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# Adenine nucleotides inhibit recombinant N-type calcium channels *via* G protein-coupled mechanisms in HEK 293 cells; involvement of the $P2Y_{13}$ receptor-type

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**1** N-type $Ca^{2+}$ channel modulation by an endogenous P2Y receptor was investigated by the whole-cell patch-clamp method in HEK 293 cells transfected with the functional rabbit N-type calcium channel.

**2** The current responses ($I_{Ca(N)}$) to depolarizing voltage steps were depressed by ATP in a concentration-dependent manner. Inclusion of either guanosine 5′-*O*-(3-thiodiphosphate) or pertussis toxin into the pipette solution as well as a strongly depolarizing prepulse abolished the inhibitory action of ATP.

**3** In order to identify the P2Y receptor subtype responsible for this effect, several preferential agonists and antagonists were studied. Whereas the concentration–response curves of ADP and adenosine 5′-*O*-(2-thiodiphosphate) indicated a higher potency of these agonists than that of ATP, $\alpha,\beta$-methylene ATP, UTP and UDP were considerably less active. The effect of ATP was abolished by the P2Y receptor antagonists suramin and $N^6$-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-$\beta,\gamma$-dichloromethylene-ATP, but not by pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid, 2′deoxy-$N^6$-methyladenosine-3′,5′-diphosphate or 2-methylthio AMP.

**4** Using reverse transcription and polymerase chain reaction, mRNA for the $P2Y_1$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$ and $P2Y_{13}$ receptor subtypes, but not the $P2Y_2$, and $P2Y_{12}$ subtypes, was detected in HEK 293 cells.

**5** Immunocytochemistry confirmed the presence of $P2Y_1$, and to a minor extent that of $P2Y_4$, but not of $P2Y_2$ receptors.

**6** Hence, it is tempting to speculate that $P2Y_{13}$ receptors may inhibit N-type $Ca^{2+}$ channels *via* the $\beta\gamma$ subunits of the activated $G_i$ protein.
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**Keywords:** N-type calcium channel; $P2Y_{13}$ receptor; G protein; HEK 293 cell; presynaptic inhibition

**Abbreviations:** $\alpha,\beta$-meATP, $\alpha,\beta$-methylene ATP; ADP-$\beta$-S, adenosine 5′-*O*-(2-thiodiphosphate); AR-C69931MX, $N^6$-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-$\beta,\gamma$-dichloromethylene-ATP; bp, base pair; HEK, human embryonic kidney; $IP_3$, inositol 1,4,5-trisphosphate; 2-MeSAMP, 2-methylthio AMP; 2-MeSATP, 2-methylthio ATP; MRS 2179, 2′deoxy-$N^6$-methyladenosine-3′,5′-diphosphate; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid; PTX, pertussis toxin; RT–PCR, reverse transcription and polymerase chain reaction; TEA, tetraethylammonium; VACC, voltage-activated calcium channel

## Introduction

ATP is not only a ubiquitous enzyme cofactor and energy source of the cell, but also a potent extracellular signaling molecule involved in the mediation of numerous physiological processes (Burnstock & Williams, 2000; Illes *et al*., 2000). ATP (ADP)- and/or UTP (UDP)-sensitive receptors are classified into two types belonging to the ligand-gated ionotropic family (P2X) and the metabotropic, G-protein-coupled family (P2Y) (Ralevic & Burnstock, 1998; Chizh & Illes, 2000; Nörenberg & Illes, 2000). In humans, eight different P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$; Burnstock & Williams, 2000; von Kügelgen & Wetter, 2000; Barnard & Simon, 2001; Communi *et al*., 2001; Abbracchio *et al*., 2003) have been detected until now.

In the central nervous system, ATP is released as a cotransmitter with a number of classic transmitters such as noradrenaline and acetylcholine (von Kügelgen & Starke, 1991; Burnstock, 1999). At peripheral and central noradrenergic neurons, both noradrenaline and its cotransmitter ATP inhibit their own release by activating presynaptically located $\alpha_2$-adrenergic and P2Y autoreceptors (von Kügelgen *et al*., 1993; Poelchen *et al*., 2001).

Recent studies classified voltage-activated calcium channels (VACCs) into three groups: high voltage-activated, which included L, N, P and Q types, intermediate (R type) and low voltage-activated (T-type) (Davila, 1999; Varadi *et al*., 1999).

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VACCs consist of four distinct subunits, $\alpha_1$, $\alpha_{2\delta}$, $\beta$ and $\gamma$ (Dolphin, 1998; Varadi *et al*., 1999). Whereas the $\alpha_1$ subunit forms the pore through which $Ca^{2+}$ enters the cell, the other subunits may play a functional role in modifying the kinetic properties of the channel (Bleakman *et al*., 1995). Coexpression of the neuronal human brain $\alpha_{1B}$ subunit with $\alpha_2$ and $\beta$ subunits in HEK 293 cells produced a functional N-type $Ca^{2+}$ channel, which was irreversibly blocked by $\omega$-conotoxin GVIA (Williams *et al*., 1992).

During the last few years, inhibition of N-type calcium channels has received a lot of attention because of their involvement, for example, in the regulation of neurotransmitter release (Wheeler *et al*., 1994; Powell *et al*., 2000). One important form of N-type $Ca^{2+}$ current ($I_{Ca(N)}$) modulation is the strongly voltage-dependent channel inhibition by receptor-coupled, heterotrimeric G proteins (Herlitze *et al*., 1996; Ikeda, 1996). The G proteins usually involved in this process belong to the pertussis toxin (PTX)-sensitive G proteins $G_i$ and $G_o$ (Zamponi & Snutch, 1998). An inhibition of $I_{Ca(N)}$ after activation of $P2Y_{12}$ receptors has been described in brain capillary endothelial cells (Simon *et al*., 2002) and in a neuronally differentiated rat pheochromocytoma (PC12) cell line (Kulick & von Kügelgen, 2002; Kubista *et al*., 2003). A similar effect has been reported for rat sympathetic neurons after stimulation of $P2Y_1$, $P2Y_2$, $P2Y_4$ or $P2Y_6$ receptors that were transfected additionally to the physiological P2Y receptor endowment of these cells (Filippov *et al*., 1998; 1999; 2000; 2003).

Native HEK 293 cells express endogenous P2Y receptors (Schachter *et al*., 1997; Moore *et al*., 2001; Fischer *et al*., 2003), but do not possess either P2X receptors or high VACCs (McNaughton & Randall, 1997). In the present study, a HEK 293 cell line transfected with the rabbit N-type $Ca^{2+}$ channel (Fujita *et al*., 1993) was used to investigate the modulation by human P2Y receptors of this channel type. Thereby, for the first time, selective effects on N-type $Ca^{2+}$ currents could be investigated without the need of functional isolation of the native channel from all other types of voltage-activated $Ca^{2+}$ currents by means of physiological or pharmacological procedures. It is suggested that, although both $P2Y_1$, $P2Y_4$, $P2Y_6$ and $P2Y_{13}$ receptors are present in these cells, only the $P2Y_{13}$ receptor is coupled to N-type $Ca^{2+}$ channels *via* a PTX-sensitive G protein. Preliminary accounts of the data have appeared previously in abstract form (Wirkner *et al*., 2002).

## Methods

### *Transfection procedures and culturing of HEK 293 cells*

The plasmid pcDNA3 1/zeo(+) (Invitrogen, Carlsbach, CA, U.S.A.) was cleaved by *Eco*RI and ligated with cDNA encoding the rabbit N-type calcium channel $\alpha_{1B}$ subunit to yield cDNA3.1/zeo(+)-$\alpha_{1B}$ (Fujita *et al*., 1993). The plasmid pDouble one (IDEC, Osaka, Japan) was also cleaved by *Eco*RI and then ligated with the 1.6 kb *Eco*RI fragment from pCAS14 containing the rabbit muscle calcium channel $\beta_1$ cDNA to yield pDouble one-$\beta_1$. Afterwards, the plasmid pDouble one-$\beta_1$ was cleaved by *Mun*I and ligated with the 3.5 kb *Eco*RI fragment from pCAS7 containing the rabbit skeletal muscle calcium channel $\alpha_{2\delta}$ subunit cDNA to yield pDouble one-$\alpha_2$-$\beta_1$.

