Structure Activity Relationships for Derivatives of Adenosine-5′-Triphosphate as Agonists at P_2 Purinoceptors: Heterogeneity Within P_{2X} and P_{2Y} Subtypes

Geoffrey Burnstock
Department of Anatomy and Developmental Biology, University College London, London, United Kingdom

Bilha Fischer
Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, Maryland

Charles H.V. Hoyle
Department of Anatomy and Developmental Biology, University College London, London, United Kingdom

Michel Maillard
Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, Maryland

Airat U. Ziganshin
Department of Anatomy and Developmental Biology, University College London, London, United Kingdom

Kazan Medical Institute, Kazan, Russia

Antonia L. Brizzolara
Department of Anatomy and Developmental Biology, University College London, London, United Kingdom

Amy von Isakovics
Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, Maryland

José L. Boyer
Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, North Carolina

T. Kendall Harden
Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, North Carolina

Kenneth A. Jacobson
Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, Maryland

Abstract

Address reprint requests to Dr. Kenneth A. Jacobson, Building 8A, Room, B1A-17, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892..

Strategy, Management and Health Policy				
Venture Capital Enabling Technology	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

The structure-activity relationships for a variety of adenine nucleotide analogues at P_{2X}- and P_{2Y}-purinoceptors were investigated. Compounds formed by structural modifications of the ATP molecule including substitutions of the purine ring (C2, C8, N1, and N^6-substituents, and a uridine base instead of adenine), the ribose moiety ($2'$ and $3'$-positions), and the triphosphate group (lower phosphates, bridging oxygen substitution, and cyclization) were prepared. Pharmacological activity at P_{2Y}-purinoceptors was assayed in the guinea pig taenia coli, endothelial cells of the rabbit aorta, smooth muscle of the rabbit mesenteric artery, and turkey erythrocyte membranes. Activity at P_{2X}-purinoceptors was assayed in the rabbit saphenous artery and the guinea-pig vas deferens and urinary bladder. Some of the analogues displayed selectivity, or even specificity, for either the P_{2X}- or the P_{2Y}-purinoceptors. Certain analogues displayed selectivity or specificity within the P_{2X}- or P_{2Y}-purinoceptor superfamilies, giving hints about possible subclasses. For example, 8-(6-aminohexylamino)ATP and $2',3'$-isopropylidene-AMP were selective for endothelial Pzypurinoceptors over P_{2Y}-purinoceptors in the guinea pig taenia coli, rabbit aorta, and turkey erythrocytes. These compounds were both inactive at P_{2X}-purinoceptors. The potent agonist N^6-methyl ATP and the somewhat less potent agonist $2'$-deoxy-ATP were selective for P_{2Y}-purinoceptors in the guinea pig taenia coli, but were inactive at P_{2X}-purinoceptors and the vascular P_{2Y}-purinoceptors. $3'$-Benzylamino-$3'$-deoxyATP was very potent at the P_{2X}-purinoceptors in the guinea pig vas deferens and bladder, but not in the rabbit saphenous artery and was inactive at P_{2Y} receptors. These data suggest that specific compounds can be developed that can be utilized to activate putative subtypes of the P_{2X}- and P_{2Y}-purinoceptor classes.

Keywords

ATP; purinoceptors; smooth muscle; nucleotides; phospholipase C

INTRODUCTION

Since the initial proposition of "the purinergic hypothesis" [Burnstock 1972, 1976], ATP has been implicated as a neurotransmitter at autonomic neuro-muscular junctions in several organs [for review see Hoyle, 1992], within autonomic ganglia [Evans et al., 1992] and within the central nervous system [see Hoyle and Burnstock, 1991b; Edwards et al., 1992]. In many situations it appears that ATP is released as a cotransmitter, often with noradrenaline or acetylcholine [Burnstock, 1976, 1990; Hoyle and Burnstock, 1991b; Hoyle, 1992]. The receptor through which ATP acts originally was classified as a P_2-purinoceptor [Burnstock, 1978], but as the physiological roles of purinergic receptors have been evaluated it has become apparent that ATP acts on several P_2-purinoceptor subtypes, i.e., P_{2X}-, P_{2Y}-, P_{2Z}-, P_{2T}-, and P_{2U}-purinoceptors [Burnstock and Kennedy, 1985; Gordon, 1986; Kennedy, 1990; Hoyle and Burnstock, 1991a,b; O'Connor et al., 1991; Hoyle, 1992; Abbracchio et al., 1993; Cusack, 1993]. P_{2X}- and P_{2Y}-purinoceptors are widely distributed subtypes, being found on smooth muscle cells of the cardiovascular, gastrointestinal, and genitourinary systems, and cardiac muscle, and many diverse cell types including endothelial cells, hepatocytes, erythrocytes, pancreatocytes, pulmonary alveolar cells, autonomic ganglionic neurons, sensory neurons, and also within the central nervous system [see Dubyak and Fedan, 1990; Hoyle and Burnstock, 1991b; Hoyle, 1992]. P_{2X}-receptors are activated by α,β-methylene ATP and apparently consist of ligand-gated ion channels [Benham and Tsien, 1987; Benham, 1989; Bean, 1992]. P_{2Y}-receptors are activated by 2-methylthio-ATP

and are linked to second messengers via G-proteins. The principal second messenger system activated by P_{2Y}-receptors is the metabolism of phosphatidyl inositol [Harden et al., 1988; Pirotton et al., 1987; Håggblad and Heilbronn, 1987], leading to liberation of intra-cellular calcium, activation of protein kinase C, and increases in cytoplasmic calcium. P_{2Z}-purinoceptors are thought to be activated by the tetrabasic form of ATP, ATP^{4-}. They mediate permeabilization of the plasmalemma, and are typically found in mast cells, and many other blood cells [Cockcroft and Gomperts, 1979a,b, 1980; Hoyle and Burnstock, 1991b; Hoyle, 1992]. P_{2T}-purinoceptors appear to have a distribution limited to platelets and their megakaryocyte progenitors; at these receptors the principal agonist is ADP, and ATP is a competitive antagonist [Gordon, 1986]. P_{2U}-purinoceptors are found on neutrophils and many other cell types. Like P_{2Y}-purinoceptors, they are also coupled to phospholipase C via G-proteins [Dubyak and el-Moatassim, 1993], but unlike P_{2Y}-receptors are activated not only by ATP but also by UTP [see Cusack, 1993].

Some other subclasses of P_2-purinoceptor have been proposed, for example, P_3, P_{3R}, and P_{2S} subtypes [Shinozuka et al., 1988; Wiklund and Gustaffson, 1988; von Kugelgen and Starke, 1990], but these have not been unambiguously delineated. Finally, the P_{2N} subtype may be synonymous with P_{2U} [Abbracchio et al., 1993], and it has been suggested that they be considered a subclass of a P_{2Y}-purinoceptor superfamily in view of their coupling to G-proteins. Sequence information [Lustig et al., 1993; Webb et al., 1993] will be essential for receptor classification.

