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RESEARCH PAPER

Metabotropic P2Y receptors inhibit $P2X_3$ receptor-channels via G protein-dependent facilitation of their desensitization

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Background and purpose: The aim of the present study was to investigate whether the endogenous metabotropic P2Y receptors modulate ionotropic $P2X_3$ receptor-channels.
Experimental approach: Whole-cell patch-clamp experiments were carried out on HEK293 cells permanently transfected with human $P2X_3$ receptors (HEK293-h$P2X_3$ cells) and rat dorsal root ganglion (DRG) neurons.
Key results: In both cell types, the $P2Y_{1,12,13}$ receptor agonist, ADP-β-S, inhibited $P2X_3$ currents evoked by the selective agonist, α,β-methylene ATP (α,β-meATP). This inhibition could be markedly counteracted by replacing in the pipette solution the usual GTP with GDP-β-S, a procedure known to block all G protein heterotrimers. $P2X_3$ currents evoked by ATP, activating both P2Y and P2X receptors, caused a smaller peak amplitude and desensitized faster than those currents evoked by the selective $P2X_3$ receptor agonist α,β-meATP. In the presence of intracellular GDP-β-S, ATP- and α,β-meATP-induced currents were identical. Recovery from $P2X_3$ receptor desensitization induced by repetitive ATP application was slower than the recovery from α,β-meATP-induced desensitization. When G proteins were blocked by intracellular GDP-β-S, the recovery from the ATP- and α,β-meATP-induced desensitization were of comparable speed.
Conclusions and Implications: Our results suggest that the activation of P2Y receptors G protein-dependently facilitates the desensitization of $P2X_3$ receptors and suppresses the recovery from the desensitized state. Hence, the concomitant stimulation of $P2X_3$ and P2Y receptors of DRG neurons by ATP may result both in an algesic effect and a partly counterbalancing analgesic activity.
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Keywords: P2Y receptor; $P2X_3$ receptor; G protein; desensitisation; HEK293 cells; DRG

Abbreviations: ADP-β-S, adenosine 5-[β-thio]diphosphate trilithium salt; AR-C6993MX, N^6-(2-methylthioethyl)-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene-ATP; DMEM, Dulbecco's modified Eagle's medium; DRG, dorsal root ganglion; $G\alpha_{i/o}$, G protein $\alpha_{i/o}$ subunit; $G\alpha_{q/11}$, G protein $\alpha_{q/11}$ subunit; $G\beta\gamma$, G protein $\beta\gamma$ subunit; GDP-β-S, guanosine 5-[β-thio]diphosphate; GTP-γ-S, guanosine 5-[γ-thio]triphosphate; HEK, human embryonic kidney; h$P2X_3$R, recombinant human $P2X_3$ receptor; MRS 2179, 2-deoxy-N^6-methyladenosine 3,5-diphosphate; 2-MeSADP, 2-methylthio-adenosine 5-diphosphate; 2-MeSAMP, 2-methylthio-adenosine 5-monophosphate; α,β-meATP, α,β-methylene adenosine 5′-triphosphate; τ_{des}, desensitization time constant; $\tau_{on(10-90\%)}$, onset time constant; PKC, protein kinase C; PTX, pertussis toxin; TRPV1, transient receptor potential cation channel, subfamily V, member 1

Introduction

P2X receptors ($P2X_{1-7}$) are cation-conducting channels that are activated by extracellular adenosine 5′-triphosphate (ATP) (Khakh, 2001; Vial *et al*., 2004). They mediate membrane depolarization as well as Ca^{2+} influx and play a role in several physiological and pathological conditions from fast excitatory synaptic transmission to nociception (Robertson *et al*., 2001; Köles *et al*., 2005). $P2X_3$ receptor-channels are stimulated by ATP in the micromolar range, activate and desensitize rapidly (within tens of milliseconds), and recover from desensitization slowly (within several minutes; Chizh and Illes, 2001). This receptor type is almost

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exclusively expressed in sensory neurons (Chen *et al*., 1995), mostly in $P2Y_1$- and TRPV1-coexpressing nociceptors (Vulchanova *et al*., 1998; Gerevich *et al*., 2004), and plays an important role in the development of both acute and chronic pain states (Burnstock, 1996; Chizh and Illes, 2001). It has been shown that the inflammatory mediators, substance P and bradykinin, potentiate $P2X_3$ currents via phosphorylation of an N-terminal protein kinase C (PKC) site or alternatively an associated protein (Paukert *et al*., 2001). Moreover, low concentrations of nucleotides in the extracellular space appear to phosphorylate PKC sites on the ectodomain of the $P2X_3$ receptor, and thereby lead to an increased sensitivity of the receptor towards high agonist concentrations and accelerate the rate of desensitization (Wirkner *et al*., 2005; Stanchev *et al*., 2006). It has also been reported that low concentrations of agonists are able to detain the receptor in the desensitized state, which is manifested as an inhibition of the $P2X_3$ currents by low agonist concentrations (Pratt *et al*., 2005; Sokolova *et al*., 2006).

The P2Y receptor family consists of G protein-coupled receptors which are activated by extracellular ATP. Up to date, eight mammalian P2Y receptor-subtypes ($P2Y_{1,2,4,6,11,12,13,14}$) have been cloned (Burnstock, 2004). These receptors are stimulated by low concentrations of ATP (in the nanomolar range); ATP binding triggers second-messenger cascades and subsequent signaling of relatively long duration (for several seconds; Illes and Ribeiro, 2004). P2Y receptors are known to modulate a number of voltage- and ligand-gated membrane channels on the somato-dendritic region of neurons (Lechner and Boehm, 2004), such as M-type K^+ channels (Filippov *et al*., 2004), voltage-activated calcium channels (Filippov *et al*., 2003; Gerevich *et al*., 2004), NMDA receptors (Luthardt *et al*., 2003), as well as vanilloid-, pH- and heat-sensitive TRPV1 receptors (Moriyama *et al*., 2003).

Recent preliminary data of our group indicate that $P2Y_1$ and $P2X_3$ receptors of rat dorsal root ganglion (DRG) neurons may negatively interact with each other (Gerevich *et al*., 2005). The aim of the present study was twofold. Firstly, to reproduce these findings at recombinant human $P2X_3$ receptors ($hP2X_3R$) permanently expressed in the human embryonic kidney (HEK) 293 cell line and secondly, to find out whether a G protein-dependent promotion of the desensitization of $P2X_3$ receptors is the reason for this negative interaction. In conclusion, we suggest that minute concentrations of ATP may activate P2Y receptors, which in turn facilitate the desensitization of $P2X_3$ receptors previously triggered by ATP occupation of high-affinity binding sites at the $P2X_3$ receptor.

Methods

Culturing of HEK293-$hP2X_3$ cells

Methods of maintenance of HEK293 cells and their stable transfection with $hP2X_3R$ cDNA have been described previously (Fischer *et al*., 2003). Cells were kept in Dulbecco's modified Eagle's medium (DMEM) also containing 25 mM HEPES, 110 $\mu g\,ml^{-1}$ sodium pyruvate, 1 $mg\,ml^{-1}$ D-glucose, 4 $\mu g\,ml^{-1}$ pyridoxine (Life Technologies, Karlsruhe, Germany), 2 mM L-glutamine, 1% non-essential amino acids (NEAA) (all Sigma, Deisenhofen, Germany), 10% fetal bovine serum and 50 $\mu g\,ml^{-1}$ geneticin (both from Life Technologies) at 37°C and 10% CO_2 in humidified air. They were plated on 35 mm plastic dishes (Sarstedt, Nürnberg, Germany) for electrophysiological recordings.

