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Attenuation of the P2Y receptor-mediated control of neuronal Ca^{2+} channels in PC12 cells by antithrombotic drugs

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1 In PC12 cells, adenine nucleotides inhibit voltage-activated Ca^{2+} currents and adenylyl cyclase activity, and the latter effect was reported to involve $P2Y_{12}$ receptors. To investigate whether these two effects are mediated by one P2Y receptor subtype, we used the antithrombotic agents 2-methylthio-AMP (2-MeSAMP) and N^6-(2-methyl-thioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene-ATP (AR-C69931MX).

2 ADP reduced A_{2A} receptor-dependent cyclic AMP synthesis with half maximal effects at 0.1–0.17 μM. In the presence of 30 μM 2-MeSAMP or 100 nM AR-C69931MX, concentration response curves were shifted to the right by factors of 39 and 30, indicative of pA_2 values of 6.1 and 8.5, respectively.

3 The inhibition of Ca^{2+} currents by ADP was attenuated by 10–1000 nM AR-C69931MX and by 3–300 μM 2-MeSAMP. ADP reinhibited Ca^{2+} currents after removal of 2-MeSAMP within less than 15 s, but required 2 min to do so after removal of AR-C69931MX.

4 ADP inhibited Ca^{2+} currents with half maximal effects at 5–20 μM. AR-C69931MX (10–100 nM) displaced concentration response curves to the right, and the resulting Schild plot showed a slope of 1.09 and an estimated pK_B value of 8.7. Similarly, 10–100 μM 2-MeSAMP also caused rightward shifts resulting in a Schild plot with a slope of 0.95 and an estimated pK_B of 5.4.

5 The inhibition of Ca^{2+} currents by 2-methylthio-ADP and ADPβS was also antagonized by AR-C69931MX, which (at 30 nM) caused a rightward shift of the concentration response curve for ADPβS by a factor of 3.8, indicative of a pA_2 value of 8.1.

6 These results show that antithrombotic drugs antagonize the inhibition of neuronal Ca^{2+} channels by adenine nucleotides, which suggests that this effect is mediated by $P2Y_{12}$ receptors.
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Abbreviations: AR-C69931MX, N^6-(2-methyl-thioethyl)-2-(3,3,3- trifluoropropylthio)-β,γ-dichloromethylene-ATP; CGS 21680, 2-p-(2-carboxyethyl)phenethylamino-5′-N-ethylcarboxamido-adenosine; I_{Ca}, voltage-gated Ca^{2+} currents; MRS 2216, 2-chloro-N^6-methyldeoxyadenosine 3′,5′-biphosphate; NGF, nerve growth factor; RO 20-1724, 4-(3-butoxy-4-methoxybenzyl)imidazoline-2-one; VACC, voltage-activated Ca^{2+} channel

Introduction

Voltage-activated Ca^{2+} channels (VACCs) mediate transmembrane Ca^{2+} entry in response to membrane depolarization and thereby contribute to a multitude of physiological processes, such as muscle contraction, endocrine secretion and gene expression. In neurons, N, P, and Q types of VACCs are involved in excitation secretion coupling and thus in synaptic transmission (Catterall, 2000). These neuronal VACCs are modulated by a plethora of neurotransmitters which act *via* appropriate G protein-coupled receptors and thereby influence neuronal signalling. The modulation of VACCs has been investigated most frequently in sympathetic neurons (Hille, 1994). There, receptor-dependent activation of G proteins leads to an inhibition of Ca^{2+} currents either through a voltage-dependent interaction of G protein $\beta\gamma$ subunits with VACCs (Zamponi & Snutch, 1998) or *via* the generation of diffusible second messengers (Hille, 1994) and subsequent activation of protein kinases (Boehm *et al*., 1996). In sympathetic nerve terminals, the G protein-mediated inhibition of VACCs is the decisive mechanism in the presynaptic autoregulation of sympatho-effector transmission by the cotransmitters ATP and noradrenaline (Boehm & Kubista, 2002).

At least seven different mammalian DNA sequences have been identified that code for putative G protein coupled nucleotide receptors ($P2Y_{1,\ 2,\ 4,\ 6,\ 11,\ 12,\ 13}$; Ralevic & Burnstock, 1998; Hollopeter *et al*., 2001; Zhang *et al*., 2001; Communi *et al*., 2001). Amongst these, $P2Y_1$ (Tokuyama *et al*., 1995), $P2Y_{11}$ (Communi *et al*., 1997), $P2Y_{12}$ (Hollopeter *et al*., 2001; Zhang *et al*., 2001), and $P2Y_{13}$ (Communi *et al*., 2001) are activated by adenine nucleotides, whereas $P2Y_6$ is activated by uridine nucleotides (Chang *et al*., 1995). $P2Y_2$ (Lustig *et al*., 1993) and $P2Y_4$ (Communi *et al*., 1995) receptors, in contrast, are sensitive to both adenine and uridine nucleotides. Five of these receptors, namely $P2Y_{1,\ 2,\ 4,\ 6,}$ and $_{12}$, have been detected in rats (Ralevic & Burnstock, 1998; Hollopeter *et al*., 2001). In heterologous expression

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systems, all P2Y receptor subtypes of the rat, with the exception of $P2Y_{12}$, couple to phospholipase C and mediate nucleotide-dependent increases in intracellular inositol phosphates (Ralevic & Burnstock, 1998). Activation of the $P2Y_{12}$ receptor, in contrast, mediates an inhibition of adenylyl cyclase (Hollopeter *et al*., 2001). Recombinant rat $P2Y_{1,2}$ and $_6$ receptors inhibit VACCs as well as M-type K^+ (K_M) channels when expressed in sympathetic neurons (Brown *et al*., 2000).

The rat phaeochromocytoma cell line PC12 is ontogenetically related to sympathetic neurons. These cells develop a neuronal phenotype upon treatment with nerve growth factor (NGF; Greene & Tischler, 1976), and express various types of neuronal VACCs (Liu *et al*., 1996). In PC12 cells, ADP was found to inhibit Ca^{2+} currents (I_{Ca}) in a voltage-dependent manner (presumably *via* G protein $\beta\gamma$ subunits), but at that time the pharmacological characteristics of the receptor involved did not correspond to any of those of molecularly defined P2Y receptor subtypes (Vartian & Boehm, 2001). More recently, we were able to show that PC12 cells express $P2Y_{12}$ receptors, activation of which reduced the accumulation of cyclic AMP (Unterberger *et al*., 2002). Previously, the inhibition of adenylyl cyclase activity *via* $P2Y_{12}$ receptors had been reported for thrombocytes (Hollopeter *et al*., 2001), endothelial (Simon *et al*., 2001), and glioma cells (Jin *et al*., 2001), but not for neurons or neuroendocrine cells. The fact that the nucleotide sensitivities for both, the inhibition of I_{Ca} and the reduction in cyclic AMP, were similar raises the question as to whether a single P2Y receptor subtype mediated both effects. In order to clarify this, we used 2-MeSAMP and AR-C69931MX and determined their effects on the inhibition of adenylyl cyclase and I_{Ca} by adenine nucleotides in PC12 cells. Previously, these drugs have been reported to reduce platelet aggregation by a blockade of $P2Y_{12}$ receptors (Jantzen *et al*., 1999; Hollopeter *et al*., 2001; Turner *et al*., 2001). The present results show that these antithrombotic drugs attenuate not only the ADP-dependent inhibition of adenylyl cyclase, but also that of neuronal VACCs.

