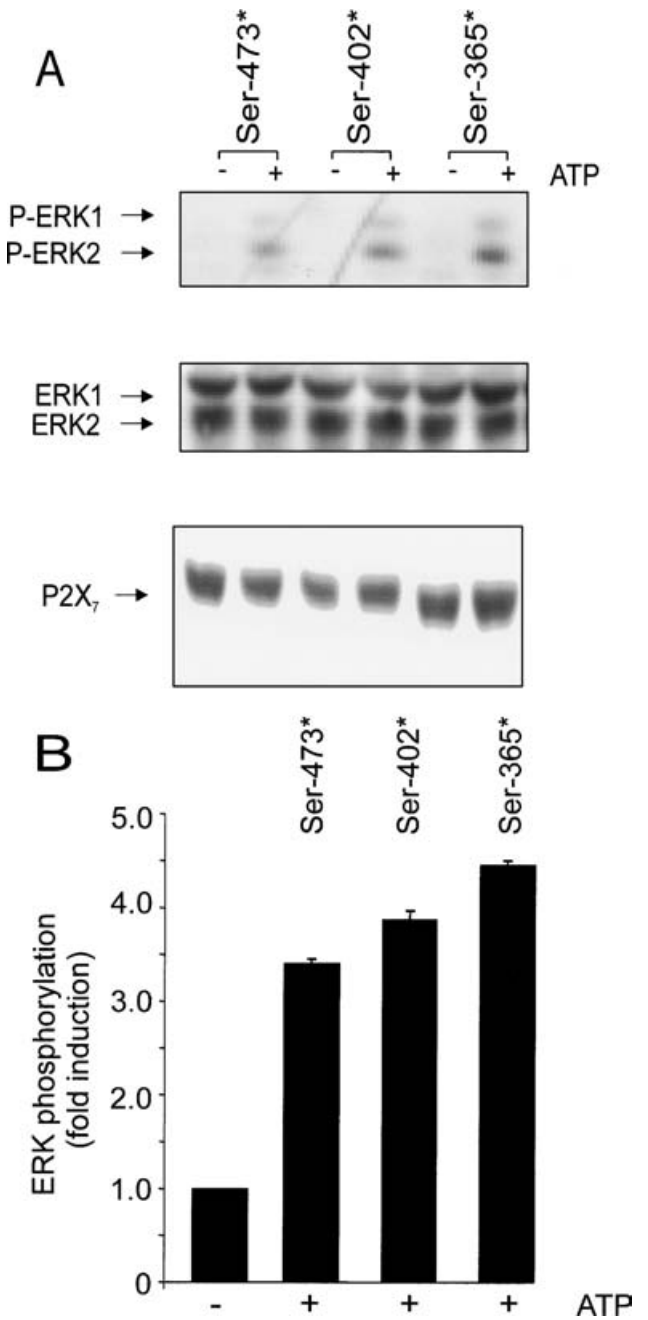

Figure 5 ERK1/ERK2 is activated by C-terminally truncated $P2X_7$ receptor

HEK-293 cells were transiently transfected with $P2X_7$ receptors at which a stop codon (*) was inserted at the position indicated. 48 h after transfection the cells were stimulated with 300 μM ATP for 5 min and then were lysed. (**A**) Western blot showing the active ERK1/ERK2 as well as control blots for the amount of ERK1/ERK2 and the $P2X_7$ receptor with and without stimulation with ATP. One of at least three independent experiments is shown. (**B**) Total ERK activation was quantified for three experiments and plotted (see the Experimental section).

(ΔN-EGFP-$P2X_7$). The ΔN-EGFP-$P2X_7$ mutants were stimulated with ATP in protocols similar to earlier experiments, and Figure 6 shows the effect on ERK1/ERK2. The important observation made with this construct was that lost its ability to activate ERK1/ERK2. We checked the expression of the $P2X_7$-receptor chimera using EGFP fluorescence and the results are shown in Figures 4(J)–4(L). The ΔN-EGFP-$P2X_7$ mutants showed an EGFP fluorescence distribution similar to the full-length receptor

