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RESEARCH PAPER

Agonist potency at $P2X_7$ receptors is modulated by structurally diverse lipids

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Background and purpose: The $P2X_7$ receptor exhibits a high degree of plasticity with agonist potency increasing after prolonged receptor activation. In this study we investigated the ability of lipids to modulate agonist potency at $P2X_7$ receptors.
Experimental approach: A variety of lipids, including lysophosphatidylcholine, sphingosylphosphorylcholine and hexadecylphosphorylcholine were studied for their effect on $P2X_7$ receptor-stimulated ethidium bromide accumulation in cells expressing human recombinant $P2X_7$ receptors and on $P2X_7$ receptor-stimulated interleukin-1β (IL1β) release from THP-1 cells. The effects of the lipids were also assessed in radioligand binding studies on human $P2X_7$ receptors.
Key results: At concentrations (3-30 μM) below the threshold to cause cell lysis, the lipids increased agonist potency and/or maximal effects at $P2X_7$ receptors in both ethidium accumulation and IL1β release studies. There was little structure activity relationship (SAR) for this effect and sub-lytic concentrations of Triton X-100 partially mimicked the effects of the lipids. The lipids caused cell lysis and increased intracellular calcium at higher concentrations (30-100 μM) which complicated interpretation of their effects in functional studies. However, the lipids (3-100 μM) also increased agonist potency 30-100 fold in radioligand binding studies.
Conclusions and implications: This study demonstrates that a diverse range of lipids increase agonist potency at the $P2X_7$ receptor in functional and binding studies. The broad SAR, including the effect of Triton X-100, suggests this may reflect changes in membrane properties rather than a direct effect on the $P2X_7$ receptor. Since many of the lipids studied accumulate in disease states they may enhance $P2X_7$ receptor function under pathophysiological conditions.
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Abbreviations: AA, arachidonic acid; $\alpha\beta$-meATP, $\alpha\beta$-methylene-ATP; ATPγS, adenosine-5′-*O*-(3-thiotriphosphate); $\beta\gamma$-meATP, $\beta\gamma$-methylene-ATP; BSA, bovine serum albumin; BzATP, 2′- and 3′-*O*-(4benzoylbenzoyl) ATP; compound-17, *N*-[2-({2-[(2-hydroxyethyl)amino]ethyl}amino)-5-quinolinyl]-2-tricyclo[3.3.1.1^{3,7}]dec-1-ylacetamide; Eoelfosine, 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine; FLIPR, fluorometric imaging plate reader; HPC, hexadecylphoshorylcholine; IL1β, interleukin-1β; LDH, lactate dehydrogenase; LysoPC C12:0, lauroyl L-α-lysophosphatidylcholine; LysoPC C14:0, myristoyl L-α-lysophosphatidylcholine; LysoPC C16:0, palmitoyl L-α-lysophosphatidylcholine; LysoPC C18:0, stearoyl L-α-lysophosphatidylcholine; LysoPE, L-α-lysophosphatidylethanolamine; LysoPI, L-α-lysophosphatidylinositol; LysoPS, oleoyl L-α-lysophosphatidylserine; 2MeSATP, 2-methylthio-ATP; PAF, platelet-activating factor; PC, L-α-phosphatidylcholine; PLA_2, phospholipase A_2; PS, L-α-phosphatidylserine; SPAP, secreted placental alkaline phosphatase; SPC, sphingosylphosphorylcholine

Introduction

The P2X receptors are a family of ligand-gated cation channels gated by extracellular ATP. The seven family members identified to date can assemble in various homomeric and heteromeric combinations to form cation channels permeable to calcium, sodium and potassium ions (North, 2002). Activation of several P2X receptor types also leads to changes in the ionic permeability of the channel and result in cells becoming permeable to relatively high molecular weight dyes (400–800 Da), such as ethidium bromide (ethidium). This was originally observed with the P2Z receptor, subsequently identified as being the $P2X_7$ receptor (Surprenant *et al*., 1996), and more recently with the $P2X_2$, $P2X_4$ and $P2X_5$ receptor types (Khakh *et al*., 1999; Virginio *et al*., 1999; Bo *et al*., 2003). Initially it was suggested that this reflected dilation of the P2X channel pore (Surprenant *et al*., 1996), but more recent studies suggest that, for the $P2X_7$ receptor, this may reflect coupling to the pannexin hemi-channel (Pelegrin and Surprenant, 2006).

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While all members of the P2X receptor family function as ligand-gated cation channels, the $P2X_7$ receptor appears to have more complex functional properties. Thus, it interacts with a wide range of cellular proteins (Kim *et al*., 2001) and also activates a diverse range of cellular responses including activation of phospholipase A_2 (PLA_2), phospholipase D, mitogen-activated protein kinase and nuclear factor-kappa B (North, 2002). Furthermore, $P2X_7$ receptor activation can lead to membrane blebbing, cell fusion and cause cell death by a variety of mechanisms, including apoptosis and necrosis (North, 2002). The reason why $P2X_7$ receptors are unique among the family in activating such a diverse range of cellular events is not known, but it may be a consequence of the much longer C terminus of the $P2X_7$ receptor.

One of the other notable features of the $P2X_7$ receptor is its plasticity. Several studies have shown that activation of the receptor leads to time-dependent persistent changes in its permeability, kinetics and agonist sensitivity (Surprenant *et al*., 1996; Chessell *et al*., 1997; Hibell *et al*., 2000, 2001; Chakfe *et al*., 2002). The change in kinetics, particularly the change in closure time, is accompanied by an increase in agonist potency and so the effects of activation on kinetics and potency may be linked (Hibell *et al*., 2001).

The mechanisms underlying the various forms of plasticity of the $P2X_7$ receptor have not been elucidated, although it is possible that second messenger systems activated following $P2X_7$ receptor activation may be involved. Thus, activation of $P2X_7$ receptors can lead to tyrosine phosphorylation and this can affect agonist potency (Adinolfi *et al*., 2003). Furthermore, $P2X_7$ receptor activation can lead to activation of PLA_2 (Alzola *et al*., 1998) which can generate both arachidonic acid (AA) and lysolipids such as lysophosphatidylcholine (LysoPC). In this respect, AA (Alloisio *et al*., 2006) and LysoPC (Takenouchi *et al*., 2007) have recently been shown to increase agonist potency or effects at $P2X_7$ receptors.

In this study we initially investigated the effects of LysoPC, and other lysolipid products of PLA_2, on $P2X_7$ receptors and found that these agents could enhance $P2X_7$ receptor function. To determine if this effect was specific to LysoPC, or could be mimicked by other lipids that affect membrane properties such as fluidity or elasticity, we evaluated a range of other lipids including alkylphosphocholines, palmitoyl carnitine and Triton X-100. We found that all of these amphiphilic agents could increase $P2X_7$ receptor function, suggesting that function of this receptor may be sensitive to membrane environment and raising the possibility that $P2X_7$ receptor function may be modulated by membrane lipids *in vivo*.

Methods

Ethidium accumulation in HEK293 cells expressing recombinant $P2X_7$ receptors

Studies were performed as described previously (Michel *et al*., 2006). HEK293 cells expressing rat, mouse or human recombinant $P2X_7$ receptors were grown in poly-L-lysine-coated, 96-well plates (Biocoat, Becton Dickinson, Oxford, UK) for 18–24 h. The assay buffer comprised (in mM): HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid)) 10, *N*-methyl-D-glucamine 5, KCl 5.6, D-glucose 10, $CaCl_2$ 0.5 (pH 7.4) and was supplemented with either 280 mM sucrose (sucrose buffer) or 140 mM NaCl (NaCl buffer). In some studies, the assay buffer was further supplemented with 0.1% bovine serum albumin (BSA) or 1 mM $MgCl_2$ and 1 mM $CaCl_2$.

Cells were washed by aspiration and addition of 350 μl of assay buffer. This was aspirated and 50 μl of assay buffer containing the lipids was added. After 20 min incubation at room temperature, 50 μl of agonist (various concentrations) and ethidium (100 μM final assay concentration) was added. In some studies, agonist was omitted in order to study the effects of lipids on basal accumulation of ethidium. Incubations were continued for various times until approximately 10–40% of the maximal attainable level of ethidium accumulation had occurred. In sucrose buffer, the incubation times were 2, 2 and 10 min when studying rat, human and mouse receptors, respectively. In NaCl buffer, incubation times with agonist were 8 and 16 min when studying rat and human receptors, respectively. Reactions were rapidly terminated by addition of 25 μl of 1.3 M sucrose assay buffer containing 5 mM Reactive black 5. Cellular accumulation of ethidium was determined by measuring fluorescence from below the plate with a FlexStation (Molecular Devices, Wokingham, UK) using an excitation wavelength of 530 nm and emission wavelength of 620 nm.

Effect of lipids on lactate dehydrogenase release from cells

To measure the effect of lipids on cell integrity, the release of lactate dehydrogenase (LDH) from cells was measured. For studies on HEK293 cells, the method for measuring ethidium accumulation in NaCl buffer described above was used except that ethidium and ATP were omitted and the incubation medium from the wells was transferred to a V-bottomed 96-well plate at the end of the incubation period. This was centrifuged at 250 *g* for 5 min to eliminate any contaminating cells, and LDH content in the supernatant was determined using a Promega Cytotox96 kit (Promega, Southampton, UK) according to the manufacturer's instructions with LDH content being measured from a standard calibration curve. For studies on THP-1 cells, the incubations were performed as described for measuring interleukin-1β (IL1β) release (see below) except that 96-well V-bottomed plates were used and reactions were terminated by addition of ice-cold buffer containing 5 mM $MgCl_2$. Plates were centrifuged at 250 *g* for 5 min and LDH in the supernatant was measured as described for the HEK293 cells.

Radioactive calcium accumulation in HEK293 cells expressing rat $P2X_2$ or mouse $P2X_4$ receptors

HEK293 cells expressing rat recombinant $P2X_2$ or mouse recombinant $P2X_4$ receptors were grown in poly-L-lysine-coated CytoStar T plates (Perkin Elmer, Beaconsfield, UK) for 18–24 h. Studies were performed using sucrose assay buffer. Cells were washed with 350 μl of assay buffer before addition of 50 μl of either assay buffer or lipid solutions and the cells were incubated at room temperature for 20 min before

adding 50 μl of buffer containing 3.7 kBq of radioactive $CaCl_2$ (^{45}Ca) and ATP. Incubations were continued for 4 min and were terminated by aspiration and addition of 350 μl of assay buffer containing 80 mM $CaCl_2$. This solution was aspirated and a further 350 μl of the same buffer was added and aspirated. Cellular accumulation of ^{45}Ca was determined by measuring the radioactivity retained on the plates using a Perkin Elmer TopCount (Perkin Elmer).