These two expression plasmids, cDNA3.1/zeo(+)-$\alpha_{1B}$ carrying the cDNAs encoding the rabbit N-type calcium channel $\alpha_{1B}$ subunit and pDouble one-$\alpha_2$-$\beta$ carrying the rabbit calcium channel $\alpha_{2\delta}$ and $\beta_1$ subunits, were introduced into HEK 293 cells using the calcium phosphate method. HEK 293-N26 cells were maintained in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, and were selected with 400 $\mu$g ml$^{-1}$ active G418 (GibcoBRL, Eggenstein, Germany) and 250 $\mu$g ml$^{-1}$ zeocin. Afterwards, the transfectants were screened for the expression of calcium channel subunits ($\alpha_{1B}$, $\alpha_{2\delta}$ and $\beta_1$) by Northern blot analysis and the binding assay, using $^{125}$I-$\omega$-conotoxin GVIA (NEN Life Science Products, Zaventem, Belgium).

Then, the cells were cultured in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum and 2 mM glutamine (Sigma, Deisenhofen, Germany) and 400 $\mu$g ml$^{-1}$ G418. The cells were splitted 1 : 12 and replated into new flasks (Sarstedt, Nürnberg, Germany) two times a week. A 0.25% trypsin solution (GibcoBRL) was used to separate the cells for passage.

### *Recording of $Ca^{2+}$ currents*

N-type $Ca^{2+}$ currents were recorded by the conventional whole-cell patch-clamp method. The external solution contained (mM): tetraethylammonium chloride (TEA) 120, KCl 3, $MgCl_2$ 1.5, $CaCl_2$ 5, HEPES 10 and glucose 11 (pH was adjusted to 7.4 with TEA-OH). In some experiments, $CaCl_2$ was substituted by $CoCl_2$ (2 mM). Patch electrodes (3–5 M$\Omega$) were filled with a solution consisting of (mM): CsCl 110, $MgCl_2$ 3, HEPES 40, EGTA 3, Mg-ATP 1.5, Li-GTP 0.3 (pH adjusted to 7.4 with CsOH). When indicated, GTP was substituted with either GTP-$\gamma$-S or GDP-$\beta$-S (0.3 mM each). In some experiments, the concentration of EGTA was decreased from 3 to 0.3 mM and 2.7 mM CsCl was added to the pipette solution. This manipulation leads to a decrease of the $Ca^{2+}$-buffering capacity.

Cells were voltage-clamped with an Axopatch 200B amplifier (Axon Instruments, Union City, CA, U.S.A.). The seal resistance of the whole-cell configuration was routinely $\approx$10 G$\Omega$. Residual pipette capacitance was compensated in the cell-attached configuration using the negative circuit of the amplifier. Whole-cell capacitance was $23.8 \pm 1.1$ pF ($n = 216$). Series resistance ($R_s$) was calculated as $\tau$ ($1/C$), where $\tau$ was the time of constant for decay of the whole-cell capacity transient. The average values of $\tau$ and $R_s$ were $286 \pm 7$ $\mu$s and $12.2 \pm 0.2$ M$\Omega$, respectively. $R_s$ was reduced by 40–80% using the inbuilt series resistance compensation circuit of the amplifier. The liquid junction potential ($V_{LJ}$) between the bath and pipette solutions at 22°C was calculated according to Barry (1994); it was found to be $-9.3$ mV. Holding potential values given in this study were corrected for $V_{LJ}$. The steady-state holding potential was $-90$ mV. $Ca^{2+}$ channel currents were evoked routinely with a 100 ms depolarizing rectangular test pulse to $-10$ mV. Unless otherwise stated, step depolarizations to test potentials were delivered every 20 s; these stimulation rates were sufficiently low to minimize cumulative inactivation of the N-type $Ca^{2+}$ channel. Current amplitudes were measured isochronically 10 ms from the onset of the test pulse near to the peak of the current. In some experiments, strong depolarizing prepulses to $+120$ mV from a holding

potential of −90 mV were applied for 20 ms. The interval between the prepulse and the test pulse was 5 ms.

Currents were filtered at 5 kHz using the built-in low-pass Bessel filter of the amplifier, and sampled at 10 kHz *via* a Digidata 1200 interface (Axon Instruments). All recordings were corrected for linear capacitance and leakage currents by the −P/4 subtraction function of the pClamp 8.0 software, used also for data acquisition and analysis (Axon Instruments). Curve fits and figures were made with the help of Sigma Plot 6.0 (SPSS, Erkrath, Germany). The $IC_{50}$ values were calculated based on fits using the following logistic function

$$I = I_{min} + (I_{max} - I_{min})[1 + (IC_{50} + agonist)^n]$$

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 agonist reducing $I_{max}$ by 50%. Higher concentrations of adenosine 5′-*O*-(2-thiodiphosphate) (ADP-$\beta$-S) and $\alpha,\beta$-methyleneadenosine 5′-triphosphate ($\alpha,\beta$-meATP) than 100 $\mu$M were considered to be pharmacologically meaningless, and therefore not investigated.

### *Substance application*

Stock solutions (1 or 10 mM) of P2 receptor agonists and antagonist were prepared in external solution, stored at −20°C and diluted immediately before use. The stock solution (1 mM) of $\omega$-Conotoxin GVIA was dissolved in 100 mg ml$^{-1}$ bovine serum albumin (Sigma) and stored at −20°C; further dilutions to the final concentration of 1 $\mu$M were made with an external medium.

Agonists were applied for 1–2 min by local superfusion using a fast, pressurized drug-application device system (DAD12, Adams and List, Westbury, NY, U.S.A.). HEK 293-N26 cells were continuously superfused with the standard external solution by one pressure-independent valve of this system. Each concentration of P2 receptor agonists was applied to individual HEK 293-N26 cells. Thereby, a possible desensitization developing during cumulative or repeated applications of increasing agonist concentrations was avoided. Only cells in which current amplitudes recovered to control values after washout were included in the evaluation.

The effects of various P2 receptor antagonists were investigated by applying the prototypic agonist ATP (300 $\mu$M) for 1 min before as well as 9 min after superfusion with suramin, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), 2′deoxy-$N^6$-methyladenosine-3′,5′-diphosphate (MRS 2179), 2-methylthioadenosine 5′-monophosphate (2-MeSAMP) and $N^6$-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-$\beta,\gamma$-dichloromethylene-ATP (AR-C69931MX). The antagonist potencies were quantified by measuring the inhibitory effect of ATP after antagonist superfusion for 10 min. $\omega$-Conotoxin GVIA (1 $\mu$M) was also pressure-applied onto the cells. In some experiments, activated PTX was intracellularly applied *via* the patch pipette. Immediately before use, PTX (2 $\mu$g ml$^{-1}$) was dissolved in pipette solution containing dithiothreitol (5 mM) and incubated for 30 min at 37°C. Then, the pipette solution containing $\beta$-nicotinamide adenine dinucleotide (NAD, 5 mM) was added in a 1 : 1 volume. The final concentration of PTX was 1 $\mu$g ml$^{-1}$. Nonactivated PTX was used as a negative control.