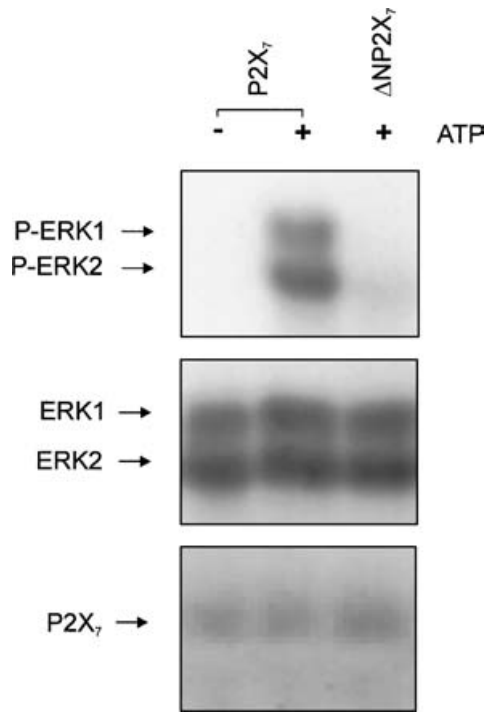

Figure 6 ΔN-$P2X_7$ receptor mutants do not activate ERK1/ERK2

HEK-293 cells were transiently transfected with the full-length and N-terminally deleted $P2X_7$ receptor. After 48 h, the cells were activated by ATP and subsequently lysed and subjected to Western blotting. Western blots show the active phospho-ERK1/ERK2, control blots for the amount of ERK1/ERK2, and the $P2X_7$ receptor. One of at least three independent experiments is shown.

and C-terminal mutants, and also similar cell morphology. That is, the ΔN-EGFP-$P2X_7$ mutant was incorporated into the plasma membrane just like the other $P2X_7$ receptors.

Time- and concentration-dependent activation of ERK1/ERK2

In the following experiments we investigated the time course of ERK1 and ERK2 activation after a single application of ATP. Figure 7 shows that ERK2 was activated within 1 min and maximally at 5 min. On the other hand, phosphorylation of ERK1 was only weak until 5–10 min of stimulation with ATP (300 μM). Similar asynchronous activation of ERK1 and ERK2 was also observed with other receptors, but the explanation is still pending [33,34]. Figure 7 also shows that phosphorylation of ERK1/ERK2 reached plateau after about 10 min of stimulation of ATP.

In one set of experiments, we investigated the effect of ATP on ERK1/ERK2 activation in HEK-293 cells expressing the $P2X_7$ receptor and the C-terminally truncated receptor Ser-365* (Figure 8). Within 5 min stimulation of both receptors with different concentrations of ATP between 0.003 and 3 mM mediated similar activation of ERK1/ERK2, indicating that the last 230 amino acids of the $P2X_7$ receptor did not affect binding of ATP to the receptor, nor activation of ERK1/ERK2. The maximum response for both receptors was obtained with 3 mM ATP. In HEK-293 cells expressing an empty vector, no effect on ERK1/ERK2 activation was detected (results not shown).

Ca^{2+} transients mediated by $P2X_7$ receptors

Activation of $P2X_7$ receptors gives rise to Ca^{2+} transients that are due predominantly to Ca^{2+} influx via the cation-selective

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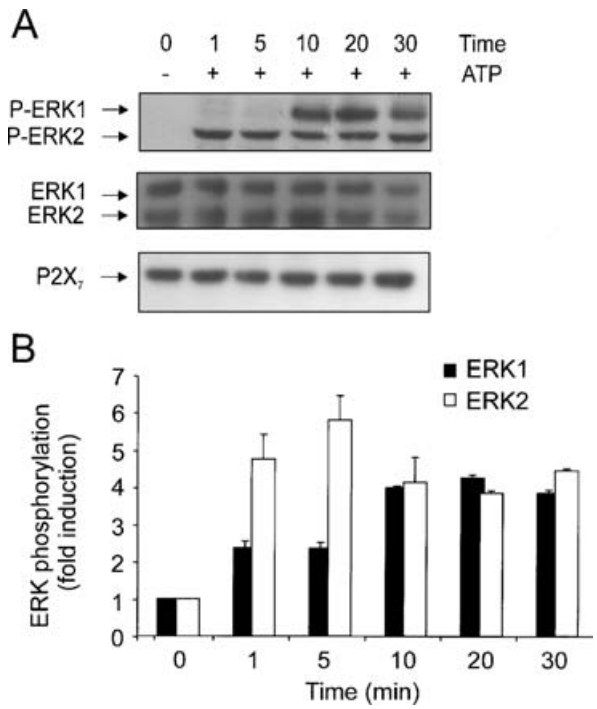

