G-protein-coupled receptor regulation of P2X₁ receptors does not involve direct channel phosphorylation

Catherine VIAL, Andrew B. TOBIN and Richard J. EVANS¹

Department of Cell Physiology and Pharmacology, University of Leicester, University Road, Leicester, LE1 9HN U.K.

P2X₁ receptors for ATP are ligand-gated cation channels, which mediate smooth muscle contraction, contribute to blood clotting and are co-expressed with a range of GPCRs (G-protein-coupled receptors). Stimulation of G_{α_q}-coupled mGluR1α (metabotropic glutamate receptor 1α), P2Y₁ or P2Y₂ receptors co-expressed with P2X₁ receptors in *Xenopus* oocytes evoked calcium-activated chloride currents (*I*_{ClCa}) and potentiated subsequent P2X₁-receptor-mediated currents by up to 250 %. The mGluR1α-receptor-mediated effects were blocked by the phospholipase C inhibitor U-73122. Potentiation was mimicked by treatment with the phorbol ester PMA. P2X receptors have a conserved intracellular PKC (protein kinase C) site; however, GPCR- and PMA-mediated potentiation was still observed with point mutants in which this site was disrupted. Similarly, the potentiation by GPCRs or PMA was unaffected by chelating the intracellular calcium rise with BAPTA/AM [bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis-(acetoxymethyl ester)] or the PKC inhibitors Ro-32-0432 and bisindolylmaleimide I, suggesting that the regulation

does not involve a calcium-sensitive form of PKC. However, both GPCR and PMA potentiation were blocked by the kinase inhibitor staurosporine. Potentiation by phorbol esters was recorded in HEK-293 cells expressing P2X₁ receptors, and radiolabelling of phosphorylated proteins in these cells demonstrated that P2X₁ receptors are basally phosphorylated and that this level of phosphorylation is unaffected by phorbol ester treatment. This demonstrates that P2X₁ regulation does not result directly from phosphorylation of the channel, but more likely by a staurosporine-sensitive phosphorylation of an accessory protein in the P2X₁ receptor complex and suggests that *in vivo* fine-tuning of P2X₁ receptors by GPCRs may contribute to cardiovascular control and haemostasis.

Key words: G-protein-coupled receptor (GPCR), metabotropic glutamate receptor, phosphorylation, protein kinase C (PKC), P2X receptor, P2Y receptor.

INTRODUCTION

Extracellular ATP functions as a signalling molecule through the activation of P2X receptors [1]. Sources of ATP include co-release with classical neurotransmitters, e.g. noradrenaline [2], and from damaged cells. P2X receptors are involved in processes as diverse as the regulation of interleukin 1β secretion from macrophages to contributing to pain sensation [3–5]. Seven P2X receptor genes have been identified, and functional receptors are thought to form from the homo- or hetero-trimeric assembly of subunits that gives rise to a number of distinct phenotypes [6]. P2X₁ receptors are expressed in a range of cell types, and P2X₁ homomeric channels have functional roles in the neuronal control of smooth-muscle contraction [7,8] and the regulation of arterial tone [9]. In addition, P2X₁ receptors play a significant role in the regulation of blood clotting, as shown by the P2X₁-receptor-knockout mouse that is protected against thromboembolism [10]. P2X₁ receptor regulation may therefore provide an important mechanism for the control of vascular tone and haemostasis, and our understanding of cardiovascular disease states.

One striking feature of both native and recombinant homomeric P2X₁ receptors is that responses desensitize during agonist application and take several minutes to recover [11]. When the conventional whole-cell recording mode is used to record from single arterial smooth muscle cells, responses to repeated applications of ATP show marked decreases in amplitude [12]. However, reproducible responses can be recorded when dialysis of the cell is limited by recordings using the permeabilized patch-clamp technique [12] or with sharp microelectrodes [11]. These results suggest that the properties of P2X₁ receptors may be regulated by a diffusible factor [12]. Further evidence that P2X₁ receptors may be regulated comes from studies in arteries where P2X-receptor-mediated responses were potentiated following endothelin-receptor stimulation [13].

P2X receptors comprise a novel class of ligand-gated cation channels with two transmembrane domains that line the ion-conducting pore, intracellular N- and C-termini, which contribute to the time-course of responses, and a large extracellular ligand-binding loop. One conserved feature throughout the family of P2X receptors is a consensus sequence for PKC (protein kinase C)-mediated phosphorylation (Thr-Xaa-[Arg/Lys]) in the intracellular N-terminal domain of the receptor, suggesting that this is a regulatory feature throughout the P2X receptor family. PKC-dependent regulation of the time-course of desensitization of P2X₂ receptors has been reported previously [14], and we have shown that mutation to disrupt the conserved PKC site in the P2X₁ receptor leads to an increase in the speed of desensitization during agonist application, as well as a decrease in the peak amplitude of the response [15]. Activation of the G-protein-coupled bradykinin and substance P receptors, and phorbol ester treatment, potentiated P2X₃-receptor-mediated responses without changing the time-course of response [16]. For the P2X₃ receptor, it was unclear whether phosphorylation was directly of the receptor or of an associated protein. Recent studies on P2X₇ receptors have shown that their function may be indirectly regulated by phosphorylation of other proteins in the P2X₇ receptor complex [17] and other ion

Abbreviations used: DAG, diacylglycerol; GPCR, G-protein-coupled receptor; *I*_{ClCa}, calcium-activated chloride current; iso-PPADS, pyridoxalphosphate-6-azophenyl-2',5'-disulphonate; mGluR1α, metabotropic glutamate receptor 1α; NMDA, *N*-methyl-D-aspartate; PKC, protein kinase C; PLC, phospholipase C; PTK, protein tyrosine kinase.

¹ To whom correspondence should be addressed (email rje6@le.ac.uk).

channels, e.g. NMDA (*N*-methyl-D-aspartate) receptors have been shown to be regulated by indirect phosphorylation of associated proteins [18]. Therefore it seems likely that there may be more than one mechanism by which phosphorylation regulates P2X receptor function.

P2X₁ receptors are co-localized with a range of G α_q -coupled receptors, including nucleotide-sensitive P2Y receptors; for example, the P2X₁ receptor is co-expressed with ADP-sensitive P2Y₁ receptors in platelets [19] and with ATP/UTP-sensitive P2Y₂ receptors in arterial smooth muscle [20]. This raises the possibility that GPCRs (G-protein-coupled receptors) that signal through the activation of G α_q subunits and the PLC (phospholipase C) pathway to generate DAG (diacylglycerol) and stimulate PKC may regulate P2X₁ receptor function. In the present study, using recombinant receptors, we have investigated the role of GPCR activation on P2X₁ receptor function. G α_q -coupled P2Y₁, P2Y₂ and mGluR1 α (metabotropic glutamate receptor 1 α) receptors and phorbol ester stimulation potentiated P2X₁-receptor-mediated currents through the activation of a staurosporine-sensitive pathway. The potentiation results not from increased trafficking of P2X₁ receptors to the cell surface or phosphorylation of the conserved PKC site on the P2X₁ receptor, but most likely through kinase action at an associated regulatory protein. These results demonstrate that P2X₁ receptors are subject to regulation by GPCRs, and this may have important consequences for our understanding of the control of arterial resistance and haemostasis.