Methods

Cell culture

PC12 cells were plated onto collagen-coated (Biomedical Technologies Inc., Stoughton, MA, U.S.A.) culture dishes (NUNC, Roskilde, Denmark) and were kept in OptiMEM (Life Technologies, Vienna, Austria) supplemented with 0.2 mM L-glutamine (HyClone, Aalst, Belgium), 25 000 IU/l penicillin and 25 mg l^{-1} streptomycin (Sigma, Vienna, Austria), 5% foetal calf serum, and 10% horse serum (both Life Technologies, Vienna, Austria). Once per week, cell cultures were split, and the medium was exchanged twice per week. In order to increase I_{Ca} of PC12 cells through neuronal differentiation (Vartian & Boehm, 2001), the horse serum was reduced to 5%, foetal calf serum was omitted from, and recombinant human β-nerve growth factor (NGF, 50 ng ml^{-1}, R&D Systems Inc., Wiesbaden, Germany) was included in, the medium for 5–6 days. Prior to the recording of I_{Ca}, PC12 cells were detached from culture dishes and replated at low density (about 30 000 cells per dish) in 35 mm dishes.

Determination of cyclic AMP

The accumulation of cyclic AMP in PC12 cell cultures was determined as described before; since differentiation of PC12 cells did not alter the ADP-dependent inhibition of adenylyl cyclase, nor the expression of $P2Y_{12}$ receptors (Unterberger *et al*., 2002), these experiments were performed in cultures not treated with NGF. PC12 cells were incubated in medium containing 2.5 μCi ml^{-1} tritiated adenine to label the cellular purines. After 12 h, the medium was replaced by a buffer (mM NaCl 120, KCl 3, $MgCl_2$ 2, $CaCl_2$ 2, glucose 20, HEPES 10, adjusted to pH 7.4 with NaOH) containing 100 μM of the phosphodiesterase inhibitor Ro-20-1724 [4-(3-butoxy-4-methoxybenzyl) imidazolidin-2-one] and 1 U ml^{-1} adenosine deaminase. Dishes were then kept at room temperature for about 90 min. Thereafter, the cells were incubated in the above buffer containing the adenosine A_{2A} receptor agonist 2-p-(2-carboxyethyl)phenethylamino-5′-N-ethylcarboxamido-adenosine (CGS 21680) for 15 min at room temperature to stimulate adenylyl cyclase. ADP was present together with CGS 21680, and P2Y receptor antagonists were added 10 min before the addition of ADP. The stimulation period was terminated by exchanging the buffer for 1 ml of 2.5% perchloric acid containing 100 μM non-labelled cyclic AMP followed by a 20 min incubation at 4°C.

Cyclic AMP was separated from the other purines by a two column chromatographic procedure (Johnson *et al*., 1994). One tenth of each sample obtained as described above was used for the determination of the total radioactivity. The remaining 900 μl were neutralized by addition of 100 μl 4.2 M KOH and applied to Dowex 50 columns (AG 50W-X4; Bio-Rad, Vienna, Austria) which were then rinsed with 3 ml H_2O. The eluate obtained by the subsequent application of 8 ml H_2O was directly poured onto alumina columns (Bio-Rad, Vienna, Austria), which were then washed with 6 ml H_2O. Finally, cyclic AMP was eluted with 4 ml imidazole buffer (20 mM imidazole in 0.2 M NaCl; pH 7.45). Radioactivity within the samples obtained was determined by liquid scintillation counting.

The radioactivity in the fraction of cyclic AMP was expressed as percentage of the total radioactivity incorporated in the cells. Stimulation of PC12 cells with the adenosine A_{2A} receptor agonist CGS 21680 caused a reproducible increase in these values of cyclic AMP, but the extent of basal and stimulated cyclic AMP synthesis may vary between different preparations (Unterberger *et al*., 2002). Therefore, the values of cyclic AMP obtained in the presence of CGS 21680 were normalized to the values obtained in its absence within the same preparation (normalized to basal). To quantify the inhibition of stimulated cyclic AMP accumulation by ADP, values obtained in its presence were expressed as percentage of the values obtained in the presence of CGS 21680 only (% of control).

Electrophysiology

Whole-cell currents were recorded at room temperature (20–24°C) from PC12 cells 48–96 h after replating at low density, according to published procedures (Vartian & Boehm, 2001), using an Axopatch 200B amplifier and the Pclamp 6.0 hard- and software (Axon Instruments, Foster City, CA, U.S.A.).

Currents were low-pass filtered at 5 kHz, digitized at 10–50 kHz, and stored on an IBM compatible computer. Traces were analysed off-line by the Clampfit program (Axon). Patch electrodes were pulled (Flaming-Brown puller, Sutter Instruments, Novato, CA, U.S.A.) from borosilicate glass capillaries (Science Products, Frankfurt/Main, Germany) and filled with a solution consisting of (mM) CsCl 130, tetraethylammonium chloride 20, $CaCl_2$ 0.24, glucose 10, HEPES 10, EGTA 5, Mg-ATP 2, and Li-GTP 2, adjusted to pH 7.3 with KOH, to yield tip resistancies of 2–3 MΩ. The external bathing solution contained (mM) NaCl 120, tetraethylammonium chloride 20, KCl 3, $MgCl_2$ 2, $CaCl_2$ 5, glucose 20, HEPES 10, adjusted to pH 7.3 with KOH. This combination of solutions results in small liquid junction potentials of about +2 mV which, however, were neglected during I_{Ca} recordings. Drugs were applied *via* a DAD-12 drug application device (Adams & List, Westbury, NY, U.S.A.) which permits a complete exchange of solutions surrounding the cells under investigation within less than 100 ms (Boehm, 1999).

Whole-cell I_{Ca} was elicited by 30 ms depolarizations from a holding potential of −80 mV to 0 mV at a frequency of 4 min^{-1}. Leakage currents were corrected for by using an on-line leak subtraction protocol which applies four hyperpolarizing pulses prior to the depolarization to 0 mV in order to determine the extent of leakage. I_{Ca} was quantified by measuring peak current amplitudes during the depolarization to 0 mV. To account for time-dependent changes in I_{Ca}, drug effects were evaluated by evoking currents in the presence of test drugs (B) and by comparing them to control currents recorded before (A) and after (washout, C) the application of the drugs, according to the equations: 200B/(A+C) = % of control current, or 100−(200B/[A+C]) = % inhibition (Vartian & Boehm, 2001).