Measurement of intracellular calcium using a fluorometric imaging plate reader

HEK293 cells stably expressing the human or rat $P2X_7$ receptor were plated at 30 000 cells per well in black-walled, clear-bottomed, 96-well plates (Costar, High Wycombe, UK) 24 h before use, and incubated under 5% CO_2 at 37°C. Cells were loaded with the calcium-sensitive fluorescent dye Fluo-4AM (2 μM) for 2 h at room temperature and then washed four times in Tyrodes buffer (mM: NaCl 145, KCl 2.5, HEPES 10, D-glucose 10, $CaCl_2$ 0.5, pH 7.4). Thereafter, lysolipids were added to the plates and changes in intracellular calcium were monitored on a fluorometric imaging plate reader (FLIPR) (Molecular Devices) by measuring cell-associated fluorescence (excitation wavelength of 488 nm and emission wavelength of 540 nM).

Radioligand binding studies on human recombinant $P2X_7$ receptors

The binding assays were conducted using tritium-labelled *N*-[2-({2-[(2-hydroxyethyl)amino]ethyl}amino)-5-quinolinyl]-2-tricyclo[3.3.1.1^{3,7}]dec-1-ylacetamide (compound-17), as described previously (Michel *et al*., 2007). Briefly membranes obtained from HEK293 cells expressing the human $P2X_7$ receptor were incubated with [^{3}H]compound-17 (2 nM) and other additions in polypropylene tubes in a final volume of 200 μl of 50 mM Tris HCl, 0.01% BSA assay buffer, pH 7.4, at room temperature. Non-specific binding was defined using 10 μM unlabelled compound-17. Reactions were conducted at room temperature for the indicated times before separating bound and free radioligand by vacuum filtration, using a Brandell cell harvester, through 96-well GF/B glass fibre filters (Perkin Elmer) pretreated with 0.3% polyethyleneimine before use. Filters were washed with 3 × 2 ml aliquots of ice-cold water containing 10 mM $CaCl_2$. Filters were dried, 50 μl of microscint O (Perkin Elmer) was added and radioactivity bound was determined using a Perkin Elmer Topcount scintillation counter. The c.p.m. values from the Topcount were converted to d.p.m. values using an externally generated quench curve. In some studies, the assay buffer was supplemented with 0.1% BSA, 140 mM NaCl or 1 mM $CaCl_2$ and 1 mM $MgCl_2$.

P2X agonist-stimulated interleukin-1β release from lipopolysaccharide-treated THP-1 cells

Studies were performed as described previously (Buell *et al*., 1998). Briefly, THP-1 cells were pretreated for 18 h with 10 μg ml^{-1} of lipopolysaccharide. Cells were harvested, resuspended in NaCl buffer containing 0.1 mM $CaCl_2$, warmed to 37°C and 50 μl (150 000 cells) was added to each well of 96-well plates containing 25 μl of lipids and either 25 μl ATP, 2′- and 3′-*O*-(4benzoylbenzoyl) ATP (BzATP) or nigericin at 37°C. Unless indicated, reactions were routinely stopped after 30 min incubation at 37°C by addition of 100 μl of ice-cold Dulbecco's modified Eagle's medium (DMEM) media containing 10% fetal bovine serum. The cells were centrifuged at 150 *g* for 5 min and 15 μl of the supernatant was removed for determination of mature IL1β content using an A549 cell bioassay that only detects mature, biologically active, IL1β (Buell *et al*., 1998). The assay uses genetically modified A549 cells containing a recombinant secreted placental alkaline phosphatase (SPAP) transgene that is activated by endogenous IL1β receptors present in the A549 cells. Confluent flasks of A549 cells were harvested by incubation in Trypsin-Versene solution for 10 min, centrifuged at 250 *g* for 5 min, resuspended in DMEM media containing 10% fetal bovine serum at a cell density of 1×10^6 cells ml^{-1} and 100 μl of cells added to 96-well tissue culture plates containing THP-1 cell supernatants or IL1β standards. The cells were incubated at 37°C overnight in a humidified atmosphere (95% air: 5% CO_2), incubated at 60°C for 20 min to inactivate endogenous phosphatase activity and cooled to room temperature. SPAP activity was measured by addition of 150 μl of 1 M diethanolamine, 280 mM NaCl, 5 mM $MgCl_2$ buffer (pH 9.5) containing 10 mM para-nitro phenylphosphate, and the rate of change in absorbance at 405 nm was measured in a 96-well plate spectrophotometer for 2 min. The SPAP activity in unknown samples was converted to absolute units of IL1β from a calibration curve generated using recombinant human IL1β.

For studies using ATP as agonist, the THP-1 cell supernatants were incubated with 20 μl of apyrase (1 U ml^{-1}) for 10 min before addition of A549 cells in order to eliminate ATP from the sample. IL1β levels were determined from a calibration curve constructed using human recombinant IL1β. In some studies, the cell supernatants were incubated with 500 ng ml^{-1} of a neutralizing antibody to IL1β to confirm that activity in the bioassay was due the presence of IL1β.

Data analysis

For ethidium accumulation studies, the data were normalized to the basal ethidium accumulation measured in the absence of agonist and lipid (0%) and the maximal agonist stimulated ethidium accumulation measured in the absence of lipid (100%). In studies to compare effects of agonists (Figure 3), the data were normalized to the maximal response to ATP obtained in the absence of LysoPC. In these studies, the maximal agonist stimulated ethidium accumulation was at least fivefold, and usually 10–30 fold, the basal level of ethidium accumulation.

In studies on cell lysis, the LDH release was expressed as a percentage of the release produced by 0.9% Triton X-100. Basal LDH release was not subtracted from the data in the normalization calculations. In studies measuring changes in intracellular calcium, the data are presented in terms of arbitrary fluorescence units and were not converted to absolute calcium concentrations. As an internal control, the response to 100 μM carbachol was also measured as this produced comparable responses to those achieved by activation of the $P2X_7$ receptors in these cell lines.

For radioligand binding studies, specific binding was calculated as the difference between total and non-specific binding. When presenting the effect of lipids, the data were normalized to the specific binding measured in the absence of lipids in order to enable lipid effects on specific binding to be illustrated.

For studies measuring IL1β release from THP-1 cells, data were expressed as a percentage of the maximal BzATP, ATP or nigericin-stimulated IL1β release measured in the absence of lipids or in terms of the absolute amount of IL1β released. Basal IL1β release from THP-1 cells was not subtracted from the data in the normalization calculations.

All data were analysed using GraphPad Prism version 3.0 (GraphPad Software Inc., San Diego, USA). For testing significance of effects, a one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test was used.

Materials

Unless stated, all studies with LysoPC were performed using the palmitoyl (C16:0) form. Lauroyl L-α-lysophosphatidylcholine (LysoPC C12:0), myristoyl L-α-lysophosphatidylcholine (LysoPC C14:0), palmitoyl L-α-lysophosphatidylcholine (LysoPC C16:0), stearoyl L-α-lysophosphatidylcholine (LysoPC C18:0), hexadecylphoshorylcholine (HPC), 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (Eoelfosine), sphingosylphosphorylcholine (SPC), palmitoyl-DL-carnitine (palmitoyl carnitine), platelet-activating factor (PAF) and lyso-PAF were dissolved in 50% ethanol/water at 10 or 30 mM. L-α-lysophosphatidylethanolamine (LysoPE, lysocephalin), oleoyl L-α-lysophosphatidylserine (LysoPS, C18:1, sodium salt), L-α-lysophosphatidylinositol (LysoPI, from liver, sodium salt), L-α-phosphatidylcholine (PC) and L-α-phosphatidylserine (PS) were dissolved in assay buffer at 1 mM. PC, PS and LysoPE were sonicated to disperse the lipid. ATP, $\alpha\beta$-methylene-ATP ($\alpha\beta$-meATP), $\beta\gamma$-methylene-ATP ($\beta\gamma$-meATP), adenosine-5′-*O*-(3-thiotriphosphate) (ATPγS), ADP, BzATP, 2-methylthio-ATP (2MeSATP) and UTP were dissolved in water or assay buffer at 10 mM. Nigericin was dissolved in ethanol (100%) at 10 mM.

Apyrase (grade VII), BSA, carbachol, ionomycin, all nucleotides, LysoPC (all forms), LysoPE, lipopolysaccharide, nigericin, PC, PS, palmitoyl carnitine, phosphorylcholine, saponin, SPC and Triton X-100 were obtained from Sigma (Poole, UK). Fluo4-AM was obtained from Invitrogen (Paisley, UK). LysoPS and LysoPI were obtained from Avanti Polar lipids (Instruchemie BV, Delfzyl, The Netherlands). HPC and eoelfosine were obtained from Alexis Chemicals (Nottingham, UK). Human recombinant IL1β and the neutralizing IL1β antibody were obtained from R&D Systems (Abingdon, Oxon, UK). Compound-17 and [^{3}H]compound-17 were obtained as described previously (Michel *et al*., 2007).

Results

LysoPC enhances recombinant P2X$_7$ receptor-mediated cellular ethidium bromide accumulation

Initially we studied the effects of LysoPC on P2X$_7$ receptor-stimulated ethidium accumulation in HEK293 cells expressing recombinant P2X$_7$ receptors. Since it is not possible to measure this response in NaCl-containing solutions for all species orthologues, we evaluated the effects of LysoPC in both sucrose and NaCl-containing buffers to enable comparisons between species.

LysoPC produced a concentration-dependent increase in BzATP potency at human P2X$_7$ receptors in either sucrose or NaCl buffer (Figures 1a and b). LysoPC also produced a concentration-dependent increase in ATP potency in both sucrose (data not shown) and NaCl (Figure 1c) assay buffer. The effects of LysoPC were observed at concentrations (10 and 30 μM) which do not cause cell lysis (see below) and were mainly manifested as an increase in agonist potency. In all studies, LysoPC (100 μM) produced even more marked increases in agonist effects (for example, see Figures 1b and c). However, in several studies, this was also accompanied by a significant increase in basal ethidium accumulation and so in those studies the effects of 100 μM LysoPC was not presented (for example, Figures 1a and b).