EXPERIMENTAL

Reagents

ATP, adenosine 5'-[α,β -methylene]triphosphate (α,β -meATP), UTP, ADP, L-glutamic acid, PMA, cytochalasin D and insulin were obtained from Sigma. ADP was treated with hexokinase in a high-glucose-containing saline at pH 8, as described previously [21], to remove contaminating levels of ATP. U-73122, U-73343, BAPTA/AM [bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester)], staurosporine, bisindolylmaleimide I, Ro-32-0432 and genistein were obtained from Calbiochem (Merck Biosciences, Nottingham, U.K.). Iso-PPADS (pyridoxalphosphate-6-azophenyl-2',5'-disulphonate) was from Tocris Cookson (Bristol, U.K.).

Cloning and mutagenesis of the human P2X₁ receptor

The human P2X₁ plasmid construct used in the present study has been described previously [22]. Point mutations in the human P2X₁ plasmid construct were introduced using the QuikChange™ mutagenesis kit (Stratagene, Amsterdam, The Netherlands) according to the manufacturer's instructions. In order to disrupt the consensus PKC motif present in the human P2X₁ receptor amino acid sequence, Thr¹⁸ was mutated to valine and Arg²⁰ was mutated to isoleucine or alanine. Introduction of the correct mutation and the absence of spontaneous mutation was confirmed by DNA sequencing (Automated ABI Sequencing Service, Leicester University, Leicester, U.K.).

Expression of recombinant receptors in *Xenopus* oocytes

The human mGluR1 α receptor was a gift from Professor S. R. Nahorski (University of Leicester, Leicester, U.K.), and human P2Y₁ and P2Y₂ receptors were a gift from Professor T. K. Harden (University of North Carolina School of Medicine, Chapel Hill, NC, U.S.A.). pcDNA3.1 vectors (Invitrogen, Paisley, U.K.) containing either P2X₁ mutant, wild-type P2X₁, mGluR1 α , P2Y₁, P2Y₂ or P2X₂ receptors were linearized. Sense-strand cRNAs

were generated from these linearized plasmids with the T7 mMessage mMachine™ kit [Ambion (Europe), Huntingdon, Cambs., U.K.). Defolliculated oocytes were injected with 50 nl (50 ng) of cRNA using an Inject + Matic microinjector (J. Alejandro Gaby, Geneva, Switzerland). When two receptors were co-injected, the final quantity of injected cRNA was always 50 ng/oocyte with a 1:1 ratio. Oocytes were maintained at 18 °C in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM sodium pyruvate and 5 mM Hepes, pH 7.6) before recording 3–6 days later.

Electrophysiological recordings

Two-electrode voltage-clamp recordings were made from oocytes using a GeneClamp 500B amplifier (Axon Instruments, Union City, CA, U.S.A.) as described previously with barium replacing calcium in the extracellular solution [22]. Membrane currents were recorded at a holding potential of –60 mV, and were acquired using a Digidata 1200 analogue-to-digital converter with pClamp 8 acquisition software (Axon Instruments). ATP or α,β -meATP (100 μ M each) was applied with a U-tube perfusion system [22] at 5 min intervals, whereas all the other reagents were bath-perfused, except iso-PPADS, which was also present at the appropriate concentration in the U-tube application of ATP. Repeated exposures of agonist were separated by 5 min in order to allow recovery from receptor desensitization. When looking at the potentiation of the P2X₁ receptor, glutamate (100 μ M), ADP (3 μ M), UTP (100 μ M), PMA (100 nM) or insulin (1 μ M) was bath-perfused for 5 min between the fourth and the fifth stimulations of the P2X₁ receptor by ATP or α,β -meATP via the U-tube. There was batch-to-batch variation in the degree of potentiation by glutamate (mean ratio 1.47–2.81); therefore, in all studies, any comparisons between different treatments were made between oocytes from a single batch. Similar variation in the degree of potentiation of other ion channels in oocytes has been reported previously [18].

Cell culture and transfections

Native HEK-293 cells and HEK-293 cells subcloned after transfection of wild-type P2X₁ (P2X₁cl-1 cells) were maintained in minimal essential medium with Earle's Salts (with GlutaMAX™ I) supplemented with 10 % foetal bovine serum and 1 % non-essential amino acids (Invitrogen) at 37 °C in a humidified atmosphere of 5 % CO₂ and 95 % air. P2X₁cl-1 cells were maintained under permanent selection in 600 μ g/ml G418 (Invitrogen). For patch-clamp studies, P2X₁cl-1 cells were attached to glass coverslips and kept in culture for a maximum of 3 days. For direct phosphorylation experiments, P2X₁cl-1 cells were transiently transfected (when indicated) with M₃ muscarinic receptors [23] using LIPOFECTAMINE™ 2000 Reagent (Invitrogen)/Opti-MEM (Invitrogen) and used as an internal control for phosphorylation.

Patch-clamp recordings

Conventional whole-cell patch-clamp experiments were performed at a holding potential of –60 mV at room temperature (21 °C) as described previously [9]. Agonists were rapidly applied via a U-tube.

Receptor phosphorylation

Receptor phosphorylation in intact cultured cells was carried out essentially as described previously [23,24]. P2X₁cl-1 cells were transiently transfected with the human M₃-muscarinic receptor. These transfected cells, or native non-transfected HEK-293 cells, were grown in six-well plates. Cells were incubated with

Figure 1 Activation of mGluR1 α potentiates P2X₁ receptor responses

(a) Repeated applications of ATP every 5 min (100 μ M, drug application is indicated by a black bar) induced a transient inward current in oocytes expressing either P2X₁ receptor alone or with the mGluR1 α . When steady-state P2X₁ responses to ATP were obtained, glutamate (100 μ M, drug application is indicated by a hatched bar) was applied in the bath-perfusion system for 5 min. Glutamate induced $I_{\text{GluR1}\alpha}$ and potentiated a P2X₁ receptor response to a subsequent application of ATP only in oocytes expressing mGluR1 α (lower panel). No potentiation was observed in oocytes expressing the P2X₁ receptor alone (upper panel). (b and c) Summary data showing the potentiation of P2X₁ receptor responses by mGluR1 α activation (fifth response/fourth response potentiation ratio) with (closed symbols) and without (open symbols) glutamate addition. (d) Pre-treatment (5 min) with glutamate (100 μ M) of oocytes co-expressing mGluR1 α and P2X₁ receptors significantly increased the amplitude of the first P2X₁ response to ATP compared with the untreated oocytes ($n = 5-11$). WT, wild-type.

50 μ Ci/ml of [³²P]orthophosphate (Amersham Biosciences) for 1 h in phosphate-free KHB (Krebs/Hepes buffer) (10 mM Hepes, pH 7.4, 118 mM NaCl, 4.3 mM KCl, 1.17 mM MgSO₄ · 7H₂O, 1.3 mM CaCl₂, 25 mM NaHCO₃ and 11.7 mM glucose). PMA (0.1 μ M) was added for 10 min, and reactions were terminated by aspiration and the addition of 1 ml of RIPA buffer [10 mM Tris, pH 7.4, 10 mM EDTA, 500 mM NaCl, 1% (v/v) Nonidet P40 and 0.5% (w/v) sodium deoxycholate]. Lysates were pre-cleared by centrifugation at 20000 g for 3 min, and anti-(M₃-muscarinic receptor) antibody [23] or anti-(P2X₁ receptor) antibody (Alomone Labs, Jerusalem, Israel) was added before incubation for 60–90 min on ice. Immunocomplexes were then isolated on Protein A–Sepharose (Amersham Biosciences). Samples were washed four times with TE buffer (10 mM Tris, pH 7.4 and 2.5 mM EDTA) and resolved by SDS/PAGE (8% gels). Gels were stained with 0.2% (w/v) Coomassie Blue to ensure that there was equal immunoprecipitation. The gels were then dried and phosphoproteins were visualized by autoradiography.