### *Reverse transcription and polymerase chain reaction*

Total RNA was isolated from HEK 293-N26 cells ($\approx 1.1 \times 10^6$ cells) 4 days after plating in culture dishes using a Total Quick RNA mini kit (BIOZOL, Eching, Germany). Twice, contamination with genomic DNA was removed by repeated digestion with RNase-free DNase I (Roche; 50 U) for 2 h at 37°C. According to standard protocols, first-strand cDNA was synthesized by reverse transcription of 1 $\mu$g of total RNA with Superscript™-II reverse transcriptase (Life Technologies). cDNA fragments of the various P2Y receptors were amplified by the use of a set of sense and antisense primers specific for the following receptor subtypes: $PY_1$, $PY_2$, $PY_4$ and $PY_6$ (Jin *et al*., 1998); $PY_{11}$ (Conigrave *et al*., 2001); $PY_{12}$ (Hollopeter *et al*., 2001) and $PY_{13}$ (Communi *et al*., 2001). PCRs were run on a PTC-200 Thermocycler (MJ Research, Boston, MA, U.S.A.) in a final volume of 25 $\mu$l containing 1 $\mu$l of the first-strand cDNA, 1 u Ampli-Taq DNA Polymerase (Perkin-Elmer), and (anti)sense primers (200 nM, each) specific for the respective P2Y receptor subtypes after initial denaturation for 3 min at 95°C with 25 cycles ($P2Y_{13}$) or 35 cycles ($P2Y_1$–$P2Y_{12}$). The annealing temperatures were 54°C, 55°C, 60°C, 58°C, 57°C, 57°C and 50°C for $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$, respectively. Negative controls lacking first-strand cDNA were run in parallel; positive controls were performed with cDNA from peripheral blood leukocytes or brain tissue. The amplification products were detected by ethidium bromide staining subsequent to agarose gel (1.5%) electrophoresis with 10 $\mu$l of PCR product.

### *Immunohistochemistry*

After fixation, washing with Tris-buffered saline (TBS, 0.05 M; pH 7.6) and blocking with 5% fetal calf serum (FCS), the cells were incubated with the rabbit anti-P2Y receptor antibodies (anti-$P2Y_1$: 1 : 1500, SmithKline Beecham Pharmaceuticals, Harlow, Essex, U.K.; anti-$P2Y_2$: 1 : 1000, Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung and Blood Institute, Bethesda, MD, U.S.A.; anti-$P2Y_4$: 1 : 1000, Alomone Labs, Jerusalem, Israel) with 0.1% Triton X-100, 5% FCS in TBS for 12 h at 4°C. The preparations were washed three times for 5 min each in TBS, and were then incubated with the secondary antibody Cy3-conjugated goat anti-rabbit IgG (1 : 800; Jackson ImmunoResearch, West Grove, PA, U.S.A.) in 5% FCS in TBS for 2 h. After intensive washing, the cultures were dehydrated in a series of graded ethanol, processed through *n*-butylacetate and covered with entellan (Merck, Darmstadt, Germany). The data obtained with the anti-$P2Y_1$ and anti-$P2Y_2$ receptor antibodies were confirmed by using similar antibodies from a commercial source (Alomone Labs); in this case, just as with the anti-$P2Y_4$ receptor antibody, the supplier's certificate of analysis states that, in the appropriate Western blots, only a single band was observed. Control experiments were carried out without primary antibody or by preadsorption of the antibody with the immunizing peptides.

Microphotographs were taken by using a fluorescence microscope (Axioskop; Zeiss, Oberkochen, Germany; fluorescence filter set: BP 450–490, FT 510; LP 520) equipped with the Zeiss Axio Vision System 2.0.

*Drugs*

The following drugs and chemicals were used: ATP, adenosine 5′-diphosphate (ADP), uridine 5′-triphosphate (UTP), uridine 5′-diphosphate (UDP), α,β-meATP, 2-MeSAMP, 2-MeSATP, ADP-β-S, PTX, ω-conotoxin GVIA, guanosine 5′-*O*-(3-thiodiphosphate) (GDP-β-S), guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S), nicotine adenosine dinucleotide (NAD), PPADS, suramin and MRS 2179 (Sigma, Deisenhofen, Germany). Samples of ATP (99% ATP, 1% ADP) and UTP (82% UTP, 18% UDP) were checked for purity by HPLC before use. AR-C69931MX was a gift from AstraZeneca (M. Wayne, Wilmington, DE, U.S.A.).

*Statistics*

Data are presented as means ± s.e.m. of $n$ experiments. Statistical comparisons were made by unpaired Student's $t$-test (two groups) or by one-way analysis of variance (three or more groups), followed by the Bonferroni $t$-test. A probability level of 0.05 or less was considered to be statistically significant.

## Results

*$Ca^{2+}$ channel currents and their voltage-dependent inhibition by ATP*

When depolarizing voltage steps were applied in 10 mV increments to untransfected HEK 293 cells, from a holding potential of −90 mV and every 20 s, no inward current response was observed up to a maximal depolarization of +70 mV ($n=25$). Hence, under our conditions, native HEK 293 cells did not possess VACCs.

To investigate whether the properties of the transfected rabbit N-type calcium channels in HEK 293-N26 cells correspond to the pattern of native N-type calcium channels, voltage–current ($I$–$V$) relations between −90 and +50 mV were determined by applying a fast (280 ms) depolarizing ramp from a holding potential of −90 mV (Figure 1a). Maximal currents were usually observed after a depolarization to −10 mV. ATP (300 μM) did not alter the holding current, but caused a voltage-dependent inhibition of $I_{Ca(N)}$ ($n=8$). In addition, a slight and reversible shift of the peak current to the right was observed in the presence of ATP.

In all subsequent experiments, test pulses were delivered every 20 s from the holding potential of −90 to −10 mV for 100 ms. Pressure application of ω-conotoxin GVIA (1 μM) for 120 s nearly abolished $I_{Ca(N)}$ (99.1 ± 0.3% inhibition; $n=5$); this effect was not reversed by washout for 10 min. The application of an external medium onto cells which lacked the normal $Ca^{2+}$ (5 mM) but contained $Co^{2+}$ (2 mM) also led to complete blockade of the inward current within 20 s (Figure 1b). Recovery to control current amplitudes was observed after washout for 60 s (7.8 ± 13.7% inhibition; $n=6$).

*Effects of P2 receptor agonists and antagonists*

Pressure application of ATP (30–1000 μM) to HEK 293 cells transfected with the rabbit N-type $Ca^{2+}$ channel caused a concentration-dependent inhibition of $Ca^{2+}$ currents (Figure 2A and B). A typical reversible slowing of the current kinetics was observed in the presence of higher concentrations of ATP (Figure 2Ab). A near steady-state inhibition was observed 60 s after starting the application of ATP; the inhibition was stable over a further 60 s and was reversed by washout within 20 s.