Preparation of DRG neuronal cultures

One-day-old Wistar rats (own breed) were used in the study. The animals were killed under CO_2 and decapitated to obtain cell cultures of DRG neurons. The isolation and culturing procedures of thoracic and lumbar DRG cells have been described in detail previously (Himmel *et al*., 2002; Gerevich *et al*., 2004). DRG cells were plated at a density of 3×10^4 cells onto 35 mm plastic dishes coated by poly-L-lysine (25 $\mu g\,ml^{-1}$) (Sarstedt). They were kept in DMEM, 35 mM total glucose, 2.5 mM L-glutamine, 15 mM HEPES, 50 $\mu g\,ml^{-1}$ gentamicin, 5% fetal bovine serum (Life Technologies), 30 $ng\,ml^{-1}$ nerve growth factor, 10 $\mu g\,ml^{-1}$ insulin, 5.5 $\mu g\,ml^{-1}$ transferrin and 5 $ng\,ml^{-1}$ selenium (Sigma). Primary cultures of rat DRG neurons were maintained for 2–4 days in a humidified atmosphere (37°C, 5% CO_2) before experimentation.

Whole-cell patch-clamp recordings

Whole-cell patch-clamp recordings were performed 2–6 days after the splitting of permanently transfected HEK293 cells, and 2–4 days after the plating of rat DRG neurons, at room temperature (20–22°C), using an Axopatch 200B patch-clamp amplifier (Molecular Devices, Union City, CA, USA). Patch pipettes (3–5 MΩ) for both HEK293 cells and DRG neurons were filled with intracellular solution of the following composition (in mM): 135 CsCl, 2 $MgCl_2$, 20 HEPES, 11 EGTA, 1 $CaCl_2$, 1.5 Mg-ATP and 0.3 Li-GTP, (pH 7.3) adjusted with CsOH. As stated below, Li-GTP (300 μM), guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S; 300 μM) or guanosine 5′-*O*-(3-thiodiphosphate) (GDP-β-S; 300 μM), respectively, were added to the pipette solution. In some of the experiments, activated *Pertussis* toxin was included in the pipette solution (Wirkner *et al*., 2004). The external recording solution consisted of (in mM) 140 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES and 11 glucose (pH 7.4) adjusted with NaOH. After the whole-cell configuration was established, an equilibrium period of 10 min was allowed to elapse for establishing adequate solution exchange between the patch pipette and the cell. All recordings were made at a holding potential of −70 mV. Data were filtered at 2 kHz with the inbuilt filter of the Axopatch 200B, digitized at 5 kHz and stored on a laboratory computer using a Digidata 1200 interface and pClamp 8.0 software (Molecular Devices).

Drugs were dissolved in external solution and applied by pressure, locally to single cells, using a DAD12 superfusion system (Adams and List, Westbury, NY, USA; 10–90% rise time of the junction potential at an open pipette tip was 60–80 ms). In studies of activation and desensitization, more rapid solution change was used (SF-77B Perfusion Fast-Step, Warner Instruments, Hamden, CT, USA). With this system,

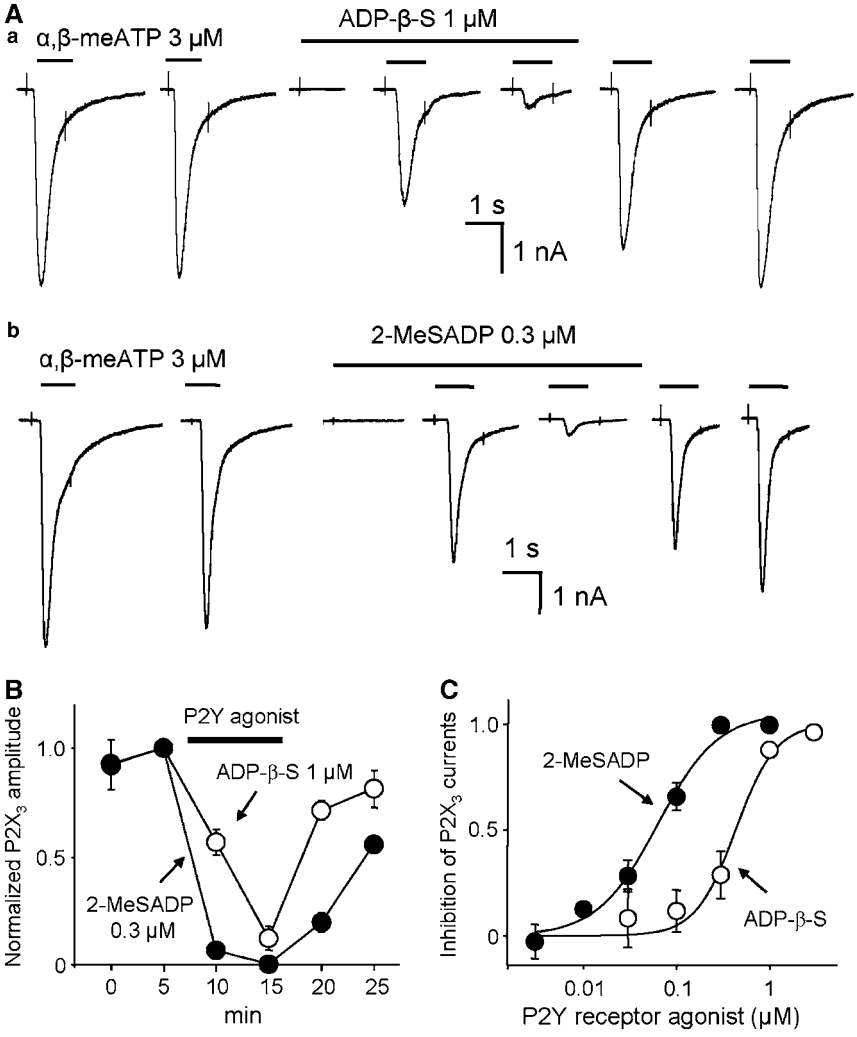

Figure 1 Inhibition of current responses to the $P2X_3$ receptor agonist α,β-meATP by the $P2Y_{1,12,13}$ receptor agonists ADP-β-S and 2-MeSADP. $P2X_3$ receptor ($P2X_3R$) currents were evoked by 3 μM α,β-meATP in HEK293 cells transfected with the human (h)$P2X_3R$. **A**, 2.5 min after a stable current was established, 1 μM ADP-β-S (**Aa**) and 0.3 μM 2-MeSADP (**Ab**) were washed in and applied for 7.5 min. None of these agonists evoked inward currents at concentrations submaximally inhibiting the $P2X_3$ channels. (**B**) $P2X_3$ current amplitudes expressed as mean$\pm$s.e.m. ($n=5$–10) for P2Y receptor agonist concentrations identical to those shown in (**A**). (**C**) Concentration–response curves for inhibition of $P2X_3$ currents by ADP-β-S and 2-MeSADP. Each point was collected from 5–10 individual cells after a 7.5-min P2Y agonist application.

the 10–90% rise times were 1–4 ms. Concentration–response curves for the $P2X_3$ receptor-agonistic effect of ADP-β-S and 2-methylthio adenosine 5-diphosphate (2-MeSADP) at both HEK293-h$P2X_3$ cells and rat DRG neurons were constructed by applying, every 5 min, increasing concentrations of these agonists for 1 s. To analyze the inhibitory effect of P2Y receptor activation on $P2X_3$ channels, a submaximal concentration of α,β-methylene ATP (α,β-meATP; in HEK293-h$P2X_3$ cells 3 μM and in DRG neurons 10 μM) was applied six times for 1 s with 5 min intervals. After the recording of two α,β-meATP-induced inward currents of the same size, low concentrations of the preferential P2Y receptor agonists 2-MeSADP or ADP-β-S (see Figure 1), or ATP itself were applied to stimulate the P2Y receptors, for 7.5 min, starting 2.5 min after the second α,β-meATP administration. Two α,β-meATP currents were recorded during and two further ones after the application of the P2Y receptor agonists or ATP. Data are presented as percentage$\pm$s.e.m. of the amplitude of the α,β-meATP current immediately before starting the application of P2Y receptor agonists (reference value). To estimate the effects of ADP-β-S and 2-MeSADP on the $P2X_3$ receptor currents, the fourth α,β-meATP current was compared to the reference value and expressed in its percentage.

