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A New Class of Compounds, Peptide Derivatives of Adenosine 5'-Carboxylic Acid, Includes Inhibitors of ATP Receptor-Mediated Responses

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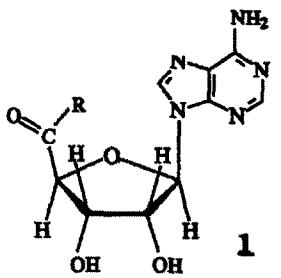
Abstract—A new type of ligand for the study of P_2-purinergic receptor subtypes was synthesized by combining and modifying conventional nucleoside chemistry with Fmoc solid phase peptide synthesis techniques. The tri- and tetra-aspartic acid derivatives of adenosine-5'-carboxylic acid (AdoCAsp$_3$ and AdoCAsp$_4$) were found to act as weak agonists at P_2-purinergic receptors, (activated by ATP and UTP respectively) present on C6 glioma cells. AdoCAsp$_4$ induced inositol 1,4,5-trisphosphate formation in the C6 cells with an EC_{50} of 73 μM. In addition, AdoCAsp$_4$ was found to inhibit ($IC_{50} \approx 80$ μM) ATP-induced cytosolic [Ca^{2+}] transients in these glioma cells. The glycine derivative, AdoCGly, increased evoked release of noradrenaline from mouse vas deferens slices, probably due to the blockade of presynaptic P_2-autoreceptors. The possibility that aspartic, glutamic or γ-carboxyglutamic residues may be used to replace phosphate groups on an ATP receptor ligand, opens up new ways in ligand design.

Introduction

This paper describes the synthesis and biological activity of conjugates of peptides and adenosine 5'-carboxylic acid (**1**), a new class of ligands (Figure 1) for P_2-purinergic receptors (P_2Rs). P_2Rs are present in the plasma membranes of many different cells.[1] Dependent on the P_2R subtype, they are activated by preferentially endogenous ATP, UTP or by ADP.[1] P_2R subtypes are currently classified mainly on a pharmacological basis.[2,3] Recently, two G-protein-coupled ATP receptor subtypes, P_{2y1}R and P_{2u}R, have been characterized by molecular cloning.[4,5] ATP acts as a fast transmitter at synapses between neurons in the coeliac ganglion[6] and in the central nervous system (CNS),[7] by opening ion channels of an ionotropic P_2R subtype. More often, ATP seems to act as a modulator, in the CNS and the peripheral nervous system (PNS) as well as on various cells of different origin (for recent reviews, see References 1–3). Synthesis of P_2R ligands has aimed at subtype-specific high affinity ligands and has provided many agonists, though very few with the desired properties (see, however, Fischer *et al.*[8]). Compounds resistant to enzyme-induced degradation by ectonucleotidases and -phosphatases have been obtained.[2,8,9] In contrast, the search for selective antagonists has so far been futile, a fact that severely limits investigation on the physiological role of the P_2-purinergic receptor subtypes. This lack of subtype-specific P_2-receptor blockers prompted us to study the possibilities of an unconventional approach to the synthesis of P_2R ligands. Applying Schwyzer's theory for peptide receptor ligands, stating that a 'message' and an 'address' part is necessary for high affinity ligand action[10] to ATP, we assumed that the message for receptor activation resides in the adenine–ribose moiety, while receptor affinity is affected by alterations in the triphosphate chain. If the introduced ligand substituent groups interact with, or sterically hinder, functional groups



1

R(1)	R^1 = -Asp
R(2)	R^2 = -Asp-Asp
R(3), AdoCAsp$_3$	R^3 = -Asp-Asp-Asp
R(4), AdoCAsp$_4$	R^4 = -Asp-Asp-Asp-Asp
R(5)	R^5 = -Gly-Asp-Asp-Asp
R(6)	R^6 = -Lys-Asp-Asp
R(7)	R^7 = -Lys
R(8)	R^8 = -Lys-Lys-Lys
R(9)	R^9 = -Gly-Lys-Lys
R(10), AdoCGly	R^{10} = -Gly
R(11)	R^{11} = -Gly-Gly-Gly

Figure 1. Synthesized conjugates of peptides and adenosine 5'-carboxylic acid (**1**). Numbering and abbreviation of compounds and structures of their peptide parts are shown.

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in the receptor that are necessary for the conformational change associated with its activation the ligand would act as an antagonist. Following this reasoning for the synthesis of **1**, a novel class of ATP analogues was designed. In these analogues, the phosphate groups of ATP were replaced by peptides diversified with respect to their molecular size, hydrophobicity and charge, in order to determine structure–activity relationships.

Results

Synthesis and analysis

New ATP analogues (**1**) were prepared according to the reaction scheme outlined in Figure 2 and described in detail in the Experimental Section. The peptide chain was assembled and adenosine 5'-carboxylic acid (**2**) attached to it on a Wang-type polystyrene resin by using conventional Fmoc solid phase peptide synthesis (SPPS). High performance liquid chromatography (HPLC) methods used for purification of the products yielded compounds with > 98 % purity.