The cells being investigated were continuously superfused with control solutions or with solutions containing agonists and/or antagonists at the concentrations indicated. To evaluate the effects of P2Y receptor antagonists (2-MeSAMP, AR-C69931MX), cells were first exposed to these agents for at least 30 s, and then ADP or other nucleotides were applied together with antagonists, again for at least 30 s. As the extent of inhibition of I_{Ca} exerted by nucleotides may vary between different cells, the inhibitory effects of different agonist concentrations, whether in the absence or presence of antagonists, were normalized to the inhibition caused by the very same agonist at 100 μM (relative inhibition of I_{Ca}; Vartian & Boehm, 2001).

Statistics

The extent as well as the concentration dependence of the nucleotide-induced inhibition of adenylyl cyclase or I_{Ca} may vary between different batches of PC12 cells (Unterberger *et al*., 2002; Vartian & Boehm, 2001). Therefore, changes in the concentration response curves for agonists in the presence of increasing antagonist concentrations were investigated in one batch of PC12 cells per one antagonist. All data represent arithmetic means ± s.e.mean; *n* represent numbers of single neurons in electrophysiological experiments and of culture dishes in cyclic AMP assays. Concentration–response curves were fitted to experimentally obtained data by the ALLFIT programme (De Lean *et al*., 1978).

Materials

[2,8-^{3}H]adenine (specific activity 32 Ci $mmol^{-1}$) was obtained from NEN (Vienna, Austria). Na-ADP, Mg-ATP, Li-GTP, 4-(3-butoxy-4-methoxybenzyl)imidazoline-2-one (RO 20-1724), 3′,5′-cyclic AMP, and adenosine 5′-O-(2-thiodiphosphate) (ADPβS) from Sigma (Vienna, Austria); 2-methylthio-AMP and -ADP, as well as 2-p-(2-carboxyethyl)-phenethylamino-5′-N-ethylcarboxamido-adenosine (CGS 21680) from RBI (Natick, MA, U.S.A.); tetrodotoxin from Latoxan (Rosans, France). 2-Chloro-N^6-methyldeoxyadenosine 3′,5′-biphosphate (MRS 2216) was a gift of Dr K.A. Jacobson (NIDDK, Bethesda, MD, U.S.A.). N^6-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene-ATP (AR-C69931MX) was kindly provided by AstraZeneca R&D Charnwood (Loughborough, U.K.).

Results

To reveal whether the adenine nucleotide-dependent inhibition of both, adenylyl cyclase and I_{Ca}, in PC12 cells was mediated by one P2Y receptor subtype, the antithrombotic $P2Y_{12}$ receptor antagonists 2-MeSAMP (Jantzen *et al*., 1999; Hollopeter *et al*., 2001) and AR-C69931MX (Turner *et al*., 2001) were used in cyclic AMP accumulation assays, on one hand, and in whole-cell patch-clamp experiments, on the other hand.

Inhibition of adenylyl cyclase

After labelling of cellular purines of PC12 cells with [^{3}H]-adenine, 272 ± 54 c.p.m. (n = 18) were retrieved within the fraction of cyclic AMP, which corresponded to 0.041 ± 0.008% of the total radioactivity in the cultures. These and all subsequent values were obtained in the presence of the phosphodiesterase inhibitor RO 20-1724. Thus, any drug-induced alteration will reflect changes in adenylyl cyclase activity rather than in cyclic AMP degradation (Unterberger *et al*., 2002). Exposure of PC12 cells to 1 μM of the A_{2A} adenosine receptor agonist CGS 21680 for 15 min caused a 25 fold increase in cellular cyclic AMP, and this effect was reduced in the presence of 0.01 to 100 μM ADP in a concentration-dependent manner. The inhibitory actions of the nucleotide were half maximal at 0.1 μM (Figure 1A) and 0.17 μM (Figure 1B), respectively, which closely corresponds to the values reported before (0.24 μM; Unterberger *et al*., 2002). In the presence of 30 μM 2-MeSAMP, the concentration–response curve for the ADP-mediated inhibition of adenylyl cyclase was shifted to the right and the effect was half maximal at 3.9 μM. The maximum of inhibition achieved by ADP, however, remained unchanged (Figure 1A). Assuming a competitive type of antagonism and thus applying the equation log (CR-1) = pA2 + log [B] (where CR is the ratio of equieffective agonist concentrations in the presence and absence of the antagonist concentration B, respectively (Arunlakshana & Schild, 1959) revealed an apparent affinity of 2-MeSAMP in the low micromolar range with a pA_2 value of 6.1.

To corroborate these data, we also used the more potent and selective antagonist AR-C69931MX. In the presence of 100 nM of this agent, the concentration–response curve for

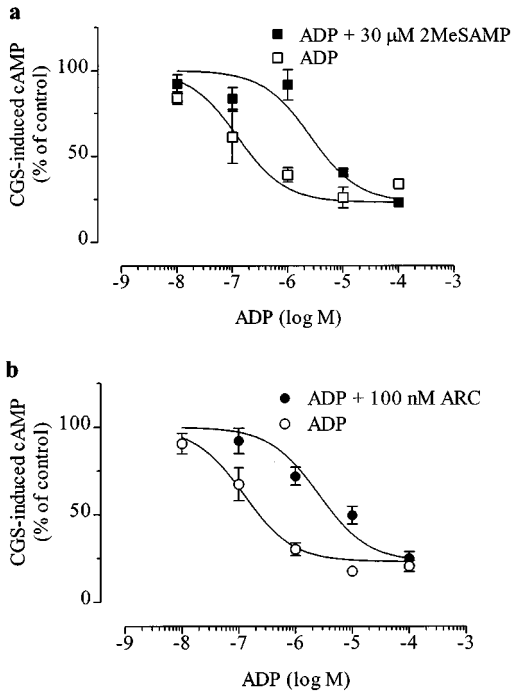

Figure 1 2-MeSAMP and AR-C69931MX antagonize the inhibition of adenylyl cyclase by ADP. After loading with [^{3}H]adenine, PC12 cells were incubated in RO 20-1724 (100 μM) for 105 min. During the last 15 min of this incubation period, 1 μM CGS 21680 was present either alone or together with the indicated concentrations of ADP (open symbols). The amount of radioactivity retrieved within the fraction of cyclic AMP (cAMP) was calculated as percentage of the total radioactivity in the cell cultures, and values obtained in the presence of CGS 21680 were normalized to the data obtained in its absence (normalized to basal). Results obtained in the presence of ADP are then expressed as percentage of those obtained in its absence (% of control; see Methods). (A) In the cultures represented by filled squares, 30 μM 2-MeSAMP were present, whereas this antagonist was lacking in the cultures represented by open symbols. $n=6-9$. (B) In the cultures represented by filled circles, 100 nM AR-C69931MX were present, whereas this antagonist was lacking in the cultures represented by open symbols. $n=6-9$.