For the comparison of drug effects in the presence and absence of intracellular GDP-β-S/GTP-γ-S, experiments were made always on the same day and from the same culture dish. Thus, cells were arbitrarily selected either for recording with normal GTP containing patch pipettes or with GTP-β-S/GTP-γ-S containing patch pipettes. Thereby, spontaneous variations in $P2X_3$ current amplitudes or their sensitivity to P2Y receptor-inhibition could be considerably reduced.

Concentration–response curves to determine the inhibitory effects of ADP-β-S and 2-MeSADP on α,β-meATP-induced currents were fitted using the following logistic function: $I=I_{max}-I_{min}/[(1+(IC_{50}+\text{agonist})^n)]$, where I is the steady-state inhibition produced by the agonist, I_{max} and I_{min} are

the maximal and minimal inhibition, respectively, n is the Hill coefficient and IC_{50} is the concentration of the agonist producing 50% of I_{max}. Concentration–response curves to determine the agonistic effects of ADP-β-S, 2-MeSADP, ATP and α,β-meATP at $P2X_3$ receptors were fitted by a logistic function similar to that described above, with the exception that E_{max} and E_{min} were the maximal and minimal current amplitudes and EC_{50} the concentration of the agonist producing 50% of E_{max}.

In experiments investigating the kinetics of $P2X_3$ currents, α,β-meATP (3 μM) and ATP (3 μM) were applied to HEK293 cells for 20 s. The amount of data sampled was reduced by a factor of 5 by means of the pClamp 8.0 software and the decay phases of the curves were fitted by Origin software (OriginLab, Northampton, MA, USA) in order to obtain the desensitization time-constants (τ_{des1}, τ_{des2}). The onset time constants ($\tau_{on(10–90\%)}$) were calculated from the individual recordings, under the assumption that in spite of the relatively slow local application they give a rough approximation of the kinetics of channel opening. In experiments examining the recovery of $P2X_3$ receptors from desensitization, HEK293 cells were stimulated repetitively with α,β-meATP and ATP at 3 μM (5-s pulses, each) with a progressive increase in the interpulse intervals. The recovery from desensitization was best fitted by using a sigmoidal function (see also Sokolova *et al*., 2004):

$$I = I_{max} + \frac{I_{max} - I_{min}}{1 + e^{(t_{50} - t)/b}}$$

where I_{max} and I_{min} are the start and finish levels during recovery, t is the time, t_{50} is the time to regain 50% of maximally recovered currents and b is the slope factor giving the change in time per e-fold change in recovery.

Data analysis

Data were analyzed off-line using pClamp 8.0. software (Molecular Devices). Figures show mean $\pm$ s.e.m. values of n experiments. Student's t-test or one-way ANOVA followed by Bonferroni's *post hoc* test were used for statistical analysis. A probability level of 0.05 or less was considered to reflect a statistically significant difference.

Materials and drugs

The following pharmacological agents were used: adenosine 5-triphosphate disodium salt (ATP), α,β-methylene ATP lithium salt (α,β-meATP), 2-methylthio-AMP triethylammonium salt (2-MeSAMP), 2-methylthio-ADP trisodium salt (2-MeSADP), adenosine 5-[β-thio]diphosphate trilithium salt (ADP-β-S), guanosine 5-triphosphate lithium salt (GTP), guanosine 5-[β-thio]diphosphate trilithium salt (GDP-β-S), guanosine 5-[γ-thio]triphosphate tetralithium salt (GTP-γ-S), *Pertussis* toxin (Sigma); 2-deoxy-N^6-methyladenosine 3,5-diphosphate diammonium salt (MRS 2179) (Tocris, Bristol, UK); N^6-(2-methylthioethyl)-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene-ATP (AR-C6993MX; cangrelor) (Jayne Prats, The Medicines Company, Waltham, MA, U.S. A.). All drugs were prepared as a concentrated stock solution in distilled water and were diluted to final concentration in external medium.

Results

Depression of α,β-meATP-induced currents in HEK293-hP2X$_3$ cells by ADP-β-S and 2-MeSADP

HEK293 cells permanently transfected with $hP2X_3$Rs exhibited a rapidly activating and desensitizing inward current in response to a 1-s application of the $P2X_3$ receptor agonist α,β-meATP at a submaximal concentration of 3 μM (Figure 1A). Cells not transfected with $P2X_3$ receptors failed to respond to α,β-meATP at any concentration used (Fischer *et al*., 2003). The responses to α,β-meATP applied at 5-min intervals were reproducible, indicating that complete recovery from receptor desensitization occurred during this time. After recording two α,β-meATP-induced currents of the same size, we superfused the $P2Y_{1,12,13}$ receptor agonists ADP-β-S (0.03–3 μM) and 2-MeSADP (3 nM–1 μM), to investigate whether ADP-sensitive, G protein-coupled ATP receptors can modulate the α,β-meATP currents (Figure 1). These ADP analogs, at the concentrations tested, did not evoke any inward current. However, both agonists significantly and concentration-dependently inhibited the α,β-meATP responses, although 2-MeSADP was approximately seven times more potent than ADP-β-S. The IC_{50} values for ADP-β-S and 2-MeSADP were 434 ± 103 and 63 ± 11 nM, respectively ($n = 5$–10; Figure 1C). The inhibitory effects of both ADP-β-S and 2-MeSADP developed slowly, with an apparent maximum 7.5 min after the beginning of their application (Figure 1A and B).

Although ADP-β-S and 2-MeSADP are known to be selective P2Y receptor agonists (Burnstock, 2004), we investigated whether they also have some agonistic effects on $hP2X_3$ receptors. They were, therefore, applied at increasing concentrations (0.3–300 μM) for 1 s onto HEK293-$hP2X_3$ cells. Both P2Y receptor agonists were able to induce inward currents by opening $P2X_3$ receptor channels, however, only at much higher concentrations than those necessary for the inhibition of $P2X_3$ currents (Figure 2a). In fact, the EC_{50} value of ADP-β-S to induce inward currents was 110 ± 1 μM ($n = 9$), and thereby more than 1000 times higher than its IC_{50} value for $P2X_3$ receptor inhibition. Comparing the inward currents evoked by ADP-β-S with those evoked by ATP (0.3–100 μM) or α,β-meATP (0.3–100 μM) demonstrated that the preferential P2Y receptor agonist had a considerably lower potency on the $P2X_3$ receptor than ATP ($EC_{50} = 3.3 \pm 0.3$ μM; $n = 9$; $P < 0.05$) and α,β-meATP ($EC_{50} = 3.6 \pm 0.4$ μM; $n = 6$; $P < 0.05$) (compare Figure 2a with Figure 5b and c). In addition, the maximum current induced by ADP-β-S ($I_{max} = 0.65 \pm 0.01$ nA; $n = 9$) was much lower than that induced by ATP ($I_{max} = 4.08 \pm 0.14$ nA; $n = 9$; $P < 0.05$) or α,β-meATP ($I_{max} = 4.85 \pm 0.19$ nA; $n = 6$; $P < 0.05$). As, up to a concentration of 300 μM, we did not reach a maximum of the concentration–response curve of 2-MeSADP for $P2X_3$ current-induction, no EC_{50} value could be calculated. However, it is quite clear that 2-MeSADP also inhibits the $P2X_3$ currents evoked by α,β-meATP at about 1000-fold lower concentrations than those inducing currents by itself at this receptor-type.