The classification of ATP receptors has been difficult due to a multiplicity of biological effects and inconsistencies in the potency of "selective" agents [Inoue and Nakazawa, 1992; Silinsky, 1989]. We surveyed the activity of both known and novel derivatives of ATP in an effort to determine structure activity relationships of ATP analogues acting at P_2-purinoceptors. We examined the selectivity of derivatives of ATP with modified purine, ribose, or triphosphate moieties, in a variety of pharmacological assay systems known to possess P_{2X}- and P_{2Y}-purinoceptors. Pharmacological assays at P_{2Y}-purinoceptors [Boyer et al., 1989] included stimulation of the production of inositol phosphates in turkey erythrocytes, relaxation of the guinea pig taenia coli, endothelium-dependent relaxation of the rabbit aorta, and endothelium-independent relaxation of the rabbit mesenteric artery. Pharmacological assays at P_{2X}-purinoceptors [Hoyle and Edwards, 1992; Hoyle et al., 1990; Burnstock and Warland, 1987a; Fischer et al., 1993] included contraction of the rabbit saphenous artery and contraction of the guinea pig vas deferens and urinary bladder.

METHODS

Synthesis of Novel ATP Analogues

New compounds were characterized (and resonances assigned) by proton nuclear magnetic resonance using a Varian GEMINI-300 FT-NMR spectrometer. Nucleotides were characterized also by ^{31}P NMR in D_2O using H_3PO_4 as an external reference on a Varian-ASM 100 300-MHz spectrometer. Samples (pD ranged from 5 to 7) were treated with CHELEX-100 (Bio-Rad, Richmond, CA) prior to spectral measurement. Synthetic intermediates were characterized on a Finnigan MAT mass spectrometer by chemical ionization mass spectrometry (NH_3) and high resolution mass spectrometry by Dr. L. Pannell and N. Whittaker (NIDDK). Nucleotides were desorbed from a glycerol matrix under FAB (fast atom bombardment) conditions using 6KV Xe atoms on a JEOL SX102 spectrometer.

Compounds **1, 4, 5, 7, 9, 10, 14, 15, 16, 19, 20, 21**, and **22** were obtained from Sigma Chemical Co. (St. Louis, MO). The purity of compound **22** was found to be >95% by HPLC. Compounds **2** and **3** were obtained from Boehringer-Mannheim (Indianapolis, IN).

Compound **8** (S$_p$ isomer) was obtained from Amersham, International (Amersham, UK). 3-(**4**-hydroxyphenyl)propionic acid N-hydroxysuccinimide ester was purchased from Pierce Chemical (Rockford, IL). 2-(6-cyanohexyl)thio-ATP, **llb**, was synthesized as described [Zimmet et al., 1993; Fischer et al., 1993]. Analytical TLC plates and silica gel (230–400 mesh) were purchased from VWR (Bridgeport, N J). Silica gel 100 C$_{18}$-reversed phase was obtained from Fluka (Ronkonoma, NY). Purification of nucleotides was achieved on DEAE-A25 Sephadex columns as described below. Where needed, final purification was done on a Hewlett Packard 1090 HPLC system using a semipreparative SynChropak RP-100 5 μ column (1 × 25 cm, Syn-Chrom, Inc., Lafayette, IN) and a linear gradient of a 0.1 M triethylammonium acetate buffer (TEAA, pH = 7–8) and acetonitrile (see below) with a flow rate of 3 m1/min ([triethylammonium]^4 salt isolated). For analytical purposes, a nucleotide/nucleoside 7U column (250 mm × 4.5 mm, Alltech Associates, Inc., Deerfield, IL) was used applying the same gradient as above at a 1 ml/min flow rate. ATP derivatives were generally >91% pure.

Nucleoside 5′-Triphosphates

The procedure for nucleoside 5′-triphosphate synthesis was adapted from Kovacs and Ötvös [1988], and Moffat [1964].

Preparation of tri-*n*-butylammonium pyrophosphate solution for triphosphate synthesis: Sodium pyrophosphate decahydrate (6.69 g, 0.015 mol) in water (100 ml) was stirred at room temperature for 10 min until a clear solution was attained. The latter was passed through a column of activated Dowex 50WX-8 200 mesh, H$^+$ form (40 ml wet resin, 720 meq). The column was washed with deionized water until neutral. The column eluate was collected in a flask (250 ml) containing tributylaniine (7.14 ml, 0.03 mol) and EtOH (75 ml) stirring at 0°C. The solution became cloudy during elution and clear when all of the free amine was consumed. Lyophilization yielded a viscous oil. The latter was dissolved in EtOH and evaporated under high vacuum (bath temperature 35–40°C). The process was repeated three times using dry dimethylformamide (30 ml) as the solvent, resulting in a thick oil which was dissolved in dry dimethylformamide (30 ml) and stored cold over activated molecular sieves.

Preparation of triethylammonium bicarbonate (TEAB) buffer: A 1 M solution was prepared by adding dry ice to a 1 M triethylamine solution in a flask covered tightly by a balloon for approximately 2 h until the pH reached 7.5.

Phosphorylation was carried out as described below for compound 18 with purification initially using ion-exchange chromatography. Typically, DEAE-Sephadex A-25, was swelled in 1.0 M NaHCO$_3$. After equilibrating the column with deionized water (75 ml), the lyophilized residue of the reaction mixture dissolved in a minimum of water was applied. A solvent gradient of 0–400 or 600 mM TEAB buffer in 1,000 or 600 ml was used to elute the triphosphates. In some cases 0–0.5 M NH$_4$HCO$_3$ buffer in 600 ml was used instead. Elution was monitored using a flow UV detector (ISCO, UA-5, 280 nm). The appropriate fractions were pooled and lyophilized. Successive lyophilization to remove TEAB yielded a product with a "clear glass" appearance. When NH$_4$HCO$_3$ buffer was used, lyophilization gave rise to a white solid.

Following ion-exchange chromatography and subsequent lyophilization, the product was taken up in a small volume of water. An analytical quantity of this solution was examined by high pressure liquid chromatography (HPLC) to determine retention time and degree of separation from contaminants. Further purification was carried out as needed on a semipreparative column. The HPLC-system consisted of an Alltech-nucleoside 7 μ column (either 250 × 4.5 mm or a Synchropak RP-100 5 μ semipreparative 250 × 10 mm column),

equipped with an Alltech Adsorbosphere HSC18 50 precolumn (20 mm); eluant A: 0.1 M triethylammonium acetate (pH 7.4); and eluant B: acetonitrile. A gradient from 5% B to 22% B in 20 min was applied. The flow rate was 1 ml/min or 3 ml/min for the analytical and semipreparative columns, respectively. Detection: UV absorption at 254 nm, using a Hewlett-Packard diode array detector.