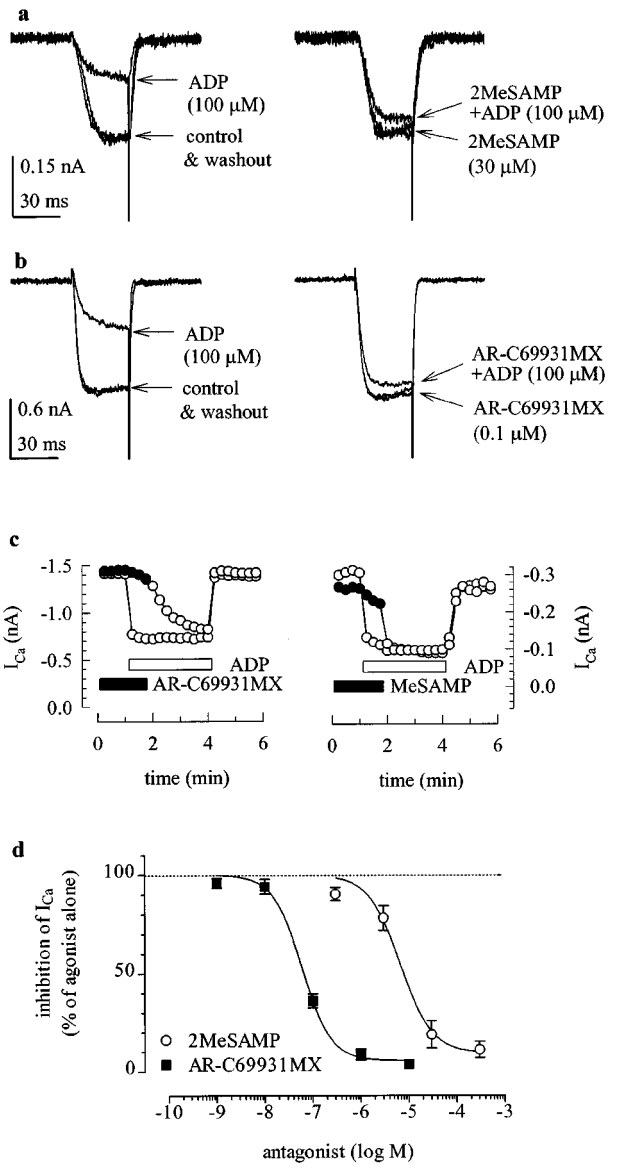

Figure 2 2-MeSAMP and AR-C69931MX antagonize the inhibition of voltage-activated Ca^{2+} currents by ADP. Ca^{2+} currents were evoked in differentiated PC12 cells by 30 ms depolarizations from -80 to 0 mV applied once every 15 s. (A) The current traces shown left were obtained before (control), during and after (washout) the application of 100 μM ADP. Thereafter, 30 μM 2-MeSAMP were present, and ADP (100 μM) was applied again in the continuing presence of this antagonist; the traces shown right were obtained again before, during and after the application of ADP. (B) ADP (100 μM) and AR-C69931MX (0.1 μM) were applied to another differentiated PC12 cell in the manner described for 2-MeSAMP in A. (C) Time course of peak Ca^{2+} current amplitudes: ADP (100 μM, white bar), AR-C69931MX (10 μM, black bar, left), and 2-MeSAMP (300 μM, black bar, right) were present as indicated by the bars. Note that ADP achieved inhibition of I_{Ca} immediately when 2-MeSAMP was removed, but required about 2 min to inhibit I_{Ca}, when AR-C69931MX was removed. (D) Concentration-dependence of the antagonism of 2-MeSAMP (0.3 to 300 μM) and AR-C69931MX (1 nM to 10 μM) versus the inhibition of I_{Ca} by ADP (100 μM). Currents were elicited and drugs were applied as described in A; $n=5-6$.

the ADP-dependent inhibition of cyclic AMP accumulation was again shifted to the right without any significant change in the maximal effect exerted by ADP (Figure 1B). The ratio of equieffective ADP concentrations in the presence and absence of AR-C69931MX was 29.6 which corresponds to a pA_2 value of 8.5. Thus, AR-C69931MX is more than 100 fold more potent an antagonist at the receptor mediating the inhibition of adenylyl cyclase than 2-MeSAMP.

Inhibition of I_{Ca}

Depolarization of differentiated PC12 cells from -80 to 0 mV elicited rapidly activating I_{Ca} with amplitudes in the nano-ampere range, as reported previously (Vartian & Boehm, 2001). In the presence of 100 μM ADP, activation kinetics were slowed and maximal current amplitudes were reduced by about one half (Figure 2A). To obtain preliminary evidence as to whether this effect might involve the same receptor as the inhibition of adenylyl cyclase, ADP (100 μM) was applied in the continuous presence of 2-MeSAMP (30 μM) which almost abolished the inhibitory action of ADP without causing *per se* an alteration in current amplitudes (Figure 2A). Likewise, the ATP analogue AR-C69931MX (0.1 μM) did not alter I_{Ca}, but largely attenuated its inhibition by ADP (Figure 2B). In contrast to these

results, the selective $P2Y_1$ receptor antagonist MRS 2216 (1 μM; Nandanan *et al.*, 1999) failed to attenuate the inhibition of I_{Ca} by 100 μM ADP (36.2 + 8.8% inhibition in the absence and 40.1 + 11.9% inhibition in the presence of MRS 2216, respectively; $n = 3$).

To further compare the actions of the two anti-thrombotic agents, we investigated their dissociation from the receptor mediating the inhibition of I_{Ca} by ADP (Figure 2C). In the presence of 100 μM ADP, AR-C69931MX (10 μM) required more than 2 min to dissociate from the receptor, thus permitting maximal inhibition by ADP. 2-MeSAMP (300 μM), in contrast, was entirely replaced by ADP within less than 15 s, and I_{Ca} was immediately reduced to the same extent as without previous application of an antagonist. This indicates a large difference in dissociation constants of these two antagonists which should therefore display greatly varying affinities. Indeed, when determining full concentration–response curves for the antagonistic effects of 2-MeSAMP and AR-C69931MX versus the inhibition of I_{Ca} by 100 μM ADP, the half maximal concentrations were 6.7 μM for 2-MeSAMP and 0.06 μM for AR-C69931MX, respectively (Figure 2D). Thus, AR-C69931MX is at least 100 fold more potent in attenuating the ADP-dependent inhibition of I_{Ca} than 2-MeSAMP.

The inhibition of I_{Ca} by ADP has been reported to be mimicked by ADPβS and 2-MeSADP, but not by uridine nucleotides; the two adenine nucleotides caused half maximal inhibition at 9 and 0.04 μM, respectively (Vartian & Boehm, 2001). To find out whether these agonists acted *via* the same receptor as ADP, they were applied first alone and then together with AR-C69931MX. As shown in Figure 3, this antagonist attenuated the inhibitory actions of both of these agonists. When tested against maximally active concentrations of ADPβS (100 μM) and 2-MeSADP (1 μM), AR-C69931MX (1 nM to 10 μM) attenuated the inhibition of I_{Ca} within the same range of concentrations, namely with IC_{50} values of 0.06 and 0.07 μM, respectively. Thus, AR-C69931MX attenuated the inhibition of I_{Ca} irrespective of which agonistic nucleotide has been used.