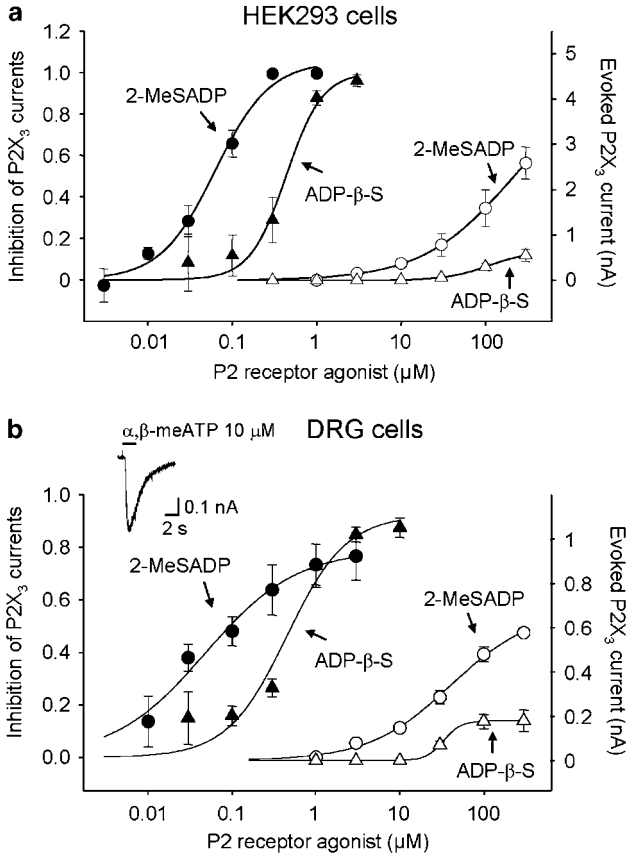

Figure 2 Inhibition of α,β-meATP-induced currents by P2Y receptor agonists at more than 1000-fold lower concentrations than required for activation. For inhibition curves (filled symbols), ADP-β-S and 2-MeSADP were applied for 7.5 min to depress $P2X_3$ currents evoked by α,β-meATP (3 μM, HEK293; 10 μM, DRG cells). The depression of the α,β-meATP-induced currents was plotted against the agonist concentrations. Concentration–response curves for activation of $P2X_3$ currents are plotted on the same graph (open symbols). Data points were collected at 5 min intervals for both agonists and were fitted with the Hill sigmoidal equation. (**a**) Results from HEK293 cells permanently transfected with $hP2X_3$ receptors. (**b**) Results from rat DRG neurons expressing native rat $P2X_3$ channels. The inset shows an original recording with the effect of α,β-meATP (10 μM) in a DRG neuron. Values shown are means$\pm$s.e.m. from 4–9 cells in both (**a**) and (**b**).

Depression of α,β-meATP-induced currents in rat DRG neurons by ADP-β-S and 2-MeSADP

We repeated the same experiments on rat small-diameter (20–35 μM) DRG neurons known to express native $P2X_3$ receptors. α,β-meATP at the submaximal concentration of 10 μM evoked rapidly desensitizing currents (Figure 2b, inset). ADP-β-S and 2-MeSADP, applied in the same way as to HEK293-$hP2X_3$ cells, concentration-dependently inhibited the α,β-meATP induced currents (Figure 2b). The IC_{50} values of ADP-β-S and 2-MeSADP to block $P2X_3$ currents were 450 ± 167 ($n=4$–10) and 48 ± 14 nM ($n=4$–6), respectively. These values are in accordance with the EC_{50} values of these agonists at rat $P2Y_1$, but not $P2Y_{12}$ receptors (Vöhringer *et al.*, 2000; Simon *et al.*, 2002), suggesting the involvement of the former receptor-type.

To assess the activation by the preferential P2Y receptor agonists of $P2X_3$ receptors in DRG neurons, ADP-β-S and 2-MeSADP (1–300 μM, each) were applied for 1 s at increasing concentrations. Both P2Y receptor agonists were able to open the $P2X_3$ receptor-channels and to induce inward currents. The EC_{50} value of ADP-β-S (40.5 ± 8.1 μM, $n=9$) was more than 1000 times higher than its IC_{50} value for $P2X_3$ receptor inhibition (Figure 2b). Moreover, ADP-β-S had clearly lower potencies at $P2X_3$ receptor channels than ATP ($EC_{50}=4.4\pm1.2$ μM) and α,β-meATP ($EC_{50}=4.4\pm0.7$ μM; Figure 5a). Once more, it was not possible to determine a clear maximum effect of 2-MeSADP at the highest concentration tested (300 μM). However, this agonist also appeared to be about 1000 times more active for $P2X_3$ receptor inhibition than for the induction of $P2X_3$ currents.

Characterization of the P2Y receptor-subtype involved in HEK293-hP2X3 cells

HEK293 cells were reported to endogenously express mRNA and protein for all known P2Y receptors with exception of the $P2Y_{12}$ subtype (Moore *et al.*, 2001; Fischer *et al.*, 2003; Wirkner *et al.*, 2004). As we used, in our experiments, ADP-β-S and 2-MeSADP, two structural analogs of ADP with high selectivity for $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors, we have concentrated our attention on these receptor subtypes. However, the selective antagonists MRS 2179 (10 μM; $P2Y_1$), 2-meSAMP (100 μM; $P2Y_{12}$) and AR-C69931MX (1 μM; $P2Y_{12/13}$) all failed to antagonize the inhibitory effect of ADP-β-S at $P2X_3$ receptors (Figure 3A).

Depression of α,β-meATP-induced currents in HEK293-hP2X3 cells by ADP-β-S, 2-MeSADP and low concentrations of ATP in the presence of intracellular GDP-β-S and GTP-γ-S

P2Y receptors transmit extracellular signals to downstream effectors by activating heterotrimeric G protein complexes. We next examined whether these G proteins might mediate the inhibitory effect of P2Y agonists, by replacing the standard GTP (300 μM) in the pipette solution with an equimolar quantity of the non-hydrolyzable GDP analogue GDP-β-S. As illustrated in Figure 3B loading of GDP-β-S via the patch pipette into HEK293 cells transfected with $hP2X_3$ receptors clearly attenuated the ADP-β-S-induced inhibition of the α,β-meATP (3 μM) currents. In addition, dialysis of the cell interior with the non-hydrolyzable GTP analog, GTP-γ-S (300 μM), resulted in an almost complete failure of ADP-β-S (1 μM) to depress $P2X_3$ receptor currents (Figure 3Bb). Neither GTP-γ-S (GTP pipette; 3.0 ± 0.5 nA, $n=7$; GTP-γ-S pipette; 2.5 ± 0.3 nA, $n=8$, $P>0.05$) nor GDP-β-S (GTP pipette; 2.2 ± 0.3 nA, $n=15$; GDP-β-S pipette; 1.9 ± 0.3 nA, $n=12$, $P>0.05$; see also Figure 5c) altered the current amplitudes when included in the pipette solution. As it is known that GDP-β-S blocks all G protein heterotrimers, whereas GTP-γ-S causes their long-lasting activation with a subsequent resistance to further stimulatory effects (Takago *et al.*, 2005), the results indicate that G proteins are involved in the inhibitory effect of the P2Y receptor agonist ADP-β-S at $P2X_3$ receptor channels. It is not known, why GTP-γ-S failed to decrease the α,β-meATP-induced current amplitudes. Finally, we tried to find out which G protein α-subunit is involved, and therefore applied activated *Pertussis* toxin