3′,5′-Cyclic-β-γ-methylene adenosine 5′-triphosphate (6)

3′,5′-cyclic-AMP free acid (0.067 g, 0.2 mmol) was suspended in MeOH (15 ml) and water (7 ml). Tributylamine was added (0.048 ml, 0.2 mmol) and the clear solution was stirred for 1.5 h at room temperature. The solvent was removed and the residue was dried under high vacuum. The latter was dissolved in dry N,N-dimethylformamide (DMF) (5 ml) together with carbonyldiimidazole (0.173 g, 5 eq.) under N_2 atmosphere. The clear solution was stirred at room temperature for 43 h. Dry methanol (51 µl, 8 eq) was added and after 1 h a solution of methylene diphosphate tributylammonium salt in DMF (2 eq in 3 ml) was added dropwise and a white precipitate was formed. The solution was stirred at room temperature for 24 h and then concentrated under vacuum. The solid residue was dissolved in a minimal amount of water and loaded on a DEAE-A25 Sephadex column (15 × 1.5 cm) applying a gradient of 0.05 M to 0.5 M NH_4HCO_3 (275 ml of each). The relevant fractions were pooled and lyophilized. Final purification by HPLC applying TEAA: acetonitrile gradient 95:5 to 78:22 in 20 min. Retention time of product: 8.6 min. ^{1}H NMR (D_2O) δ: 8.24 (s, 1H, H-8), 8.21 (s, 1H, H-2), 6.17 (s, 1H, H-1′), 4.55 (d, J = 2.0 Hz, 1H, H-2′), 4.47 (m, 1H, H-3′), 4.31 (m, 3H, H-4′ + H-5′), 2.01 (t, J = 19.3 Hz, 2H, PCH_2P). FAB: 485 (MP^{-4} $3H^+$).

N[6]-Methyl-adenosine 5′-triphosphate trisammonium salt (12)

N[6]-Me-adenosine hydrate (Research Biochemicals Int., Natick, MA) was dried in vacuo at 90°C for 20 h. The reaction to form the triphosphate was carried out as described for **18** beginning with 0.3 mmol of the nuckoside. Thin layer chromatography (TLC) (propanol: H_2O: NH_4OH 11:2:7) indicated the formation of the 5′-triphosphate (R_f 0.13) as the sole product. The product was isolated using a Sephadex DEAE A-25 column, eluting with a gradient of 0–0.65 M TEAB (500 ml of each). Further purification was achieved using HPLC applying a linear gradient of 0–30% acetonitrile in 0.05 M NH_4HCO_3 in 15 min (retention time 6.6 min). ^{31}P NMR (D_2O) δ: −9.0 (br.s), −10.8 (s), −22.1 (br.s) ppm. FAB: 520 (M^{-4} + $3H^+$).

8-Bromoadenosine 5′-triphosphate trisammonium salt (13)

The reaction to form the triphosphate from 8-bromoadenosine was carried out as described for **18** on a 0.14 mmol (nucleoside) scale. 8-broino ATP trisammonium salt was obtained in 66% yield (60.7 mg) after chromatography using 0–0.65M NH_4HCO_3 gradient (total volume 600 ml). Retention time was 11.8 min using a linear gradient of TEAA: acetonitrile 95:5 to 78:22 in 20 min. ^{1}H NMR (D_2O) δ: 8.26 (s, 1H, H-2), 6.15 (d, 1H, J = 6.2 Hz, H-1′), 5.25 (t, 1H, J = 6.2 Hz, H-2′), 4.66 (m, 1H, H-3′), 4.3 (m, 314, H-4′, H-5′) ppm. ^{31}P NMR δ: −10.55 (m), −13.68 (d, J = 20 Hz), −25.1 ("t") ppm. High resolution (res) FAB for $C_{10}H_{14}O_{13}N_5$ (81 Br)P_3 and $C_{10}H_{14}O_{13}N_5$ (79 Br)P_3: [calculated (calcd.)] 583.8984, 585.8966; found 583.8998, 585.8993(M^{-4} + $3H^+$).

5-Fluorouridine 5′-triphosphate tris triethylammonium salt (18)

This was a typical nucleoside phosphorylation reaction (see above for preparation of reagents and product purification).

To a solution of 5-fluorouridine (dried in a vacuum oven at 50°C for 10 h, 0.05 g, 0.19 mmol) in trimethylphosphate (1.9 ml) was added Proton Sponge® (0.06 g, 0.275 mmol).

After stirring for 15 min at 0°C, phosphorous oxychloride (distilled, 44 μl, 0.46 mmol) was added dropwise, and the mixture was stirred at 0°C for 2 h. A mixture of Bu_3N (0.188 ml) and $(Bu_3NH^+)_2\ P_2O_7H_2^{-2}$ in dimethylformamide (0.5 M, 2.5 ml, 1.25 mmol) was added at once, and after 2 min the reaction was quenched with TEAB solution (0.2M, 18.8 ml). The solution was stirred at room temperature for 45 min and then lyophilized to yield a semi-solid. TLC (on silica gel, propanol: H_2O: 28% NH_4OH 11: 2: 7) indicated the formation of the triphosphate ($R_f = 0.12$). The residue was applied to a Sephadex DEAE-A25 column, which was eluted using a linear gradient of 0–0.6 M NH_4HCO_3 (300 ml of each), to obtain the mono and triphosphate products. 5-fluorouridine 5′-monophosphate monoammonium salt (28) was obtained in 49% yield (34.5 mg). It was further purified by HPLC using a linear gradient of TEAA: acetonitrile 95:5 to 78:22 in 20 min. Retention time was 3.8 min. ^1H (NMR (D_2O) δ: 8.15 (d, J = 6.3 Hz, 1H, H-6), 5.95 (dd, J = 4.4, 1.4 Hz, 1H, H-1′), 4.33 (quintet, 2H, H-2′ + H-3′), 4.26 (m, 1H, H-4′), 4.08 (ABq split into q, 2H, H-5′). ^{31}P NMR δ: –9.49 (s). High res. FAB: calcd. for: $C_9H_{10}N_2O_9PF$: 341.0186, found: 341,0197. ($M^{-2} + H^+$). 5-Fluorouridine 5′-triphosphate trisammonium salt (17) was obtained in 36% yield (39 mg). ^1H NMR (D_2O) δ: 8.12 (d, J = 6.4 Hz, 1H, H-6), 5.97 (dd, J = 5, 1.5 Hz, H-1′), 4.45 ("t", J = 5 Hz, 1H, H-2′), 4.38 ("t", J = 5 Hz, 1H, H-3′), 4.26 (m, 3H, H-4′ + H-5′) ppm. ^{31}P NMR (D_2O) δ: –9.78 (m), –13.87 (d, J = 20 Hz), –24.92 (t, J = 20 Hz). High res. FAB: calcd. for $C_9H_{14}FN_2O_{15}P_3$: 501.9591, found: 501.9531 ($M^{-4} + 3H^+$). Retention time was 4.5 min using a linear gradient of TEAA: acetonitrile from 95:5 to 78:22 in 20 min.