Figure 7 Time dependence of ERK1 and ERK2 activation via the P2X$_7$ receptor

HEK-293 cells were transiently transfected with the P2X$_7$ receptor and stimulated with ATP (300 μM). Reaction was stopped at the given intervals, and each batch of cells was lysed and processed as described above. (**A**) Western blot showing the active ERK1/ERK2, control blots for the amount of ERK1/ERK2, and the P2X$_7$ receptor. The image is representative of three individual preparations. (**B**) ERK1 and ERK2 activation was quantified and plotted as means $\pm$ S.E.M. from three experiments.

channel of the receptor. To test whether the P2X$_7$ mutants were functional, Ca^{2+} signals were monitored in P2X$_7$-expressing cells (see Figures 2I and 2J). Figure 9(A) shows the mean Ca^{2+} transients in response to BzATP in HEK-293 cells expressing the full-length P2X$_7$ receptor. Interestingly, the ΔN-EGFP- P2X$_7$-receptor mutant gave rise to significantly larger Ca^{2+} transients (Figure 9B), despite the fact that this mutant receptor was not able to cause ERK1/ERK2 activation (Figure 6). On the other hand, the C-terminally truncated receptor Ser-365* caused very small Ca^{2+} transients (Figure 9C) in response to BzATP, very similar to that elicited by the empty vector (Figure 9D), yet ERK1/ERK2 activation was intact with the C-terminal mutant (Figure 5). As expected, the empty vector gave rise only to very small Ca^{2+} transients (Figure 9D), most likely due to non-specific effects of BzATP on endogenous P2 receptors that HEK-293 cells express. HEK-293 cells always responded well to ATP, which stimulated endogenous P2X and P2Y receptors.

Activation of ERK1/ERK2 in the rat pancreas

In order to address the question of whether the P2X$_7$ receptor is able to activate ERK1/ERK2 in a native tissue, we set up the following experiments on a pancreatic cell suspension. Since pancreatic ducts comprise less than 5% of the pancreatic tissue and are difficult to isolate to purity [6], we used a cell suspension from whole pancreas that included acini and ducts. Pancreas also expresses other P2 receptors and ectonucleotidases, such as CD39, and therefore we used an ATP analogue, BzATP, for

Table 1 Summary of the effects of the P2X$_7$-receptor constructs on ERK1/ERK2 activation and Ca^{2+} transients

ND, not determined.

Receptor	ERK activation	Ca^{2+} transients
pEGFP-P2X$_7$	+	+
pEGFP-C2	−	−/+
ΔN-EGFP-P2X$_7$	−	++
pEGFP-P2X$_7$-Ser-365*	+	−/+
pEGFP-P2X$_7$-Ser-402*	+	ND
pEGFP-P2X$_7$-Ser-473*	+	ND

P2X$_7$-receptor stimulation. Figure 10 shows that stimulation of the pancreas cell suspension for 5 min with BzATP (100 μM) resulted in an approx. 4-fold activation of ERK1 and ERK2. Since pancreatic acinar cells do not have P2X$_7$ receptors [7], the effect of BzATP is due to activation of P2X$_7$ receptors in pancreatic duct cells. These findings are very comparable with ERK activation in transiently transfected HEK-293 cells (see Figure 3).

DISCUSSION

In this study we used heterologous expression system to transfect rat P2X$_7$ receptor into HEK-293 cells and follow its insertion into the plasma membrane using EGFP-P2X$_7$ chimerae. We also followed the activation of MAP kinases ERK1 and ERK2 and using several receptor mutants established that the N-terminus, not the C-terminus, was important in ERK activation. In contrast, the C-terminal mutant lost its ability to mediate Ca^{2+} influx, while the N-terminal mutant caused normal or even enhanced Ca^{2+} influx (Table 1). Thus the Ca^{2+}-channel property of the receptor is divorced from its intracellular signalling to ERK1/ERK2. Furthermore, we show that similar ERK1/ERK2 activation occurs in a pancreatic epithelium that contains endogenous P2X$_7$ receptors.