The structures of the synthesized compounds were verified by the following analytical methods. Analytical data for the conjugate R(4) showed that the determined (time of flight mass spectrometry) molecular weight corresponded to the calculated weight (741). It has an absorbance maximum in UV spectrum at 258 nm with $\varepsilon = 15000\ M^{-1}cm^{-1}$ (characteristic for adenosine derivatives), and it carries a high negative charge at pH = 7.0 [R(4) has a longer retention time in an anion exchange column than ATP and the conjugates **1** with a smaller number of aspartic residues]. ^1H and ^{13}C NMR spectra confirmed the structure of R(4). Four doublets of proton signals (δ 7.95, 8.08, 8.34 and 8.95) originating from NH protons of four amide bonds and the broad two-proton singlet at δ 8.11 (free exocyclic NH_2 group at C6 position of adenine base) indicated the right order of the peptide chain and showed that no acylation of the C6 amino group has occurred. Two singlets at δ 8.33 and 8.50 confirmed that the adenine base of the conjugate had remained unchanged during coupling and deprotection steps. ^{13}C NMR spectrum revealed the presence of signals for all carbons in the adenine, ribose and peptide parts of the molecule. The low field region signals with δ 169 – 180 correspond to nine carboxyl carbons of the conjugate.

Biological testing of compounds

Effect on ATP-induced cytosolic [Ca^{2+}] peak levels. The new peptide derivatives of adenosine-5'-carboxylic acid were tested for presumptive agonist/antagonist properties at C6 glioma P_{2y}- and P_{2u}-purinoceptors. We have earlier shown the presence of both receptor subtypes on these cells by differential desensitization using 2-methylthio-ATP (2-MeS-ATP) and UTP.[11] Nucleotide-induced cytosolic [Ca^{2+}] transients consist of two components: an initial mobilization of Ca^{2+} from internal stores (shown in the absence of external Ca^{2+}), followed by a sustained, slowly decreasing second phase resulting from influx of extracellular Ca^{2+}, and a subsequent return to basal levels (approximately 100 nM). The tri- and tetra aspartic acid derivatives of adenosine 5'-carboxylic acid, $AdoCAsp_3$ [R(3)] and $AdoCAsp_4$ [R(4); Figure 3 shows a typical recording], were found to act as weak agonists of C6 glioma P_2Rs (the latter was more potent). Figure 4a shows that $AdoCAsp_4$ (100 μM and 200 μM) significantly reduced (by 45 % and 73 %, respectively) ATP-induced (20 μM) cytosolic [Ca^{2+}] increases. Single measurements also showed that $AdoCAsp_4$ (50–200 μM) attenuated ATP-induced responses in a concentration dependent manner, with an IC_{50} of approximately 80 μM. Additionally, $AdoCAsp_4$ (100 μM) was found to diminish UTP-induced Ca^{2+} increase by ≈ 55 % (Figure 4b). UTP was by itself a much less potent agonist than ATP (see Figures 4a and 4b).

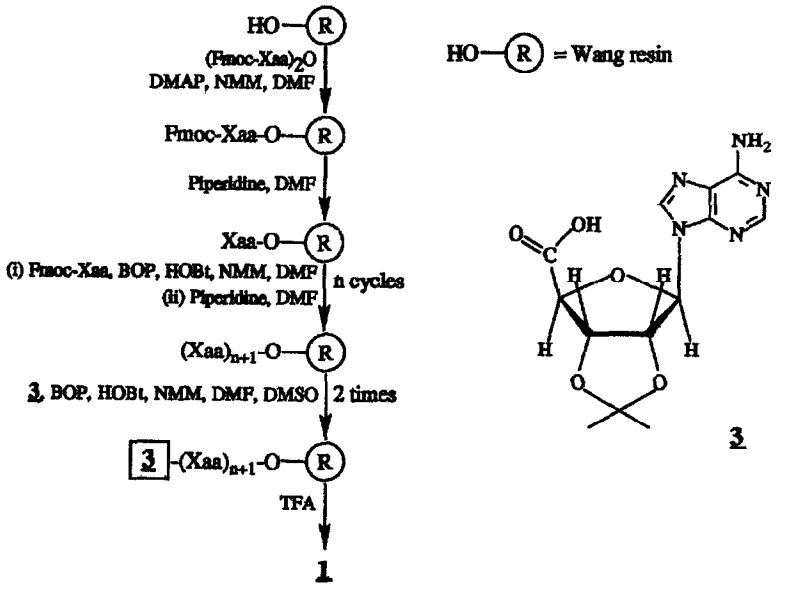


Figure 2. Synthetic procedures used for preparation of conjugates (**1**). 3 = 2',3'-Isopropylideneadenosine 5'-carboxylic acid.