Data analysis

Data are shown as means $\pm$ S.E.M. Differences between the means were determined by the appropriate Student's t test and were considered significant when $P < 0.05$.

RESULTS

Activation of mGluR1 α potentiates P2X₁ receptor responses

ATP evoked desensitizing inward currents in *Xenopus* oocytes expressing recombinant P2X₁ receptors as reported previously [22]. The initial response to a supra-maximal concentration of ATP (100 μ M) had a peak current amplitude of 11164 ± 660 nA ($n = 31$). When ATP (100 μ M) was re-applied 5 min later, the peak response was reduced by approx. 50%; however, subsequent applications at 5 min intervals gave essentially reproducible responses (no significant difference from the second response; $n = 5$; Figures 1a, upper panel, and 1b) indicating that a 5 min recovery period gave a steady-state level of recovery from receptor desensitization. Our previous studies on vascular smooth muscle had suggested that this recovery from receptor desensitization may involve a diffusible factor [12] and could possibly be associated with PKC activation. To test whether a G α_q -coupled receptor could regulate this re-sensitization process, we co-expressed the human P2X₁ receptor with human mGluR1 α and determined the effect of mGluR1 α activation with glutamate. Human mGluR1 α was chosen initially as this gives robust responses in oocytes [25]. Oocytes have provided an excellent simple model system for studying recombinant receptor regulation by kinases and receptors

including studies on P2X receptors [14,16] and NMDA receptors [26,27].

Responses evoked by ATP (100 μ M) were the same for cells co-expressing P2X₁ and mGluR1 α receptors as for the P2X₁ receptor alone, showing that expression of the glutamate receptor itself had no detectable effect on the properties of P2X₁ receptors. To determine whether mGluR1 α activation regulated the P2X₁-receptor-mediated response, we applied glutamate for 5 min between the fourth and fifth ATP applications. Glutamate (100 μ M) evoked a large inward current (9055 ± 870 nA; $n = 6$); this results from a $G\alpha_q$ -subunit-mediated increase in intracellular calcium and the subsequent activation of endogenous oocyte calcium-activated chloride channels and is consistent with that described previously [25] (glutamate had no effect on oocytes injected with only P2X₁ receptors; $n = 5$). Glutamate also potentiated significantly the amplitude of subsequent ATP-evoked responses [defined as a ratio of P2X₁ current amplitude measured after glutamate (fifth application of ATP) to that measured before glutamate (fourth application)] with a potentiation ratio of 2.38 ± 0.15 compared with 0.94 ± 0.05 for the control ($n = 6$ and 5 respectively; Figures 1a–1c) with essentially no effect on the time-course of responses.

The glutamate effect was similar when outward P2X₁ currents were evoked from a holding potential of +60 mV (potentiation ratio 1.37 ± 0.06 ; $n = 5$), indicating that the potentiation does not show strong voltage-dependence, and a similar potentiation was observed when using a sub-maximal ATP concentration (1 μ M) (results not shown). The potentiation by glutamate often increased P2X₁ responses to amplitudes similar to those of the initial response, suggesting that mGluR1 α activation may speed recovery from desensitization. The amplitude of the first P2X₁ current in the presence or absence of a 5 min pre-treatment with glutamate (100 μ M) in oocytes co-expressing mGluR1 α and P2X₁ receptors was significantly larger in oocytes subject to glutamate pre-treatment (12257 ± 490 nA; $n = 11$) compared with non-glutamate-treated oocytes (8552 ± 55 nA; $n = 11$) (Figure 1d). These results suggest that activation of the mGluR1 α increases the peak amplitude of the P2X₁-receptor-mediated response.

Time-dependent P2X₁ potentiation by mGluR1 α

To investigate the time-course of the potentiation by mGluR1 α activation, we applied glutamate for only 30 s before the fifth application of ATP. Interestingly, this had no potentiating effect on the fifth P2X₁ receptor response (Figure 2a, ii); however, the subsequent application of ATP (sixth) 5 min later resulted in a dramatic increase of the P2X₁ response (Figures 2a, ii, and 2b). This indicates that P2X₁ potentiation requires a significant delay following mGluR1 α activation, and suggests that the potentiation results from the activation of a second-messenger pathway and not a direct interaction of the mGluR1 α and P2X₁ receptors, as has been suggested for direct cross-talk between metabotropic dopamine receptors and GABA_A (γ -aminobutyric acid A) receptor channels [28].

$G\alpha_q$ -coupled P2Y₁ and P2Y₂ receptors potentiate P2X₁ responses

To determine whether other $G\alpha_q$ -coupled receptors could potentiate P2X₁ receptor responses, P2Y₁ or P2Y₂ receptors were co-expressed with the P2X₁ receptor. ADP-sensitive P2Y₁ and UTP-sensitive P2Y₂ receptors can also be activated by ATP. Therefore, in these studies, α, β -meATP (100 μ M) was used to activate P2X₁ receptors (this agonist is ineffective at P2Y₁ and P2Y₂ receptors). For oocytes co-expressing the P2X₁ and P2Y₁ receptors, ADP (3 μ M) was applied after the fourth response to α, β -meATP and

Figure 2 Potentiation of P2X₁ responses by mGluR1 α is time-dependent

(a) When steady-state P2X₁ responses to ATP were obtained, glutamate (100 μ M, drug application is indicated by a hatched bar) was applied in the bath-perfusion system either 5 min (i) or 30 s (ii) before the next ATP application. Glutamate induced I_{ClCa} s (when co-expressed with the P2X₁ receptor) and, when applied for 5 min before ATP, potentiated the subsequent P2X₁ receptor response (i). When glutamate was applied for 30 s before the fifth stimulation of P2X₁ receptors by ATP (100 μ M, drug application is indicated by a black bar), it had no effect on the fifth P2X₁ response, but potentiated the sixth P2X₁ response 5 min later (ii). (b) Summary of experiments showing that P2X₁ potentiation requires a delay following mGluR1 α activation. Pre-application of glutamate 5 min (i) and 30 s (ii) before subsequent ATP responses. Open bars, no glutamate; grey bars, with glutamate stimulation ($n = 3$ –6).

evoked a large I_{ClCa} (calcium-activated chloride current) (5187 ± 1071 nA; $n = 7$) (as described previously [29]) and potentiated the fifth α, β -meATP-elicited responses (1.25 ± 0.07 ; $n = 7$, compared with 0.98 ± 0.01 for the control; $n = 6$, $P < 0.01$). For oocytes co-expressing the P2X₁ and P2Y₂ receptors, UTP (100 μ M) was applied after the fourth response to α, β -meATP and evoked an I_{ClCa} (1439 ± 417 nA; $n = 5$) (as described previously [30]) and potentiated the fifth α, β -meATP-elicited responses (1.17 ± 0.04 ; $n = 5$, compared with 0.94 ± 0.01 for the control; $n = 3$, $P < 0.01$). The differences between the mean amplitude of I_{ClCa} s and the level of potentiation between mGluR1 α , P2Y₁ and P2Y₂ receptors most likely reflects differences in the expression level of the GPCRs and their coupling to second-messenger systems. ADP or UTP had no effect on subsequent α, β -meATP-evoked responses in oocytes expressing only P2X₁ receptors ($n = 3$ for each). These studies indicate that several

Gα_q-linked receptors can potentiate P2X₁-receptor-mediated responses. Given the more robust potentiation by mGluR1α, this was chosen to investigate the Gα_q-mediated regulation of P2X₁ receptors in more detail.