LysoPC C14:0, LysoPC C18:0 and LysoPC C18:1 (10 and 30 μM) increased responses to ATP to the same extent as LysoPC C16:0 but LysoPC C12:0 had no significant effect at 10 or 30 μM (data not shown). All further studies with LysoPC focused on the use of the C16:0 form.

The effects of LysoPC were not restricted to the human P2X$_7$ receptor as LysoPC increased ATP potency at mouse P2X$_7$ receptors when studied in sucrose buffer (Figure 1d) and also increased ATP responses in cells expressing rat P2X$_7$ receptors (see below).

Effect of other lipids on P2X$_7$ receptor-stimulated ethidium accumulation

To investigate the effect of other lipids on human P2X$_7$ receptors, we examined their ability to enhance responses to a sub-maximal concentration of ATP (Figures 2a and b). Among the lysolipids, LysoPS (Figure 2a) and LysoPI (data not shown) produced a similar enhancement of ATP effect as LysoPC, but LysoPE had no significant effect at 30 μM (Figure 2a). Other phosphocholine-containing lipids such as HPC, SPC and palmitoyl carnitine (Figure 2a) as well PAF, lyso PAF and eoelfosine (data not shown) also increased responses to ATP when present at concentrations of 10–30 μM. Additional studies showed that HPC, SPC and LysoPS (10 and 30 μM) increased ATP potency and produced similar effects to LysoPC (Figure 1) in both sucrose and NaCl buffer (data not shown).

Similar to LysoPC, most of the other lipids increased basal ethidium accumulation by 20–40% at a concentration of 100 μM (data not shown) and so the maximal concentration of lipid presented in these studies was 30 μM. SPC (1–30 μM) produced a small decrease (2–3%) in basal ethidium accumulation while LysoPS and HPC produced a small increase (5–10%) in basal ethidium accumulation at 10–30 μM (Figure 2b). In contrast, LysoPS, HPC, SPC and palmitoyl carnitine increased the response to a threshold concentration of ATP (0.5 mM) from approximately 4–5% of the control ATP maximal response to 40–250% of the control ATP maximal response (Figure 2a). PC and PS (1–100 μM), low concentrations of choline (1–100 μM) and phosphoryl-

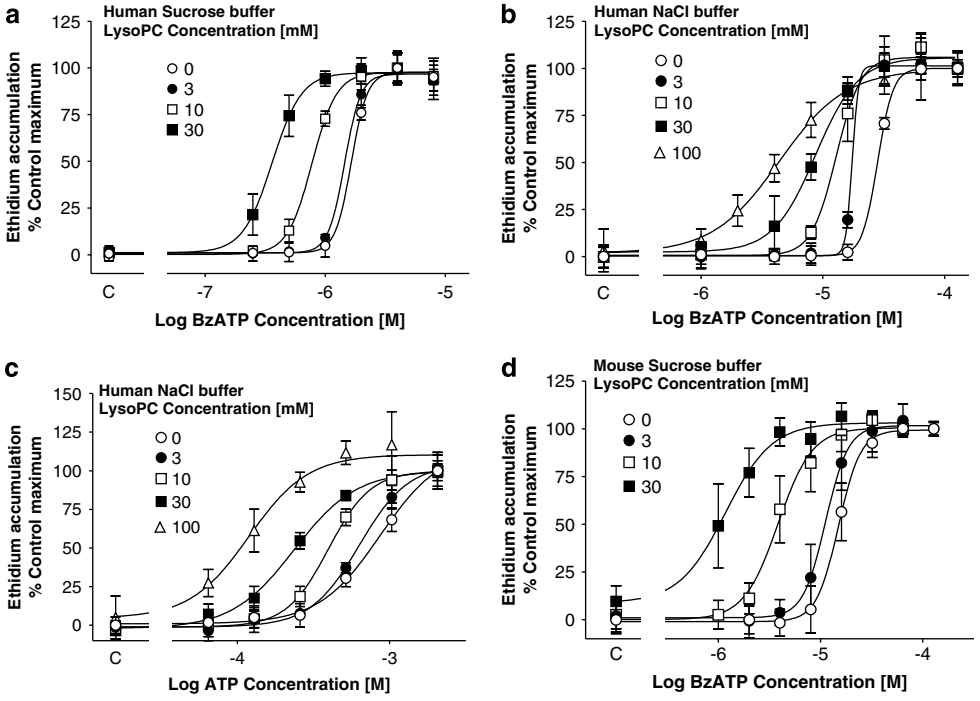

Figure 1 The effect of LysoPC on P2X$_7$ receptor-stimulated ethidium accumulation in HEK293 cells expressing human or mouse recombinant P2X$_7$ receptors. Cells were pre-incubated with LysoPC for 20 min at room temperature before adding agonist and ethidium. Cellular ethidium accumulation was measured in a fluorescence plate reader after a further incubation for 2, 8, 8 or 32 min in (**a**), (**b**), (**c**) and (**d**), respectively. (**a**) Effect of LysoPC on BzATP-stimulated ethidium accumulation in cells expressing human P2X$_7$ receptors when using sucrose assay buffer. (**b**) Effect of LysoPC on BzATP-stimulated ethidium accumulation in cells expressing human P2X$_7$ receptors when using NaCl assay buffer. (**c**) Effect of LysoPC on ATP-stimulated ethidium accumulation in cells expressing human P2X$_7$ receptors when using NaCl assay buffer. (**d**) Effect of LysoPC on BzATP-stimulated ethidium accumulation in cells expressing mouse P2X$_7$ receptors when using sucrose assay buffer. In each figure, the data are expressed relative to the maximal agonist stimulated ethidium accumulation in the absence of LysoPC. For the normalization, the 0% value was that measured in the absence of agonist and LysoPC. Control values (*C*) obtained in the absence of agonist are shown on the abscissa. The data are the mean ± s.e.m. of three to four experiments. LysoPC, lysophosphatidylcholine; BzATP, 2′- and 3′-*O*-(4benzoylbenzoyl) ATP.

choline (1 μM–1 mM) had no effect on agonist potency at human P2X$_7$ receptors (data not shown).

HPC and SPC (10–30 μM) also increased responses to BzATP at mouse P2X$_7$ receptors (data not shown). Furthermore, LysoPC, LysoPI, LysoPS (Figure 2c) as well as HPC and SPC (data not shown) increased responses to ATP at rat P2X$_7$ receptors at concentrations of 10–100 μM. These lipids significantly increased basal ethidium accumulation in cells expressing rat P2X$_7$ receptors at 100 μM but the increase (approximately 10% of the maximal response to ATP) was less marked than in cells expressing the human P2X$_7$ receptor (approximately 20–40% of the control ATP maximal response).

All of the lipids that increased P2X$_7$ receptor-mediated responses were amphiphiles. To explore if simpler amphiphilic compounds could also affect P2X$_7$ receptor function, we examined the effect of Triton X-100. Concentrations of Triton X-100 (0.00225 and 0.00675% v v^{-1}) that did not cause cell lysis (see below) increased ATP potency at both human (data not shown) and rat (Figure 2d) P2X$_7$ receptors. At a concentration of 0.0225% v v^{-1} Triton X-100 caused appreciable cell lysis (see below) and there was no detectable agonist-stimulated ethidium accumulation in the presence of this concentration of Triton X-100 (Figure 2d). The reduction in maximal ethidium accumulation produced by Triton X-100 most likely reflects its ability to dislodge cells from the surface of the 96-well plates and to lose their cell contents as the assay only detects ethidium accumulation in adherent cells that still contain their cell contents. Saponin (data not shown) had no effect on ATP potency at concentrations above or below the concentration that caused maximal cell lysis (0.0165 mg ml^{-1}, see below).

In view of the marked increase in intracellular calcium produced by LPC and SPC (Figure 5), the effect on agonist potency of other agents such as uridine triphosphate (UTP) and ionomycin, which increase intracellular calcium, was explored. However, neither UTP (100 μM) nor ionomycin (1–10 μM) had any affect on ATP- or BzATP-stimulated ethidium accumulation (data not shown).

Modulation by LysoPC of P2 agonist potency at recombinant P2X$_7$ receptors

LysoPC also increased the potency of other P2 agonists to stimulate ethidium accumulation in cells expressing human recombinant P2X$_7$ receptors (Figure 3). Thus, 2MeSATP and ATPγS (128 μM) produced modest responses on their own while $\alpha\beta$-meATP, $\beta\gamma$-meATP and ADP were without effect at concentrations up to 128 μM. However, following a 20 min pre-incubation with LysoPC (30 μM), all agonist effects were