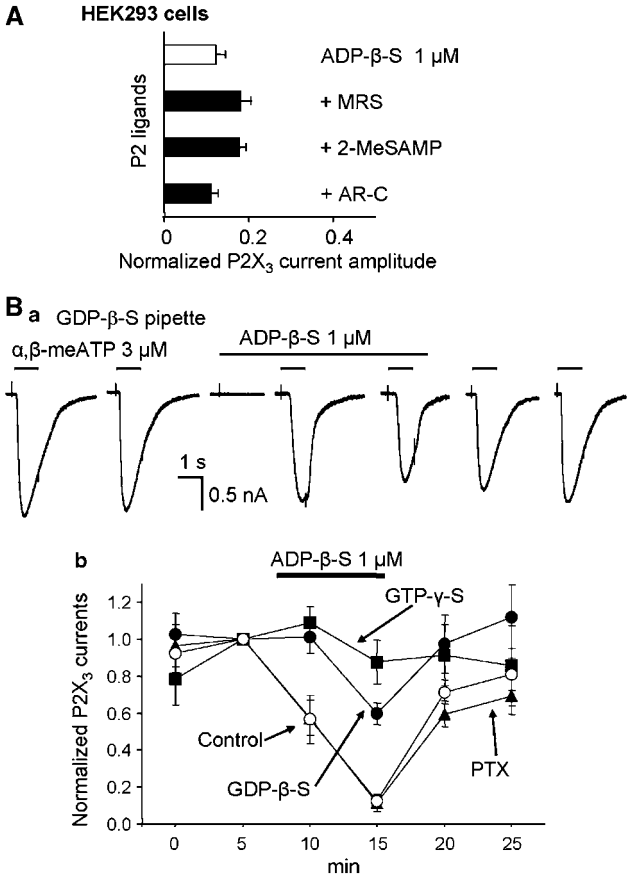

Figure 3 Inhibition of α,β-meATP-induced currents by P2Y receptor agonists in the presence of $P2Y_{1,12,13}$ receptor antagonists; blockade of G protein heterotrimers prevents the inhibitory interaction between α,β-meATP and ADP-β-S. HEK293 cells were permanently transfected with hP2X$_3$ receptors. (**A**) P2X$_3$ currents were evoked by 3 μM α,β-meATP in HEK293-hP2X$_3$ cells. Bars represent the normalized amplitude (mean$\pm$s.e.m.) of P2X$_3$ currents after 7.5 min ADP-β-S application expressed in percentage of the last α,β-meATP-current before ADP-β-S application. The open bar indicates the control inhibition of P2X$_3$ currents by ADP-β-S at 1 μM both in the presence (MRS 2179, 10 μM; 2-meSAMP, 100 μM; AR-C69931MX, 1 μM) and absence of antagonists. (**B**) P2X$_3$ currents were evoked by 3 μM α,β-meATP in HEK293 cells transfected with the hP2X$_3$ receptors. HEK293-P2X$_3$ cells were loaded with equimolar GDP-β-S, instead of the usual 300 μM GTP (**Ba**) via the patch pipette for at least 15 min to block G proteins. After this procedure, ADP-β-S no longer inhibited P2X$_3$ receptor currents. (**Bb**), P2X$_3$ current amplitudes are expressed as mean$\pm$s.e.m. ($n=8$–12) before, during and after the application of 1 μM ADP-β-S; recording was with patch pipettes containing GDP-β-S (300 μM), GTP-γ-S (300 μM) or *Pertussis* toxin (PTX; 1 μg ml^{-1}). GDP-β-S and GTP-γ-S, but not PTX blocked the inhibitory effect of ADP-β-S.

(1 μg ml^{-1}), a blocker of G$\alpha_{i/o}$, into the intracellular solution. As *Pertussis* toxin did not interfere with the ADP-β-S effect, the participation of G$\alpha_{i/o}$ is unlikely (Figure 3Bb).

Recently, it has been shown that long-lasting application of nanomolar ATP concentrations inhibits P2X$_3$ receptor-channels via binding to a high affinity site of the receptor (Sokolova *et al*., 2004, 2006; Pratt *et al*., 2005). To investigate whether the activation of G proteins by ATP facilitates this effect, we desensitized the P2X$_3$ receptors with a 7.5-min application of ATP (30–100 nM; Figure 4). This low concentration markedly inhibited the α,β-meATP (10 μM)-induced

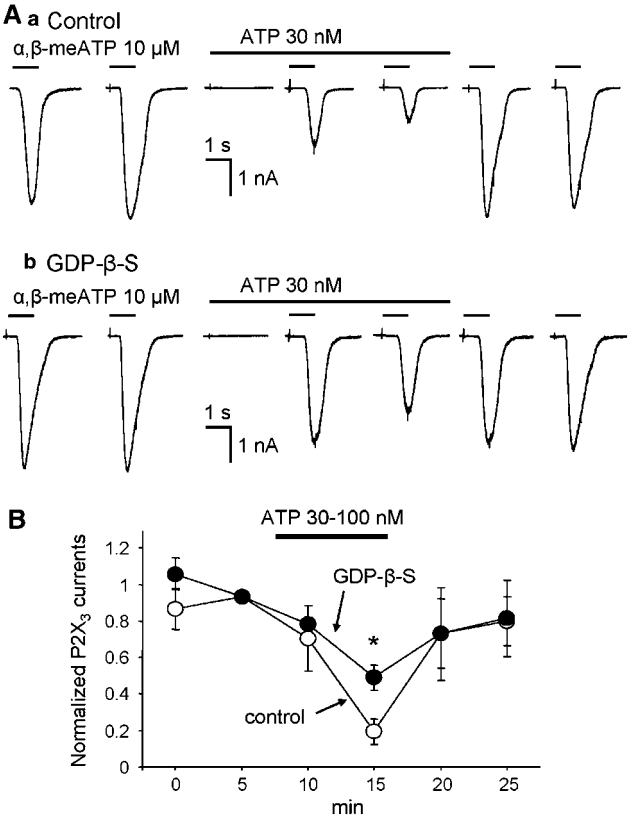

Figure 4 Blockade of G protein heterotrimers modulates the inhibitory effect of low ATP concentrations on the P2X$_3$ currents. HEK293 cells were permanently transfected with hP2X$_3$ receptors. (**A**) P2X$_3$ currents were evoked by 3 μM α,β-meATP. Where indicated, HEK293-P2X$_3$ cells were loaded instead of the regular 300 μM GTP (**Aa**) with equimolar GDP-β-S (**Ab**). (**B**) P2X$_3$ current amplitudes expressed as mean$\pm$s.e.m. ($n=5$) before, during and after the application of 30–100 nM ATP; recording was with patch-pipettes containing GDP-β-S (300 μM) or GTP (300 μM; control). Mean$\pm$s.e.m. of five cells. *$P<0.05$; statistically significant difference from the respective control current.

P2X$_3$ currents in HEK293-hP2X$_3$ cells (Figure 4Aa and B). However, in cells loaded with GDP-β-S (300 μM), the same concentration of ATP depressed the effect of α,β-meATP with a lower efficiency (Figure 4Ab and B).

α,β-meATP- and ATP-induced currents in rat DRG neurons and HEK293-hP2X$_3$ cells in the presence of intracellular GDP-β-S

In another set of experiments, we applied increasing concentrations of ATP and α,β-meATP onto HEK293-hP2X$_3$ and DRG cells to establish concentration–response curves and to measure the maximal amplitudes of the evoked P2X$_3$ currents (Figure 5). In HEK cells, the ATP-induced I_{max} became significantly higher, when GDP-β-S replaced GTP in the intracellular solution (Figure 5b), whereas the I_{max} of α,β-meATP did not change under these conditions (Figure 5c). Similarly, in DRG cells, ATP had a lower maximum effect than α,β-meATP (Figure 5a). However, when the cells were loaded with GDP-β-S, ATP became as effective in inducing P2X$_3$ current as α,β-meATP.