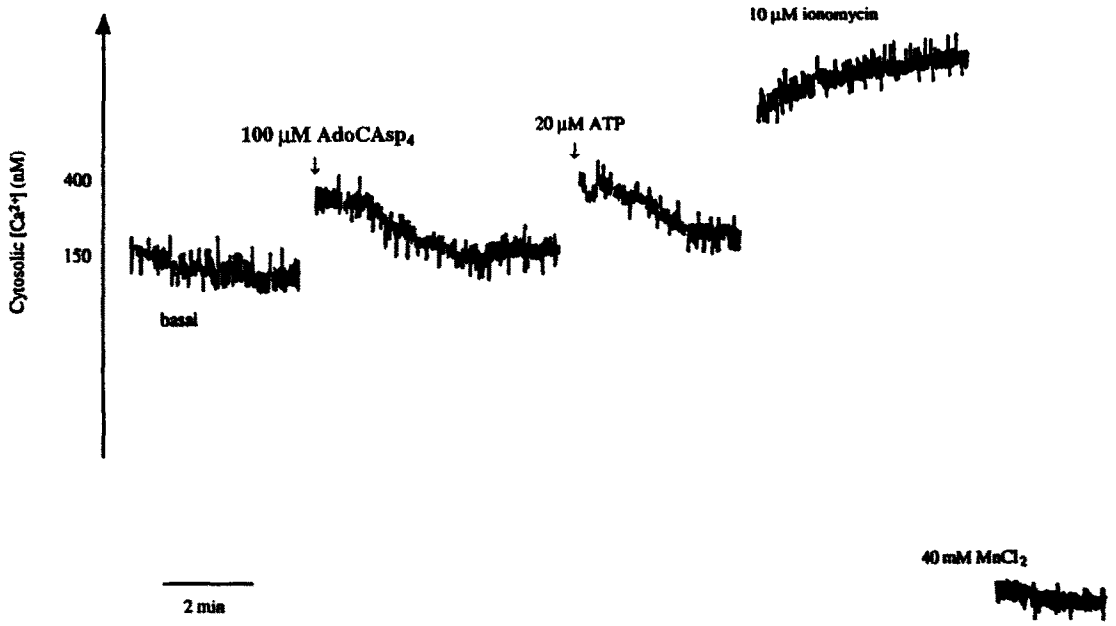


Figure 3. Addition of 100 μM $AdoCAsp_4$ [R(4)] increased cytosolic [Ca^{2+}] in C6 glioma cells and attenuated the subsequent ATP-induced peak.

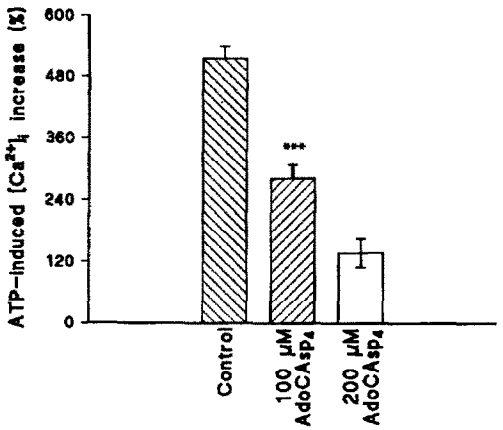


Figure 4a. Percent ATP-induced $[Ca^{2+}]_i$ increase over basal levels. Following a 5 min preincubation of the C6 cells with $AdoCAsp_4$ [R(4); 100 or 200 μM] 20 μM ATP-induced cytosolic [Ca^{2+}] peak levels were significantly reduced. Data are shown as mean ± SEM (hatched bars) and range (open bar). For the control bar n = 3, for 100 μM $AdoCAsp_4$ (n = 7), 200 μM $AdoCAsp_4$ (n = 2).

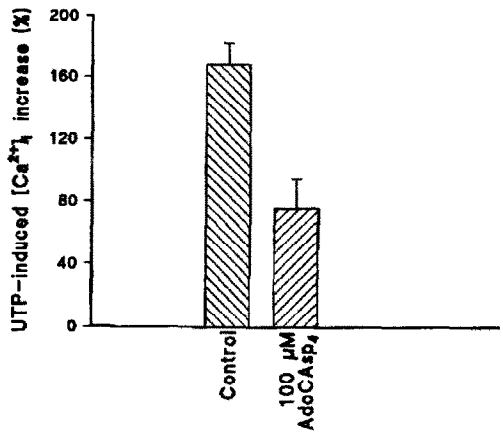


Figure 4b. Percent UTP-induced $[Ca^{2+}]_i$ increase over basal levels. Significant reduction of 50 μM UTP-induced $[Ca^{2+}]_i$ peak levels was observed after a 5 min preincubation with 100 μM $AdoCAsp_4$ [R(4); n = 3].