3′-Acetylamino-3′-deoxyadenosine 5′-triphosphate (23)

3′-Amino-3′-deoxyadenosine 5′-triphosphate (22, 15 mg, 26 mmol) and potassium carbonate (21 mg, 0.13 mmol) were dissolved in 2 ml of water. Sodium sulfosuccinimidyl acetate (36 mg, 0.13 mmol, Pierce Chemical) in 0.2 ml of dimethylsulfoxide (DMSO) was added at 0°C, and the reaction was allowed to proceed at room temperature for 24 h. The product was purified by repeated injections on HPLC using a Synchropak RP-100 column (1 × 25 cm) applying a linear gradient of acetonitrile 5–22% TEAA (0.85%/min, flow rate 3 ml/min, t = 8.5 min). The appropriate fractions were collected and lyophilized to dryness. The product was obtained as a triethylammonium salt (8.5 mg, 39%). UV: $\lambda_{max} = 259$ nm. High resolution MS for $C_{12}H_{18}N_6O_{13}P_3$: calcd. 547.0145, found 547.0170 (MH_3^-).

3′-[3-(4-Hydroxyphenyl)propionylamino]-3′-deoxyadenosine 5′-triphosphate (24)

3′-Amino-3′-deoxyadenosine 5′-triphosphate (6 mg, 11 mmol) and potassium carbonate (7.2 mg, 55 mmol) were dissolved in 0.8 ml of water. 3-(4-hydroxyphenyl) propionic acid N-hydroxysuccinimide ester (27 mg, 0.1 mmol) in 0.4 ml of DMSO was added at room temperature, and the mixture was stirred for 24 h at room temperature. The product was purified by the procedure described for compound 23, t = 14 min (4.3 mg obtained, 41% yield). UV: $\lambda_{max} = 259$ nm. High resolution MS for $C_{19}H_{24}N_6O_{14}P_3$: calcd. 653.0563, found 653.0596 (MH_3^-).

3′-N-Benzylamino-3′-deoxyadenosine 5′-triphosphate (25)

3′-Amino-3′-deoxyadenosine 5′-triphosphate (22, 15 mg, 26 mmol) and potassium carbonate (20 mg) were dissolved in 2 ml of water. Excess benzaldehyde (0.5 ml) was added, and the mixture was stirred vigorously at room temperature. After 4 h, excess benzaldehyde was extracted with chloroform. The aqueous solution was cooled to 0°C, and 25 mg of sodium cyanoborohydride were added portionwise over 0.5 h, and the mixture was stirred for an additional 2 h at 0°C. Reaction with benzaldehyde and reduction with $NaBH_3CN$ were repeated twice to ensure higher yields of 25. The product was purified by the procedure described for compound 23, t = 13 min. The yield was 25% of 25

(triethylamine salt), based on unrecovered starting material (30% reisolated). The ^{1}H NMR spectrum was consistent with the proposed structures. UV: λ_{max} = 259 nm. High resolution MS for $C_{17}H_{22}N_6O_{12}P_3$: calcd. 595.0551, found 595.0509 (MH$_3^-$).

3′-N-Benzylamino-3′-deoxyadenosine 5′-monophosphate (29) was obtained by treating **25** in a pH = 4.5 formic acid soution at 100°C for 4 h (FAB [pos]/nitrobenzyl alcohol matrix): m/z 438 MH$_2^+$, 302 MH$_2^+$-adenine, 136 adenine + H$_2^+$. This fragmentation pattern was definitive proof that N-alkylation had occurred on the sugar moiety.

2′,3′-Isopropylidene adenosine 5′-triphosphate tetraammonium salt (26)

The reaction to prepare the 5′-triphosphate from 2′,3′isopropylideneadenosine (Aldrich Chemical Co., St. Louis, MO) was carried out as described for **18** on a 0.3 mmol (nucleoside) scale. The product was isolated by chromatography on a Sephadex DEAE-A25 column using a gradient of 0–0.5 M NH$_4$HCO$_3$. Both mono and triphosphate derivatives were isolated. 2′,3′-isopropylidene AMP ammonium salt (27) was obtained in 16% yield (22 mg). Retention time was 10.2 min applying a linear gradient of TEAA: acetonitrile 95:5 to 60:40 in 20 min. ^{1}H NMR (D$_2$O) δ: 8.50 (s, 1H, H-8), 8.25 (s, 1H, H-2), 6.28 (d, J = 3.4 Hz, 1H, H-1′), 5.41 (dd, J = 6.1, 3.4 Hz, 1H, H-2′), 5.19 (dd, J = 6.1, 2Hz, 1H, H-3′), 4.64 (br.s, 1H, H-4′), 3.97 (dd, J = 7.5, 3.9 Hz, 2H, H-5′), 1.67 (s, 3H, Me), 1.45 (s, 3H, Me). FAB: 387 (M^{-2} + H$^+$). 2′,3′-isopropylidene ATP tetraammonium salt (26) was obtained in 30% yield (55 mg). ^{1}H NMR (D$_2$O) δ: 8.48 (s, 1H, H-8), 8.24 (s, 1H, H-2), 6.29 (d, J = 3.4Hz, 1H, H-1′), 5.40 (dd, J = 6,3.4Hz, 1H, H-2′), 5.26 (dd, **J** = 6,1.5Hz, IH, H-37, (H-4′ was hidden by the water peak), 4.20 (m, 2H, H-57, 1.69, 1.44 (s, 3H, Me) ppm. 31P NMR 6: −5.4(br.s), −6.2(br.s), −20.6(br.s) ppm. High res. FAB for $C_{13}H_{19}N_5O_{13}P_3$: calcd. 546.0192, found: 546.0162 (M^{-4} + 3H$^+$). Retention time was 3.4 min applying a linear gradient of TEAA: acetonitrile 80:20 to 40:60 in 20 min.

Pharmacological Assays

Stimulation of inositol phosphate formation by ATP analogues was measured in turkey erythrocyte membranes as described [Harden et al., 1988; Boyer et al., 1989]. Briefly, 1 ml of washed turkey erythrocytes was incubated in inositol-free medium (Dulbecco's modified eagle medium [DMEM] Gibco, Grand Island, NY) with 0.5–1 mCi of 2-[^{3}H]*myo*-inositol (20 Ci/mmol; American Radiolabelled Chemicals, Inc., St. Louis, MO) for 18–24 h in a humidified atmosphere of 95% air, 5% CO$_2$, at 37°C. Erythrocyte ghosts were prepared by rapid lysis in hypotonic buffer (5 mM sodium phosphate, pH 7.4, 5 mM MgCl$_2$, 1 mM EGTA) as described (12). Phospholipase C activity was measured in 25 μ1 of [^{3}H]inositol-labeled ghosts (≈ 175 μg of protein, 200–500,000 cpm/assay) in a medium containing 424 μM CaCl$_2$, 0.91 mM MgSO$_4$, 2 mM EGTA, 115 mM KCI, 5 mM KH$_2$P0$_4$, and 10 mM Hepes, pH 7.0. Assay (200 μ1 final volume) contained 1 μM GTPγS and the indicated concentrations of nucleotide analogues. Ghosts were incubated at 30°C for 5 min, and total [^{3}H]inositol phosphates were quantitated after purification by anion exchange chromatography as previously described [Harden et al., 1988].