The green fluorescence of the EGFP-P2X$_7$-receptor chimera shows that the full-length receptors are inserted into the plasma membrane and cells have morphology just like the untransfected HEK-293 cells (Figure 2). Fusion of EGFP to the N-terminus of the P2X$_7$ receptor does not interfere with insertion of the receptor into the plasma membrane and receptors couple to intracellular signalling (see below). In addition, all C-terminal mutants and N-terminal mutants are also inserted into the plasma membrane (Figure 4). Indirectly, these results indicate that the signal sequence for plasma membrane targeting is not in the terminal parts of the receptor and/or that other trafficking proteins might be involved in receptor targeting, as also suggested recently [16]. Our studies with rP2X$_7$-receptor mutants are in contrast with human P2X$_7$ C-terminal mutants that do not target to the plasma membrane of HEK-293 cells [32].

One important observation in our study is the localization of P2X$_7$-receptor chimerae, and the healthy appearance of transfected cells. All our C- and N-terminal P2X$_7$-receptor mutants target to the plasma membrane, with the most intense fluorescence at the sites of contact with the substratum in filopodia and in contact with other cells. In many HEK-293 cells P2X$_7$ receptors form small clusters within or just below the plasma membrane (Figures 2 and 4). Similar cluster formation has been observed with antibody detection of P2X$_7$ receptors in astrocytes [35] and transfected HEK-293 cells [36]. Other P2X receptors, such as P2X$_1$, P2X$_2$, P2X$_3$ and P2X$_6$, also show clusters in various

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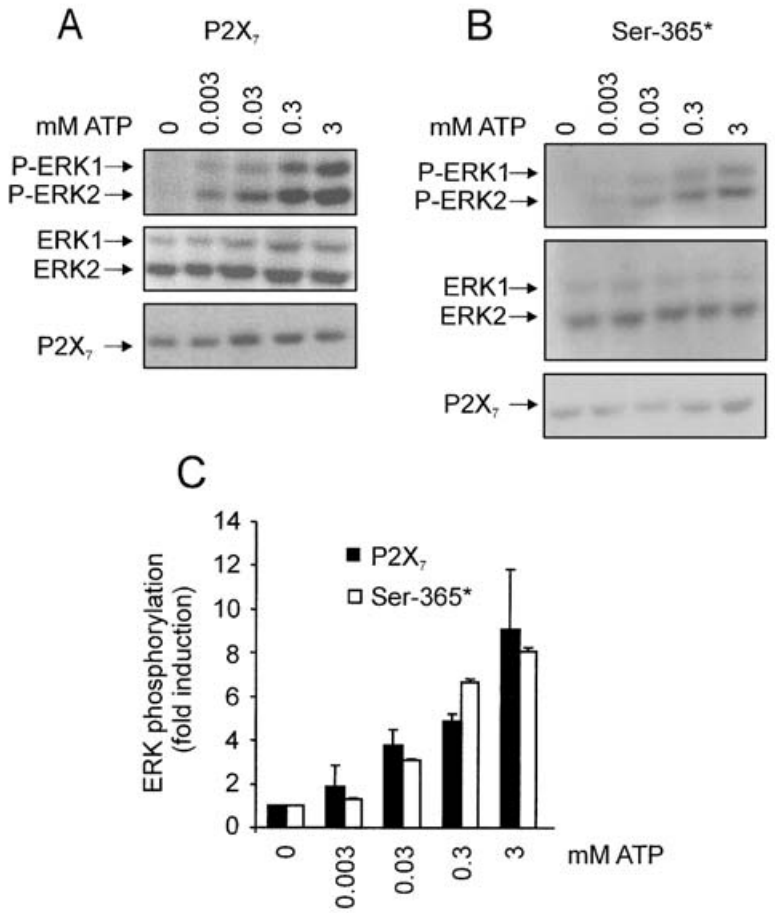