Agonist-induced inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] formation. The P_2R subtypes of C6 glioma cells, $P_{2y}R$ and $P_{2u}R$, appear to belong to the family of G protein-coupled receptors, with seven putative transmembrane segments.[4,5] Their agonists activate several signal transduction pathways: formation of Ins(1,4,5)P_3 via $G_{i/o}$ and G_q coupled inositol phospholipid-specific phospholipase C as well as mobilization of intracellular Ca^{2+} and release of eicosanoids.[11,12] Concentration–effect relationships were established for ATP, 2-MeS-ATP, UTP, $AdoCAsp_3$ and $AdoCAsp_4$ (Figure 5; representative of 2–3 separate experiments) and EC_{50} values were calculated to be 56 μM for ATP, 38 μM for 2-MeS-ATP, 53 μM for UTP and 73 μM for $AdoCAsp_4$. The EC_{50} for ATP-induced Ins(1,4,5)P_3 formation was in the same range as for the ATP-induced cytosolic [Ca^{2+}] increase.[11] Maximal response to ATP produced a 6-fold increase of Ins(1,4,5)P_3 over resting levels, derivatives were less active and the agonist potency order was ATP > 2-MeS-ATP > $AdoCAsp_4$ > UTP. Full concentration–effect curves were not obtained for $AdoCAsp_3$; even with up to 1 mM of the compound a plateau phase was not reached.

Metabolic stability. Presence of the substrate and metabolites was determined after a 3 h incubation with C6 cells at 37 °C by HPLC analysis. Degradation of the new conjugates was < 5 % during the experiments. The conjugates were comparable in stability to some of the previously synthesized 2-alkylthio derivatives of ATP.[9]

Effect on presynaptic P_2R. Endogenous ATP[13,14] and ATP analogues such as 2-methylthio-ATP[15] activate a presynaptic P_2R which inhibits neurotransmitter release from noradrenergic nerve terminals in mouse vas deferens. 2-Cyclohexylthio-ATP[9] inhibited the evoked release of noradrenaline (Figure 6a), indicating an agonist activity of this compound at the presynaptic P_2R. In contrast, the peptide derivative AdoCGly caused a significant (22 %)

increase in noradrenaline release (Figure 6b) suggesting that this derivative has antagonist activity, similar to that of suramin.[13,14] The presynaptic P_2R on mouse vas deferens is assumed to be a P_{2y} subtype.[14,15]

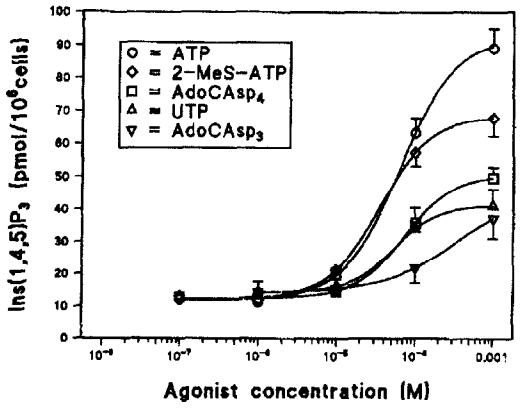
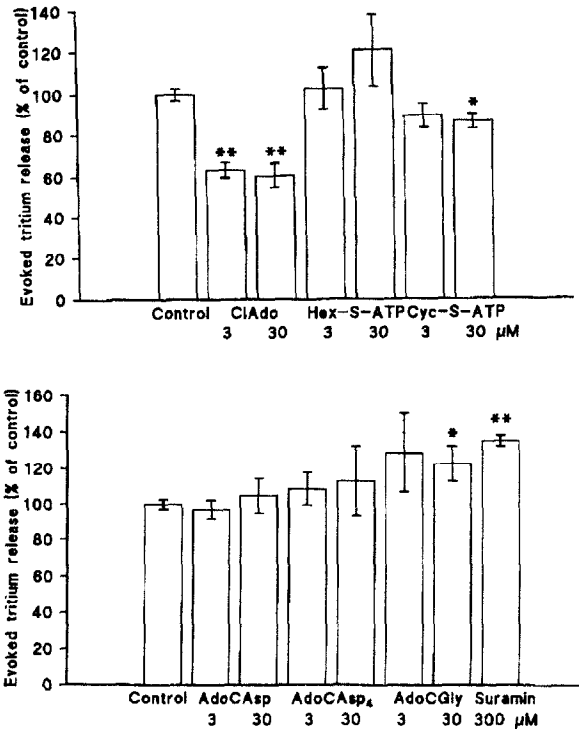


Figure 5. Concentration–dependence of agonist-induced Ins(1,4,5)P_3 formation. Data are expressed as mean ± SD from one representative experiment (n = 3).



Figures 6a and b. Mouse vas deferens slices preincubated with [^{3}H]noradrenaline: effect on evoked overflow (60 pulses, 1 Hz; n = 4–10). Asterisks indicate significant difference from control measurements. Hex-S-ATP = 2-hexenylthio-ATP and Cyc-S-ATP = 2-cyclohexylthio-ATP.