Relaxant responses mediated via P$_{2y}$-purinoceptors were examined in the guinea pig taenia coli, rabbit aorta, and mesenteric artery using standard methods [Hoyle and Edwards, 1992; Burnstock and Warland, 1987b; Rubino et al., 1992; Fischer et al., 1993]. Contractile responses mediated via P$_{2x}$-purinoceptors were examined in the guinea pig urinary bladder and vas deferens, and rabbit saphenous artery, also using standard methods [Hoyle and Edwards, 1992; Hoyle et al., 1990; Burnstock and Warland, 1987a; Fischer et al., 1993].

RESULTS

Synthesis of ATP Analogues

Nucleotide analogues were synthesized chemically or obtained from commercial sources. The analogues and their biological activities are listed in Table 1, which also includes previously published data. Sites on the molecule of adenosine $5'$-triphosphate that were modified include substitutions of the purine ring (uridine instead of adenine and C2, C8, N1, and N^6-substituents), the ribose moiety ($2'$ and $3'$-positions), and the triphosphate group (lower phosphates, oxygen substitution, and cyclization). Thus, the effects on potency at purinoceptors of various functional groups (e.g., N_6-amino and $2'$ and $3'$-hydroxyl groups of ATP) and substitutions that alter the preference for the syn vs. anti conformation (e.g., 8-bromo) were explored.

The modified adenine nucleosides were phosphorylated by a standard method [Kovacs and Ötvös, 1988], using phosphorous oxychloride followed by bis(tributy1ammonium)pyrophosphate addition, and purified by ion exchange chromatography and/or HPLC. Spectral proof of structure to which the analogues were subjected included NMR (proton and phosphorous) and fast atom bombardmant (FAB) mass spectrometry (including high resolution for determination of elemental composition of each product). Purity was determined by HPLC.

Compound **6**, a cyclized analogue of β,γ-methylene ATP (**5**), was prepared from $3',5'$-cyclic AMP tributylammonium salt (Fig. 1), which was activated by carbonyldiimidazole and condensed with methylene diphosphonate bis(tributy1ammonium) salt [Ott et al., 1967; Hecht and Kozarich, 1973].

Derivatives of $3'$-amino-$3'$-deoxy-ATP (**22**, Fig. 2) were prepared by acylation of the amino group or its reductive alkylation with benzaldehyde and cyanoborohydride [Morr et al., 1975], followed by purification by HPLC. Both N-acetyl (**23**) and N-*p*-hydroxyphenylpropionyl (**24**) derivatives are included, with the latter being derived from a prosthetic group for radiolabeling (Bolton-Hunter reagent) so that the conjugate may serve as a substrate for iodination [Jacobson et al., 1992].

Pharmacological Activity

The activity of the ATP analogues evaluated in various P_{2x}- and P_{2y}-purinoceptor systems is summarized in Table 1. Dose response curves are shown in Figure 3 (metabolism of phosphatidyl inositol in turkey erythrocytes) and Figures 4–6 (smooth muscle assays).

Triphosphate group modifications

Reduction in the number of phosphates in the ATP molecule caused a marked reduction in activity at P_{2x}-purinoceptors in the vas deferens: adenosine $5'$-diphosphate (**2**) was less potent than ATP, and adenosine $5'$-monophosphate (**3**) was without effect. At urinary bladder P_{2x}-purinoceptors ADP was equipotent with ATP, and AMP was inactive. At p_{2y}-purinoceptors in the turkey erythrocyte ADP was 3-fold less potent than ATP, and AMP was inactive (Fig. 3A). In the taenia coli ADP was equipotent to ATP, and AMP was much less potent. Thus, neither the P_{2y}- nor P_{2x}-purinoceptors tested in these preparations exhibited much selectivity for ATP vs. ADP.

Replacement of a bridging oxygen with a methylene group, to form α,β-methylene ATP (4) or β,γ-methylene ATP (5) resulted in a reduced activity in the taenia coli, but markedly enhanced activity in the urinary bladder and vas deferens. [^{3}H]α, β-methylene ATP has been used as a high affinity radioligand at vas deferens P_{2X} receptors [Bo et al., 1992]. In

the erythrocyte preparation α,β-methylene ATP was a very weak, yet full agonist, and β,γ-methylene ATP was nearly inactive at 100 µM (Fig. 3A). Replacement of a bridging oxygen with an imido group to form β,γ-imido ATP (7), had an effect similar to methylene replacement in the taenia and vas deferens, but it was equipotent with ATP in the erythrocytes.

Thiophosphate modifications also affected activity. The *S*-isomer of ATP-α-S (8), was approximately 10-fold more potent than ATP in the taenia coli [Burnstock et al., 1984], but at the turkey erythrocyte P_{2Y}-receptor it was 2-fold less potent than ATP. Phosphorothioate substitution on the terminal phosphates of ATP or ADP, i.e., **9** and **10**, respectively, enhanced potency relative to the parent compound at P_{2Y}-receptors on turkey erythrocyte but not in the taenia coli. ADP-β-S (**10**) was approximately 140 times more potent than ATP, and 400 times more potent than ADP in the turkey erythrocyte model (Fig. 3A).

The phosphorothioate compounds did not have significantly greater potency than ATP at the P_{2X}-purinoceptor in either the urinary bladder or vas deferens [Burnstock et al., 1985, 1985.

Compound **6**, cyclized α,β-methylene ATP, was without effect in all seven types of preparation.

Base modifications

In general, modifications of the purine nucleus in ATP at the C8 or N^6 position were not well tolerated by P_{2X}-purinoceptors, where compounds so modified were without effect at the highest concentrations tested (usually 30 µM). At the P_{2Y}-purinoceptors in the mesenteric artery, taenia coli, and erythrocyte preparations, most compounds formed by C8 or N^6 substitutions were either inactive or of much lower potency than ATP. 8-Bromo-ATP (**13**), shown previously to be active at urinary bladder P_{2X} receptors [Welford et al., 1987], was inactive at vas deferens P_{2X} receptors and at vascular P_{2Y} receptors. However, N^6-methyl ATP (**12**), although inactive in vascular smooth muscle, was equipotent with ATP in the taenia coli and inactive in P_{2X} preparations. This compound was also only slightly less potent (7-fold) than ATP for stimulation of inositol phosphate formation in erythrocyte membranes (Fig. 3B). In addition, both 8-(6-amino-hexylamino)ATP (**14**) and adenosine-N1-oxide triphosphate (**15**) were much more potent than ATP as agonists at the endothelial receptor in the aorta. However, they had no activity at the mesenteric artery P_{2Y}-receptor and were less potent than ATP at the turkey erythrocyte P_{2Y}-purinoceptor (Fig. 3B). While the values for the aorta represent activation of P_{2Y} receptors on endothelial cells, the P_{2Y} receptors on the mesenteric artery are located on the smooth muscle [Pearson and Gordon, 1989; Ralevic and Burnstock, 1991].