Figure 8 Concentration–response curve for pEGFP-P2X$_7$-Ser-365*

HEK-293 cells were transiently transfected with either the full-length P2X$_7$ receptor (**A**) or the receptor with the Ser-365* mutation (**B**). The cells were then stimulated for 5 min with one of the following ATP concentrations 0.003, 0.03, 0.3 and 3 mM. Subsequently, cells were lysed and prepared for Western blotting. The blots are representative of three individual preparations. (**C**) ERK1/ERK2 activation quantified and plotted as means ± S.E.M. from three experiments.

transfected and native cells [36,37]. Notably, in several new studies with transfected HEK-293 cells no clusters of P2X$_7$ receptors were observed [13,16,38]. However, the cell morphology in those studies is quite different to ours, as cells were rounded and bleb easily. In addition to clustering at the plasma membrane, native and heterologously expressed P2X receptors are also located intracellularly (e.g. Figure 4C) [16,31,39,40], and these may provide buffer for receptor supply and retrieval.

The present study in the recombinant system clearly shows that the P2X$_7$ receptors lead to phosphorylation of ERK1/ERK2, and these are activated within a few minutes (Figure 3). After a transient peak in ERK2 activation, ERK1/2 activation was sustained within the 30 min of the experiment. Since the P2X$_7$ receptors would desensitize and/or ATP would be broken down by ectonucleotidases, it is most likely that the ERK activation, once turned on, was persistent. These results are consistent with several recent reports, which also show that P2X$_7$ receptors activate ERKs in astrocytes, Jurkat cells and even parotid acini [25,27,35].

The key observations in our study are with the P2X$_7$-receptor mutants, which show that the N-terminus and not the C-terminus is involved in cell signalling to ERKs, as the ΔN P2X$_7$ receptors lose the ability to activate ERK1/ERK2 (Figures 5 and 6). Regarding the C-terminus of the P2X$_7$ receptor, it is thought to be involved in the lytic pore formation in some expression systems, but not in others [11,16]. A recent study by Kim and co-workers [23] on rat P2X$_7$ receptor expressed in HEK-293 cells indicated that many cytoskeletal proteins interact with the C-terminal part. Our results with the C-terminally truncated mutants show that this part of the receptor (amino acids 365–595) is not involved in ERK signalling, as the partial or full truncation of the C-terminal part still fully activates ERK1/ERK2 (Figure 5). This activation of ERK1/ERK2 by the C-terminal truncation Ser-365* was concentration-dependent in the same manner as for the full-length receptor (Figure 9). However, since Kim and co-workers [23] show that Tyr-343 mutation prevents phosphorylation of the receptor protein tyrosine phosphatase β, it is conceivable that the last part of the predicted transmembrane segment, or the very beginning of the C-terminal segment, might be important in interaction with some proteins. Nonetheless, our studies with the ΔN-EGFP-P2X$_7$ mutant show clearly that although this mutant was inserted into the plasma membrane (Figure 4) it could not activate ERKs (Figure 6). Nevertheless, N-terminal deletion did not abolish Ca^{2+} transients (influx), which in fact were higher than in cells expressing the full receptor (Figure 9). On the other hand, C-terminal deletion diminished Ca^{2+} transients (Figure 9), yet ERK1/ERK2 stimulation was unaffected.