Further functional evaluation. The new peptide derivatives of adenosine 5'-carboxylic acid were also tested for activity in several other relevant biological systems. None of the derivatives showed agonist or antagonist activity on adenosine A_{2a}-receptor-mediated cyclic AMP–formation in PC12 phaeochromocytoma cells. The A_2-agonist 5'-*N*-ethylcarboxamidoadenosine (NECA) was used as a reference. No protein kinase inhibition by the peptide derivatives (protein kinase A, protein kinase C and casein kinase II) in the presence of substrate peptide and ATPγ[^{32}P] was observed. Finally, the peptide derivatives were ineffective also as inhibitors of adenylate kinase, an enzyme possessing two nucleotide binding sites.[16]

Discussion

The need for subtype-specific P_2R antagonists prompted us to try a new approach. We chose to design compounds where the phosphate groups of ATP had been substituted for various amino acid residues or short peptides, such as anionic aspartate residues. The rationale behind this approach was that it can be assumed that the phosphate groups of ATP interact with cationic amino acid residues, i. e. arginine, lysine or histidine, in their receptors. Phosphate and carboxylate groups have been shown to interact in a similar way with the guanidinium group of arginine.[17] The introduced amino acid residues, differing in charge, hydrophobicity, volume and hydrogen bonding ability, might interact differentially with functional groups of the receptors thereby changing the affinity, specificity or agonist/antagonist properties of the ligand. SPPS methods may be used together with nucleoside synthesis methods to generate numbers of ATP analogues as single new and pure compounds or as 'chemical libraries' that can be tested for pharmacological activity.

Among the synthesized analogues, $AdoCAsp_4$ [R(4)] was found to inhibit ATP-induced increases in cytosolic Ca^{2+} in C6 glioma cells with an IC_{50} value of approximately 80 μM. This should be compared to existing antagonists to P_2-purinergic receptors, such as the non-selective P_{2x}/P_{2y}-antagonist suramin (100 μM–1 mM) which blocks responses to P_2R agonists in guinea-pig urinary bladder and taenia coli smooth muscle[18], and Reactive Blue 2, an anthraquinone sulphonic acid derivative, which appears to inhibit rabbit mesenteric artery P_{2y}-receptors selectively, within a limited concentration interval in the micromolar range.[19] The structure of $AdoCAsp_4$ shows similarities with ATP in overall charge, but the spatial arrangement of the charges is likely to be very different. The inhibitory action of $AdoCAsp_4$, which has an IC_{50} value of 80 μM, implies that the aspartic acid carboxylic anions contribute to the increased affinity in the interaction compared to, for example, AMP and adenosine, which are inactive in the C6 cells. Ion pairing of receptor guanidinium (or ammonium) groups with the five carboxylate groups of the four aspartate residues of $AdoCAsp_4$ and is probably different from that of the ATP triphosphate moiety.

Cloned P_2Rs contain, in their putative extracellular loops and surface of the predicted seven transmembrane helices, thirteen ($P_{2y1}R$) to seventeen ($P_{2u}R$) positively charged amino acid residues (of which six and nine residues, respectively, are Arg, and the rest Lys or His), which may contribute to ion pairing of the negatively charged ligand. It is obvious that the main structural difference between adenosine and ATP resides in the phosphate moiety. Comparison of the surface charge of adenosine receptors

($AdoA_1R$, $AdoA_{2a}R$, $AdoA_{2b}R$) with those in P_2Rs reveals that there are fewer positively charged residues (only a third as many), and that there is only one Arg residue present (in the A_1R). Negative surface charges (aspartic or glutamic acid) are few in the P_2Rs (7–11), which still leaves a net positive charge in the extracellular domain, available for interaction with a negatively charged ligand, whereas the adenosine receptors have a net negative charge at the receptor extracellular surface (except the A_1R which has one positive net charge). The excess of cationic putative ligand binding sites of the P_2R extracellular surface provides a possible structural basis for the agonist/antagonist activity of $AdoCAsp_4$ compared to ATP which probably interacts with different cationic residues.

The two cloned P_2R subtypes have significantly different primary sequences in their putative extracellular loops, suggesting that a structural basis for receptor subtype specificity should exist. Provided peptide nucleoside derivatives can be designed, that show a specific pattern of interaction with the amino acids in these loops, receptor subtype-specific experimental drugs could be developed.

$AdoCAsp_4$ represents a new class of ATP receptor ligands. As there is only very limited structural information about some of the receptors, there are no rational methods for prediction of ligand structure and optimal design. Further development of receptor subtype-specific high affinity ligands may therefore have to rely on the generation of relatively large numbers of new ligands or libraries of ligands and screening of these for receptor interaction. Peptide derivatives of adenosine, like $AdoCAsp_4$, have an advantage over modified nucleotides synthesized using conventional nucleotide chemistry in that the rapid and convenient methods of SPPS can be employed in the synthesis of potential ligands.

Taken together, our results corroborate the pharmacological specificity of the peptide derivatives for P_2-purinergic receptors.

Experimental Section

Synthesis and analysis

p-Benzyloxybenzyl alcohol resin (Wang resin), (benzotriazoloxy)-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP), hydroxybenzotriazole (HOBt), Fmoc-L-Asp(Bu^t), Fmoc-L-Lys(Boc), and Fmoc-Gly were purchased from Novabiochem.