Does potentiation involve trafficking of new P2X₁ receptors to the cell surface?

The amplitude of the P2X₁-receptor-mediated current in oocytes is dependent on both the single channel properties of the receptor and the number of receptors present. GPCRs have been shown to potentiate NMDA receptor currents through recruiting new receptors to the cell surface [27,31]. To determine whether or not trafficking of new P2X₁ receptors to the cell surface was responsible for the potentiation of the response to GPCR stimulation, we looked at the recovery of P2X₁ responses following application of the slowly reversible P2 receptor antagonist iso-PPADS. Iso-PPADS (100 μM, applied for 5 min after the third ATP application) essentially abolished the response to concomitant application of ATP (100 μM, fourth application); this recovered to 55.6 ± 8.7% (*n* = 3) of the control third response following a 5 min washout (the fifth application) (Figure 3). In this batch of oocytes, mGluR1α activation gave a potentiation ratio (fifth/fourth) of 2.26 ± 0.24 (*n* = 3). If the potentiation was due to effects on P2X₁ receptors already present on the cell surface, we would predict the fifth/third ratio to be 0.55 (recovery from iso-PPADS) × 2.26 (potentiation ratio) = 1.24 (Figure 3). Following mGluR1α stimulation for the 5 min washout following iso-PPADS application, the potentiation ratio (fifth/third) was 1.07 ± 0.03 and is consistent with the potentiation of the P2X₁ receptors already at the cell surface and not insertion of new P2X₁ receptors.

P2X₁ receptor potentiation is mediated by a PLC-initiated cascade

To confirm that mGluR1α-mediated potentiation of P2X₁ responses occurs via a Gα_q-initiated PLC-initiated signalling cascade, we used the PLC inhibitor U-73122 to block the hydrolysis of PtdIns(4,5)P₂ and consequently the generation of DAG and Ins(1,4,5)P₃. In oocytes co-expressing mGluR1α and P2X₁ receptors, U-73122 (10 μM, 20 min) dramatically reduced by > 90% the glutamate-evoked *I*_{ClCa} (4064 ± 1135 nA for non-treated cells to 359 ± 209 nA for U-73122-treated cells; *n* = 5 and 4 respectively, *P* < 0.05) and abolished the glutamate potentiation of the P2X₁ response (control potentiation 2.09 ± 0.23 and 0.95 ± 0.09 for the oocytes treated with U-73122; *n* = 5 and 4 respectively; Figure 4). Treatment with the inactive analogue U-73343 (10 μM, 20 min) had no effect on glutamate potentiation of the P2X₁ response and no significant effect on the glutamate-evoked chloride current (*n* = 4; Figures 4b and 4c). These results demonstrate the mGluR1α-evoked potentiation involves a PLC-mediated signalling pathway, either through a rise in intracellular calcium mediated by Ins(1,4,5)P₃ and/or activation of protein kinases.

P2X₁ potentiation is not dependent on the intracellular calcium concentration

To examine whether the potentiation of the P2X₁ response by glutamate was dependent on a rise in intracellular calcium resulting from the generation of Ins(1,4,5)P₃ by PLC activation, we used the calcium chelator BAPTA/AM. BAPTA/AM is a membrane-permeant form of BAPTA, which, following diffusion into the cell, is hydrolysed by cytosolic esterases and trapped intracellularly as the active calcium-buffering agent BAPTA. For oocytes co-expressing mGluR1α and P2X₁ receptors, BAPTA/AM (50 μM, 2 h) had no effect on the potentiation of the P2X₁ response to ATP

Figure 3 Potentiation of P2X₁ responses is not the result of the insertion of new P2X₁ receptors at the cell surface

(a) The P2 receptor antagonist iso-PPADS (100 μM) abolished the P2X₁ response to ATP (100 μM, indicated by a black bar). The P2X₁ response partially recovers after 5 min washout (upper panel). When glutamate (100 μM) was applied for 5 min after iso-PPADS treatment (100 μM), the subsequent P2X₁ response to ATP was potentiated (solid line, lower panel). The amplitude of this potentiation corresponded to the predicted amplitude which would be observed if the potentiation was only due to P2X₁ receptors already at the cell surface (dotted line). (b) Summary of experiments showing the level of glutamate-induced potentiation in control oocytes co-expressing mGluR1α and P2X₁ receptors, inhibition by iso-PPADS and washout at 5 min, and the glutamate-induced potentiation of the P2X₁ receptor response during washout. Data are plotted as the ratio (fifth application/third application), except for iso-PPADS, that corresponds to the fourth/third application ratio (*n* = 3 for all).

(potentiation ratio 1.92 ± 0.20 and 1.47 ± 0.06 for BAPTA/AM and control respectively; *n* = 6; Figures 5a and 5c). BAPTA/AM treatment also had no effect on the peak amplitude of the first P2X₁-receptor-mediated response (11 779 ± 1069 and 11 914 ± 933 nA for control and BAPTA/AM respectively; *n* = 4). The abolition of the glutamate-evoked *I*_{ClCa} (68 ± 30 nA for BAPTA/AM compared with 6612 ± 701 nA for the control; *n* = 6, *P* < 0.001; Figures 5a and 5b) confirms the effective chelation and buffering of intracellular calcium by BAPTA. These results demonstrate that the mGluR1α-mediated potentiation of the P2X₁ response does not require a change in the intracellular calcium concentration.

P2X₁ potentiation involves activation of kinase(s)

The activation of PLC leads to the generation of DAG, which is known to activate protein kinases. To investigate the

Figure 4 P2X₁ receptor potentiation by mGluR1 α is mediated by a PLC-initiated cascade

(a) Glutamate application (100 μ M, indicated by hatched bar) to oocytes co-expressing mGluR1 α and P2X₁ receptors evoked I_{CaCa} and potentiated subsequent responses to ATP (100 μ M, indicated by black bar) (left-hand panel). Both the I_{CaCa} and potentiation of the P2X₁ receptor response were abolished by treatment with the PLC inhibitor U-73122 (10 μ M, 20 min) (right-hand panel). (b) Effects of the PLC inhibitor U-73122 and its inactive analogue U-73343 (10 μ M, 20 min) on the amplitude of the I_{CaCa} . (c) Summary of experiments showing that P2X₁ potentiation was abolished by the PLC inhibitor U-73122, but was not affected by its inactive analogue U-73343. Data are plotted as potentiation ratio (fifth/fourth application of ATP) ($n = 4-5$).