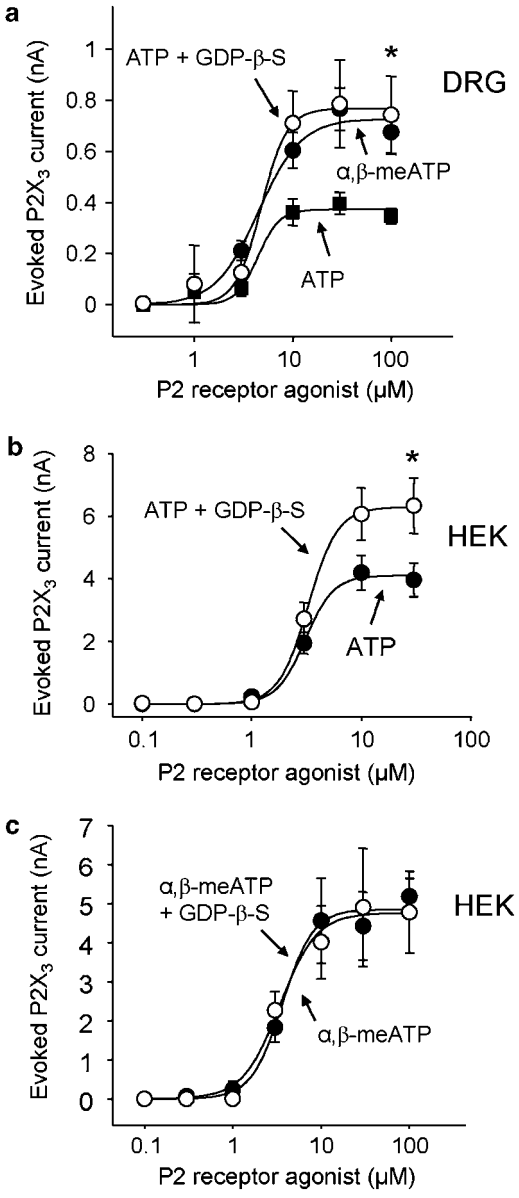

Figure 5 Activation of G proteins by P2Y receptors inhibits the peak amplitudes of $P2X_3$ currents. ATP and α,β-meATP were applied with 5 min intervals onto DRG neurons (**a**) and HEK293 cells permanently transfected with $hP2X_3$ receptors (**b** and **c**), for 1 s each at increasing concentrations to evoke $P2X_3$ currents. Where indicated, cells were loaded instead of the regular 300 μM GTP with equimolar GDP-β-S by means of the pipette solution for at least 15 min before the first agonist application. Mean $\pm$ s.e.m. of 6–9 cells. $^*P<0.05$; statistically significant difference from the respective control current.

Onset of $P2X_3$ receptor desensitization by α,β-meATP and ATP

Next, we were interested in the question whether the activation of G proteins by ATP alters the desensitization of the $P2X_3$ receptor. These receptors are known to desensitize rapidly within milliseconds and recover from desensitization slowly over within several minutes. To allow accurate measurement of time constants within the limits of the fast superfusion system, in these experiments a low agonist concentration was used. Twenty seconds of application of 1 μM α,β-meATP to HEK293-$hP2X_3$ cells induced an inward current ($\tau_{on(10-90\%)} = 240 \pm 29$ ms) that peaked at an amplitude of 2.68 ± 0.38 nA ($n = 12$ each) and decayed back to baseline indicating complete receptor desensitization (Figure 6A and C). The current decay, which represented the onset of desensitization, could be fitted bi-exponentially and had two time constants ($\tau_{des1} = 686 \pm 109$ and $\tau_{des2} = 2609 \pm 497$; $n = 12$) (Figure 6Cc and Cd). As τ_{des2} remained relatively constant under all experimental conditions, in the following, the onset of desensitization or the decay constant always means the τ_{des1} value. We next applied ATP at the same concentration (1 μM) for 20 s onto HEK293-$hP2X_3$ cells. ATP elicited inward currents much faster than α,β-meATP ($\tau_{on(10-90\%)} = 115 \pm 15$ ms, $n = 16$; $P<0.05$) (Figure 6Ab and Cb) and the amplitude of these currents was smaller (1.73 ± 0.21 nA; $P<0.5$) (Figure 6Aa and Ca) than of those evoked by α,β-meATP. Moreover, ATP currents desensitized more rapidly ($\tau_{des1} = 304 \pm 38$ ms; $P<0.05$) than α,β-meATP currents (Figure 6Ab and Cc).

We asked ourselves whether the difference between ATP- and α,β-meATP-induced desensitization is due to the activation of P2Y receptors by ATP and the subsequent activation of G protein-dependent mechanisms. To answer this question, we loaded a number of HEK293-$hP2X_3$ cells with GDP-β-S (300 μM). In these cells, ATP induced inward currents had significantly larger peak amplitudes (Figure 6Ba and Ca) and decayed back to baseline significantly slower (Figure 6Bb and Cc) than in GTP (300 μM)-loaded cells. Moreover, both the amplitude and the decay of ATP currents in the intracellular presence of GDP-β-S were comparable with the α,β-meATP-induced currents (Figure 6A–C). Finally, when we applied α,β-meATP onto cells loaded with intracellular GDP-β-S, the rate of activation, the peak amplitude and the decay of the currents did not differ significantly from those recorded from cells loaded with GTP.

Recovery of $P2X_3$ receptors from desensitization by α,β-meATP and ATP

After rapid desensitization, $P2X_3$ receptors stay in the desensitized state for considerable periods of time. To investigate the recovery from desensitization, HEK293-$hP2X_3$ cells were stimulated repetitively with 5 s pulses of α,β-meATP or ATP (10 μM each), by progressively increasing the interpulse intervals (Figure 7). During this application time ATP and α,β-meATP completely desensitized $P2X_3$ receptors. The recovery of $P2X_3$ receptors from desensitization exhibited a sigmoidal time course and was best fitted with an equation described in methods. The time course of recovery was expressed as t_{50}, namely the time to regain 50% of the maximally recovered current amplitudes. The recovery from α,β-meATP-induced desensitization was significantly faster ($t_{50} = 0.91 \pm 0.09$ min; $n = 9$) than the recovery from ATP-induced desensitization ($t_{50} = 2.33 \pm 0.20$ min; $n = 11$). To examine whether the difference in the recovery time was dependent on the ATP-induced G protein activation via P2Y receptors, we repeated the experiments with cells loaded with GDP-β-S (300 μM). In these cells, the recovery from α,β-meATP-induced desensitization did not change ($t_{50} = 1.07 \pm 0.06$ min; $n = 6$), whereas the recovery from ATP-induced desensitization became significantly faster