UV spectra were recorded on a Shimadzu UV 240 spectrometer. Molecular weights were determined with Applied Biosystems Bio-Ion 20 plasma desorption time of flight mass spectrometer. ^1H and proton decoupled ^{13}C NMR spectra were recorded in D_2O (unless noted otherwise) on a Bruker AM 200 spectrometer at 200 and 50 MHz, respectively. Chemical shifts (δ, in ppm) were measured relative to the internal reference (acetone) and converted to the tetramethylsilane scale by using acetone δ values 2.15 and 31.5 for ^1H and ^{13}C NMR shifts, respectively. Assignments of ^{13}C chemical shifts were made according to Breitmeier and Voelter.[20] HPLC equipment used consisted of LKB 2249 gradient pump, LKB 2141 variable wavelength monitor and LKB 2221 integrator. Reverse phase HPLC was performed with analytical Alltech Nucleoside 7U C_{18} (250 × 4.6 mm, flow rate 1 mL/min) or preparative Lenchrom C_{16} (250 × 16 mm, 6 mL/min) columns with linear gradient of 0–12 % acetonitrile in water (0.1 % trifluoroacetic acid, TFA) over 15 min. Mono Q HR 5/5 anion exchange column (Pharmacia) was used for analytical (linear sodium chloride gradient 0–0.4 M in 10 mM sodium phosphate buffer, pH = 7.0, 12 min, solvent system A) as well as for small-scale preparative (ammonium formate gradient 0.4–1.0 M in 10 min, system B) separations.

A typical procedure, synthesis of R(4). For the description of all principal synthetic steps we detail the synthesis of one derivative, R(4) [see Figure 2 for an outline of the synthetic scheme and the structures of compounds R(1)–R(11)].

For anchoring of the first amino acid to the resin,[21] 100 mg of polystyrene-based *p*-benzyloxybenzyl alcohol resin (Wang resin, content of hydroxyl groups 0.90 mmol/g) was packed into a Bio-Rad 2 mL Poly-Prep chromatography column which luer tip could be attached to either nitrogen pressure or a water-jet suction line. Preformed symmetric anhydride was prepared of Fmoc-Asp(Bu^t) (360 μmol) and dicyclohexylcarbodiimide (180 μmol) in 2 mL dichloromethane (DCM) and the obtained solution filtered directly into the column with the resin. *N*-methylmorpholine (NMM, 600 μmol) and *p*-dimethylaminopyridine (40 μmol) were added to the reaction mixture. After 5 h the resin was washed with DCM and free hydroxyl groups capped with acetic anhydride. The substitution level of the esterified resin was determined in the course of the quantitative removal of N-terminal Fmoc-groups by sequential treatment (5 + 15 min) with 1.5 mL of 25 % piperidine in DMF and was found to be 0.60 mmol/g.

Additional residues were coupled using standard SPPS technique with Fmoc/Bu^t protection strategy and BOP and HOBt activation.[21] A twofold excess of Fmoc-Asp(Bu^t) (120 μmol) was dissolved in a solution of NMM (400 μmol) in DMF (1.4 mL) and added to a mixture of 115 μmol of BOP and HOBt. The solution was allowed to preactivate for 4 min at room temperature and added to the resin. The reaction mixture was agitated (20 min) by allowing a weak stream of nitrogen to pass through the filter at the bottom of the column. At this point the qualitative ninhydrin (Kaiser) test showed complete coupling. The N-terminal Fmoc protection was removed with 25 % piperidine in DMF. The coupling cycle was repeated twice for the attachment of the third and fourth aspartic acid.

Coupling of 2',3'-isopropylideneadenosine 5'-carboxylic acid[22,23] (**3**) to the peptide chain was done as described above with the following modifications due to the low solubility of **3** in DMF, different reactivity of its carboxylic acid group (if compared to ordinary amino acids) and the possible acylation of the unprotected exocyclic 6-NH_2

group of adenine base. Thus, **3** (90 μmol) was dissolved in a solution of NMM (400 μmol) in DMF:DMSO (2:1, 1.4 mL), followed by addition of 85 μmol of BOP and HOBt. Alternatively, as **3** does not carry Fmoc protection, it is possible to use at this step a stronger and more hydrophobic base diisopropyl ethyl amine, which renders **3** better solubility in DMF. After 1 min of activation the solution was added to the resin. The coupling reaction was allowed to proceed for 15 min. Twofold coupling was necessary, as checked with the ninhydrin test after the first and second reaction. Side-chain protecting groups (including isopropylidene protection of 2'- and 3'-hydroxyl groups of ribose part of the compound) were removed and the conjugate cleaved from the resin by treatment with 95 % TFA (2 mL) at room temperature for 2 h. The product was analysed with HPLC to work out purification conditions. The crude conjugate R(4) contained 5 % of adenosine-5'-carboxylic acid (**2**) and some other small impurities. It was further purified on reverse phase or anion exchange columns, whereas the latter variant of HPLC is especially suitable for the purification of this highly charged hydrophilic compound. The relevant fractions were collected and lyophilized to yield a white solid. Analytical HPLC with Nucleoside 7U C_{18} column showed > 98 % purity of the product.