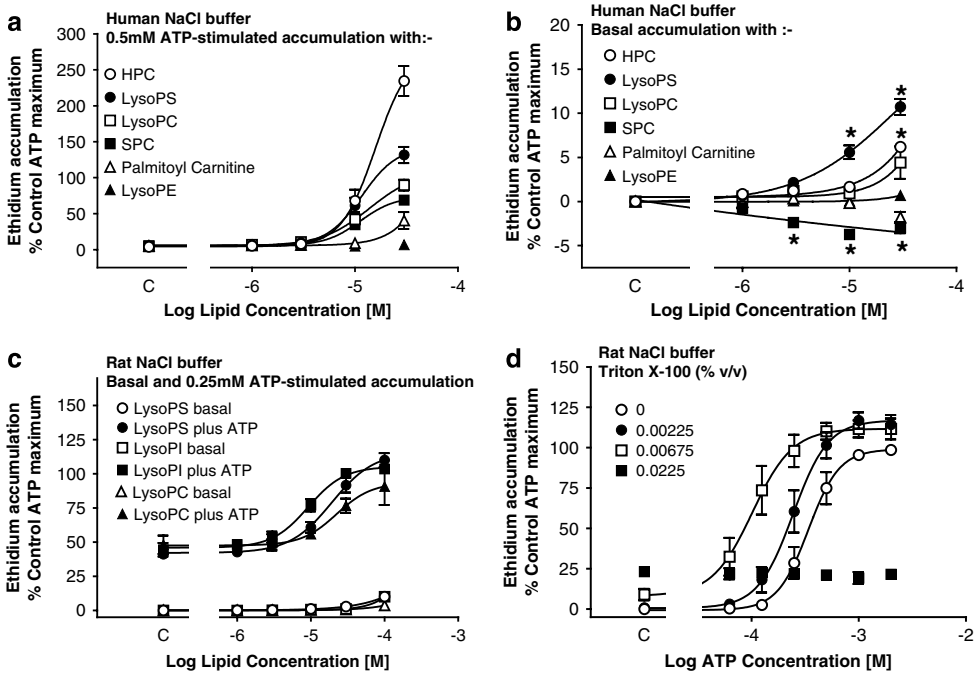

Figure 2 The effect of lipids on ATP-stimulated ethidium accumulation in HEK293 cells expressing human or rat recombinant P2X$_7$ receptors. Cells were pre-incubated with the indicated lipids for 20 min at room temperature before adding ethidium or agonist and ethidium. Cellular ethidium accumulation was measured in a fluorescence plate reader after 8 (**a** and **b**) or 4 (**c** and **d**) min. Studies were performed at room temperature in NaCl buffer. (**a**) Effect of lipids on 0.5 mM ATP-stimulated ethidium accumulation in cells expressing human P2X$_7$ receptors. (**b**) Effect of lipids on basal ethidium accumulation in cells expressing human P2X$_7$ receptors. *$P<0.05$ vs basal ethidium accumulation, one-way ANOVA followed by Dunnett's *post hoc* test. (**c**) Effect of lipids on basal and 0.25 mM ATP-stimulated ethidium accumulation in cells expressing rat P2X$_7$ receptors. (**d**) Effect of Triton X-100 on ATP-stimulated ethidium accumulation in cells expressing rat P2X$_7$ receptors. In each figure, the data are expressed relative to the maximal ethidium accumulation produced by 8 mM ATP (human P2X$_7$ receptor) or 2 mM ATP (rat P2X$_7$ receptor) in the absence of LysoPC. For the normalization, the 0% value was that measured in the absence of agonist and LysoPC. Control values (*C*) obtained in the absence of lipid or ATP are shown on the abscissa. The data are the mean$\pm$s.e.m. of three to four experiments. ANOVA, one-way analysis of variance; LysoPC, lysophosphatidylcholine.

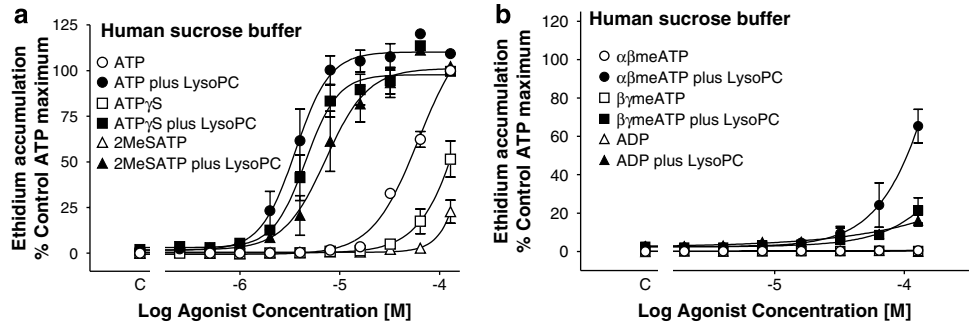

Figure 3 The effect of 30 μM LysoPC on agonist-stimulated ethidium accumulation in HEK293 cells expressing human recombinant P2X$_7$ receptors. Cells were pre-incubated in the absence or presence of 30 μM LysoPC for 20 min and then concentration–effect curves to the indicated agonists were determined in the continued absence (open symbols) or continued presence of 30 μM LysoPC (filled symbols). Studies were performed at room temperature in sucrose assay buffer. The data are expressed relative to the maximum ATP-stimulated ethidium accumulation in the absence of LysoPC. The 0% value represents ethidium accumulation in the absence of agonist and LysoPC. The data are the mean$\pm$s.e.m. of three experiments. Control values (*C*) obtained in the absence of agonist are shown on the abscissa. LysoPC, lysophosphatidylcholine.

potentiated. Indeed, in the presence of 30 μM LysoPC, ATPγS and 2MeSATP produced maximal responses comparable to ATP and even $\alpha\beta$-meATP and $\beta\gamma$-meATP produced detectable responses although we did not use high enough concentrations to determine if they could produce maximal effects. ADP also produced a small response in the presence of LysoPC but appeared to be a partial agonist. Note that these studies were conducted using a sucrose assay buffer as this enabled detection of the effects of the low potency agonists. The rank order of agonist potency in the absence of LysoPC was ATP > ATPγS > 2MeSATP. In the presence of LysoPC, the rank order was ATP > ATPγS > 2MeSATP $\gg$ $\alpha\beta$-meATP > $\beta\gamma$-meATP, although the differences between ATP, ATPγS and 2MeSATP became less pronounced.

Effect of lipids on cell lysis (LDH release)

Many of the lipids can affect cell integrity so their effect on LDH release in the HEK293 cells used in this study was examined. HPC, SPC, LysoPS, LysoPI, LysoPC had no significant effect on cell lysis in cells expressing rat or human recombinant $P2X_7$ receptors at concentrations of 1–30 μM (data not shown). However, at 100 μM, all of these lipids caused cell lysis to varying extents. Lysis was more marked in cells expressing the human $P2X_7$ receptor where the basal release of LDH was 9.8 ± 2.2% of total cell LDH and this increased ($P<0.05$, one-way ANOVA followed by Dunnett's *post hoc* test) to 21 ± 3.5, 28 ± 5.0, 28.3 ± 5.9, 52.4 ± 10.6 and 54.7 ± 8.3% in the presence of 100 μM LysoPS, LysoPC, LysoPI, SPC and HPC, respectively. In cells expressing rat $P2X_7$ receptors, the basal release of LDH was 5.3 ± 0.6% of total cell LDH and this increased ($P<0.05$, one-way ANOVA followed by Dunnett's *post hoc* test) to 12.5 ± 1.5, 14.3 ± 0.9, 13.6 ± 1.9, 16.6 ± 1.2 and 19.7 ± 1.5% in the presence of 100 μM LysoPS, LysoPC, LysoPI, SPC and HPC, respectively. The concentration–effect curve for cell lysis caused by Triton X-100 and saponin was very steep. In cells expressing rat $P2X_7$ receptors, the basal LDH release of 5.2 ± 0.7% of total cell LDH was not affected by 0.00675% Triton X-100 (5.9 ± 0.8%), but 0.0225% Triton X-100 caused almost maximal LDH release (86.7 ± 1.4%, $P<0.05$, one-way ANOVA followed by Dunnett's *post hoc* test). Similarly, the basal LDH release of 5.9 ± 0.8% of total cell LDH was not affected by 0.0055 mg ml^{-1} saponin (7.2 ± 1.1) but 0.0165 mg ml^{-1} saponin caused almost maximal LDH release (75.8 ± 7.1, $P<0.05$, one-way ANOVA followed by Dunnett's *post hoc* test). Similar data were obtained in cells expressing human $P2X_7$ receptors (data not shown).

Effect of SPC on $P2X_2$ and $P2X_4$ receptor-mediated ^{45}Ca accumulation

To provide some insight into the selectivity of the lipid actions on $P2X_7$ receptor-mediated responses, the effect of SPC was examined on ATP-stimulated ^{45}Ca accumulation in cells expressing $P2X_2$ and $P2X_4$ receptors. SPC was used for these studies as it produced less effect on intracellular calcium than LysoPC or HPC (see below). SPC (10 and 30 μM) produced an inhibition of ATP-stimulated ^{45}Ca accumulation in cells expressing the rat $P2X_2$ receptor (Figure 4a), but had no effect at concentrations of 1–30 μM in cells expressing mouse $P2X_4$ receptors (Figure 4b).

Effect of lipids on intracellular calcium in HEK293 cells

Several of the lipids have been shown to increase intracellular calcium (see Discussion). To determine if this also occurred in our studies, we measured lipid-induced changes in intracellular calcium in cells expressing human and rat $P2X_7$ receptors using an FLIPR. LysoPC and SPC (10 μM) produced a slow increase in intracellular calcium in HEK293 cells expressing either human recombinant $P2X_7$ receptors (Figures 5a and b) or rat recombinant $P2X_7$ receptors (Figures 5c and d). At 30 μM, both lipids produced an initial transient increase in intracellular calcium which was followed by a sustained pronounced increase in intracellular calcium. HPC produced comparable effects to LysoPC (data not shown) while the effects of LysoPC were more pronounced than those of SPC. These effects were probably not mediated by activation of $P2X_7$ receptors as they were not affected by the $P2X_7$ receptor antagonist decavanadate (30 μM) and were also observed in wild-type HEK293 cells (data not shown). In comparison to the effects of the lipids, carbachol (100 μM) produced a similar magnitude response to 30 μM SPC, but the response was transient and returned to baseline within 180 s (data not shown). There were some differences between the effects of the lipids in cells expressing human and rat $P2X_7$ receptors but this was not examined further.

Effect of LysoPC in radioligand binding studies

Since the ability of lipids to raise basal ethidium accumulation, lyse cells and increase intracellular calcium complicated interpretation of the data from the functional studies, we also examined the effects of the various lipids in radioligand binding studies. The radioligand available, [^{3}H]compound-17, only has high affinity for human $P2X_7$ receptors, so we restricted these studies to this species orthologue and focused mainly on studying the effects of LysoPC.

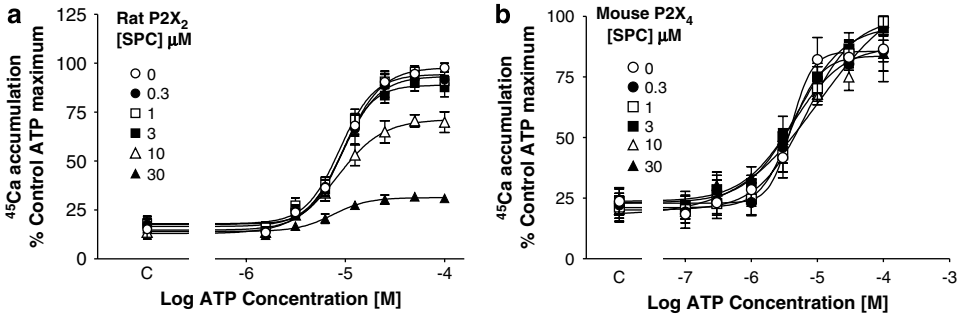

Figure 4 The effect of SPC on ATP-stimulated ^{45}Ca accumulation in HEK293 cells expressing (**a**) rat recombinant $P2X_2$ receptors or (**b**) mouse recombinant $P2X_4$ receptors. Cells were pre-incubated in the absence or presence of the indicated concentrations of SPC for 20 min and then exposed to ATP and ^{45}Ca in the absence or continued presence of SPC for a further 4 min before measuring cellular accumulation of ^{45}Ca. Studies were performed at room temperature in a sucrose assay buffer. The data are expressed relative to the maximal ATP-stimulated ^{45}Ca accumulation in the absence of SPC with the 0% value being the background radioactivity measured in the absence of ^{45}Ca. The data are the mean$\pm$s.e.m. of three experiments. Control values (*C*) obtained in the absence of ATP are shown on the abscissa. SPC, sphingosylphosphorylcholine.