possibility that mGluR1 α -mediated potentiation of the P2X₁ response might be the consequence of kinase(s) activation, we examined the effect of PMA (which mimics the action of DAG) on the potentiation of P2X₁ responses. Treatment with PMA (100 nM for 5 min before the fifth application of ATP) of oocytes expressing P2X₁ receptors significantly potentiated P2X₁ responses to ATP (2.40 ± 0.17 ; $n = 4$, compared with 0.86 ± 0.07 for the control; $n = 5$; Figure 6). The PMA regulation was slightly larger following buffering intracellular calcium to low levels with BAPTA/AM (ratio 2.57 ± 0.21 and 1.72 ± 0.17 for BAPTA/AM treatment and control respectively; $n = 4$, $P < 0.05$), and indicates that a rise in intracellular calcium is not essential for the PMA-mediated potentiation. Pre-treatment (20 min) with the broad-spectrum kinase inhibitor staurosporine (300 nM) had no effect on either the peak amplitude of the first ATP response (11857 ± 796 and 13660 ± 1277 nA for control and staurosporine respectively; $n = 6$) or the degree of run-down between the first and second responses (44 ± 1.8 and $53 \pm 7\%$ for control and staurosporine respectively; $n = 6$) and indicates that the staurosporine-sensitive pathway is not essential for the recovery of P2X₁ receptors from the desensitized state or that there is ongoing turnover of phosphorylation associated with normal receptor function. However, staurosporine essentially abolished the potentiation evoked by PMA (300 nM, 20 min) (1.11 ± 0.04 ;

Figure 5 P2X₁ potentiation is not dependent on the intracellular calcium concentration

(a) Glutamate (100 μ M, drug application is indicated by a hatched bar) was applied for 5 min to oocytes co-expressing mGluR1 α and P2X₁ receptors pre-treated with the membrane-permeant calcium chelator BAPTA/AM (50 μ M, 2 h). No glutamate-evoked I_{CaCa} s were recorded, although the potentiation of the P2X₁ response to the subsequent application of ATP (100 μ M, drug application is indicated by a black bar) remained the same (right-hand panel) as the control (left-hand panel). (b) Summary of experiments showing that glutamate-evoked I_{CaCa} s were abolished by the calcium chelator BAPTA/AM, whereas (c) P2X₁ potentiation (ratio of fifth/fourth application) was not significantly affected ($n = 6$).

$n = 4$) (Figure 6), and in oocytes co-expressing mGluR1 α and P2X₁ receptors, staurosporine inhibited glutamate potentiation of the P2X₁ response (1.29 ± 0.04 ; $n = 6$, compared with 2.81 ± 0.18 for the control; $n = 6$) dramatically, without significantly altering the glutamate-elicited chloride current (5366 ± 1363 ; $n = 6$, compared with 2584 ± 639 for the control; $n = 6$; Figure 6c). These results suggest that PMA- and mGluR1 α -mediated potentiation of the P2X₁ receptor involves the activation of a staurosporine-sensitive protein kinase.

PKC-dependent phosphorylation is not involved in potentiation of the P2X₁ response

Studies on the P2X₂ and P2X₃ receptors [14,16] have suggested that the highly conserved PKC motif (Thr-Xaa-[Arg/Lys]) located on the intracellular N-terminus of all known P2X receptors may be involved in the regulation of P2X receptors. To determine whether or not this conserved PKC consensus sequence (Thr¹⁸Pro¹⁹Arg²⁰) was involved in the regulation of the P2X₁ receptor by G α_q -coupled receptors, we generated a number of mutant receptors that would lead to the disruption of the PKC site in the P2X₁ receptor [Thr¹⁸ $\rightarrow$ Val (T18V), Arg²⁰ $\rightarrow$ Ile (R20I) and Arg²⁰ $\rightarrow$ Ala (R20A)]. All three mutants had significantly reduced peak current amplitudes compared with the control (10950 ± 795 , 70.8 ± 19 ,

Figure 6 P2X₁ potentiation involves activation of kinase(s)

(a) PMA (100 nM, 5 min) treatment of oocytes expressing P2X₁ receptor alone potentiated a steady-state P2X₁ response to ATP (100 μM, drug application is indicated by a black bar) (middle panel). This potentiation was abolished in the presence of the broad-spectrum protein kinase inhibitor staurosporine (300 nM, 20 min) (right-hand panel). The left-hand panel shows the control. (b) Summary of experiments showing that PMA potentiation of the P2X₁ response to ATP can be blocked by the broad-spectrum protein kinase inhibitor staurosporine (potentiation ratio of fifth/fourth ATP application). (c) In oocytes co-expressing mGluR1α and P2X₁ receptors, staurosporine inhibited glutamate potentiation dramatically (ratio of fifth/fourth application) of P2X₁ responses to ATP ($n = 4-6$).

6213 ± 1278 and 4879 ± 784 nA for wild-type, T18V, R20I and R20A respectively; $n = 4-7$). The mutants T18V and R20A showed a significant ($P < 0.05$) speeding of the time-course compared to the wild-type; however, the mutant R20I that would also disrupt the consensus PKC site had no effect on the time-course of the response (50% decay of peak current, 286 ± 26, 92.5 ± 35, 158 ± 40 and 240 ± 41 ms for wild-type, T18V, R20A and R20I respectively; $n = 4-7$). Recent studies have shown that the P2X₁ receptor is phosphorylated at Thr¹⁸ [32]; however, in the absence of data to show directly that dephosphorylation of the P2X₁ receptor results in an increase of the speed of decay, it is unclear whether phosphorylation or structural changes associated with the mutants are responsible for the speeding up of the time-course of the response. Glutamate potentiated the P2X₁ response for each individual P2X₁ receptor mutant co-expressed with mGluR1α (1.91 ± 0.19; $n = 4$, for R20I; 1.47 ± 0.15; $n = 4$, for R20A; and 4.22 ± 0.89; $n = 7$, for T18V, compared with 2.05 ± 0.25 for the wild-type P2X₁ receptor; $n = 4$; $P < 0.01$ for each) and no change in the glutamate-evoked chloride current amplitude was observed (results not shown). Similarly, PMA-mediated

potentiation was unaffected in the T18V mutant (potentiation ratio 1.40 ± 0.11 and 1.71 ± 0.19 for wild-type and T18V respectively; $n = 4$ and 5). These results show that mGluR1α- or PMA-dependent potentiation of the P2X₁ receptor does not involve direct phosphorylation by PKC of the P2X₁ receptor at Thr¹⁸.

Another possibility was that PKC was acting to phosphorylate the P2X₁ receptor at another site or at an accessory regulatory protein. The peak amplitude of P2X₁ receptor currents was unaffected by the PKC inhibitors Ro-32-0432 (3 μM, 2 h, peak amplitude 8363 ± 146 and 11383 ± 553 nA for control and Ro-32-0432 respectively; $n = 6$) and bisindolylmaleimide I (1 μM, 2 h, peak amplitude 11729 ± 684 and 13100 ± 1502 nA for control and bisindolylmaleimide respectively; $n = 6$ and 4). Similarly, P2X₁ receptor potentiation by mGluR1α (potentiation ratio 2.32 ± 0.22 and 1.85 ± 0.23 respectively for control and Ro-32-0432; $n = 6$, and 1.86 ± 0.17 and 1.77 ± 0.24 for control and bisindolylmaleimide respectively; $n = 6$ and 4) or PMA (potentiation ratio 1.40 ± 0.11, 1.25 ± 0.04 and 1.36 ± 0.06 for control, Ro-32-0432 and bisindolylmaleimide respectively; $n = 4$, 4 and 3) was unaffected and demonstrates that classical PKC isoforms are not involved in P2X₁ receptor regulation.

Does the regulation of P2X₁ receptors involve direct phosphorylation of the channel?