Analytical data for R(4). UV λ_{max} (H_2O, pH = 7.0) = 258 nm; MS (m/z) = 742 (M + H) and 764 (M + Na); HPLC retention times were 12.8 min (7U C_{18} column) and 9.7 min [Mono Q anion exchange column, solvent A; compared to the retention times 2.0, 4.6, 7.5 and 6.8 min for R(1), R(2), R(3) and ATP, respectively]; NMR (in DMSO-d_6, δ relative to tetramethylsilane internal standard): ^{1}H NMR, δ, 2.4–2.9 (m, 8H, 4 × H-β), 4.1–4.2 (m, 1H, H-3'), 4.41 (s, 1H, H-4'), 4.4–4.6 (m, 3H, 3 × H-α), 4.7–4.9 (m, 1H, H-2'), 6.00 (d, J = 7.6 Hz, 1H, H-1'), 7.95 (d, J = 7.7 Hz, 1H, CONH), 8.08 (d, J = 7.6 Hz, 1H, CONH), 8.11 (br s, 2H, 6-NH_2), 8.34 (d, J = 6 Hz, 1H, CONH), 8.33 and 8.50 (2 × s, 2H, H-2 and H-8), 8.95 (d, J = 8.6 Hz, 1H, CONH). ^{13}C NMR, δ, 35.8 (βC, 3C), 36.3 (βC), 48.5 (αC), 49.0 (αC), 49.3 (αC), 49.5 (αC), 72.3 and 73.1 (C3' and C2'), 84.5 (C4'), 87.5 (C1'), 119.2 (C5), 141.2 (C8), 148.7 (C2), 150.1 (C4), 154.1 (C6), 169.3, 170.0 (2C), 170.1, 171.5 (2C), 171.6, 171.7 and 171.9 (9 × C=O).

Analytical data for other conjugates. All conjugates were synthesized according to the above described procedures. They showed analytical data that were consistent with their structures. Determined molecular weights of all the compounds were in accordance with the calculated weights and they had UV absorbance maximum at 258 nm. NMR spectral data for conjugates confirmed their structure:

R(3). ^{1}H NMR, δ, 2.6–2.9 (m, 6H, 3 × H-β) 4.4–4.5 (m, 1H, H3'), 4.53 (d, J = 2.8 Hz, 1H, H-4'), (H-2' and H-α hidden by water peak), 6.06 (d, J = 6.3 Hz, 1H, H-1'); 8.32 and 8.38 (2 × s, 2H, H-2 and H-8). ^{13}C NMR, δ, 36.4 (βC), 36.7 (βC), 36.8 (βC), 50.5 (αC), 50.9 (αC), 51.4 (αC), 73.3 and 73.4 (C2' and C3'), 84.2 and 89.5 (C4' and C1'), 120.6 (C5), 145.0 and 146.2 (C8 and C2), 149.5 and 151.5 (C4 and C6), 172.4, 172.7, 172.8, 174.0, 175.0, 175.1 and 175.4 (C=O, 7C).

R(7). ^{1}H NMR, δ, 1.2–1.4 (m, 2H, H-γ), 1.4–1.9 (m, 4H, H-β and H-γ), 2.81 (t, J = 7 Hz, 2H, H-ε), 4.29 (dd, J = 8 Hz and 5 Hz, 1H, H-α), 4.5–4.9 (3 × m, 3H, H-2', H-3' and H-4' overlapped by water peaks), 6.09 (d, J = 6 Hz, 1H, H-1'), 8.27 and 8.46 (2 × s, 2H, H-2 and H-8). ^{13}C NMR, δ, 23.3 (γC), 27.5 (βC), 31.5 (δC), 40.5 (εC), 54.0 (αC), 74.3 and 74.7 (C2' and C3'), 84.8 and 90.0 (C4' and C1'), 120.3 (C5), 144.3 and 146.7 (C8 and C2), 149.6 and 151.9 (C4 and C6), 172.4 and 176.6 (C=O, 2C).

R(8). ^{1}H NMR, δ, 1.2–1.5 (m, 6H, H-γ); 1.5–1.9 (m, 12H, H-β, H-δ), 2.8–3.0 (m, 6H, H-ε), 4.2–4.4 (m, 3H, H-α), 4.5–4.9 (3 × m, 3H, H-2', H-3' and H-4', overlapped by water peak), 6.21 (d, J = 5.5 Hz, 1H, H-1'), 8.37 and 8.53 (2 × s, 2H, H-2 and H-8). ^{13}C NMR, δ, 23.2 (γC), 23.3 (γC, 2C), 27.5 (βC, 3C), 31.2 (δC), 31.7 (δC), 31.9 (δC), 40.4 (εC, 3C), 54.2 (αC), 54.6 (αC), 54.8 (αC), 74.2 and 74.7 (C2' and C3'), 84.6 and 90.1 (C4' and C1'), 120.5 (C5), 144.3 and 146.7 (C8 and C2), 149.6 and 151.9 (C4 and C6), 172.5, 174.6, 174.8 and 176.9 (C=O, 4C).