To obtain more insight into the interactions between agonists and antagonists, ADP was applied at increasing concentrations in either the absence or presence of AR-C69931MX at 10, 30 or 100 nM. As shown in Figure 4, AR-C69931MX caused a rightward shift of the concentration–response curve for the ADP-dependent inhibition of I_{Ca}, with no obvious change in the maximal effect achieved by ADP. The Schild analysis (Arunlakshana & Schild, 1959) of these data corroborated that AR-C69931MX behaved as a competitive antagonist (slope 1.09) with an apparent affinity in the low nanomolar range ($pK_B = 8.7$). The concentration–response curve for the inhibition of I_{Ca} by ADPβS was also shifted to the right in the presence of 30 nM AR-C69931MX without any noticeable alteration in the maximal inhibition of I_{Ca} (Figure 4B). The ratio of equieffective concentrations of ADPβS in the presence and absence of 30 nM AR-C69931MX, respectively, was 3.8, and the equivalent result for ADP was 4.9. As a consequence, the value log (CR-1) obtained with one concentration of AR-C69931MX versus ADPβS was almost superimposable on the linear regression of such values obtained with three concentrations of this antagonist versus ADP (Figure 4C). Furthermore, the pA_2 value for AR-C69931MX versus ADPβS (8.1) shows considerable correlation with the pK_B value (8.7) obtained with ADP as an agonist.

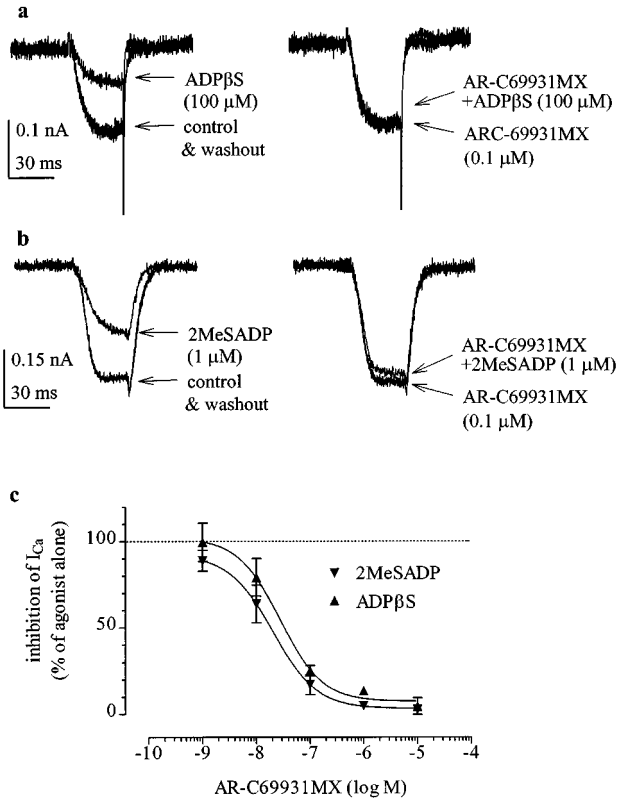

Figure 3 AR-C69931MX antagonizes the inhibition of voltage-activated Ca^{2+} currents by ADPβS and 2-MeSADP. Ca^{2+} currents were evoked in differentiated PC12 cells by 30 ms depolarizations from −80 to 0 mV applied once every 15 s. (A) The current traces shown left were obtained before (control), during and after (washout) the application of 100 μM ADPβS. Thereafter, 0.1 μM AR-C69931MX were present, and ADPβS (100 μM) was applied again in the continuing presence of this antagonist; the traces shown right were obtained again before, during and after the application of ADPβS. (B) 2-MeSADP (1 μM) and AR-C69931MX (0.1 μM) were applied to another differentiated PC12 cell in the manner described in A. (C) Concentration-dependence of the antagonism between AR-C69931MX (1 nM – 10 μM) and ADPβS (100 μM) or 2-MeSADP (1 μM). Currents were elicited and drugs were applied as described in A; $n = 2-5$.

Increasing concentrations of ADP were also applied in the absence and presence of 10 to 100 μM 2-MeSAMP, and the inhibition of I_{Ca} was determined. As observed for AR-C69931MX, these concentrations of 2-MeSAMP shifted the concentration–response curve for the inhibition of I_{Ca} by ADP to the right (Figure 5A). When these results were subjected to a Schild analysis (Figure 5B), a slope of 0.95 indicated an underlying competitive mechanism, and a pK_B value of 5.4 corroborated an apparent affinity of 2-MeSAMP in the low micromolar range.

Discussion

Amongst the G protein-coupled nucleotide (P2Y) receptors that are known to be expressed in the rat (i.e. $P2Y_{1,\ 2,\ 4,\ 6,}$ and $_{12}$), the $P2Y_{12}$ subtype is peculiar, as it is the only receptor that interacts with inhibitory G proteins in order to

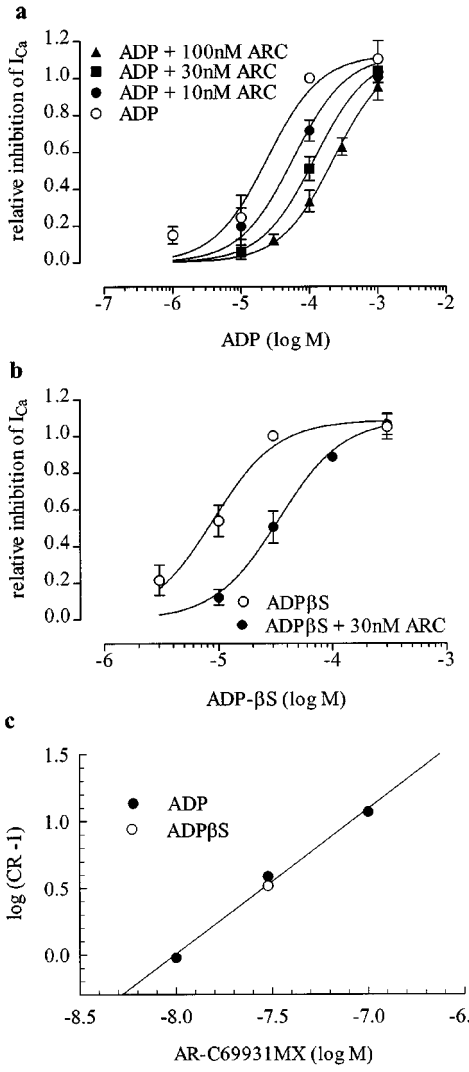

Figure 4 Antagonism between AR-C69931MX and ADP or ADPβS in the inhibition of voltage-activated Ca^{2+} currents. Ca^{2+} currents were determined in, and drugs were applied to, differentiated PC12 cells as described in Figures 2 and 3. (A) Concentration-dependence for the inhibition of I_{Ca} by increasing concentrations of ADP applied either alone or in the continuing presence of 10, 30 or 100 nM AR-C69931MX; $n=4-6$. (B) Concentration-dependence for the inhibition of I_{Ca} by increasing concentrations of ADPβS applied either alone or in the continuing presence of 30 nM AR-C69931MX; $n=4-6$. (C) Schild plot of the data shown in A and B. The slope of the linear regression was 1.09 and the estimated pK_B was 8.7.