On the other hand substitution at C-2, as in 2-(6-cyanohexy1)thio ATP (**11b**), as reported previously [Fischer et al., 1993], produced an agonist more potent than ATP in all systems except the p_{2X} system of the saphenous artery, and it was by far the most potent agonist in several p_{2Y}-purinoceptor preparations (Figs. 3B, 4). Although it was approximately 100 times potent than ATP at urinary bladder P_{2X}-receptors (Fig. 6A) it produced tonic contractions rather than the phasic contractions of ATP. In the presence of indomethacin (which inhibits prostaglandin synthesis) compound **11b** produced contractions that were less tonic, and with a lower potency than ATP under the same conditions.

Compound **18**, 5-fluoro-UTP, was only a weak agonist in the P_{2Y} systems of turkey erythrocytes (Fig. 3B) and guinea pig taenia coli, and was inactive at P_{2Y}-receptors in the rabbit mesenteric artery. However, in the rabbit aorta P_{2Y}-receptors it was more potent than ATP. In the P_{2X} systems of the rabbit saphenous artery and the guinea pig vas deferens

compound **18** was inactive. At guinea pig bladder P_{2X}-receptors it was more potent than ATP (Fig. 6A).

Ribose modifications

Kemoval of either free hydroxyl group (at $2'$- or $3'$-positions) resulted in the loss of activity in most but not all of the smooth muscle assays. Compound **19**, $2'$-deoxy-ATP, was roughly equipotent with ATP in the taenia coli (Fig. 4), and was 7-fold less potent than ATP in the turkey erythrocyte (Fig. 3C). Compound **20**, the related isomer $3'$-deoxy-ATP (also known as cordycepin $5'$-triphosphate), was less potent than $2'$-deoxy-ATP at P_{2Y}-purinoceptors (Fig. 3C), but was as active as ATP, or nearly so, in the vas deferens and urinary bladder P_{2X}-purinoceptor systems. Curiously, compound **21**, $2',3'$-dideoxy-ATP, was ten-fold less potent than ATP at taenia coli P_{2Y} receptors (Fig. 4) and weakly active at turkey erythrocyte P_{2Y} receptors (Fig. 3C). Compound **21** was also active at vas deferens P_{2X} receptors ($\approx$ATP) and at urinary bladder P_{2X} receptors (>ATP).

Substitutions at the $3'$-position produced various results. Compound **23**, $3'$-acetylamino-$3'$-deoxy-ATP, was more potent than ATP at rabbit mesenteric artery P_{2Y}-receptors but was inactive at 10 μM at rabbit aorta P_{2Y}-receptors. In contrast, a related compound, $3'$[3-(4-hydroxyphenyl)propionylamino]-$3'$-deoxy-ATP (**24**), was considerably more potent than ATP in the aorta, but inactive in the mesenteric artery. Both these compounds were nearly inactive at P_{2Y}-receptors in the taenia coli and in turkey erythrocyte membranes. The simpler derivative, $3'$-amino-ATP (**22**), also provided a contrast, being approximately 15 times more potent than ATP in the erythrocyte (Fig. 3C), and almost as potent as ATP in the taenia coli (Fig. 4). At P_{2X}-receptors compounds **22,23**, and **24** were more or less as potent as ATP in the guinea pig bladder (Fig. 6A) and vas deferens (Fig. 6B), but inactive in the saphenous artery.

Compound **25**, $3'$-benzylamino-$3'$-deoxyATP, was the most potent agonist at vas deferens P_{2X}-receptors (Fig. 6A) and also highly potent at urinary bladder P_{2X}-receptors (Fig. 6B). This compound was inactive in another P_{2X}-system (saphenous artery) and either inactive or only a weak agonist in P_{2Y}-systems (e.g., turkey erythrocytes, Fig. 3C).

The homologous compounds $2',3'$-isopropylidene-ATP (**26**) and $2',3'$-isopropylidene-AMP (**27**) had unexpectedly different profiles of activity. Neither was particularly active at either P_{2X}- or P_{2Y}-purinoceptors, but the AMP derivative was a potent agonist at the rabbit endothelial P_{2Y}-purinoceptor.

DISCUSSION

We have identified ATP derivatives of high potency and in some cases selectivity as p_{2X}- and p_{2Y}-purinoceptor agonists. Several compounds were inactive in the P_{2X}-purinoceptor systems (e.g., **12, 14, 15, 19**, and **27**), and several (**6,16,25**) were inactive in the P_{2Y}-purinoceptor systems. Further analyses of the relative affinities of these agonists in different tissue preparations provides evidence for heterogeneity within the P_{2X}- and P_{2Y}-purinoceptor subclasses.

Modifications of ATP that greatly reduce or abolish activity within most P_{2X} and P_{2Y} preparations are: N1,N6-etheno modification (**16**), removal of $3'$-hydroxyl (**20**), and cyclizing the phosphate to the $3'$-position (compound **6**, which also contained a β,γ-methylene modification). Substitution at the purine N6 or 1-position is tolerated at P_{2Y}, but not P_{2X} receptors. 8-bromo-ATP (**13**), although reported previously to be somewhat active at P_2-purinoceptors [Satchell and Maguire, 1975], was relatively ineffective at the receptors tested in this study. This may be a result of a change in the conformational preference about

the glycosidic bond to *syn* in compound **13**. In contrast, compound **14**, which is also 8-substituted but with a less bulky NH substituent, was very potent at one class of P_{2Y} receptors. It will be important to carry out conformational energy calculations on these derivatives.

Of the compounds used in this study, several (compounds **14, 15, 19, 23, 24, 25**, and **27**) could prove useful in evaluating subtypes of P_2-purinoceptors. Compound **14** (8-[6-aminohexylamino]ATP) and compound **15** (adenosine N1-oxide $5'$-triphosphate) were specific for P_{2Y}-purinoceptors, and selective for the endothelial receptors of the rabbit aorta vs. the mesenteric artery and taenia coli (also somewhat active in turkey erythrocytes). Compound **27** (isopropylidene-AMP) had a similar profile, except that it appeared to be specific for the aortic endothelial P_{2Y}-purinoceptor. Compound **24**, the *p*-hydroxyphenylpropionyl derivative, also showed a high degree of selectivity for the aortic endothelial P_{2Y}-receptor vs. other P_{2Y} systems. Compound **19** ($2'$-deoxyATP) was specific for another P_{2Y}-receptor system, being equipotent with ATP in the taenia coli, and seven times less potent than ATP in the turkey erythrocyte, but being inactive in the five other preparations, implying a selectivity for the taenia coli and erythrocyte P_{2Y}-receptors. An amino group instead of hydroxyl group at the $3'$-position (**22**) generally enhanced potency at P_2 receptors, and acylation or alkylation of tht amine markedly affected potency and selectivity. Although the N-acetyl derivative (**23**) was active in the vas deferens and urinary bladder, the only P_{2Y}-purinoceptor at which it had any appreciable activity was that in the rabbit mesenteric artery. Compound **25** ($3'$-benzylamino-$3'$-deoxyATP) was the only compound that showed specificity for P_{2X}-purinoceptors, and was active only in the vas deferens and urinary bladder. In both these preparations it was substantially more potent than ATP. It appears that there is bulk tolerance at the $3'$-position of ATP at P_{2X}-receptors in general and in a more restricted fashion at P_{2Y}-receptors of the rabbit aorta.