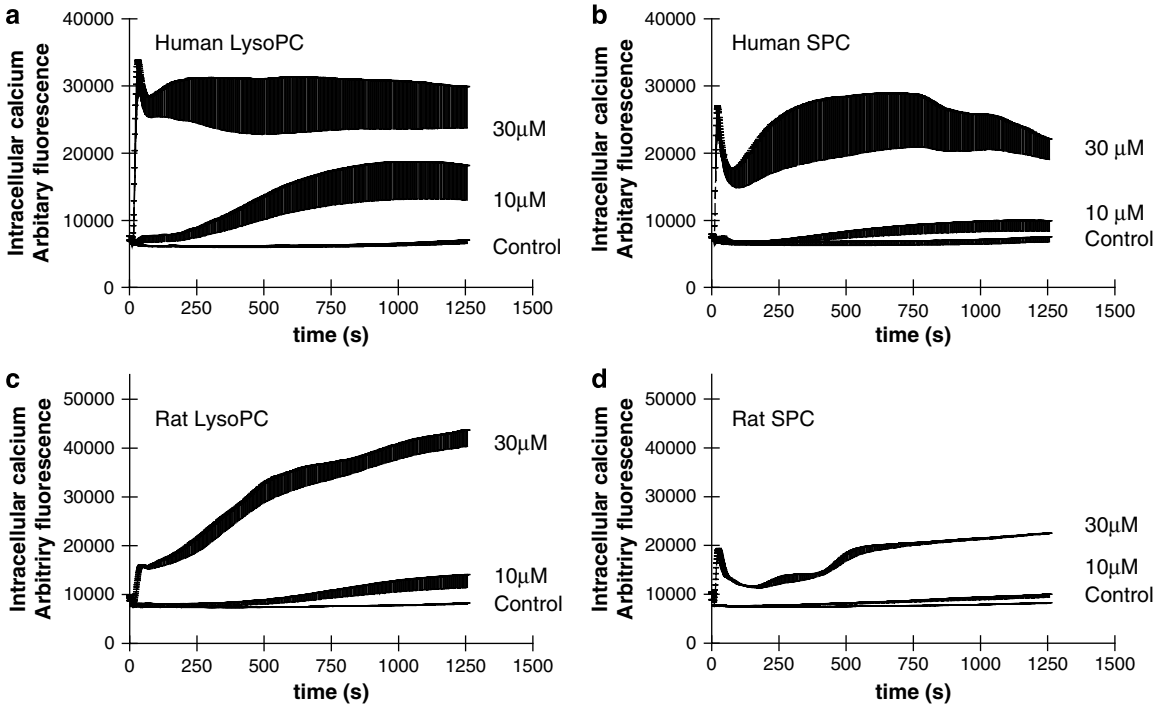

Figure 5 The effect of LysoPC and SPC on intracellular calcium in HEK293 cells expressing human recombinant P2X$_7$ receptors (**a** and **b**) or rat recombinant P2X$_7$ receptors (**c** and **d**). Cells were loaded with the calcium-sensitive dye, Fluo-4 AM. LysoPC or SPC was added and changes in Fluo-4 fluorescence were followed using an FLIPR. Studies were performed at room temperature in an NaCl-based tyrodes buffer containing 0.5 mM $CaCl_2$ and no added $MgCl_2$. The data are presented as arbitrary fluorescence units. For comparison, 100 μM carbachol produced an increase of 20 000 arbitrary fluorescence units. The data are the range from two experiments, one performed in quadruplicate and the other in sextuplicate. LysoPC, lysophosphatidylcholine; SPC, sphingosylphosphorylcholine; FLIPR, fluorometric imaging plate reader.

LysoPC enhanced the potency of ATP to inhibit the binding of [^{3}H]compound-17 to human P2X$_7$ receptors (Figure 6a). The threshold concentration of LysoPC for this effect was between 3 and 10 μM. There appeared to be a plateau of effect as 30 and 100 μM produced the same increase in ATP potency (approximately 100-fold). There was a trend for the higher concentration of LysoPC to affect control levels of radioligand binding, but this was not significant ($P>0.05$, one-way ANOVA followed by Dunnett's *post hoc* test).

The potentiation of ATP effects by LysoPC can also be illustrated by plotting its ability to enhance the inhibition of binding produced by a fixed concentration of ATP (Figure 6b). For this figure, the level of binding measured in the presence of either a threshold concentration of ATP (0.3 μM) or an approximately IC_{50} concentration of ATP (3 μM) was plotted at each concentration of LysoPC (Figure 6b). For comparison, the data were normalized, so that specific binding in the presence of ATP and absence of LysoPC was assigned a value of 100%. LysoPC produced a concentration-dependent increase in the inhibition of binding produced either by a threshold concentration of ATP (0.3 μM) or by an approximate IC_{50} concentration of ATP (3 μM) (Figure 6b). As would be expected from the concentration-dependent increases in ATP potency produced by LysoPC (Figure 6a), the effects of LysoPC appear more pronounced against the higher concentration of ATP (Figure 6b).

Among the various forms of LysoPC, the greatest effects were observed with LysoPC C16:0 and LysoPC C18:0, with lesser effects of LysoPC C14:0 and LysoPC C12:0 (Figures 7a and b). Note that for these experiments, LysoPC C16:0 was included as a standard and these data are separate to that shown in Figure 6.

Effect of other lipids in radioligand binding studies

The other lipids effective at increasing ATP or BzATP potency in functional studies produced similar effects to LysoPC in the binding studies. In particular, HPC, SPC and palmitoyl carnitine increased ATP potency at human P2X$_7$ receptors at concentrations from 10 to 100 μM (Figures 7c and d). In more detailed studies, similar to those depicted in Figure 6a, these compounds produced identical effects to LysoPC in that they did not affect control levels of specific binding but increased ATP potency in a concentration-dependent manner (data not shown).

LysoPC (30 μM) also increased the potency of BzATP as well as several other P2 agonists that, in the absence of LysoPC, had little effect on binding (Figure 8). For several P2 agonists (2MeSATP, ADP, ATPγS, $\alpha\beta$-meATP and $\beta\gamma$-meATP), LysoPC also increased the maximal inhibition of binding. The rank order of agonist potency in the absence or presence of LysoPC was BzATP > ATP > ATPγS > 2-MeSATP $\geqslant$ ADP > $\alpha\beta$-meATP $\geqslant$ $\beta\gamma$-meATP. UTP had no effect on binding in the absence of LysoPC, but in its presence produced approximately 25% inhibition of binding.

The effects of LysoPC on ATP potency in the binding studies were also observed in the presence of more physio-

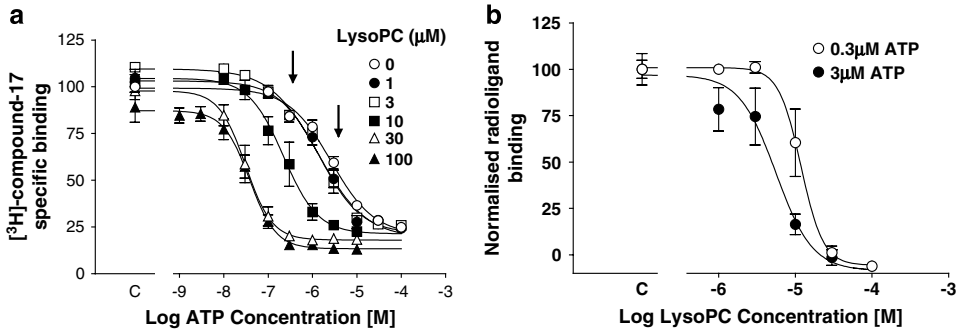

Figure 6 The effect of LysoPC on ATP inhibition of [^{3}H]compound-17 binding to membranes from human P2X$_7$ receptor-expressing HEK293 cells. All reagents were incubated for 1 h at room temperature before measuring specific binding. Non-specific binding was defined using 10 μM compound-17. (**a**) Inhibition curves to ATP measured in the absence and presence of the indicated concentrations of LysoPC. The two arrows represent the ATP concentrations that were used to prepare the data shown in (**b**). The data are expressed relative to the level of specific binding measured in the absence of lipids. Control values (*C*) obtained in the absence of ATP are shown on the abscissa. (**b**) The effect of LysoPC on ATP-inhibition of [^{3}H]compound-17 binding measured at the two ATP concentrations indicated in (**a**). For this figure, the data have been normalized with the level of binding measured in the presence of ATP and absence of LysoPC being assigned a value of 100% and the level of binding measured in the presence of both ATP and 100 μM LysoPC being assigned a value of 0%. Control values (*C*) obtained in the absence of LysoPC are shown on the abscissa. The data are the mean$\pm$s.e.m. of three experiments. LysoPC, lysophosphatidylcholine; compound-17, *N*-[2-({2-[(2-hydroxyethyl)amino]ethyl}amino)-5-quinolinyl]-2-tricyclo[3.3.1.1^{3,7}]dec-1-ylacetamide.