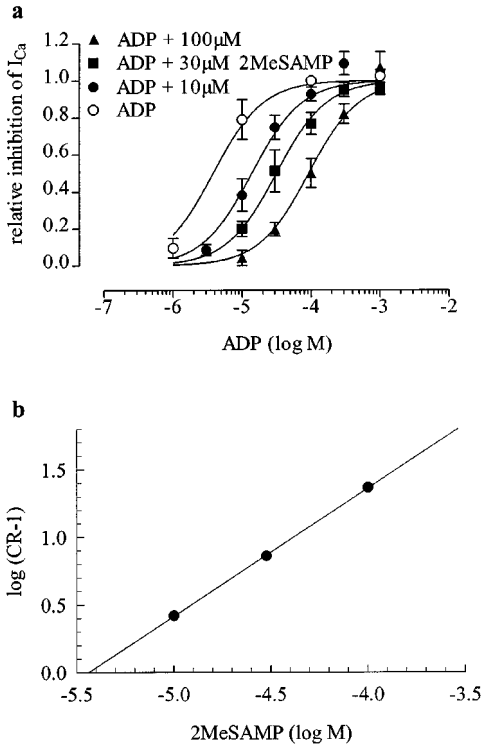

Figure 5 Antagonism between 2-MeSAMP and ADP in the inhibition of voltage-activated Ca^{2+} currents. Ca^{2+} currents were determined in, and drugs were applied to, differentiated PC12 cells as described in Figures 2 and 3. (A) Concentration-dependence for the inhibition of I_{Ca} by increasing concentrations of ADP applied either alone or in the continuing presence of 10, 30 or 100 μM 2-MeSAMP; $n=5-6$. (B) Schild plot of the data shown in A. The slope of the linear regression was 0.95 and the estimated pK_B was 5.4.

inhibit adenylyl cyclase (Ralevic & Burnstock, 1998; Hollopeter *et al*., 2001). The most important action $P2Y_{12}$ receptors mediate *in vivo* is platelet aggregation, and mice deficient in this receptor subtype thus display prolonged bleeding times (Foster *et al*., 2001). However, inhibition of adenylyl cyclase is not the mechanism by which $P2Y_{12}$ receptors mediate their prothrombotic activity, and other signalling mechanisms, such as activation of integrins, PI3 kinase and Rap1, have been suggested to be involved in this effect (Gachet, 2001; Lova *et al*., 2002). By using PC12 cells, an experimental model typically used to study neuronal receptors (Greene & Tischler, 1976), we now present results suggesting that natively expressed $P2Y_{12}$ receptors may also couple to neuronal VACCs.

Adenine nucleotides were found before to inhibit VACCs in PC12 cells, and the rank order of agonist potency was the following: 2-MeSADP = 2MeSATP > ADPβS > ADP = ATP. Uridine nucleotides, in contrast, were ineffective, and the inhibitory action of ADP was antagonized only by reactive blue 2, but not by any other prototypical P2 receptor antagonist, such as A3P5P, PPADS, or suramin (Vartian & Boehm, 2001). This order of agonist potency is similar to those of recombinant $P2Y_{12}$ receptors (Hollopeter *et al*., 2001; Zhang *et al*., 2001; Takasaki *et al*., 2001), but reactive blue 2 blocks many P2 receptor subtypes, including $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_{12}$ (Hansmann *et al*., 1997; Bogdanov *et al*., 1998; Takasaki *et al*., 2001). To establish whether $P2Y_{12}$ receptors might be involved in the regulation of VACCs by adenine nucleotides, we now used 2-MeSAMP and AR-C69931MX, antagonists at recombinant $P2Y_{12}$ receptors (Hollopeter *et al*., 2001; Takasaki *et al*., 2001).

In analogy to platelets (Hollopeter *et al*., 2001; Foster *et al*., 2001) and glioma cells (Jin *et al*., 2001), the ADP-dependent inhibition of adenylyl cyclase activity in PC12 cells was suggested to be mediated by $P2Y_{12}$ receptors (Unterberger *et al*., 2002). In support of this conclusion, both 2-MeSAMP (30 μM) and AR-C69931MX (100 nM) caused a rightward shift of the concentration–response curve for the ADP-dependent reduction of the cyclic AMP accumulation elicited by the A_{2A} receptor agonist CGS 21680. As the maximal inhibition by ADP was not altered, one may infer a

competitive mechanism. The pA_2 values (Arunlakshana & Schild, 1959) calculated on the basis of this assumption were 6.1 for 2-MeSAMP and 8.4 for AR-C69931MX. Thus, these two antagonists block the P2Y receptors mediating the inhibition of adenylyl cyclase in PC12 cells with apparent affinities in the low micromolar and nanomolar range, respectively.

When whole-cell I_{Ca} of PC12 cells was reduced by 100 μM ADP, this effect was attenuated by 2-MeSAMP at 3–300 μM and by AR-C69931MX at 0.01–10 μM. Moreover, 2-MeSAMP required less than 15 s to diffuse from the receptor in order to permit reinhibition of I_{Ca} by ADP, whereas AR-C69931MX required about 2 min to do so. These results indicated that both agents blocked the receptor mediating the ADP-dependent modulation of VACCs and suggested that AR-C69931MX is equipped with a considerably higher affinity. To investigate the interaction between ADP and the two antagonists with respect to the inhibition of I_{Ca} in further detail, increasing concentrations of the agonistic nucleotide were applied in the absence and presence of several concentrations of these antagonists. As with the ADP-induced inhibition of cyclic AMP synthesis, there was no change in the maximal effect of ADP, but the concentration–response curves were shifted to the right. Application of the analysis introduced by Arunlakshana & Schild (1959) indicated underlying competitive mechanisms with pKB values for the two antagonists of 5.4 (2-MeSAMP) and 8.7 (AR-C69931MX), respectively. Thus, the two antagonists interfered with the ADP-dependent inhibition of I_{Ca} in the same range of concentrations as with the P2Y receptor-mediated inhibition of adenylyl cyclase. These results indicate that in PC12 cells, one single P2Y receptor subtype mediates not only an inhibition of adenylyl cyclase, but also of VACCs.

AR-C69931MX displayed an about 1000 fold higher affinity for the receptor mediating the inhibition of I_{Ca} in PC12 cells than 2-MeSAMP. At recombinant $P2Y_{12}$ receptors, AR-C69931MX is also about 1000 fold more potent an antagonist than 2-MeSAMP (Takasaki *et al*., 2001). Moreover, the apparent affinities of AR-C69931MX and 2-MeSAMP for the receptor mediating the modulation of VACCs in PC12 cells (K_B values of 2 nM and 4 μM, respectively) correlate well with their reported potencies in blocking heterologously expressed $P2Y_{12}$ receptors (half maximal inhibition at 2 nM and 5 μM, respectively; Takasaki *et al*., 2001; Hollopeter *et al*., 2001). Taken together, these data suggest that it was $P2Y_{12}$ receptors that mediated the inhibition of both, adenylyl cyclase and VACCs.