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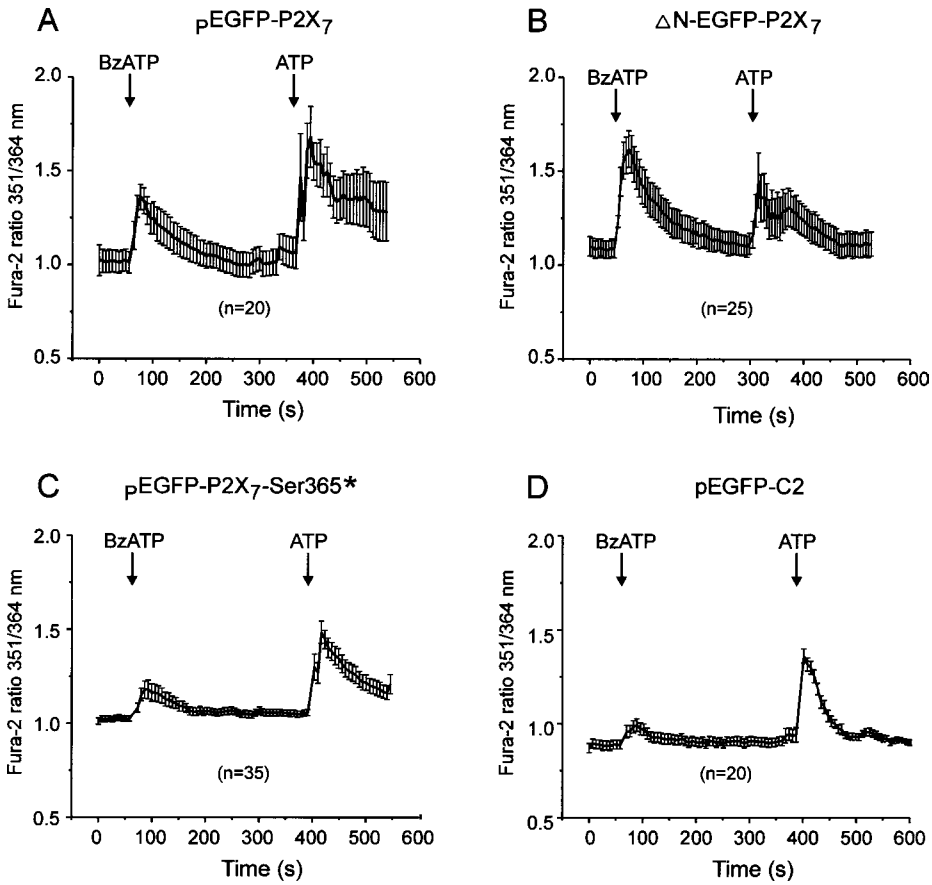

Figure 9 Ca^{2+} influx via the $P2X_7$ receptor

Fura-2 fluorescence ratio 351/364 nm was used to monitor Ca^{2+} signalling in HEK-293 cells transfected with various $P2X_7$ receptor constructs that showed green fluorescence (see Figure 2). HEK-293 cells were stimulated with BzATP (0.1 mM) and ATP (0.1 mM). Panels show Ca^{2+} responses in cells transfected with the full-length $P2X_7$ receptor (**A**), the ΔN-EGFP-$P2X_7$ receptor mutant (**B**), the $P2X_7$ C-terminal mutant Ser-365* (**C**) and the empty vector (**D**). Each panel shows measurements from 4–7 independent experiments, where from each five GFP fluorescent and responding cells recorded in one scan were used for calculations of mean $\pm$ S.E.M.

In a recent study Smart and co-workers [41] show that C-terminal deletion at residue 360 also stopped Ca^{2+} transients, channel and pore function of the channel, most probably because the receptor did not get to the membrane. There were also single mutations further along the C-terminus that produced the same effect. In our case (with truncation at 365), the receptor did target to the membrane and coupled to ERK signalling (Figures 4 and 5). Thus taken together, our study with N- and C-terminal mutants shows that ERK signalling and Ca^{2+} transients are independent, being associated with N- and C-termini, respectively. Therefore, different parts of the receptor are involved in the channel/Ca^{2+}-influx property of the receptor and signalling to ERKs.