Hydrogen bonding ability is apparently not essential at the $2'$- and $3'$-positions for activity at P_2 receptors. The $2',3'$-dideoxy ATP (**21**) was considerably active at both P_{2X}- and P_{2Y}-receptors. The greater potency of compound **21** vs. **20** ($3'$-deoxyATP) at bladder P_{2X}-receptors is unexplained, but may be related to conformational (e.g., *exo* vs. *endo*) effects. Also, the $2',3'$-isopropylidene-AMP (**27**), which is lacking hydrogen bond donor ability, was active at P_{2Y}-receptors of the rabbit aorta. The inactivity of the corresponding triphosphate (**26**) is unexplained. Perhaps the binding mode of this bulky analogue in the receptor binding site is different from ATP itself. Compound **27** was not tested for antagonistic properties.

One of the most potent P_{2Y}-purinoceptor agonists is 2-methylthioATP, **11a** [Satchell and Maguire, 1975; Burnstock and Kennedy, 1985], which is much less potent than ATP at P_{2U}-receptors. The enhanced potency at both P_{2X}- and P_{2Y}-purinoceptors and stability of long chain functionalized congeners of 2-Me-SATP have been reported previously [Fischer et al., 1993; Zimmet et al., 1993]. In the present study the most potent compound in all of the P_{2Y}-purinoceptor assays proved to be a 2-alkylthio derivative of ATP (**11b**), which displayed nanomolar potency. Unlike in our previous study [Fischer et al., 1993], in which we showed a close correlation between the potency of a series of eleven 2-alkylthio ATP derivatives in the turkey erythrocyte preparation and the guinea pig taenia coli, in the present study such a correlation was not maintained in series of analogues with other types of modifications. For example, ADP-β-S (**10**) has a very high affinity for the turkey erythrocyte P_{2Y}-purinoceptor, which leads to its development as a radioligand used in receptor studies [Cooper et al., 1989; van Galen et al., 1992], but has no greater affinity for the P_{2Y}-purinoceptor in the guinea pig taenia coli than ATP or ADP.

The receptors in the vas deferens and urinary bladder appeared to be very similar with no purine derivative having a large differential effect in the two tissues. Three compounds (**13, 16**, and **26**) were inactive in the vas deferens while having a limited activity in the urinary bladder. However, the pyrimidine compounds UTP (**17**) and 5-F-UTP (**18**) were as potent as or more potent than ATP in the bladder, yet were without effect in the vas deferens. Whether this is indicative of a true pyrimidine receptor, or whether the P_{2X}-purinoceptor in the bladder recognizes pyrimidine nucleotides has yet to be evaluated.

It is interesting to note that none of the agents had much of an effect on the rabbit saphenous artery. In this vessel α,β-methylene ATP (**4**) is a potent constrictor [Burnstock and Warland, 1987a], as it is also in the vas deferens and urinary bladder [Burnstock and Kennedy, 1985]. However, compounds with modifications that made them potent agonists in the vas deferens and urinary bladder (e.g., compounds **11b** and **25**) were at best weak agonists in the saphenous artery.

Long-chain 2-alkylthioATP analogues, which are highly potent at P_{2Y}-purinoceptors, were previously shown to resist degradation by nucleotidases [Zimmet et al., 1993], and this will greatly enhance utility of compounds such as **11b** as selective pharmacological tools. Since these compounds are of nanomolar potency at turkey erythrocyte p_{2Y}-receptors [Fischer et al., 1993], they may serve as the basis for the design of molecular probes for ATP receptors. Such probes, potentially including radioligands, fluorescent probes, immobilized ligands for affinity chromatography, affinity labels, and covalently reactive ligands could be obtained using a functionalized congener approach, as has been demonstrated for other classes of purine receptors [Jacobson and Daly, 1991]. Compound **14**, which contains an amino group linked via a chain at the 8-position, may serve as the basis for functionalized congeners selective for P_{2X} receptors such as occur in the rabbit aorta. Compound **22**, which contains an amino group on the ribose, may serve as the basis for functionalized congeners active at P_{2X} receptors in the guinea pig bladder and at P2Y receptors in the rabbit aorta.

In conclusion, it is apparent that the P_2-purinoceptors in the seven tissues are all different from one another in their pharmacological profile. Within the P_{2X}-purinoceptor and P_{2Y}-purinoceptor families there are further subtypes that can be distinguished by the selective actions of ATP derivatives. The potentially most noteworthy results from this study, in addition to the recognition of the heterogeneity of P_2-purinoceptor subtypes are: (1) the high potency (particularly at P_{2Y}-purinoceptors) of 2-alkylthio derivatives of ATP; (2) the high selectivity of 8-(6-aminohexylamino)ATP and 2′,3′-isopropylidene ATP for endothelial P_{2Y}-purinoceptors; (3) the selectivity of the potent agonist N^6-methyl ATP and the somewhat less potent agonist 2′-deoxy-ATP at P_{2Y}-purinoceptors in the taenia coli; and (4) the high potency of 3′-benzylamino-3′-deoxyATP at P_{2X}-purinoceptors in the guinea pig vas deferens and bladder, but not rabbit saphenous artery P_{2X}-purinoceptors. Whether this selectivity is species or tissue dependent is not yet known.

Acknowledgments

REFERENCES

Abbracchio MP, Cattabeni F, Fredholm BB, Williams M. Purinoceptor nomenclature: A status report. Drug Dev Res. 1993; 28:207–213.

Allsup DJ, Boarder MR. Comparison of P2 purinergic receptors of aortic endothelial cells with those of adrenal medulla: Evidence for heterogeneity of receptor subtype and of inositol phosphate response. Mol Pharmacol. 1991; 38:84–91. [PubMed: 2164632]

Bean BP. Pharmacology and electrophysiology of ATP-activated ion channels. Trends Pharmacol Sci. 1992; 13:87–90. [PubMed: 1374198]

Benham CD. ATP activated channels gate calcium entry in single smooth muscle cells dissociated from rabbit ear artery. J Physiol. 1989; 419:689–701. [PubMed: 2559977]

Benham CD, Tsien RW. A novel receptor-operated Ca^{2+} - permeable channel activated by ATP in smooth muscle. Nature. 1987; 328:275–278. [PubMed: 2439921]

Bo XN, Simon J, Burnstock G, Barnard EA. Solubilization and molecular size determination of the P(2X) purinoceptor from rat vas deferens. J Biol Chem. 1992; 267:17581–17587. [PubMed: 1387637]

Boyer JL, Downes CP, Harden TK. Kinetics of activation of phospholipase C by P_2-purinergic receptor agonists and guanine nucleotides. J Biol Chem. 1989; 264:884–90. [PubMed: 2910869]

Burnstock G. Purinergic nerves. Pharmacol Rev. 1972; 24:509–581. [PubMed: 4404211]

Burnstock G. Do some nerve cells release more than one transmitter? Neuroscience. 1976; 1:239–248. [PubMed: 11370511]

Burnstock, G. A basis for distinguishing two types of purinergic receptor. In: Straub, RW.; Bolis, L., editors. Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach. Raven Press; New York: 1978. p. 107-118.