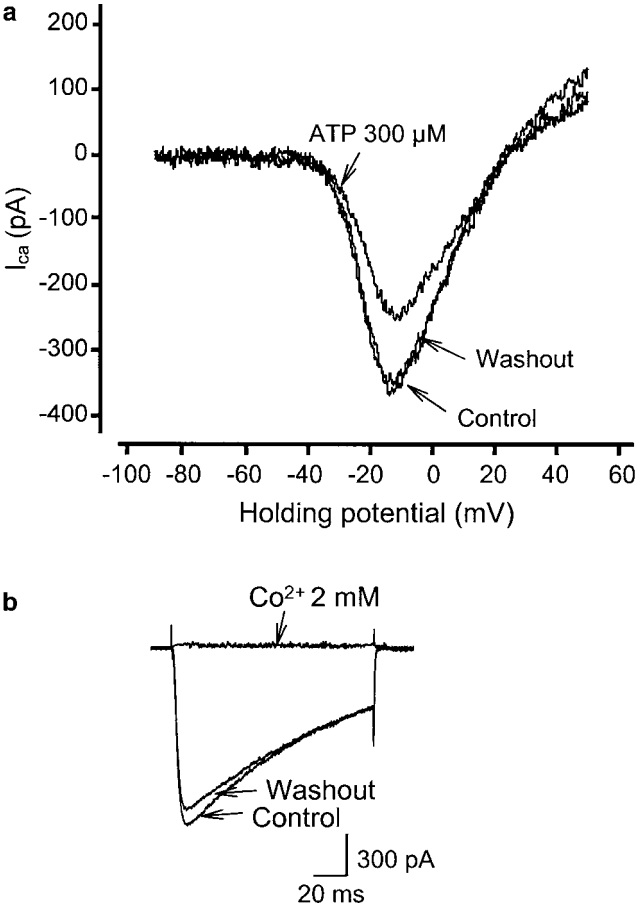


**Figure 1** Characterization of the properties of the N-type calcium channel in HEK 293-N26 cells. Voltage-dependent inhibition by ATP and effects of $Co^{2+}$. (a) Depolarizing voltage ramps of 280 ms duration from a holding potential of −90 to 50 mV were applied to evoke inward currents both in the absence and presence of ATP (300 μM), as well as after its washout. Application of ATP results in a slight shift of the voltage–current ($I$–$V$) relationship to the right. (b) Cells were depolarized to −10 mV from a holding potential of −90 mV for 100 ms every 20 s to induce inward currents. Substitution of $Ca^{2+}$ by $Co^{2+}$ (2 mM; $n=6$) in the superfusion medium abolished the current in this cell. The effect of a $Ca^{2+}$-free medium supplemented with $Co^{2+}$ was reversed by washout.

In addition to ATP, cells were exposed to a series of further P2 receptor agonists (Figure 3a and b). The concentration–response curves of the $P2Y_{1,12,13}$ receptor agonists ADP (0.1–1000 μM) and ADP-β-S (0.1–100 μM) were remarkably similar and clearly differed from the concentration–response curve of ATP (1–1000 μM), which acts in addition at $P2Y_{2,11}$ receptors. $IC_{50}$ values were calculated for ADP and ATP, where the full concentration–response curves were established. Based on these values, ADP (6.7 μM) had a higher potency than ATP (116.6 μM) itself. Further agonists with selectivities for certain receptor types such as α,β-meATP (1–100 μM; $P2X_{1,3}$), UTP and UDP (1–1000 μM each; $P2Y_{2,4,6}$) inhibited $I_{Ca(N)}$ maximally by about 10–20%.

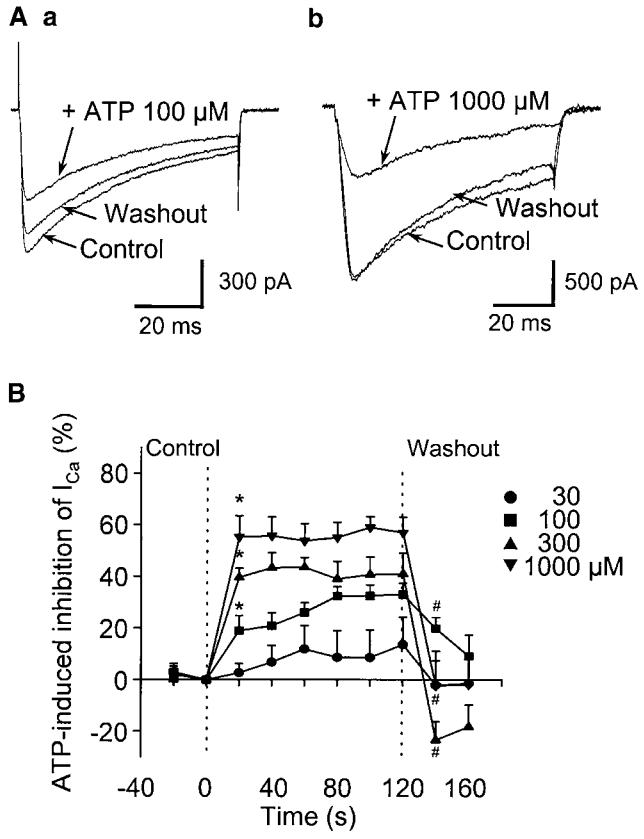


**Figure 2** Time- and concentration-dependence of the inhibition by ATP of N-type calcium channels in HEK 293-N26 cells. Cells were depolarized to −10 mV from a holding potential of −90 mV for 100 ms every 20 s to induce inward currents. Current amplitudes were measured 10 ms after the onset of the test pulse. (A) After recording two stable control currents, ATP (30–1000 $\mu$M) was applied onto separate cells for 120 s each, followed by washout. (a) Representative tracings before (control) during and 40 s after (washout) the application of ATP (100 $\mu$M) for 1 min. (b) Representative tracings before, during and 40 s after the application of ATP (1000 $\mu$M) for 1 min. (B) Means ± s.e.m. of 5–11 experiments similar to those shown in (A). The data were normalized with respect to the second control current recorded before the application of ATP. $^{*}P<0.05$; statistically significant difference from the second control current. $^{\#}P<0.05$; statistically significant difference from the effect of ATP after 120 s application.

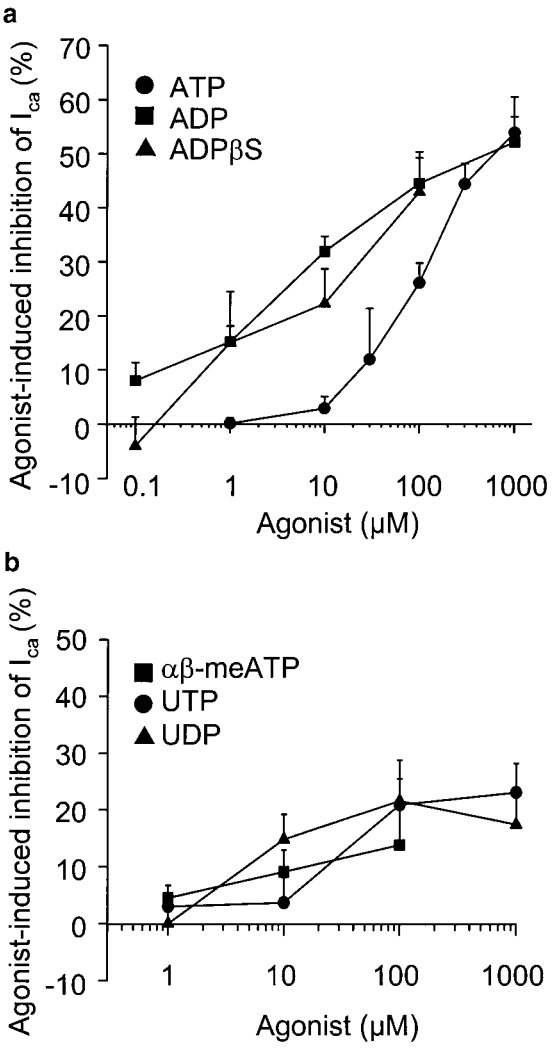


**Figure 3** Concentration-dependent inhibition by P2 receptor agonists of N-type calcium channels in HEK 293-N26 cells. Cells were depolarized to −10 mV from a holding potential of −90 mV for 100 ms every 20 s to induce inward currents. After recording of two stable control currents, increasing concentrations of various P2 receptor agonists were applied onto separate cells for 60 s each, followed by washout. Only cells that fully recovered are included. The agonist-induced depression of $I_{Ca(N)}$ was calculated in comparison with the second control current amplitude. (a) Concentration–response relationships for ATP ($n=5$–11), ADP ($n=5$–8) and ADP-$\beta$-S ($n=6$–8). (b) Concentration–response relationships for $\alpha,\beta$-meATP ($n=5$–7), UDP ($n=7$) and UTP ($n=7$–11). Means ± s.e.m. of $n$ experiments are shown in (a) and (b).