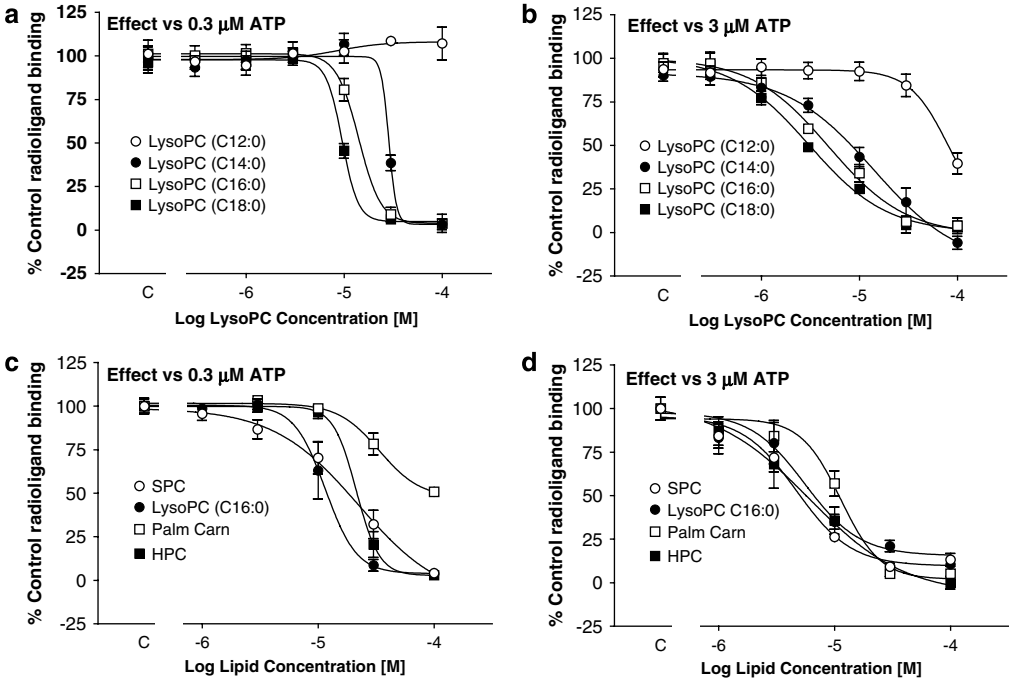

Figure 7 The ability of lipids to enhance ATP inhibition of [^{3}H]compound-17 binding to membranes from human P2X$_7$ receptor-expressing HEK293 cells. Various concentrations of the lipids were incubated with membranes, radioligand and either 0.3 μM (**a** and **c**) or 3 μM (**b** and **d**) ATP for 1 h at room temperature before measuring specific binding. Non-specific binding was defined using 10 μM compound-17. For these figures, the data have been normalized with the level of binding measured in the presence of ATP and absence of lipid being assigned a value of 100% and the level of binding measured in the presence of the indicated concentration of ATP and 100 μM LysoPC being assigned a value of 0%. Control values (*C*) obtained in the absence of LysoPC or lipid are shown on the abscissa. The data are the mean$\pm$s.e.m. of three experiments. LysoPC, lysophosphatidylcholine; compound-17, *N*-[2-({2-[(2-hydroxyethyl)amino]ethyl}amino)-5-quinolinyl]-2-tricyclo-[3.3.1.1^{3,7}]dec-1-ylacetamide.

logical relevant concentrations (1 mM) of $CaCl_2$ and $MgCl_2$ (Figure 9b) as well as in the presence of 140 mM NaCl (Figure 9c), although the ions changed the shape of the ATP inhibition curves making comparisons complicated (Figure 9a shows data from Figure 6a for comparison). Since lysolipids are known to bind to serum albumin, we evaluated the effect of BSA on the enhancement of ATP potency produced by LysoPC. BSA (0.1%) reduced the ability of 30 μM LysoPC to increase ATP potency but had no effect on the ability of 100 μM LysoPC to increase ATP potency (Figure 9d). As in previous studies (Figure 6a), there was a trend for 100 μM LPC to reduce total binding but this was not significant.

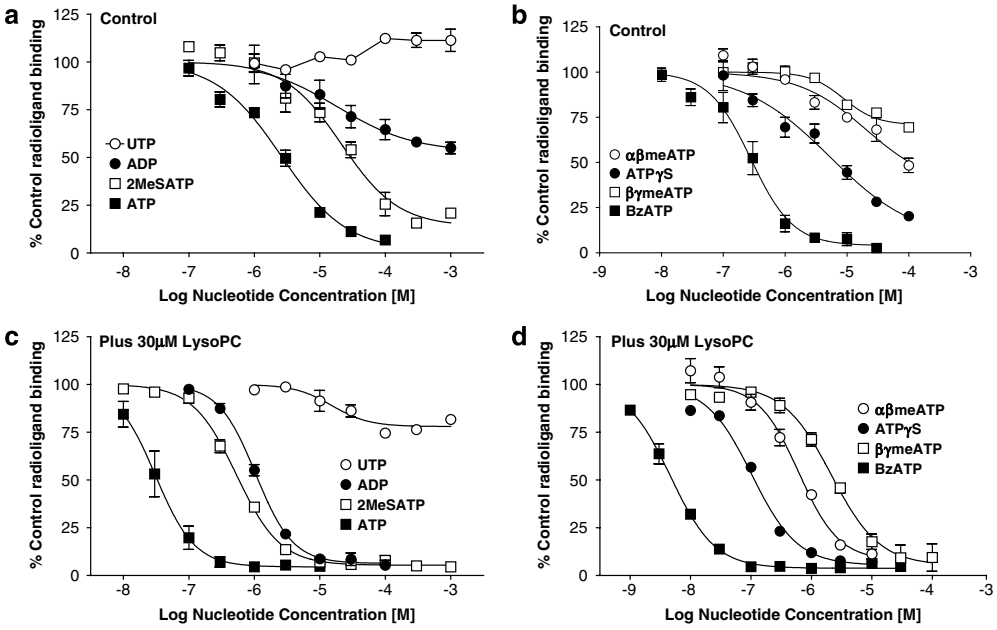

Figure 8 The effect of 30 μM LysoPC on P2 agonist inhibition of [^{3}H]compound-17 binding to membranes from human P2X$_7$ receptor-expressing HEK293 cells. Various concentrations of the P2 agonist were incubated with membranes, radioligand and either 0 (**a** and **b**) or 30 μM (**c** and **d**) LysoPC for 1 h at room temperature before measuring specific binding. NSB was defined using 10 μM compound-17. (**a**) and (**c**) show data for UTP, ADP, 2MeSATP and ATP. (**b**) and (**d**) show data for $\alpha\beta$-meATP, ATPγS, $\beta\gamma$-meATP and BzATP. The data are the mean$\pm$s.e.m. of three experiments. LysoPC, lysophosphatidylcholine; compound-17, *N*-[2-({2-[(2-hydroxyethyl)amino]ethyl}amino)-5-quinolinyl]-2-tricyclo[3.3.1.1^{3,7}]dec-1-ylacetamide; 2MeSATP, 2-methylthio-ATP; BzATP, 2′- and 3′-*O*-(4benzoylbenzoyl) ATP.

Effect of lysolipids on P2X$_7$ receptor-mediated IL1β release from THP-1 cells

To determine if the lipid effects observed at recombinant P2X$_7$ receptors could be observed at native P2X$_7$ receptors, we evaluated their effects on P2X agonist-stimulated IL1β release from human THP-1 cells, a human monocytic leukaemia cell line. Previous studies have shown that this response is mediated exclusively through activation of the P2X$_7$ receptor (Buell *et al*., 1998). Note that for these studies we co-incubated with lipid and P2X agonist rather than pre-incubating with the lipids for 20 min as the data were more reproducible in this format.

BzATP produced a more rapid and marked release of IL1β from THP-1 cells than ATP (Figure 10a). At a concentration of 10 μM, LysoPC, LysoPI, LysoPS, HPC and SPC increased BzATP potency (Figure 10b) and also increased ATP-stimulated IL1β release to levels comparable to those observed with BzATP (data not shown). These studies were performed in buffer containing low concentrations of $CaCl_2$ and no $MgCl_2$ to enable quantitative analysis of the effects of the lipids on BzATP potency. However, the lipids also enhanced responses to ATP in the presence of more physiologically relevant concentrations of $CaCl_2$ and $MgCl_2$ (both 1 mM; Figures 10c and d). Thus, LysoPC, LysoPI and LysoPS enhanced responses to 1 mM ATP 7–10-fold (Figure 10c), while SPC and HPC enhanced effects of 1 mM ATP by 20–30-fold (Figure 10d). When these studies were repeated in cells pre-treated with 1 μM compound-17, there was no response to either BzATP or ATP in the presence or absence of the lipids (data not shown).

The lipids produced even more pronounced effects at 30 μM, but at this concentration several lipids stimulated a low level of ILβ release and reduced the maximal response to BzATP (data not shown). In addition, most lipids caused cell lysis at concentrations greater than 10 μM although the extent varied between the lipids with effects of SPC and LysoPS being much less pronounced (Figure 11a). The lipids did not significantly affect nigericin-induced IL1β release (Figure 11b) except for LysoPS, which significantly enhanced the response to 3 μM nigericin ($P<0.05$, one-way ANOVA followed by Dunnett's *post hoc* test). However, this increase in IL1β was marginal and the extent of this potentiation was considerably less than observed against responses to ATP or BzATP (cf Figures 10b and 11b).

Triton X-100 caused lysis of THP-1 cells although its concentration-effect curve was very steep (Figure 11c) with no significant lysis at 0.00675% v v^{-1} but almost complete lysis at 0.0225% v v^{-1}. BzATP-stimulated IL1β release was enhanced by the sub-lytic concentration of Triton X-100 (0.00675% v v^{-1}, Figure 10d). The higher concentration of 0.0225% v v^{-1} Triton X-100, which caused cell lysis, elevated basal IL1β release slightly and BzATP did not enhance IL1β release further in its presence. The elevation in basal IL1β release produced by 0.0225% v v^{-1} Triton X-100 was not prevented by compound-17 (1 μM), suggesting that it was not P2X$_7$ receptor-mediated. However, all responses in the absence or presence of 0.00675% Triton X-100 were blocked by 1 μM compound-17 (data not shown).