The physiological relevance of $P2X_7$-receptor-mediated ERK1/ERK2 activation is provided by our studies on pancreatic cells. For our study we chose ATP and BzATP concentrations that were optimal for stimulation of pancreatic duct $P2X_7$ receptors in physiological solutions and in the range of ATP released by pancreatic acini [6,8,42]. Apart from ERK1/ERK2 activation in the HEK-293 cell expression system, similar ERK activation also occurs in the native pancreatic tissue (Figure 10). Since pancreatic ducts, but not pancreatic acini, express the $P2X_7$ receptors [7], we assume that the ERK signal would originate from pancreatic ducts. $P2X_7$-receptor stimulation in pancreatic ducts evokes Na^+ and Ca^{2+} influx sustained for up to 5 min, and potentiates secretin-evoked fluid secretion persisting for 10–15 min [6,9]. Activation of ERK1/ERK2 falls well within this time frame. However, at this stage we do not know whether the ERK activation is involved directly in the secretory processes. A study on rat medullary thick ascending limb showed that stimulation of ERK1/ERK2 (via nerve growth factor) decreases the Na^+/H^+ exchanger activity [43]. However, our new studies on pancreatic ducts show that ATP does not affect Na^+/H^+ exchanger or any other H^+/HCO_3^- transporters present [44]. Possibly, ERK stimulation is involved in more general cellular processes, including cytoskeleton reorganization or proliferation, metabolism and genomic effects. In pancreatic acini (which do not have $P2X_7$ receptors), ERK activation initiated via the cholecystokinin receptor also influences cell growth, and by phosphorylation of Hsp27 and Hsp28 it can affect actin cytoskeleton, and thus secretory events [29]. In parotid acini, which do have $P2X_7$ receptors that stimulate ion transporters [17], Bradford and Soltoff [25] showed very recently that $P2X_7$ receptors activate protein kinase D and ERK1/ERK2 in protein kinase C-dependent signalling and suggested that, at least for protein kinase D activation, extracellular Ca^{2+} is not essential.

Taken together, the important outcome of this study on ERK and $P2X_7$ activation and distribution is that the $P2X_7$ receptor does not only behave as a 'killer' receptor, but can be associated with 'positive' effects. Some positive effects of $P2X_7$ receptors

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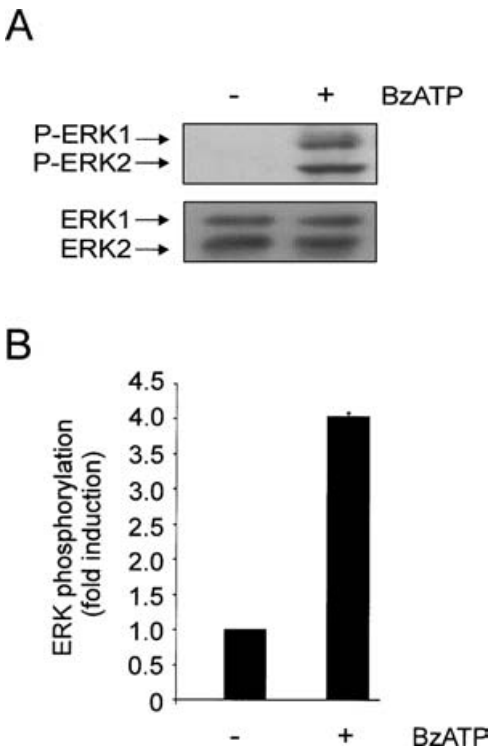

Figure 10 Activation of ERK1 and ERK2 in pancreas

Rat pancreas was digested with collagenase and the cell suspension was stimulated with BzATP (0.1 mM) for 5 min. Subsequently cells were lysed and prepared for Western blotting. (**A**) Western blots show the active ERK1/ERK2 and control blot for the amount of ERK1/ERK2. The image is representative of three independent experiments. (**B**) Total ERK activation was quantified and plotted.

have recently been appreciated, and include increased chemokine MCP-1 (chemoattractant protein-1) expression in astrocytes and increased proliferation rate of lymphoid cells [26,35,45]. Our CLSM results indicate that $P2X_7$ receptors are strategically placed in areas of contact, especially in filopodia (Figures 2 and 4), and it would be reasonable to suggest that they are involved in cytoskeleton reorganization, as ERK stimulation also indicates. The study of Kim and co-workers [23] shows that $P2X_7$ receptors might be associated with several cytoskeletal proteins, and their study implies that it is the C-terminus that is involved in intracellular signalling. However, our study shows that the N-terminus is important in ERK stimulation, and this does not require a receptor-mediated increase in intracellular Ca^{2+}, which is associated with the C-terminus.

In conclusion, in a heterologous expression system $P2X_7$-receptor stimulation lead to activation of ERK1 and ERK2, an event which is independent of Ca^{2+} influx through the receptor channel. In addition, in native pancreas the $P2X_7$ receptor mediates similar effects on ERK1/ERK2 activation, and its relevance to epithelial transport remains to be elucidated.

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