In the following experiments, a selected concentration of the prototypic agonist ATP (300 $\mu$M) was applied for 1 min before as well as 9 min after superfusion with various P2 receptor antagonists. The antagonist potencies were quantified by measuring the inhibitory ATP effect after antagonist superfusion for 10 min. In the absence of antagonists, the effect of ATP was reproducible 10 min after its first application (Figure 4Aa and B). The nonselective P2 receptor antagonist suramin (30 $\mu$M) and the $P2Y_{12,13}$-selective antagonist AR-C6993MX (1 $\mu$M) markedly inhibited the depression by ATP (Figure 4Ab and B). In contrast, further receptor-selective antagonists such as PPADS (30 $\mu$M; $P2X_{1,2,3,5}/P2Y_1$), MRS 2179 (30 $\mu$M; $P2Y_1$) and 2-MeSAMP (100 $\mu$M; $P2Y_{12}$) failed to interfere with ATP (300 $\mu$M) (Figure 4B).

Since both agonist and antagonist studies unequivocally indicated the involvement of an ADP-preferring P2Y receptor, ADP rather than ATP was used as an agonist in the following experiments. Two subsequent applications of ADP (100 $\mu$M) for 1 min each and with 9 min intervals inhibited $I_{Ca}$ by 56.2 ± 6.3 and 40.8 ± 4.1%, respectively ($n=5$). When MRS 2179 (30 $\mu$M) was present in the superfusion medium for 9 min before and during the second application of ADP (100 $\mu$M), there was no interaction of the antagonist with the ADP-induced inhibition of $I_{Ca}$ (59.2 ± 5.1 and 51.8 ± 4.8% inhibition, respectively; $n=6$; $P>0.05$). Hence, N-type $Ca^{2+}$ channels were definitely not affected *via* $P2Y_1$ receptor-activation.

### *Characterization of the G protein*

Since none of the P2 receptor agonists altered the holding current of HEK 293-N26 cells, the presence of an endogenous

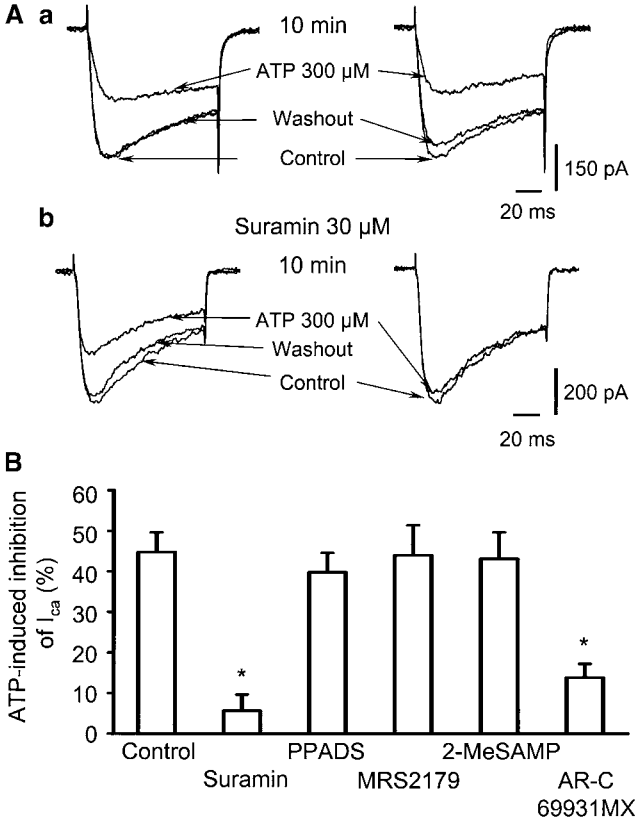


**Figure 4** Effects of P2 receptor antagonists on the ATP-induced inhibition of N-type $Ca^{2+}$ channels in HEK-N26 cells. Cells were depolarized to −10 mV from a holding potential of −90 mV for 100 ms every 20 s to induce inward currents. ATP (300 $\mu$M) was applied for 1 min before as well as 9 min after superfusion with various P2 receptor antagonists. The antagonistic potencies were quantified by measuring the inhibitory effect of ATP after antagonist superfusion for 10 min. (A) Representative tracings. (a) In the absence of antagonists, the ATP effect was reproducible 10 min after its first application. Complete recovery occurred upon washout. (b) Suramin (30 $\mu$M) abolished the ATP-induced inhibition of $I_{Ca(N)}$. (B) Means ± s.e.m. of the ATP-induced inhibition of $I_{Ca(N)}$ both in the absence (control, $n=8$) and presence of antagonists (suramin 30 $\mu$M, $n=6$; PPADS 30 $\mu$M, $n=7$; MRS 2179 30 $\mu$M, $n=5$; 2-MeSAMP 100 $\mu$M, $n=7$; AR-C69931MX 1 $\mu$M, $n=5$). $^*P<0.05$; statistically significant difference from the time-matching control current.

P2X receptor can be unequivocally excluded (see also Moore *et al*., 2001). In order to investigate the transduction mechanism of a G-protein-coupled P2Y receptor possibly involved in the blockade of $I_{Ca(N)}$, instead of GTP, GDP-$\beta$-S (300 $\mu$M), an inhibitor of G-protein-dependent processes (Sternweis and Pang, 1990), was added to the standard pipette solution. Immediately after establishing whole-cell configuration, ATP (300 $\mu$M) exerted its usual inhibitory effect. However, 15 min later, when the cell was microdialyzed by GDP-$\beta$-S, ATP failed to cause any depression of $I_{Ca(N)}$ (1.2 ± 1.1% inhibition, $n=12$; Figure 5Ab), suggesting the participation of a G protein in the modulation of $Ca^{2+}$ channels (compare with the time-matching control effect of ATP recorded with the standard GTP-containing micropipette; 40.6 ± 5.4%, $n=12$; Figure 5Aa).

In another series of experiments, GTP was substituted by GTP-$\gamma$-S (300 $\mu$M, Figure 5B), that irreversibly activates G proteins (Gilman, 1987). In fact, GTP-$\gamma$-S caused a slowly

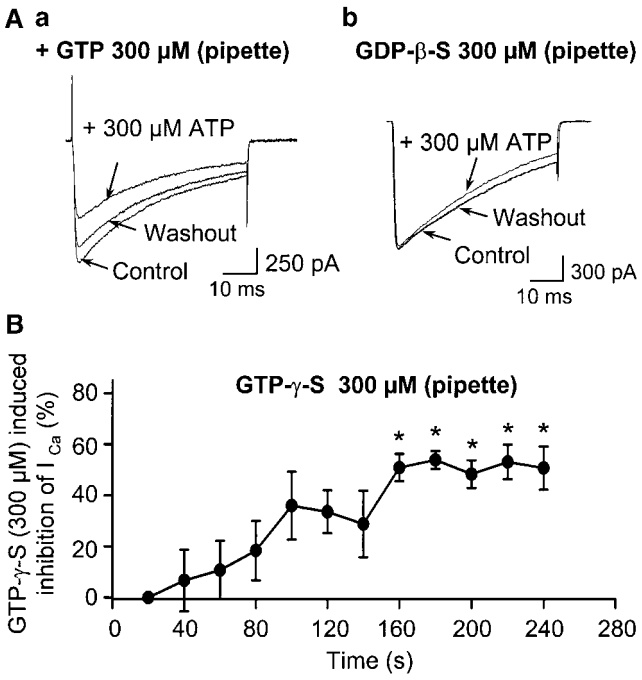


**Figure 5** Effect of intracellular GDP-$\beta$-S on the ATP-induced inhibition of N-type $Ca^{2+}$ channels in HEK 293-N26 cells; inhibitory effect of intracellular GTP-$\gamma$-S. Cells were depolarized to −10 mV from a holding potential of −90 mV for 100 ms every 20 s to induce inward currents. (A) Representative tracings. (a) Current responses before (control) during and 40 s after (washout) the application of ATP (300 $\mu$M) for 1 min; a standard pipette solution containing GTP (300 $\mu$M) was used. (b) Current responses recorded with a pipette solution containing GDP-$\beta$-S (300 $\mu$M) instead of GTP. The experimental protocol was identical with that used in (a). (B) The pipette solution contained GTP-$\gamma$-S (300 $\mu$M) instead of the standard GTP. A slowly developing inhibition of $I_{Ca(N)}$ was observed in comparison with the second current response measured immediately after establishing whole-cell access. Means ± s.e.m. of six experiments. $^*P<0.05$; statistically significant difference from the second current response in this series.