Our results have shown that following Gα_q-linked-receptor activation, P2X₁ receptors are regulated by a staurosporine-sensitive pathway. In addition to the conserved PKC site, there are several (four) tyrosine residues in the intracellular domains of the human P2X₁ receptor, and there are a number of serine (three) and threonine (three) residues in the C-terminus, raising the possibility that phosphorylation of these residues may regulate channel function. Oocytes are an excellent model for investigating the regulation of P2X₁ receptor ion channels; however, protocols to determine phosphorylation of proteins in oocytes are not straightforward. To look at direct phosphorylation of the P2X₁ receptor, we have used established protocols for mammalian cells [23,24]. To check that similar regulatory mechanisms are present in mammalian cells, we tested the effects of PMA on P2X₁ receptor currents. PMA potentiated P2X₁-receptor-mediated responses in HEK-293 cells approx. 2-fold ($P < 0.05$) over control non-treated cells, demonstrating that there is similar regulation in mammalian cells.

HEK-293 cells were loaded with [³²P]orthophosphate; this results in radiolabelling of phosphorylated proteins. In lysates from cells stably expressing the P2X₁ receptor, immunoprecipitation with an anti-(P2X₁ receptor) antibody revealed a phosphoprotein corresponding to the P2X₁ receptor (no such band was present in non-transfected controls) (Figure 7). Stimulation with PMA (0.1 μM for 10 min) had no effect on the basal level of phosphorylation of the P2X₁ receptor. However, PMA did increase the level of phosphorylation of M₃-muscarinic receptors co-transfected into the P2X₁ receptor cell line as reported previously for the M₃-muscarinic receptor [23], indicating functional integrity of PMA-stimulated phosphorylation in these cells. These results demonstrate that the P2X₁ receptor is basally phosphorylated; however, the lack of effect of PMA on the level of phosphorylation indicates that the regulation of P2X₁ receptors by PMA is indirect and involves phosphorylation of an accessory protein that regulates P2X₁ receptor function.

Insulin- and tyrosine-kinase-mediated regulation of P2X₁ responses

Although serine/threonine phosphorylation is more commonly recognized as a mechanism to modulate the function of ion channels

Figure 7 Phosphorylation of the P2X₁ receptor

Cells stably expressing the P2X₁ receptor were transiently transfected with the human M₃-muscarinic receptor. These cells and native non-transfected HEK-293 cells (NT) were labelled with [³²P]orthophosphate and stimulated in the presence or absence of PMA (0.1 μM) for 10 min. The cells were then solubilized in RIPA buffer, and either the P2X₁ receptor (Anti-P2X₁) or the M₃-muscarinic receptor (Anti-M₃) was immunoprecipitated from the lysate using receptor-specific antibodies. The position of the phosphorylated P2X₁ receptor is shown by the arrow. The PMA-mediated phosphorylation of the M₃-muscarinic receptor was used in this experiment as a positive control and is demonstrated clearly in the right-hand panel, which is a shorter exposure of the M₃-receptor immunoprecipitation. The data shown are representative of four experiments.

and receptors, it has been shown that tyrosine phosphorylation can also be involved as, for example, in the regulation of NMDA receptors [33] and voltage-gated potassium channels [34]. Therefore we took advantage of endogenous insulin and IGF-1 (insulin-like growth factor 1) receptors and associated tyrosine kinases present in *Xenopus* oocytes to determine if the potentiation of the P2X₁ receptor could be regulated by PTKs (protein tyrosine kinases). In oocytes expressing the P2X₁ receptor, insulin (1 μM for 5 min before the fifth application of ATP) significantly potentiated the P2X₁ response to ATP (1.34 ± 0.10 ; $n = 6$, compared with 0.91 ± 0.03 for the control; $n = 4$). These results suggested that PTKs may be involved in the regulation of P2X₁ receptors, although in oocytes, insulin receptors can also couple to other kinases.

To test the involvement of PTKs in mGluR1α-mediated potentiation, we used the tyrosine kinase inhibitor genistein. Genistein (100 μM, 30 min) decreased the amplitude of the first response to ATP by > 50% (9498 ± 1983 and 3694 ± 1046 nA for control and genistein respectively; $n = 4$, $P < 0.05$), but had no effect on the glutamate-evoked potentiation ratio (1.55 ± 0.23 and 1.45 ± 0.13 for control and genistein respectively; $n = 4$). This suggests that PTK activity may contribute to the basal regulation of P2X₁ receptors, but not to the Gα_q-mediated potentiation of the receptor.

DISCUSSION

ATP acting through P2X₁ receptors is important in the control of smooth-muscle tone and platelet function. In the present study, we have shown that P2X₁-receptor-mediated responses can be potentiated by phorbol ester treatment, and following the activation of Gα_q-coupled receptors acting through a PLC-linked pathway. We show directly for the first time that the P2X₁ receptor is basally phosphorylated at multiple sites and that the

potentiation of the P2X₁ receptor appears to be mediated by phosphorylation of an accessory regulatory protein by a staurosporine-sensitive pathway, but not involving classical PKC isoforms.

Activation of mGluR1α, P2Y₁ and P2Y₂ GPCRs and the phorbol ester PMA potentiated P2X₁-receptor-mediated currents evoked by a supramaximal concentration of ATP. These effects were blocked by the kinase inhibitor staurosporine, implicating protein phosphorylation in the regulation of P2X₁ receptor function. The most likely site of action was considered to be the conserved PKC site in the N-terminus of P2X receptors [14]. However, when we disrupted the consensus site for PKC phosphorylation (mutants T18V, R20I and R20A) the P2X₁ response potentiation following PMA or mGluR1α stimulation was unaffected or slightly increased. This demonstrated that direct PKC-mediated phosphorylation of the P2X₁ receptor did not account for the potentiation of the response and suggests that the potentiation of the P2X₁ receptor following Gα_q-coupled GPCR activation or PMA involves a mechanism distinct from phosphorylation of the conserved N-terminal PKC site that has been implicated in the regulation of P2X₂ and P2X₃ receptors [14,16].

To investigate in more detail the signalling pathways associated with mGluR1α- and PMA-mediated potentiation of the P2X₁ receptor, we used a range of drugs known to interfere with particular signalling pathways. The PLC inhibitor U-73122 abolished the mGluR1α-mediated potentiation of the P2X₁ receptor, indicating that the receptor was coupling through a Gα_q-linked pathway to produce Ins(1,4,5)P₃ and DAG. Ins(1,4,5)P₃ production leads to the release of calcium from intracellular stores (as shown by the stimulation of calcium-activated chloride currents) and DAG leads to the stimulation of kinases, and is most commonly associated with the stimulation of PKC. The calcium can also act as a cofactor required for the activation of some kinases, e.g. mGluR1 regulation of NMDA receptor channels by PKC [35]. When we blocked the rise in intracellular calcium with BAPTA/AM, there was no effect on the mGluR1α- or PMA-mediated potentiation of the P2X₁ receptor, demonstrating that a rise in intracellular calcium was not essential and suggesting that calcium-sensitive PKC isoforms are not involved in the regulation. This is supported further by the fact that the broad-spectrum PKC inhibitors Ro-32-0432 and bisindolylmaleimide I had no effect on the potentiation of P2X₁ receptor responses following PMA or mGluR1α stimulation. Taken with the mutagenesis studies that disrupted the conserved PKC site on the P2X₁ receptor, these results suggest that classical PKC isoforms are unlikely to be involved in either the direct or indirect potentiation of P2X₁ receptors by PMA or following Gα_q-coupled receptor stimulation.