In these studies, IL1β release was measured using a bioassay that only detects the mature form of IL1β. To ensure that the effects of the lysolipids were not due to release of some other agent that affected the bio-assay, we confirmed that a neutralizing monoclonal antibody against IL1β could block the reporter gene responses elicited by

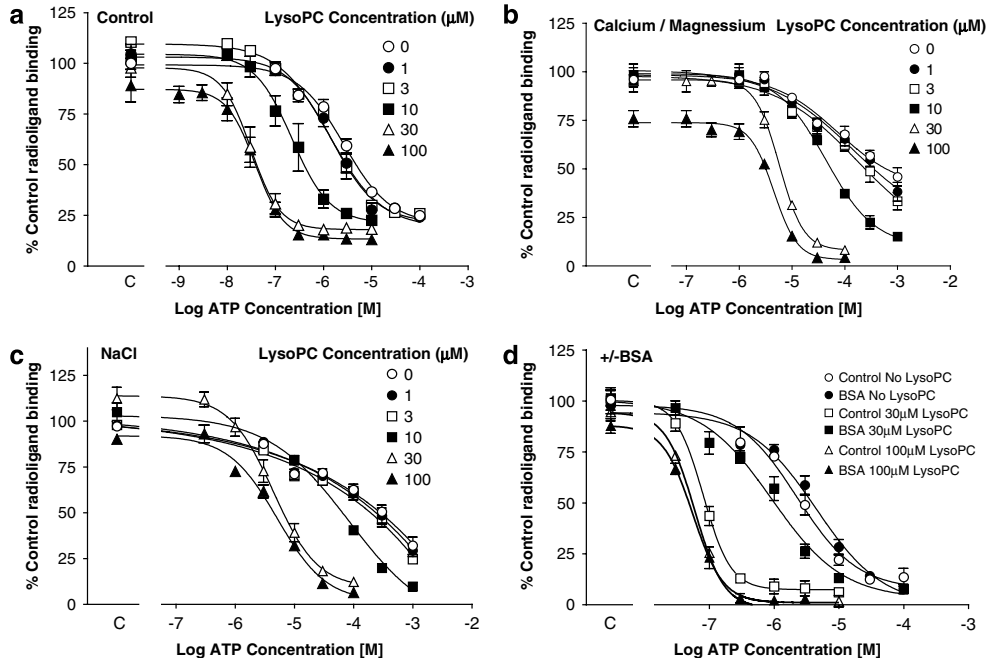

Figure 9 The effect of ions and BSA on the ability of LysoPC to enhance ATP inhibition of [^{3}H]compound-17 binding to membranes from human P2X$_7$ receptor-expressing HEK293 cells. All reagents were incubated for 1 h at room temperature before measuring specific binding. Non-specific binding was defined using 10 μM compound-17. (**a**) Inhibition curves to ATP measured in the absence or presence of the indicated concentrations of LysoPC conducted in 50 mM Tris buffer containing 0.01% BSA (data from Figure 6a). (**b**) Inhibition curves to ATP measured in the absence and presence of the indicated concentrations of LysoPC conducted in 50 mM Tris buffer containing 0.01% BSA, 1 mM $CaCl_2$ and 1 mM $MgCl_2$. (**c**) Inhibition curves to ATP measured in the absence or presence of the indicated concentrations of LysoPC in 50 mM Tris buffer containing 0.01% BSA and 140 mM NaCl. (**d**) Inhibition curves to ATP measured in the absence or presence of the indicated concentrations of LysoPC in 50 mM Tris buffer containing 0.01% BSA (control) or 0.1% BSA (BSA). In all figures, the data are expressed relative to specific binding measured in the absence of lipids. Control values (*C*) obtained in the absence of ATP are shown on the abscissa. The data are the mean$\pm$s.e.m. of 3–5 experiments. BSA, bovine serum albumin; LysoPC, lysophosphatidylcholine.

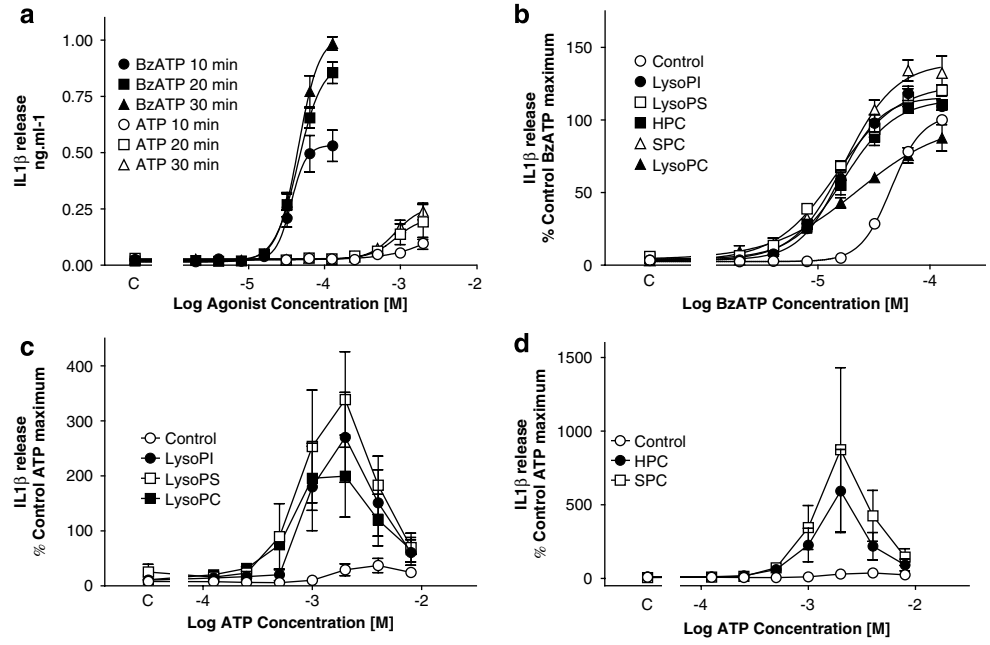

Figure 10 Effect of lipids on agonist-stimulated IL1β release from THP-1 cells. (**a**) Time course for the ability of ATP and BzATP to stimulate IL1β release in the presence of 0.1 mM $CaCl_2$. (**b**) Effect of 10 μM of the indicated lipids on BzATP-stimulated IL1β release in the presence of 0.1 mM $CaCl_2$. (**c** and **d**) Effect of 10 μM of the indicated lipids on ATP-stimulated IL1β release in the presence of 1 mM $CaCl_2$ and 1 mM $MgCl_2$. In (**a**), the data represent the absolute amounts of IL1β released. In (**b**–**d**), the data have been normalized to the maximal IL1β release obtained in the presence of agonist and absence of lipid. Basal IL1β release has not been subtracted from the normalization. Control values (*C*) obtained in the absence of ATP or BzATP are shown on the abscissa. The data are the mean$\pm$s.e.m. of three to four experiments. IL1β, interleukin-1β; BzATP, 2′- and 3′-*O*-(4benzoylbenzoyl) ATP.

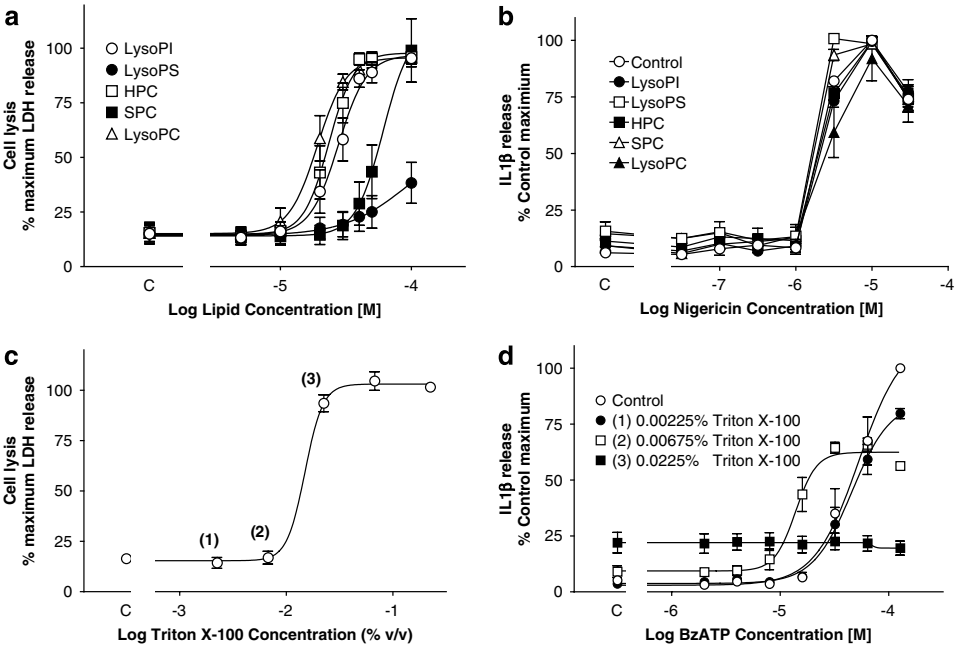

Figure 11 Effect of lipids on cell integrity and IL1β release from THP-1 cells. (**a**) Effect of lipids on LDH release from THP-1 cells. Data are expressed as a percentage of the maximal release produced by 0.9% Triton X-100. (**b**) Effect of 10 μM of the indicated lipids on nigericin-stimulated IL1β release. The data are normalized to the maximal release of IL1β produced in the absence of lipids. (**c**) Effect of Triton X-100 on LDH release from THP-1 cells. Numbers in parenthesis refer to concentrations employed in (**d**). Data are expressed as a percentage of the maximal release produced by 0.9% Triton X-100. (**d**) Effect of Triton X-100 on BzATP-stimulated IL1β release. Numbers in parenthesis refer to concentrations employed in (**c**). Data are expressed as a percentage of the maximal release produced by BzATP in the absence of Triton X-100. For each figure, the data are the mean $\pm$ s.e.m. of three to four experiments. Basal responses were not subtracted in any of the figures. Control values (*C*) obtained in the absence of lipid, nigericin, Triton X-100 or BzATP are shown on the abscissa. IL1β, interleukin-1β; LDH, lactate dehydrogenase; BzATP, 2′- and 3′-*O*-(4benzoylbenzoyl) ATP.

supernatants from cells treated with P2X$_7$ agonists and the various lysolipids (data not shown).

Discussion

The main findings of this study were that agonist potency at the P2X$_7$ receptors can be modulated by micromolar concentrations of many lipids and that this can be observed in both functional and radioligand binding studies on the recombinant P2X$_7$ receptor and in functional studies on native P2X$_7$ receptors. Since there was little structural specificity for this effect, and some of the effects could be mimicked by Triton X-100, the change in receptor function may be predominantly due to lipid-induced changes in membrane properties.