developing inhibition of $I_{Ca(N)}$ which peaked at 50.7 ± 8.5% ($n=6$), 4 min after establishing whole-cell configuration. This effect was comparable with the inhibition caused by ATP (300 $\mu$M; see above). Hence, $Ca^{2+}$ channels appeared to be blocked both by direct (internal GTP-$\gamma$-S) and indirect (external ATP) G-protein activation (Dolphin, 1998). Moreover, a strongly depolarizing pre-pulse to +120 mV (20 ms duration) markedly counteracted the effects of ATP (3.2 ± 4.8%; $n=9$; Figure 6A) and GTP-$\gamma$-S (10.3 ± 0.2%; $n=4$; Figure 6B). Further, a reduction of the EGTA concentration in the pipette solution from 3 to 0.3 mM decreases the $Ca^{2+}$ buffering capacity. However, in spite of this manipulation, the inhibitory effect of ATP (300 $\mu$M) on $I_{Ca}$ was not altered (3 mM EGTA, 51.0 ± 5.2%, $n=6$; 0.3 mM EGTA, 43.6 ± 5.0%, $n=7$).

Finally, it was tested whether the inhibition of $Ca^{2+}$ channels by P2Y receptor activation is associated with a G protein of the PTX-sensitive $G_i/G_o$ family. For this purpose, PTX was activated according to the protocol described in Methods and included into the pipette solution at a concentration of 1 $\mu$g ml$^{-1}$. Immediately after establishing whole-cell access, ATP (300 $\mu$M) depressed $Ca^{2+}$ current amplitudes as usual (Figure 7a and b). However, this effect gradually decreased and completely disappeared 60 s later. In

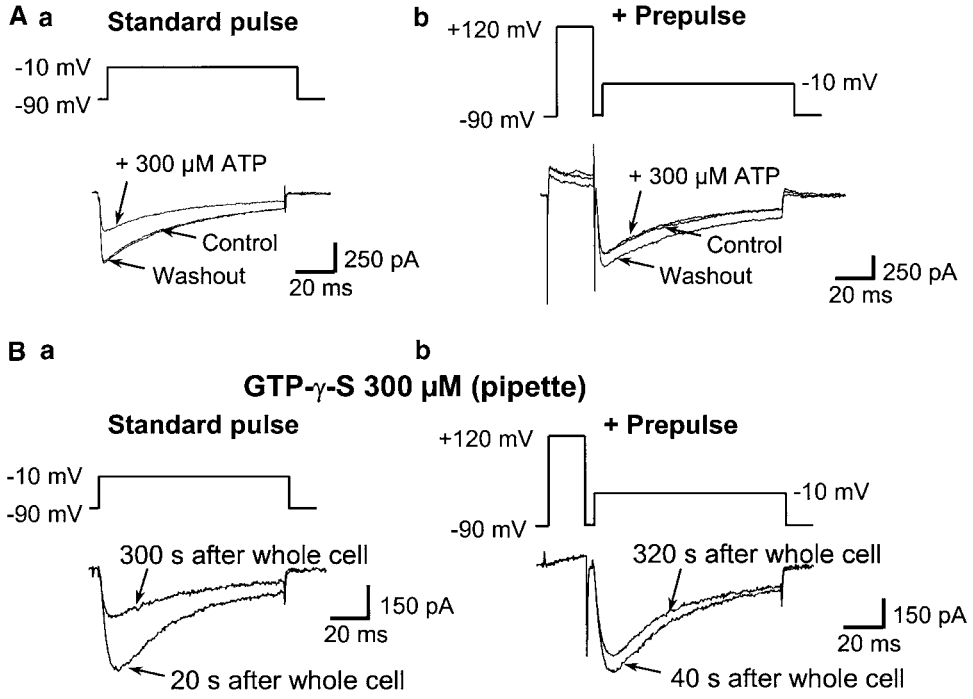


**Figure 6** Effect of a strongly depolarizing prepulse on the ATP- and GTP-$\gamma$-S-induced inhibition of N-type $Ca^{2+}$ channels in HEK 293-N26 cell. Cells were depolarized to $-10$ mV from a holding potential of $-90$ mV for 100 ms every 20 s to induce inward currents. In some experiments, the test pulse was preceded by a prepulse of 20 ms duration to $+120$ mV. The duration of the interpulse interval was 5 ms. The voltage protocols are illustrated on top of each plot. (A) Representative tracings illustrating the effect of ATP (300 $\mu$M) on $I_{Ca(N)}$ evoked by a standard pulse alone (a) or by a standard pulse preceded by a prepulse (b). (B) Representative tracings illustrating the effect of intracellular GTP-$\gamma$-S (300 $\mu$M) on $I_{Ca(N)}$ evoked by a standard pulse alone (a) or by a standard pulse preceded by a prepulse (b). The time period after establishing whole-cell configuration is indicated. Recordings both in (A) and (B) were from the same cell, respectively.

contrast, the effect of ATP was stable over time, when PTX was not activated before inclusion into the pipette (Figure 7b), suggesting the involvement of a PTX-sensitive G protein in the modulation of $Ca^{2+}$ channels by ATP.

### *Presence of P2Y receptors*

Using RT–PCR, the expression of mRNA encoding $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$ was investigated in HEK 293-N26 cells cultured under identical conditions as those cells which were subjected to electrophysiological recordings. mRNAs coding for $P2Y_1$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$ and $P2Y_{13}$ were detected by the occurrence of 528, 431, 380, 410 and 575 bp amplification products, respectively (Figure 8A). No products were generated in RNA samples not subjected to cDNA synthesis, indicating that those samples were free from genomic DNA. No evidence was obtained for the expression of $P2Y_2$ and $P2Y_{12}$ receptors (Figure 8A). With the help of immunocytochemical staining, $P2Y_1$ but not $P2Y_2$ receptor subtypes were detected; in a few cells, especially within cell aggregates, $P2Y_4$ receptor immunoreactivity was observed (Figure 8B). Since $P2Y_{13}$ receptor antibodies were not available, the search for the presence of this particular receptor was not possible.

## Discussion

Modulation of VACCs by neurotransmitters is involved in the presynaptic regulation of neurotransmission (Hille, 1992; 1994). In this respect, the inhibition of N-type $Ca^{2+}$ channels following activation of G-protein-coupled receptors seems to be particularly important. Recent studies have shown that extracellular ATP may either facilitate transmitter release *via* P2X or depress transmitter release *via* P2Y receptor activation (von Kügelgen *et al*., 1993; Boehm, 1999). The exact transduction mechanism of P2Y receptors leading to the inhibition of neurosecretion is still unknown, although recent data suggest that, in bovine adrenal chromaffin cells, a modulation of VACCs underlies the inhibitory effect of ATP on exocytosis (Powell *et al*., 2000).