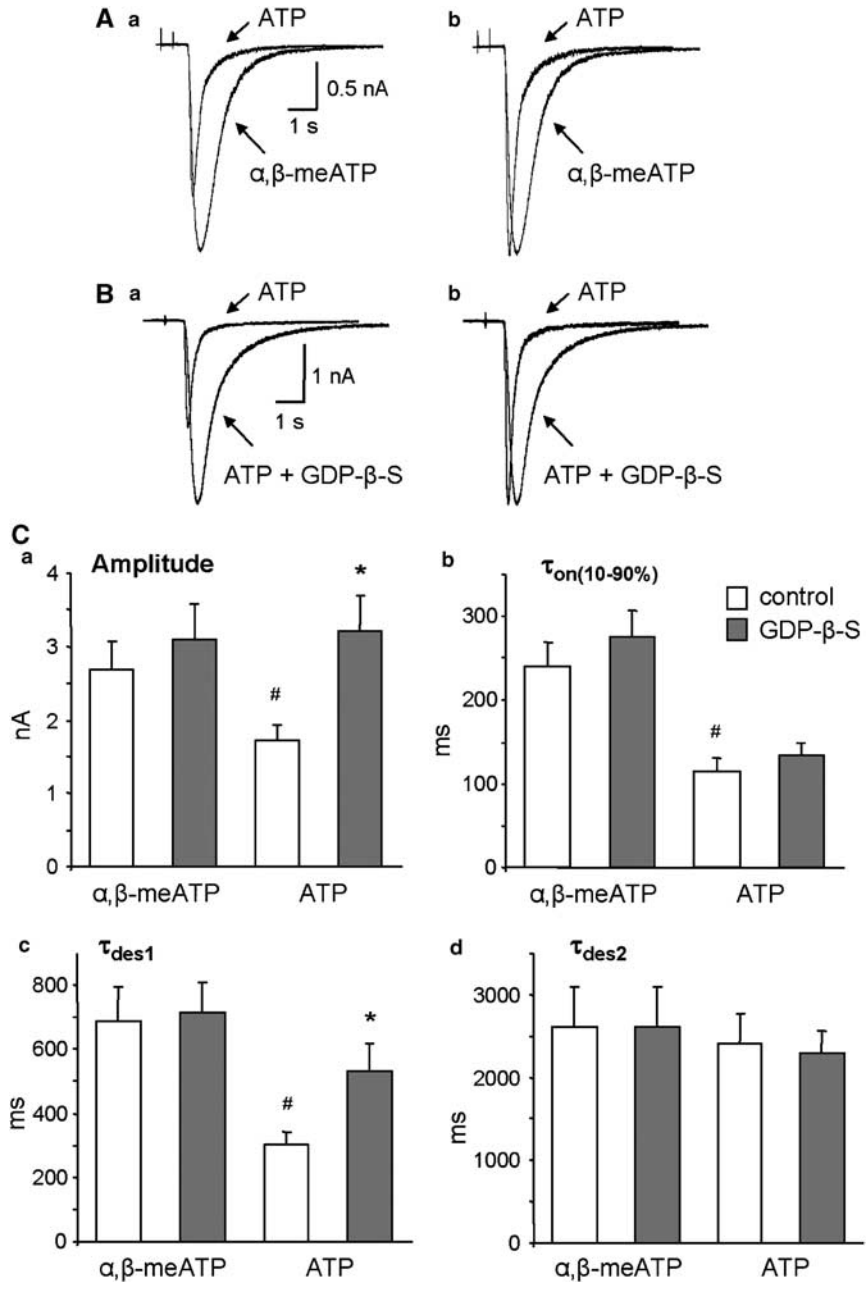

Figure 6 Activation of G proteins by P2Y receptors facilitates the desensitization of $P2X_3$ currents. (**Aa**) Representative $P2X_3$ currents induced by 20 s application of ATP or α,β-meATP (1 μM, each) in HEK293 cells transfected with $hP2X_3$ receptors. (**Ab**) The same currents as in (**Aa**) with scaled amplitudes to compare their kinetics. (**Ba**) Representative $P2X_3$ currents induced by 20 s application of 3 μM ATP in the presence and absence of intracellular GDP-β-S (300 μM). (**Bb**) The same currents as in (**Ba**) with scaled amplitudes to compare their kinetics. (**C**) Amplitude, onset ($\tau_{on(10-90\%)}$) and desensitization (τ_{des1} and τ_{des2}) rates of ATP and α,β-meATP (1 μM, each)-induced currents with and without intracellular GDP-β-S (300 μM) treatment. Mean$\pm$s.e.m. from 12–18 cells. $^{\#}P<0.05$ statistically significant difference from α,β-meATP; $^{*}P<0.05$; statistically significant difference from control ATP.

(1.63 ± 0.10 min; $n=9$; $P>0.05$) although did not reach that achieved by α,β-meATP (Figure 7Ac and C).

Discussion and conclusions

The main finding of this study is that the activation of metabotropic P2Y receptors inhibits the conductance of recombinant $hP2X_3$ receptor channels through facilitation of their desensitization and suppression of their recovery from the desensitized state. Previous experiments of our group indicate that, in small-diameter nociceptive rat DRG neurons, the $P2Y_{1,12,13}$ agonist ADP-β-S inhibits inward currents evoked by the application of the $P2X_{1,3}$ agonist α,β-meATP (Gerevich *et al*., 2005). Although in the rat and mouse DRG, there is a minor population of α,β-meATP-sensitive $P2X_1$ receptor-containing neurons, the majority of neurons bears the $P2X_3$ receptor subtype (Petruska *et al*.,

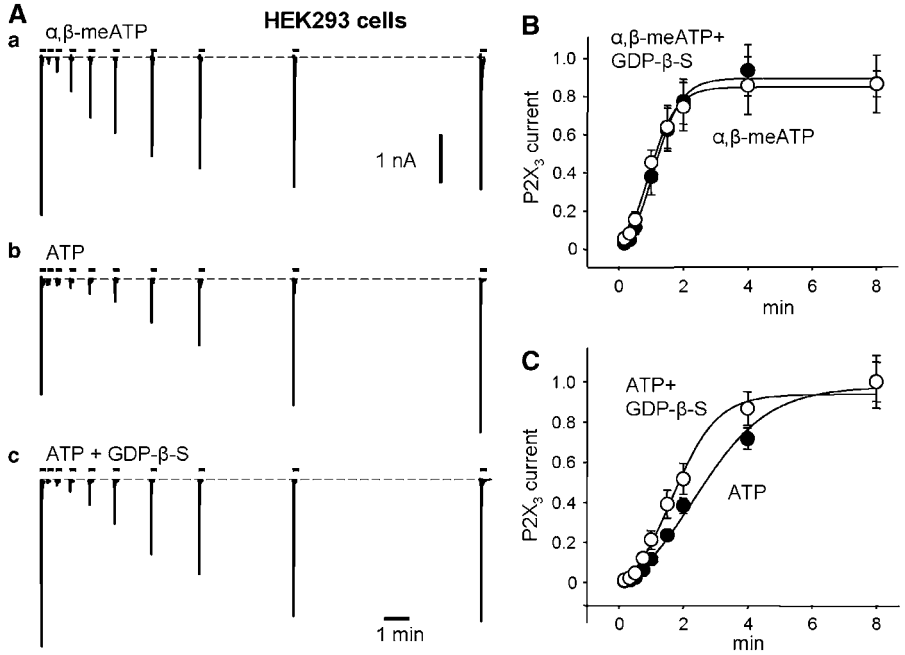

Figure 7 G protein-dependent recovery from desensitization of hP2X$_3$ receptors in HEK293 cells. (**A**) Representative currents evoked by repetitive stimulation by 5 s pulses of α,β-meATP or ATP (10 μM, each), with progressive increase in the interpulse intervals. (**B**) Recovery from α,β-meATP-induced desensitization in the presence and absence of intracellular GDP-β-S (300 μM) to block all G protein heterotrimers was fitted with a sigmoid curve (for the equation see Methods). (**C**) Recovery from ATP-induced desensitization was recorded and plotted as in (**B**).