The observed rank order of agonist potency (2-MeSADP > ADPβS > ADP) may also be indicative of a role of $P2Y_1$ receptors. In addition, heterologously expressed $P2Y_1$ receptors were shown to mediate an adenine nucleotide-dependent inhibition of VACCs in sympathetic neurons (Filippov *et al*., 2000). Thus, $P2Y_1$ receptors might also be involved in the adenine nucleotide-dependent reduction of I_{Ca} in PC12 cells. However, the inhibition of I_{Ca} by ADPβS and 2-MeSADP was also antagonized by nanomolar concentrations of AR-C69931MX, and the reduction of I_{Ca} by ADP is not attenuated by $P2Y_1$ receptor antagonists (present results and Vartian & Boehm, 2001). Most importantly, PC12 cells were reported not to express $P2Y_1$ receptors (Arslan *et al*., 2000; Unterberger *et al*., 2002). Thus, $P2Y_1$ receptors did not contribute to the inhibition of VACCs by ADP in PC12 cells.

A receptor that displays pharmacological characteristics similar to those of $P2Y_{12}$ is the $P2Y_{13}$ receptor (Communi *et al*., 2001; Zhang *et al*., 2002). Moreover, this receptor subtype is also capable of mediating an inhibition of adenylyl cyclase and might thus be involved in the effects described above. At both, the human and murine variants of the $P2Y_{13}$ receptor, 2-MeSADP, ADPβS, and ADP are more or less equipotent agonists (Communi *et al*., 2001; Zhang *et al*., 2002), whereas at recombinant $P2Y_{12}$ receptors (Takasaki *et al*., 2001) and at the receptor mediating the inhibition of VACCs in PC12 cells (Vartian & Boehm, 2001), 2-MeSADP is about 1000 fold more potent than ADP. This discrepancy may suggest that $P2Y_{13}$ receptors do not contribute to the inhibition of I_{Ca} in PC12 cells. However, neither the molecular identity nor the pharmacological characteristics of rat $P2Y_{13}$ receptors have been described. Furthermore, the affinities of either AR-C69931MX or 2-MeSAMP at recombinant $P2Y_{13}$ receptors have not been reported. Hence, it remains to be established whether $P2Y_{13}$ receptors are expressed in PC12 cells and may couple to neuronal VACCs.

Adenine nucleotides have long been recognized as important elements in haemostasis and thrombosis (Gresele & Agnelli, 2002), and the platelet aggregating activity is mediated by two P2Y receptors, $P2Y_1$ and $P2Y_{12}$. The latter receptor, in particular, is the target of well known antithrombotic drugs, such as ticlopidine and clopidogrel. More recently, 2-MeSAMP and AR-C69931MX were also shown to inhibit platelet aggregation by blocking $P2Y_{12}$ receptors (Jantzen *et al*., 1999; Turner *et al*., 2001), and the latter agent has already been used in phase II clinical trials (Gresele & Agnelli, 2002). One fact that appears to render $P2Y_{12}$ receptors particularly attractive as targets for antithrombotic drugs is their reportedly restricted tissue distribution: apart from the platelets, significant expression has been detected only in the brain, and there it appeared to be confined to glial cells (Hollopeter *et al*., 2001; Zhang *et al*., 2001). However, adenine nucleotides are also transmitters in the nervous system, and they are well known to contribute to sympatho-effector transmission (Boehm and Kubista, 2002). The present results indicate that $P2Y_{12}$ receptors may exert functions in neurons and/or neuroendocrine cells by controlling the gating of VACCs. Since $P2Y_{12}$ receptor expression was also detected in superior cervical ganglia of the rat (E. Moskvina and S. Boehm, unpublished observation), appropriate antagonists can be expected to interfere with the nucleotide signalling in the sympathetic nervous system. Therefore, care should be taken to detect the possibly subtle effects which antithrombotic drugs might exert in the autonomic nervous system.

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References

ARSLAN, G., FILIPEANU, C.M., IRENIUS, E., KULL, B., CLEMENTI, E., ALLGAIER, C., ERLINGE, D. & FREDHOLM, B.B. (2000). P2Y receptors contribute to ATP-induced increases in intracellular calcium in differentiated but not undifferentiated PC12 cells. *Neuropharmacol.,* **39,** 482–496.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol.,* **14,** 48–58.

BOEHM, S. (1999). ATP stimulates sympathetic transmitter release via presynaptic P2X purinoceptors. *J. Neurosci.,* **19,** 737–746.

BOEHM, S., HUCK, S., & FREISSMUTH, M. (1996). Involvement of a phorbol esterinsensitive protein kinase C in the α_2-adrenergic inhibition of voltage-gated Ca^{2+} current in chick sympathetic neurons. *J. Neurosci.,* **16,** 4596–4603.

BOEHM, S. & KUBISTA, H. (2002). Fine tuning of sympathetic transmitter release via ionotropic and metabotropic presynaptic receptors. *Pharmacol. Rev.,* **54,** 43–99.

BOGDANOV, Y.D., WILDMAN, S.S., CLEMENTS, M.P., KING, B.F. & BURNSTOCK, G. (1998). Molecular cloning and characterization of rat P2Y4 nucleotide receptor. *Br. J. Pharmacol.,* **124,** 428–430.

BROWN, D.A., FILIPPOV, A.K. & BARNARD, E.A. (2000). Inhibition of potassium and calcium currents in neurones by molecularly-defined P2Y receptors. *J. Auton. Nervous Syst.,* **81,** 31–36.

CATTERALL, W.A. (2000). Structure and regulation of voltage-gated Ca^{2+} channels. *Annu. Rev. Cell Dev. Biol.,* **16,** 521–555.

CHANG, K., HANAOKA, K., KUMADA, M. & TAKUWA, Y. (1995). Molecular cloning and functional analysis of a novel P2 nucleotide receptor. *J. Biol. Chem.,* **44,** 26152–26158.

COMMUNI, D., GONZALES, N.S., DETHEUX, M., BREZILLON, S., LANNOY, V., PARMENTIER, M. & BOEYNAEMS, J.M. (2001). Identification of a novel human ADP receptor coupled to Gi. *J. Biol. Chem.,* **276,** 41479–41485.

COMMUNI, D., GOVAERTS, C., PARMENTIER, M. & BOEYNAEMS, J.M. (1997). Cloning of a human purinergic receptor coupled to phospholipase C and adenylyl cyclase. *J. Biol. Chem.,* **51,** 31969–31973.

COMMUNI, D., PIROTTON, S., PARMENTIER, M. & BOEYNAEMS, J.M. (1995). Cloning and functional expression of a human uridine nucleotide receptor. *J. Biol. Chem.,* **270,** 30849–30852.

DE LEAN, A., MUNSON, P.J. & RODBARD, D. (1978). Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am. J. Physiol.,* **235,** 97–102.

FILIPPOV, A.K., BROWN, D.A. & BARNARD, E.A. (2000). The $P2Y_1$ receptor closes the N-type Ca^{2+} channel in neurones, with both adenosine triphosphates and diphosphates as potent agonists. *Br. J. Pharmacol.,* **129,** 1063–1066.