Native $P2Y_{12}$ receptors depressed VACCs in neuronally differentiated rat PC12 cells (Kubista *et al*., 2003). Moreover, $P2Y_{12}$ receptors inhibited the evoked entry of calcium through N-type calcium channels at the neuronal processes of these cells (Kulick & von Kügelgen, 2002). Finally, ATP and/or other nucleotides depressed N-type $Ca^{2+}$ currents in superior cervical ganglion cells that were additionally transfected with $P2Y_1$ (Filippov *et al*., 2000), $P2Y_2$ (Filippov *et al*., 1998), $P2Y_4$ (Filippov *et al*., 2003) or $P2Y_6$ cRNA (Filippov *et al*., 1999). We used an alternative approach in that endogenous human P2Y receptors were activated to inhibit a recombinant rabbit N-type $Ca^{2+}$ channel transfected into a HEK 293 cell line.

The HEK 293 cell line is a suitable and widely used system for the expression of VACCs. Untransfected HEK 293 cells do not contain high VACCs (McNaughton & Randall, 1997). Furthermore, $Ca^{2+}$ currents, evoked in our transfected HEK 293-N26 cells, exhibited the electrophysiological characteristics described for N-type $Ca^{2+}$ channels with a peak current of the

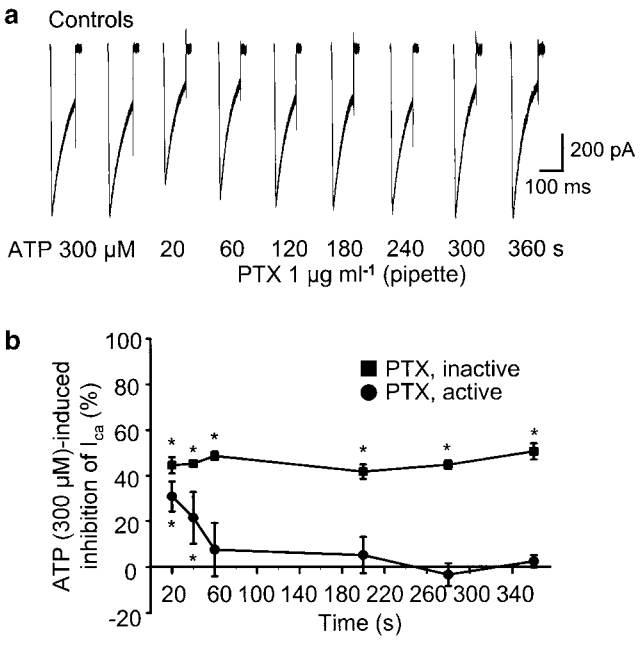


**Figure 7** Effect of intracellular PTX on the ATP-induced inhibition of N-type $Ca^{2+}$ channels in HEK 293-N26 cells. Cells were depolarized to −10 mV from a holding potential of −90 mV for 100 ms every 20 s to induce inward currents. Whole-cell recordings were performed with a pipette solution containing activated or nonactivated PTX (1 $\mu$g ml$^{-1}$). Two control currents were recorded immediately after establishing whole-cell access. Then, ATP (300 $\mu$M) was applied for 6 min. (a) Representative tracings showing time-dependent decrease of the ATP-induced inhibition after application of activated PTX *via* the pipette solution. The presence of ATP in the superfusion medium is indicated by the number of seconds. (b) ATP-induced inhibition of $I_{Ca(N)}$ with activated and nonactivated PTX in the pipette solution. Means ± s.e.m. of 5–7 experiments. *$P<0.05$; statistically significant difference from the second current response in this series.

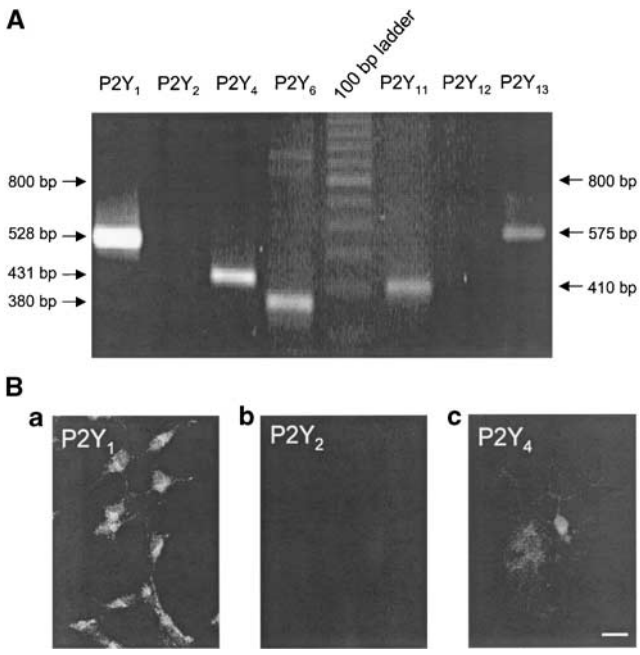


**Figure 8** P2Y receptor mRNA expression and immunohistochemistry in HEK 293-N26 cells. (A) Subsequent to total RNA extraction and RT–PCR amplification with primers specific for distinct P2Y receptor cDNA fragments ($P2Y_{13}$, 25 cycles; $P2Y_1$–$P2Y_{12}$, 35 cycles), cDNA products were analyzed by agarose gel (1.5%) electrophoresis. A representative gel with ethidium bromide-stained cDNA fragments is shown: $P2Y_1$ (528 bp), $P2Y_4$ (431 bp), $P2Y_6$ (380 bp), $P2Y_{11}$ (410) and $P2Y_{13}$ (575 bp). $P2Y_2$ and $P2Y_{12}$ transcripts were not detectable. (B) Representative fluorescence image of HEK 293-N26 cells after immunocytochemical labeling of $P2Y_1$ (a) and $P2Y_4$ receptors (c), with rabbit anti-$P2Y_1$ and $P2Y_4$ receptor antibodies (scale bar, 20 $\mu$m). $P2Y_2$ receptor immunoreactivities (b) could not be detected.

$I/V$ curve around −10 mV, tail current (McNaughton & Randall, 1997), inhibition by $Co^{2+}$ ions (Wakamori *et al.*, 1998) and sensitivity to $\omega$-conotoxin GVIA that specifically binds to the N-type $Ca^{2+}$ channel (Olivera *et al.*, 1994).

Previous studies in neurons have identified different G-protein-dependent pathways for the inhibition of $I_{Ca(N)}$ (Hille, 1994). One mechanism may involve diffusible second messengers and the subsequent activation of protein kinases (Boehm *et al.*, 1996). A second mechanism is membrane-delimited and involves neurotransmitter receptors, G proteins and VACCs. In the present experiments, ATP time- and concentration-dependently inhibited $Ca^{2+}$ currents and slightly shifted the voltage–current curve to the right. A shift in the voltage dependence of activation, a slowed activation kinetics and a reduced current amplitude of $Ca^{2+}$ currents are characteristic properties following stimulation of G-protein-coupled receptors (Bean, 1989). Channels with these characteristics are described as being 'reluctant' to open (Bean, 1989). However, a strong depolarizing pulse applied before the test pulse can reconvert 'reluctant' into 'willing' channels (Bean, 1989; Ikeda, 1991), a phenomenon called prepulse facilitation. In this study, application of a strongly depolarizing prepulse could prevent ATP-induced inhibition of the $Ca^{2+}$ current, indicating a G-protein-mediated mechanism of $Ca^{2+}$ channel modulation. Intracellular dialysis with GDP-$\beta$-S, an inhibitor of G-protein-dependent reactions (Sternweis & Pang, 1990), also prevented the inhibitory action of ATP. In order to mimic G protein stimulation by ATP, GTP-$\gamma$-S, an irreversible activator of G proteins (Gilman, 1987), was included into the pipette solution. Under these conditions, $Ca^{2+}$ channel inhibition, comparable to the degree of inhibition induced by ATP, was observed 5 min after establishing whole-cell access. It is noteworthy that prepulse facilitation reversed the GTP-$\gamma$-S-induced inhibition as well. Since PTX inhibited the effect of ATP on $I_{Ca(N)}$, the involvement of $G_{i,o}$ can be assumed in this process.