R(9). ^{1}H NMR, δ, 1.2–1.5 (m, 4H, H-γ), 1.5–1.9 (m, 8H, H-β, H-δ), 2.8–3.0 (m, 4H, H-ε), 3.93 (d, J = 17 Hz, 1H, H-α,a of Gly), 4.00 (d, J = 17 Hz, 1H, H-α,b of Gly), 4.2–4.4 (m, 2H, H-α of Lys), 4.5–4.8 (3 × m, 3H, H-2', H-3' and H-4', overlapped by water peak), 6.09 (d, J = 6 Hz, 1H, H-1'), 8.38 and 8.54 (2 × s, 2H, H-2 and H-8). ^{13}C NMR, δ, 23.0 (γC), 23.2 (γC), 27.3 (βC), 27.4 (βC), 31.1 (δC), 31.1 (δC), 31.7 (δC), 40.3 (εC, 2C), 43.2 (αC, Gly), 53.8 (αC, Lys), 54.6 (αC, Lys), 74.0 and 74.3 (C2' and C3'), 84.7 and 89.8 (C4' and C1'). 120.4 (C5), 144.6 and 146.4 (C8 and C2), 149.5 and 151.7 (C4 and C6), 171.9, 173.0, 174.9 and 176.8 (C=O, 4C).

R(10). ^{1}H NMR, δ, 3.99 (d, J = 18 Hz, 1H, Hα, a), 4.06 (d, J = 18 Hz, 1H, Hα, b), 4.6–4.9 (3 × m, 3H, H-2', H-3', H-4', overlapped by water peak), 6.21 (d, J = 6 Hz, 1H, H-1'), 8.40 and 8.55 (2 × s, 2H, H-2 and H-8). ^{13}C NMR, δ, 42.2 (αC), 74.1 and 74.3 (C2' and C3'), 84.9 and 89.8 (C4' and C1'), 121.9 (C5), 144.6 and 146.1 (C8 and C2), 149.6 and 151.5 (C4 and C6), 173.0 and 174.2 (C=O, 2C).

Biological testing of compounds

Measurement of cytoplasmic Ca^{2+} concentration in C6 glioma cells. Cells were cultured and experiments performed as previously described.[9] Briefly, cell culture dishes with a confluent layer of C6 glioma cells were washed twice with 2 mL Krebs–Ringer–Hepes (KRH) buffer (conc. in mM: NaCl 125; KCl 5; $MgSO_4$ 1.2; KH_2PO_4 1.2; $CaCl_2$ 2; glucose 6; Hepes 25; pH adjusted to 7.4). After addition of fura-2/AM (2 μM), a cell permeant Ca^{2+}-sensitive fluorescent probe,[24] cells were incubated in the dark for 1 h. The medium was changed to 2 mL fresh KRH buffer and the cells incubated for another 15 min to remove non-hydrolyzed dye. Immediately before measurement, the experimental incubation medium was replaced again and the vessels placed in the spectrofluorometer. After base line stabilization, the ligand was rapidly added under careful mixing, vessels were re-inserted within 10 s and changes in fluorescence were registered until base line levels were reached again (3–10 min). For desensitization or inhibition studies, ligands were

added sequentially without change of medium. Cytosolic [Ca^{2+}] was quantified using the Ca^{2+}-ionophore ionomycin (10 μM) and $MnCl_2$ (20 mM) to determine maximum and minimum values of fura-2 fluorescence. Calculations were made according to Grynkiewicz *et al.*[24] Significance levels were calculated using Student's *t*-test.

Determination of agonist-induced Ins(1,4,5)P_3. C6 glioma cells were seeded at a cell density of 10^4 cells/well in a 24-well plate and cultured to confluency. After pre-incubation in LiCl containing buffer (10 mM, 20 min, 37 °C) nucleotides or derivatives were added (1 min, 37 °C) and accumulated Ins(1,4,5)P_3 was determined. The assay method (performed essentially as previously described[25]) is based on competition for binding sites on an adrenocortical binding protein between [^{3}H]Ins(1,4,5)P_3 and unlabeled Ins(1,4,5)P_3 sampled from the stimulated C6 cells.

P_2R-modulated effects on evoked noradrenaline release from mouse vas deferens. Experiments were performed as described by von Kügelgen *et al.*[13] Stimulating frequency was 1 Hz, number of pulses 60. Reference compounds were 2-chloro-adenosine (ClAdo) and the non-specific P_2R antagonist suramin. ClAdo (3, 30 μM) inhibits evoked noradrenaline release due to presynaptic P_1-receptor activation (Figure 6a) while suramin (300 μM) causes increases in transmitter release because of blockage of P_2-autoinhibition of noradrenaline release (Figure 6b; see also von Kügelgen *et al.*).[13]

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