We found that a wide range of lipids could increase agonist potency or effects at human recombinant P2X$_7$ receptors in both functional and binding studies. Similar effects were observed at mouse and rat receptors in ethidium accumulation functional studies and so appear to represent a general effect on P2X$_7$ receptors. We did not examine lipid effects at all other P2X receptors types, but SPC did not enhance ATP-stimulated ^{45}Ca accumulation in cells expressing rat P2X$_2$ receptors or mouse P2X$_4$. In studies on THP-1 cells, the lipids exhibited a degree of specificity for enhancing P2X$_7$ receptor-stimulated IL1β release as they had no effect, or very little effect, on nigericin-induced IL1β release. However, it should be noted that the various lipids used in this study can affect the function of a wide range of other ion channels. LysoPC modulates TREK2 potassium channels (Lesage *et al.*, 2000), non-selective cation channels (Magishi *et al.*, 1996), sodium channels (Burnashev *et al.*, 1989), potassium channels (Kiyosue and Arita, 1986) and TRPC5 channels (Flemming *et al.*, 2006). SPC modulates ryanodine receptors (Uehara *et al.*, 1999), while palmitoyl carnitine activates calcium channels (Spedding and Mir, 1987) and increases ATP potency to inhibit Kir6.2 potassium channels (Haruna *et al.*, 2000). Lysolipids also modify gramicidin channels (Lundbaek and Andersen, 1994) and NMDA receptors (Casado and Ascher, 1998), while amphiphilic lipids such as Triton X-100 modulate γ-amino butyric acid A (GABA$_A$) receptor (Sogaard *et al.*, 2006) and sodium channel (Lundbaek *et al.*, 2004) function.

The structure activity relationship (SAR) of the lipids for increasing ATP potency at the P2X$_7$ receptor was broad as several lysolipids, SPC, palmitoyl carnitine and even synthetic alkylphosphocholines such as HPC and eoelfosine produced similar effects. The presence of a single long acyl chain in the lipid was important as neither PC nor PS affected agonist potency and, for LysoPC, effects were only observed when the acyl chain contained greater than 12–14 carbon atoms. This may suggest that membrane insertion of these lipids, with consequent disruption of the ordered structure of the lipid bilayer, is required for the lipid effects. Many of the lipids possessed a polar head group but this was not essential for activity as even Triton X-100 could affect agonist potency, albeit over a restricted concentration range.

Recently, polymyxin B (Ferrari *et al.*, 2007) has been shown to enhance $P2X_7$ receptor function and this effect also appears to be mediated via its long C16-C18 acyl chain.

In functional studies on recombinant $P2X_7$ receptors, the effects of the lipids on agonist responses were observed over quite a narrow concentration range, usually 10–30 μM, and were complicated by the ability of higher concentrations of lipids to increase basal ethidium accumulation, elevate intracellular calcium, stimulate IL1β release and cause cell lysis. However, the effects were demonstrable at sub-lytic concentrations and were also observed in radioligand binding studies so they do not appear to require cell lysis. Indeed, cell lysis very likely obscured the extent of lipid effects in the functional studies as concentrations of Triton X-100 that caused lysis inhibited $P2X_7$ receptor-mediated ethidium accumulation (Figure 2d) and IL1β release (Figure 11d). Note also that saponin caused cell lysis but did not enhance $P2X_7$ receptor-mediated responses, suggesting that these two effects are independent.

The lipids appeared to enhance responses by increasing agonist efficacy rather than affinity. Thus, in THP-1 cells, ATP was a partial agonist to stimulate IL1β release, as has also been observed in studies on P2Z receptors in human lymphocytes (Gargett *et al.*, 1997). The finding that the lipids produced a large increase in the maximal ATP-stimulated IL1β release suggests an increase in agonist efficacy rather than affinity. In the radioligand binding studies, the lipids increased ATP potency, which may suggest an effect on agonist affinity. However, it should be noted that ATP inhibits radioligand binding in an allosteric manner (Michel *et al.*, 2007) and so this could reflect an effect on affinity or efficacy. The finding that several of the agonists (ADP, $\beta\gamma$meATP) produced only partial inhibition of binding and that the lipids increased their maximal inhibition of binding would strongly suggest that they increased agonist efficacy rather than affinity. Interestingly, these functional and binding data provide a first detailed rank order of agonist potency for activating the $P2X_7$ receptor. Previously, the rank order of agonist potency at the $P2X_7$ receptor was BzATP > ATP $\geqslant$ 2MeSATP > ATPγS but this was based on data using a single concentration of agonist (Gargett *et al.*, 1997). In the present study, the rank order of agonist potency derived from the functional and binding studies was most consistent with BzATP > ATP > ATPγS > 2-MeSATP $\geqslant$ ADP $\gg$ $\alpha\beta$-meATP > $\beta\gamma$-meATP $\gg$ UTP. ADP appeared to be a partial agonist.

The mechanism by which lysolipids affected the function of the $P2X_7$ receptor is unknown. The lipids could have affected $P2X_7$ receptor function as a consequence of the multiplicity of other actions the compounds produce, through a specific interaction with the $P2X_7$ receptor or through an effect on membrane properties such as fluidity or elasticity.

Several of the lysolipids such as LysoPC, SPC and LysoPS can produce a multiplicity of cellular effects (Katz and Messineo, 1981; Meyer zu Heringdorf *et al.*, 2002) and may also activate specific G protein-coupled receptors (Xu, 2002; Sugo *et al.*, 2006) although this is controversial (Witte *et al.*, 2005; Seuwen *et al.*, 2006). Certainly, however, several of the lipids activate various intracellular enzymes (Xu, 2002) and elevate calcium in a variety of cell types (Golfman *et al.*, 1999; Jabr *et al.*, 2000), including our HEK2923 cells. However, it is unlikely that elevation of intracellular calcium contributed to the effects of the lipids in this study as neither ionomycin nor UTP increased agonist potency in ethidium accumulation studies on the recombinant $P2X_7$ receptor, yet both increase intracellular calcium in HEK293 cells (E Fonfria, unpublished observations). Furthermore, the finding that lipid effects on agonist potency could be observed in binding studies on membrane fractions would seem to rule out any lipid effects mediated through activation of cellular enzymes or changes in intracellular calcium concentration.

It also seems unlikely that any of the agents produced their effect via a specific interaction with the $P2X_7$ receptor given the very broad SAR, including the ability of Triton X-100 to mimic some of the lipid effects. All of the lipids that affected $P2X_7$ receptor function were amphiphilic (Katz and Messineo, 1981; Lundbaek and Andersen, 1994) and capable of integrating into lipid bilayers and affecting membrane properties. It has been suggested that amphiphilic lipids can modulate ion channel function by changing membrane elasticity (Lundbaek *et al.*, 2004; Sogaard *et al.*, 2006) and this may represent a plausible common mechanism of action for the effects of the lipids on the $P2X_7$ receptor. Additional studies will be needed to examine this possibility further.

The effects of the lipids on $P2X_7$ receptors only occurred at relatively high concentrations and it is unclear if these concentrations are reached *in vivo*. Certainly, total plasma LysoPC concentrations are high, being approximately 250 μM (Vuong *et al.*, 1999; Kishimoto *et al.*, 2002), and tissue concentrations of cellular LysoPC are approximately several mM (Corr *et al.*, 1982). However, the free concentration of LysoPC is difficult to assess due to its tight binding to serum. Indeed, we found that LysoPC effects were reduced by BSA, although 100 μM LysoPC still increased ATP potency in the presence of 0.1% BSA. Serum albumin concentrations are closer to 5%, but we could not evaluate such high concentrations of serum in our assays, as no functional or binding response could be measured. It is perhaps relevant to note that several of the lipids that affected $P2X_7$ receptor function are elevated in disease states or under pathophysiological conditions and, in some cases, have been implicated as being causative to the disease. Thus, LysoPC levels are elevated in ischaemia (Sobel *et al.*, 1978), psoriatic skin (Ryborg *et al.*, 1995) and in atherosclerotic plaques (see Golfman *et al.*, 1999; Kougias *et al.*, 2006). Indeed, LysoPC is a major constituent of oxidized low-density lipoprotein (Kougias *et al.*, 2006) and has been reported to stimulate IL1β production from monocytes (Liu-Wu *et al.*, 1998) and microglial cells (Stock *et al.*, 2006). In addition, palymitoyl carnitine and related carnitines (Spedding and Mir, 1987) are elevated in ischaemic tissue while SPC levels are elevated in several diseases including Niemann-Pick disease type A, a disease that is associated with excessive neuronal cell death (Berger *et al.*, 1995; Meyer zu Heringdorf *et al.*, 2002). Clearly these lipids produce multiple cellular effects at the concentrations that affect $P2X_7$ receptor function, including considerable elevation of cell calcium, so determining the relative contribution, if any, of their action on $P2X_7$ receptors will be challenging.

A further consideration concerns the potential influence of lysolipids on $P2X_7$ receptor function under non-pathophysiological conditions. Previous studies have shown that $P2X_7$ receptor function is increased in an activation-dependent manner (see Introduction) through an unknown mechanism. As $P2X_7$ receptor activation can stimulate PLA_2 (Alzola *et al*., 1998), this could generate relatively high concentrations of lysolipids in close proximity to the $P2X_7$ receptor and, perhaps in conjunction with AA (Alloisio *et al*., 2006), enhance receptor function. Intriguingly, $P2X_7$ receptor activation causes externalization of phosphatidylserine in many cell types (MacKenzie *et al*., 2001) which may favour generation of LysoPS by extracellular PLA_2. This may be important as LysoPS enhanced $P2X_7$ receptor function but, in contrast to several other lysolipids, caused minimal lysis of THP-1 cells. We are in the process of studying the role of PLA_2 in modulating $P2X_7$ receptor function.

Finally, the role of the $P2X_7$ receptor in cancer has received attention recently as many cancer cells express $P2X_7$ receptors and activation of the $P2X_7$ receptor can cause cell death (White and Burnstock, 2006). We found that HPC could also increase $P2X_7$ receptor function. This agent is used clinically for the treatment of leishmaniasis (Soto and Soto, 2006) and as an anticancer agent (Jendrossek and Handrick, 2003) and can achieve plasma concentrations of 40–80 $\mu g\,ml^{-1}$ (10–20 μM) in clinical trials for cancer (Jendrossek and Handrick, 2003). Given the role of the $P2X_7$ receptor in both the killing of parasitic (Lammas *et al*., 1997) and cancer cells (White and Burnstock, 2006), further studies to investigate the potential role of the $P2X_7$ receptor in the therapeutic effects of HPC seem to be warranted.

Overall, these studies provide further information on the function of the $P2X_7$ receptor and continue to illustrate the high degree of plasticity of this receptor. Understanding the mechanism by which these lipids affect potency and determining their physiological relevance will require further work but these results provide a tantalizing insight into how this receptor may be modulated.

Conflict of interest

The authors are employees of GSK who funded this research.

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