2000; Calvert and Evans, 2004). To substantiate these findings and to investigate the transduction mechanism of P2Y receptors, in the present study we used HEK293 cells devoid of any functional P2X receptor and possessing only those recombinant hP2X$_3$ receptor subtypes that were permanently transfected into them (Fischer *et al*., 2003). However, HEK293 cells are endowed with a wide range of functional P2Y receptors of the P2Y$_{1,2,4,13}$ subtypes (Schachter *et al*., 1997; Fischer *et al*., 2003, 2005). The P2Y$_{1,2,4}$ receptors are coupled via the α-subunits of a G$_{q/11}$ protein to the phospholipase C/inositol 1,4,5-trisphosphate cascade and thereby initiate the release of Ca^{2+} from its intracellular pools (Fischer *et al*., 2003, 2005); P2Y$_{13}$ receptors were shown to negatively couple via their $\beta\gamma$-subunits to recombinant N-type Ca^{2+} channels expressed in HEK293 cells (Wirkner *et al*., 2004).

In HEK293-hP2X$_3$ cells, ADP-β-S and 2-MeSADP had twofold effects. Firstly, at low concentrations they inhibited the P2X$_3$ currents induced by α,β-meATP application and, secondly, at high concentrations they produced inward currents via the activation of P2X$_3$ receptor-channels. These two effects were evoked by agonist concentrations differing by at least three orders of magnitude. However, more recently a sustained application of nanomolar ATP concentrations has been shown to inhibit P2X$_3$ receptor-channels by decreasing their rate of recovery from the desensitized state (Sokolova *et al*., 2004, 2006; Pratt *et al*., 2005). By analogy, very low concentrations of ATP were reported to obscure P2X$_1$ receptor responses by driving a significant fraction of the receptor pool into a long-lasting, refractory, closed state (Rettinger and Schmalzing, 2003). In consequence, the P2X$_1$ current amplitudes were depressed, as they represent an amalgam of the simultaneous activation and desensitization (Rettinger and Schmalzing, 2004). Under our experimental conditions, an additional mechanism may operate, because the P2Y receptor-agonist ADP-β-S had a much lower inhibitory potency at P2X$_3$ currents after the blockade of G protein heterotrimers by intracellular GDP-β-S or after their irreversible activation by GTP-γ-S (Sternweis and Pang, 1990).

Based on our previous results in rat DRG neurons (Gerevich *et al*., 2005), we expected that, in the human HEK293 cell line, the P2Y$_1$ receptor subtype will be the interaction partner of hP2X$_3$ receptors. However, in HEK293-hP2X$_3$ cells, neither P2Y$_1$ nor P2Y$_{12,13}$ interfered with hP2X$_3$ receptors as confirmed by the use of the selective antagonists MRS 2179 (P2Y$_1$), 2-MeSAMP (P2Y$_{12}$) and AR-C69931MX (P2Y$_{12,13}$). We were not able to identify the definite P2Y receptor subtype which was the site of action for the structural analogues of ADP; this may be either the P2Y$_6$ subtype (Burnstock, 2004) or a hitherto unidentified (possibly orphan) P2Y receptor (Abbracchio *et al*., 2005, 2006). It is noteworthy that the IC$_{50}$ values of ADP-β-S and 2-MeSADP for inhibiting α,β-meATP responses were similar to the EC$_{50}$ values of the respective agonists at human recombinant and endogenous P2Y$_1$, but not P2Y$_{12,13}$ receptors, measured both by binding studies and the modulation of second messenger pathways (Schachter *et al*., 1996; Leon *et al*., 1997; Hechler *et al*., 1998; Zhang *et al*., 2001, 2002; Simon *et al*., 2002), suggesting the involvement of a P2Y$_1$ (or P2Y$_1$-like) receptor-type. Moreover, of about 370 orphan G protein-coupled receptors responding to endogenous ligands, more than 150 remain orphan and several of these sequences are structurally related to P2Y receptors (Abbracchio *et al*., 2006).

The interaction between P2Y and $P2X_3$ receptors appeared to determine the amplitude of ATP-, but not α,β-meATP-induced currents both in HEK293-h$P2X_3$ and DRG cells. It is assumed that ATP simultaneously occupies the recognition sites of $P2X_3$ receptor channels and P2Y receptors, thereby causing two opposite effects. Hence, ATP may cause inward currents on the one hand by $P2X_3$ receptor activation and at the same time it may decrease this current amplitude by P2Y receptor-activation. In contrast to ATP, the selective $P2X_3$ receptor agonist α,β-meATP fails to occupy P2Y receptors and thereby evokes a maximum current amplitude equaling that evoked by ATP only after the blockade of heteromeric G proteins by intracellular GDP-β-S.

$P2X_3$ receptors desensitize rapidly within tens of milliseconds in the continuous presence of the agonist. Our data revealed that the negative interaction between $P2X_3$ and P2Y receptors appears to be due to a G protein-dependent increase in the rate of $P2X_3$ receptor desensitization. In fact, the desensitization rate constant (τ_{des1}) of ATP was smaller than that of α,β-meATP and this difference disappeared in the presence of intracellular GDP-β-S. Further, we investigated the recovery from desensitization, a process that takes a couple of minutes in the case of $P2X_3$ receptors. We found in agreement with others that this recovery was significantly slower with ATP than with α,β-meATP as an agonist (Sokolova *et al*., 2004, 2006; Pratt *et al*., 2005). Thus, we conclude that, in addition to the different agonist structures, affinities and the ability of the receptor to desensitize in response of low agonist concentrations, G protein-activation facilitates the desensitization of $P2X_3$ receptors and suppresses the recovery from the desensitized state. In our opinion, G protein activation may shift the energy balance of the $P2X_3$ receptor and thus ease the transition into the desensitized conformation state.

The mode of action of P2Y receptor-linked G proteins to modulate the rate of desensitization of $P2X_3$ receptor channels was not investigated in the present study. $G\alpha_{q/11}$ may stimulate via phospholipase C, the activity of protein kinase C, which directly phosphorylates the *N*-terminus (Boue-Grabot *et al*., 2000; Paukert *et al*., 2001; North, 2002) or the ecto-domain (Wirkner *et al*., 2005; Stanchev *et al*., 2006) of the P2X receptor and in consequence alters the rate of desensitization. Another possible mechanism involves $G\beta\gamma$, which may either block $P2X_3$ receptor channels in a membrane-delimited manner, analogous to that described, for example, for voltage-dependent Ca^{2+} channels (Boehm, 2003; Lechner and Boehm, 2004) or NMDA receptor-channels (Yu *et al*., 1997), or less likely, activate a G protein-coupled receptor kinase (GIRK) (Kohout and Lefkowitz, 2003) that can result in receptor desensitization.

An opposing activity of ATP via stimulatory $P2X_3$ and inhibitory P2Y receptors may represent a novel and efficient regulatory mechanism avoiding the development of overtly painful stimuli. $P2X_3$ receptors are almost exclusively expressed in sensory neurons and play important roles in the transmission of pain signals to the CNS (Chizh and Illes, 2001; North, 2004). These receptors are situated at the peripheral terminals of small-diameter sensory DRG neurons and react to the release of ATP caused by any type of noxious tissue damage (Burnstock, 1996; Kennedy *et al*., 2003). The opening of $P2X_3$ receptor channels results in rapid Ca^{2+} and Na^+ influx leading to membrane depolarization and the subsequent generation of propagated action potentials as pain signals. P2Y receptors are also present on $P2X_3$ expressing sensory neurons (Gerevich and Illes, 2004) and they are activated by low concentrations of ATP or its degradation product ADP (Ruan and Burnstock, 2003; Gerevich and Illes, 2004). ADP-β-S, a selective P2Y receptor agonist, has been shown to exhibit an analgesic effect probably through the inhibition of N-type voltage-gated calcium channels situated at the terminals of DRG neurons (Borvendeg *et al*., 2003; Gerevich *et al*., 2004). The interaction between an ATP-sensitive $P2X_3$ and an ADP-sensitive P2Y receptor may also contribute to analgesia by ADP-β-S.

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Conflict of interest

The authors state no conflict of interest.

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