Membrane-delimited pathways comprise a direct effect of the activated G protein subunit or subunits on the $Ca^{2+}$ channel itself. A key role of the $G_{\beta\gamma}$ rather than $G_\alpha$ subunits had been proposed (Herlitze *et al.*, 1996; Ikeda, 1996). A $Ca^{2+}$-dependent inactivation of the N-type $Ca^{2+}$ channel (Budde *et al.*, 2002), as a consequence of the P2Y receptor-induced increase of intracellular $Ca^{2+}$, could be excluded by reducing the usual intrapipette concentration of EGTA. Under these conditions, the calcium-buffering capacity of EGTA decreases and a $G_\alpha$-mediated triggering of the phospholipase C/inositol 1,4,5-trisphosphate ($IP_3$)/$Ca^{2+}$ pathway should lead to a larger inhibition of $I_{Ca(N)}$. However, this was apparently not the case, indicating the involvement of $G_{\beta\gamma}$ rather than $G_\alpha$ in this process.

Furthermore, it was of interest whether all types or only a single type of endogenous P2Y receptors expressed by HEK

293-N26 cells are involved in the modulation of $I_{Ca(N)}$. Until now, eight P2Y receptors with molecularly distinct properties have been described ($P2Y_{1–12}$, Burnstock & Williams, 2000; $P2Y_{13}$, Communi *et al*., 2001; $P2Y_{14}$, Abbracchio *et al*., 2003). Conflicting reports concerning the occurrence of P2Y receptor subtypes in HEK 293 cells exist. It has been suggested that HEK 293 cells exhibit $IP_3$ responses to adenine nucleotides *via* activation of $P2Y_1$ and $P2Y_2$ receptor subtypes and furthermore mRNA for the $P2Y_1$, but not for the $P2Y_4$ subtype, was detected, using RT–PCR (Schachter *et al*., 1997). In a comprehensive study, copies of $P2Y_1$, $P2Y_4$ and $P2Y_{11}$ mRNA, but not of $P2Y_2$, and $P2Y_6$ mRNA were determined (Moore *et al*., 2001). Finally, $P2Y_1$ and $P2Y_4$ receptor activation released $Ca^{2+}$ from their intracellular storage sites in HEK 293 cells (Fischer *et al*., 2003).

The present data confirm the outcome of the study of Moore *et al*. (2001) by detecting $P2Y_1$, $P2Y_4$ and $P2Y_{11}$ mRNAs in HEK 293-N26 cells using RT–PCR. In addition, $P2Y_6$ and $P2Y_{13}$ mRNA was found, whereas no evidence was obtained for the expression of $P2Y_2$ and $P2Y_{12}$ receptors. Accordingly, $P2Y_1$ and $P2Y_4$, but not $P2Y_2$ receptor immunoreactivities, were identified by an immunocytochemical approach. The reported variability in the P2Y receptor endowment of HEK 293 cells may be due to the fact that different subcultures express different sets of P2Y receptors (i.e. for $P2Y_{13}$, compare this study with Zhang *et al*., 2002).

In the present experiments, ADP and ADP-$\beta$-S were more potent than ATP; $\alpha,\beta$-meATP, UDP and UTP were weak agonists only. ADP and ADP-$\beta$-S preferentially activate the human $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptor subtypes that are practically insensitive to UTP and UDP (von Kügelgen & Wetter, 2000; Communi *et al*., 2001). ATP and UTP are equipotent on $P2Y_2$ receptors (von Kügelgen & Wetter, 2000), while the human $P2Y_4$ and $P2Y_6$ receptors are preferentially stimulated by UTP and UDP, respectively (von Kügelgen & Wetter, 2000). The low residual activity of UTP and UDP in the present study may be due to the interconversion of UDP to ADP by nucleoside diphosphokinase (Harden *et al*., 1997), and the subsequent activation of $P2Y_{13}$ receptors by ADP. The failure of $\alpha,\beta$-meATP to considerably inhibit $I_{Ca(N)}$ was not surprising, because $\alpha,\beta$-meATP is a $P2X_{1,3}$ receptor-selective agonist (Khakh *et al*., 2001).

Whereas the agonist profile of the endogenous receptor present in HEK 293-N26 cells indicates a preference for ADP, its antagonist profile conforms with a $P2Y_{13}$, but not with a $P2Y_1$ or $P2Y_{12}$ receptor. The $P2Y_1$ receptor-selective antagonists MRS 2179 (Nandanan *et al*., 1999) and PPADS (von Kügelgen & Wetter, 2000; for high concentrations of PPADS, see Marteau *et al*., 2003) did not interfere with ATP. The $P2Y_{12}$ receptor-preferential antagonist 2-MeSAMP (Hollopeter *et al*., 2001), which is a partial agonist at $P2Y_{13}$ receptors with a low antagonistic potency (Marteau *et al*., 2003), also failed to alter the ATP effect. Moreover, AR-C69931MX, with selectivities for $P2Y_{12}$ and $P2Y_{13}$ receptors (Barnard & Simon, 2001; Boeynaems *et al*., 2003; Marteau *et al*., 2003), antagonized the ATP-induced inhibition of $I_{Ca(N)}$. The incomplete blockade of the ATP response by AR-C6993MX may be due to the fact that this compound belongs to a class of antagonists which act in the nanomolar range at $P2Y_{12}$, but only in the micromolar range at $P2Y_{13}$ receptors (Boeynaems *et al*., 2003). The nonselective P2 receptor antagonist suramin was also effective. It is noteworthy that suramin has been reported not to discriminate between an endogenous adenosine receptor and the $P2Y_{13}$ receptor transfected into 1321N1 cells coexpressing also $G_{\alpha 16}$ (Marteau *et al*., 2003). In view of the somewhat higher antagonistic potency of suramin in comparison with AR-C69931MX, and the existence of the $P2Y_{11}$ receptor mRNA in HEK 293 cells, a minor contribution of this partly $G_i$-coupled receptor type (Ralevic & Burnstock, 1998) to the depression of $I_{Ca(N)}$ cannot be excluded by the present experiments.

Our finding that PTX abolishes the inhibitory effect of ATP on N-type $Ca^{2+}$ channels also assigns this effect to $P2Y_{12}$ or $P2Y_{13}$ in contrast to $P2Y_1$ receptors. Whereas $P2Y_1$ receptors couple to the PTX-insensitive $G_{q,11}$, $P2Y_{12}$ and $P2Y_{13}$ receptors couple to the PTX-sensitive $G_i$ (Ralevic & Burnstock, 1998; Hollopeter *et al*., 2001; Zhang *et al*., 2002). Since $P2Y_{13}$ receptor antibodies are not available, a direct proof for the presence of the $P2Y_{13}$ receptor protein in HEK 293 cells is still missing. However, based on the combined pharmacological and RT–PCR evidence supporting the involvement of a PTX-sensitive P2Y receptor, it is suggested that endogenous $P2Y_{13}$ receptors negatively interact with N-type $Ca^{2+}$ channels, while $P2Y_1$ and $P2Y_4$ receptors release $Ca^{2+}$ from intracellular pools subsequent to an increased production of $IP_3$ (Fischer *et al*., 2003). Recently, analysis of the distribution of the $P2Y_{13}$ receptor mRNA revealed high-level expression in tissues of several brain regions (Zhang *et al*., 2002). Hence, it appears to be appropriate to assume that a native $P2Y_{13}$ or $P2Y_{13}$-like receptor inhibits $I_{Ca(N)}$ in neurons and thereby exerts a pre- and/or postsynaptic modulatory action.

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