In addition to PKC, DAG can stimulate a range of other kinases and signalling pathways [36]. Evidence for the direct involvement of kinase(s) in the regulation of P2X₁ receptors was provided in two ways: (i) the kinase-stimulating phorbol ester PMA potentiated P2X₁-receptor-mediated responses, and (ii) mGluR1α- and PMA-mediated potentiation of P2X₁ receptors was abolished by the broad-spectrum kinase inhibitor staurosporine. Further studies will be required to determine the nature of the staurosporine-sensitive and calcium-independent kinase that mediates the potentiation of P2X₁ receptors following mGluR1α activation or PMA treatment.

In the intracellular domains of the P2X₁ receptor, there are a number of serine, threonine and tyrosine residues that could potentially be targets for receptor phosphorylation by staurosporine-sensitive kinases and mediate the regulation of P2X₁ receptors. In the present study, we have shown that the P2X₁ receptor is basally phosphorylated. Phosphorylation was still detected for the T18A mutant receptor (results not shown), indicating that residues

other than those at the conserved PKC site are phosphorylated. A previous study on the P2X₁ receptor failed to detect phosphorylation with a phosphotyrosine-specific antibody, suggesting that these residues are not phosphorylated; however, the very conservative point mutation to phenylalanine had a marked effect on channel function [37]. This suggests that either phosphotyrosine residues were not accessible to the anti-phosphotyrosine antibody or other serine and/or threonine residues in the intracellular domains are phosphorylated. In the present study, PMA treatment potentiated P2X₁-receptor-mediated responses; however, it had no detectable effect on the phosphorylation of the P2X₁ receptor. Regulation of ion channels through the phosphorylation of accessory proteins has been demonstrated for a range of receptors including the P2X₇ receptor [17] and NMDA receptors [18]. This type of mechanism for P2X₁ receptor regulation is supported by experiments with cytochalasin D that acts to disrupt cytoskeletal/intracellular protein interactions. Cytochalasin D had no effect on the peak amplitude of ATP-evoked responses (8363 ± 1416 and 7386 ± 3593 nA for control and cytochalasin D treatment respectively; $n = 6$ and 7), but reduced the level of glutamate-evoked potentiation of P2X₁ responses (cytochalasin D, $10 \mu\text{M}$, 24 h, glutamate potentiation ratio 1.57 ± 0.10 ; $n = 7$, compared with control, 2.23 ± 0.22 ; $n = 6$, $P < 0.05$). This suggests that cytoskeletal interactions with the P2X₁ receptor are involved in kinase-dependent regulation of the receptor and that the staurosporine-sensitive/kinase-dependent regulation of the P2X₁ receptor is not directly at the level of the receptor, but results from phosphorylation of an accessory protein.

What could be the mechanism for the potentiation of the P2X₁-receptor-mediated response? One of the distinguishing features of P2X₁ receptors is their rapid desensitization during continued agonist application and the requirement of several minutes for recovery of the response. In arterial smooth muscle cells, we have shown previously that recovery from desensitization was likely to be regulated by a diffusible factor [12]. In the present study, the amplitude of P2X₁-receptor-mediated responses or the pattern of response to repeated applications of ATP was unaffected by the broad-spectrum kinase inhibitor staurosporine. This suggests that recovery from desensitization is not staurosporine-sensitive/kinase-dependent and demonstrates that potentiation following mGluR1 α stimulation does not result from an increase in the speed of the recovery from the desensitized state. The present study also showed that it is unlikely that the potentiation results from the insertion of new P2X₁ receptors at the cell surface; a mechanism that has been shown to potentiate NMDA-receptor-mediated responses by mGluR1 α stimulation in oocytes [27]. The potentiation of a maximal response to ATP, and equivalent effects at approximately EC₅₀ concentrations of ATP, would rule out an effect on the agonist sensitivity of the channel and makes a change in the single channel properties most likely to account for the potentiation.

In the present study, we have shown that P2X₁-receptor-mediated responses can be potentiated by a range of G α_q -coupled receptors. This regulation is most likely regulated by the phosphorylation of an accessory protein in the P2X₁ receptor complex. The P2X₁ receptor is expressed together with GPCRs in a range of tissues and suggests that regulation of the P2X₁ receptor may be widespread. We have previously shown that P2X₁ receptors can play important roles in cardiovascular control and haemostasis. In platelets, P2X₁ receptor activation potentiates subsequent P2Y₁ receptor-mediated calcium rises [38] and this synergy may be important in the regulation of blood clotting [10]. The present study suggests that P2Y₁ receptors may also reciprocally up-regulate subsequent P2X₁-receptor-mediated responses in these cells

and indicates that GPCRs may provide a mechanism for fine-tuning of P2X₁ receptors *in vivo*.

This work was supported by the Wellcome Trust. We thank Dr E. P. Seward for comments on the manuscript.