FOSTER, C.J., PROSSER, D.M., AGANS, J.M., ZHAI, Y., SMITH, M.D., LACHOWICZ, J.E., ZHANG, F.L., GUSTAFSON, E., MONSMA, JR, F., WIEKOWSKI, M.T., ABBOMDANZO, S.J., COOK, D.N., BAYNE, M.L., LIRA, S.A. & CHINTALA, M.S. (2001). Molecular identification and characterization of the platelet ADP receptor targeted by thienopyridine antithrombotic drugs. *J. Clin. Invest.,* **107,** 1591–1598.

GACHET, C. (2001). ADP receptors of platelets and their inhibition. *Thromb. Haemost.,* **86,** 222–232.

GREENE, L.A. & TISCHLER, A.S. (1976). Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. U.S.A.,* **73,** 2424–2428.

GRESELE, P. & AGNELLI, G. (2002). Novel approaches to the treatment of thrombosis. *Trends Pharmacol. Sci.,* **23,** 25–32.

HANSMANN, G., BÜLTMANN, R., TULUC, F. & STARKE, K. (1997). Characterization by antagonists of P2-receptors mediating endothelium-dependent relaxation in the rat aorta. *Naunyn. Schmiedebergs. Arch. Pharmacol.,* **356,** 641–652.

HILLE, B. (1994). Modulation of ion-channel function by G-protein-coupled receptors. *Trends Neurosci.,* **17,** 531–536.

HOLLOPETER, G., JANTZEN, H.M., VINCENT, D, LI, G., ENGLAND, L., RAMAKRISHNAN, V., YANG, R.B., NURDEN, A., JULIUS, D. & CONLEY, P.B. (2001). Identification of the plateleted ADP receptor targeted by antithrombotic drugs. *Nature,* **409,** 202–207.

JANTZEN, H.M., GOUSSET, L., BHASKAR, V., VINCENT, D., TAI, A., REYNOLDS, E.E. & CONLEY, P.B. (1999). Evidence for two distinct G-protein-coupled ADP receptors mediating platelet activation. *Thromb. Haemost.,* **81,** 111–117.

JIN, J., TOMLINSON, W., KIRK, I.P., KIM, Y.B., HUMPHRIES, R.G. & KUNAPULI, S.P. (2001). The C6-2B glioma cell $P2Y_{AC}$ receptor is pharmacologically molecularly identical to the platelet $P2Y_{12}$ receptor. *Br. J. Pharmacol.,* **133,** 521–528.

JOHNSON, R.A., ALVAREZ, R. & SALOMON, Y. (1994). Determination of adenylyl cyclase catalytic activity using single and double column procedures. *Methods Enzymol.,* **238,** 32–56.

LIU, H., FELIX, R., GURNETT, C.A., DE WAARD, M., WITCHER, D.R. & CAMPBELL, K.P. (1996). Expression and subunit interaction of voltage-dependent Ca^{2+} channels in PC12 cells. *J. Neurosci.,* **16,** 7557–7565.

LOVA, P., PAGANINI, S., SINIGAGLIA, F., BALDUINI, C. & TORTI, M. (2002). A Gi-dependent pathway is required for activation of the small GTPase Rap1B in human platelets. *J. Biol. Chem.,* **277,** 12009–12015.

LUSTIG, K.D., SHIAU, A.K., BRAKE, A.J. & JULIUS, D. (1993). Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc. Natl. Acad. Sci. U.S.A.,* **90,** 5113–5117.

RALEVIC, V. & BURNSTOCK, G. (1998). Receptors for purines and pyrimidines. *Pharmacol. Rev.,* **50,** 413–492.

NANDANAN, E., CAMAIONI, E., JANG, S.Y., KIM, Y.C., CRISTALLI, G., HERDEWIJN, P., SECRIST, J.A., TIWARI, K.N., MOHANRAM, A., HARDEN, T.K., BOYER, J.L. & JACOBSON, K.A. (1999). Structure-activity relationships of biphosphate nucleotide derivatives as $P2Y_1$ receptor antagonists and partial agonists. *J. Med. Chem.,* **42,** 1625–1638.

SIMON, J., VIGNE, P., EKLUND, K.M., MICHEL, A.D., CARUTHERS, A.M., HUMPHREY, P.P.A., FRELIN, C. & BARNARD, E.A. (2001). Activity of adenosine diphosphates and triphosphates on a $P2Y_T$-type receptor in brain capillary endothelial cells. *Br. J. Pharmacol.,* **132,** 173–182.

TAKASAKI, J., KAMOHARA, M., SAITO, T., MATSUMOTO, M., MATSUMOTO, S.I., OHISHI, T., SOGA, T., MATSUSHIME, H. & FURUCHI, K. (2001). Molecular cloning of the platelet $P2T_{AC}$ receptor: pharmacological comparison with another ADP receptor, the P2Y1 receptor. *Mol. Pharmacol.,* **60,** 432–439.

TOKUYAMA, Y., HARA, M., JONES, E.M.C., FAN, Z. & BELL, G.I. (1995). Cloning of rat and mouse P2Y purinoceptors. *Biochem. Biophys. Res. Comm.,* **211,** 211–218.

TURNER, N.A., MOAKE, J.L. & MCINTIRE, L.V. (2001). Blockade of adenosine diphosphate receptors P2Y(12) and P2Y(1) is required to inhibit platelet aggregation in whole blood under flow. *Blood,* **98,** 3340–3345.

UNTERBERGER, U., MOSKVINA, E., SCHOLZE, T., FREISSMUTH, M. & BOEHM, S. (2002). Inhibition of adenylyl cyclase by neuronal P2Y receptors. *Br. J. Pharmacol.,* **135,** 673–684.

VARTIAN, N. & BOEHM, S. (2001). P2Y Receptor-mediated inhibition of voltage-activated Ca^{2+} currents in PC12 cells. *Eur. J. Neurosci.,* **13,** 899–908.

ZAMPONI, G.W. & SNUTCH, T.P. (1998). Modulation of voltage-dependent calcium channels by G proteins. *Cur. Op. Neurobiol.,* **8,** 351–356.

ZHANG, F.L., LUO, L., GUSTAFSON, E., LACHOWICZ, J., SMITH, M., QIAO, X., LIU, Y.H., CHEN, G., PRAMANIK, B., LAZ, T.M., PALMER, K., BAYNE, M. & MONSMA, JR, F.J. (2001). ADP is the cognate ligand for the orphan G protein-coupled receptor SP1999. *J. Biol. Chem.,* **276,** 8608–8615.

ZHANG, F.L., LUO, L., GUSTAFSON, E., PALMER, K., QIAO, X., FAN, X., YANG, S., LAZ, T.M., BAYNE, M. & MONSMA, JR, F.J. (2002). P2Y13: identification and characterization of a novel Gαi-coupled ADP receptor from human and mouse. *J. Pharmacol. Exp. Ther.,* **301,** 705–713.

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