REFERENCES

- 1 Burnstock, G. (1997) The past, present and future of purine nucleotides as signalling molecules. *Neuropharmacology* **36**, 1127–1139
- 2 Sneddon, P. and Burnstock, G. (1984) ATP as a co-transmitter in rat tail artery. *Eur. J. Pharmacol.* **106**, 149–152
- 3 Cockayne, D. A., Hamilton, S. G., Zhu, Q.-M., Dunn, P. M., Zhong, Y., Novakovic, S., Malmberg, A. B., Cain, G., Berson, A., Kassotakis, L. et al. (2000) Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X₃-deficient mice. *Nature (London)* **407**, 1011–1015
- 4 Souslova, V., Cesare, P., Ding, Y., Akopian, A. N., Stanfa, L., Suzuki, R., Carpenter, K., Dickenson, A., Boyce, S., Hill, R. et al. (2000) Warm-coding deficits and aberrant inflammatory pain in mice lacking P2X₃ receptors. *Nature (London)* **407**, 1015–1017
- 5 Tsuda, M., Shigemoto-Mogami, Y., Koizumi, S., Mizokoshi, A., Kohsaka, S., Salter, M. W. and Inoue, K. (2003) P2X₄ receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature (London)* **424**, 778–783
- 6 North, R. A. (2002) Molecular physiology of P2X receptors. *Physiol. Rev.* **82**, 1013–1067
- 7 Mulryan, K., Gitterman, D. P., Lewis, C. J., Vial, C., Leckie, B. J., Cobb, A. L., Brown, J. E., Conley, E. C., Buell, G., Pritchard, C. A. and Evans, R. J. (2000) Reduced vas deferens contraction and male infertility in mice lacking P2X₁ receptors. *Nature (London)* **403**, 86–89
- 8 Vial, C. and Evans, R. J. (2000) P2X receptor expression in mouse urinary bladder and the requirement of P2X₁ receptors for functional P2X receptor responses in the mouse urinary bladder smooth muscle. *Br. J. Pharmacol.* **131**, 1489–1495
- 9 Vial, C. and Evans, R. J. (2002) P2X₁ receptor-deficient mice establish the native P2X receptor and a P2Y₆-like receptor in arteries. *Mol. Pharmacol.* **62**, 1438–1445
- 10 Hechler, B., Lenain, N., Marchese, P., Vial, C., Heim, V., Freund, M., Cazenave, J.-P., Cattaneo, M., Ruggeri, Z. M., Evans, R. J. and Gachet, C. (2003) A role of the fast ATP-gated P2X₁ cation channel in thrombosis of small arteries *in vivo*. *J. Exp. Med.* **198**, 661–667
- 11 Valera, S., Hussy, N., Evans, R. J., Adami, N., North, R. A., Surprenant, A. and Buell, G. (1994) A new class of ligand-gated ion channel defined by P2X receptor for extracellular ATP. *Nature (London)* **371**, 516–519
- 12 Lewis, C. J. and Evans, R. J. (2000) Lack of run-down of smooth muscle P2X receptor currents recorded with the amphotericin permeabilized patch technique, physiological and pharmacological characterization of the properties of mesenteric artery P2X receptor ion channels. *Br. J. Pharmacol.* **131**, 1659–1666
- 13 La, M. and Rand, M. J. (1993) Endothelin-1 enhances vasoconstrictor responses to exogenously administered and neurogenically released ATP in rabbit isolated perfused arteries. *Eur. J. Pharmacol.* **249**, 133–139
- 14 Boue-Grabot, E., Archambault, V. and Seguela, P. (2000) A protein kinase C site highly conserved in P2X subunits controls the desensitization kinetics of P2X₂ ATP-gated channels. *J. Biol. Chem.* **275**, 10190–10195
- 15 Ennion, S. J. and Evans, R. J. (2002) P2X₁ receptor subunit contribution to gating revealed by a dominant negative PKC mutant. *Biochem. Biophys. Res. Commun.* **291**, 611–616
- 16 Paukert, M., Osteroth, R., Geisler, H.-S., Brandle, U., Glowatzki, E., Ruppersberg, J. P. and Grunder, S. (2001) Inflammatory mediators potentiate ATP-gated channels through the P2X₃ subunit. *J. Biol. Chem.* **276**, 21077–21082
- 17 Adinolfi, E., Kim, M., Young, M. T., Di Virgilio, F. and Surprenant, A. (2003) Tyrosine phosphorylation of HSP90 within the P2X₇ receptor complex negatively regulates P2X₇ receptors. *J. Biol. Chem.* **278**, 37344–37351
- 18 Zheng, X., Zhang, L., Wang, A. P., Bennett, M. V. and Zukin, R. S. (1999) Protein kinase C potentiation of *N*-methyl-D-aspartate receptor activity is not mediated by phosphorylation of *N*-methyl-D-aspartate receptor subunits. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 15262–15267
- 19 Kunapuli, S. P., Dorsam, R. T., Kim, S. and Quinton, T. M. (2003) Platelet purinergic receptors. *Curr. Opin. Pharmacol.* **3**, 175–180
- 20 Ralevic, V. and Burnstock, G. (1998) Receptors for purines and pyrimidines. *Pharmacol. Rev.* **50**, 413–492
- 21 Mahaut-Smith, M. P., Ennion, S. J., Rolf, M. G. and Evans, R. J. (2000) ADP is not an agonist at P2X₁ receptors: evidence for separate receptors stimulated by ATP and ADP on human platelets. *Br. J. Pharmacol.* **131**, 108–114
- 22 Ennion, S. J., Hagan, S. and Evans, R. J. (2000) The role of positively charged amino acids in ATP recognition by human P2X₁ receptors. *J. Biol. Chem.* **275**, 29361–29367

- 23 Budd, D. C., Willars, G. B., McDonald, J. E. and Tobin, A. B. (2001) Phosphorylation of the Gq/11-coupled M₃-muscarinic receptor is involved in receptor activation of the ERK-1/2 mitogen-activated protein kinase pathway. *J. Biol. Chem.* **276**, 4581–4587
- 24 Budd, D. C., McDonald, J. E. and Tobin, A. B. (2000) Phosphorylation and regulation of a Gq/11-coupled receptor by casein kinase 1 α . *J. Biol. Chem.* **275**, 19667–19675
- 25 Saugstad, J. A., Segerson, T. P. and Westbrook, G. L. (1996) Metabotropic glutamate receptors activate G-protein-coupled inwardly rectifying potassium channels in *Xenopus* oocytes. *J. Neurosci.* **16**, 5979–5985
- 26 Skeberdis, V. A., Lan, J., Zheng, X., Zukin, R. S. and Bennett, M. V. (2001) Insulin promotes rapid delivery of N-methyl-D-aspartate receptors to the cell surface by exocytosis. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 3561–3566
- 27 Lan, J. Y., Skeberdis, V. A., Jover, T., Grooms, S. Y., Lin, Y., Araneda, R. C., Zheng, X., Bennett, M. V. and Zukin, R. S. (2001) Protein kinase C modulates NMDA receptor trafficking and gating. *Nat. Neurosci.* **4**, 382–390
- 28 Liu, F., Wan, Q., Pristupa, Z. B., Yu, X. M., Wang, Y. T. and Niznik, H. B. (2000) Direct protein–protein coupling enables cross-talk between dopamine D5 and γ -aminobutyric acid A receptors. *Nature (London)* **403**, 274–280
- 29 Webb, T. E., Simon, J., Krishek, B. J., Bateson, A. N., Smart, T. G., King, B. F., Burnstock, G. and Barnard, E. A. (1993) Cloning and functional expression of a brain G-protein-coupled ATP receptor. *FEBS Lett.* **324**, 219–225
- 30 Lustig, K. D., Shiau, A. K., Brake, A. J. and Julius, D. (1993) Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 5113–5117
- 31 Lan, J. Y., Skeberdis, V. A., Jover, T., Zheng, X., Bennett, M. V. and Zukin, R. S. (2001) Activation of metabotropic glutamate receptor 1 accelerates NMDA receptor trafficking. *J. Neurosci.* **21**, 6058–6068
- 32 Liu, G. J., Brockhausen, J. and Bennett, M. R. (2003) P2X₁ receptor currents after disruption of the PKC site and its surroundings by dominant negative mutations in HEK293 cells. *Auton. Neurosci.* **108**, 12–16
- 33 Wang, Y. T. and Salter, M. W. (1994) Regulation of NMDA receptors by tyrosine kinases and phosphatases. *Nature (London)* **369**, 233–235
- 34 Huang, X. Y., Morielli, A. D. and Peralta, E. G. (1993) Tyrosine kinase-dependent suppression of a potassium channel by the G protein-coupled m1 muscarinic acetylcholine receptor. *Cell* **75**, 1145–1156
- 35 Skeberdis, V. A., Lan, J., Opitz, T., Zheng, X., Bennett, M. V. and Zukin, R. S. (2001) mGluR1-mediated potentiation of NMDA receptors involves a rise in intracellular calcium and activation of protein kinase C. *Neuropharmacology* **40**, 856–865
- 36 Yang, C. and Kazanietz, M. G. (2003) Divergence and complexities in DAG signaling: looking beyond PKC. *Trends Pharmacol. Sci.* **24**, 602–608
- 37 Toth-Zsamboki, E., Oury, C., Watanabe, H., Nilius, B., Vermeylen, J. and Hoylaerts, M. F. (2002) The intracellular tyrosine residues of the ATP-gated P2X₁ ion channel are essential for its function. *FEBS Lett.* **524**, 15–19
- 38 Vial, C., Rolf, M. G., Mahaut-Smith, M. P. and Evans, R. J. (2002) A study of P2X₁ receptor function in murine megakaryocytes and human platelets reveals synergy with P2Y receptors. *Br. J. Pharmacol.* **135**, 363–372

Received 10 December 2003/21 April 2004; accepted 14 May 2004

Published as BJ Immediate Publication 14 May 2004, DOI 10.1042/BJ20031910