Burnstock G. Cotransmission. The Fifth Heymans Lecture, Ghent, February 17, 1990. Arch Int Pharmacodyn Ther. 1990; 304:7–33. [PubMed: 2173506]

Burnstock G, Kennedy C. Is there a basis for distinguishing more than one type of P_2-purinoceptor? Gen Pharmacol. 1985; 16:433–440. [PubMed: 2996968]

Burnstock G, Warland JJI. A pharmacological study of the rabbit saphenous artery in vitro: A vessel with a large purinergic contractile response to sympathetic nerve stimulation. Br J Pharmacol. 1987a; 90:111–120. [PubMed: 3814914]

Burnstock G, Warland JJI. P2-purinoceptors of two sub-types in the rabbit mesenteric artery: Reactive blue 2 selectively inhibits responses mediated via the P2Y-but not the PZX-purinoceptor. Br J Pharmacol. 1987b; 90:383–391. [PubMed: 3828656]

Burnstock G, Cusack NJ, Hills JM, Mackenzie I, Meghji P. Studies on the stereoselectivity of the P_2-purinoceptor. Br J Pharmacol. 1983; 79:907–913. [PubMed: 6317121]

Burnstock G, Cusack NJ, Meldrum LA. Effects of phosphorothioate analogues of ATP, ADP and AMP on guinea pig taenia coli and urinary bladder. Br J Pharmacol. 1984; 82:369–374. [PubMed: 6733363]

Burnstock G, Cusack NJ, Meldrum LA. Studies on the stereoselectivity of the P_2-purinoceptor on the guinea pig vas deferens. Br J Pharmacol. 1985; 84:431–434. [PubMed: 2983813]

Cockcroft S, Gomperts BD. Activation and inhibition of calcium-dependent histamine secretion by ATP ions applied to rat mast cells. J Physiol. 1979a; 296:299–243.

Cockcroft S, Gomperts BD. ATP induces nucleotide permeability in rat mast cells. Nature. 1979b; 279:541–542. [PubMed: 450099]

Cockcroft S, Gomperts BD. The ATP^{4-} receptor of mast cells. Biochem J. 1980; 188:789–798. [PubMed: 6162453]

Cooper CL, Morris AJ, Harden TK. Guanine nucleotide-sensitive interaction of a radiolabeled agonist with a phospholipase C-linked P_{2Y}-purinergic receptor. J Biol Chem. 1989; 264:6202–6206. [PubMed: 2495280]

Cusack NJ. P_2 receptor: Subclassification and structure-activity relationships. Drug Dev Res. 1993; 28:244–252.

Cusack, NJ.; Hourani, SMO. Structure activity relationships for adenine nucleotide receptors on mast cells, human platelets, and smooth muscle. In: Jacobson, KA.; Daly, JW.; Manganiello, V., editors. Purines in Cellular Signalling: Targets for New Drugs. Springer; New York: 1990. p. 254-259.

Dubvak GR, el-moatassim C. Signal transduction via P_2-purinergic receptors for extracellular ATP and other nucleotides. A J Physiol. 1993; 265:C577–C606.

Dubyak GR, Fedan JS. Biological Actions of Extracellular ATP. Ann NY Acad Sci. 1990; 603

Edwards FA, Gibb AJ, Colquhoun D. ATP receptor-mediated synaptic currents in the central nervous system. Nature. 1992; 359:144–147. [PubMed: 1381811]

Evans RJ, Derkach V, Suprenant A. ATP mediates fast synaptic transmission in mammalian neurons. Nature. 1992; 357:503–505. [PubMed: 1351659]

Fischer B, Boyer JL, Hoyle CHV, Ziganshin AU, Brizzoloara AL, Knight GE, Zimmet J, Burnstock G, Harden TK, Jacobson KA. Identification of potent, selective P_{2Y}-purinoceptor agonists: Structure activity relationships for 2-thioether derivatives of adenosine-$5'$-triphosphate. J Med Chem. 1993; 36:3937–3946. [PubMed: 8254622]

Gordon JL. Extracellular ATP: Effects, sources and fate. Biochem J. 1986; 233:309–319. [PubMed: 3006665]

Håggblad J, Heilbronn E. Externally applied adenosine-$5'$-triphosphate causes inositol triphosphate accumulation in cultured chick myocytes. Neurosci Lett. 1987; 74:199–204. [PubMed: 3033552]

Harden TK, Hawkins PT, Stephens L, Boyer JL, Downes P. Phosphoinositide hydrolysis by guanosine $5'$-([gamma-thio]triphosphate)-activated phospholipase C of turkey erythrocyte membranes. Biochem J. 1988; 252:583–593. [PubMed: 2843174]

Hecht SM, Kozarich JW. A chemical synthesis of adenosine $5'$-[γ-^{32}P] triphosphate. Biochim Biophys Acta. 1973; 331:307. [PubMed: 4777661]

Hoyle, CHV. Transmission: Purines. In: Burnstock, G.; Hoyle, CHV., editors. Autonomic Neuroeffector Mechanisms. Harwood Academic Press; New York: 1992. p. 367-407.

Hoyle, CHV.; Burnstock, G. Purinergic receptors. In: Doods, HN.; Van Meel, JCA., editors. Receptor Data for Biological Experiments: A Guide to Drug Selectivity. Ellis Horwood; New York: 1991a. p. 54-61.

Hoyle, CHV.; Burnstock, G. ATP receptors and their physiological roles. In: Stone, TW., editor. Adenosine in the Nervous System. Academic Press; New York: 1991b. p. 43-76.

Hoyle CHV, Edwards GA. Activation of P_1- and P_{2Y}-purinoceptors by ADP-ribose in the guinea pig taenia coli, but not of P_{2X}-purinoceptors in the vas deferens. Br J Pharmacol. 1992; 107:367–374. [PubMed: 1422586]

Hoyle CHV, Knight GE, Burnstock G. Suramin antagonizes responses to P_2-purinoceptor agonists and purinergic nerve stimulation in the guinea pig urinary bladder and taenia coli. Br J Pharmacol. 1990; 99:617–621. [PubMed: 2331585]

Inoue K, Nakazawa K. ATP-receptor operated Ca^{2+} influx and catecholamine release from neuronal cells. News Physiol Sci. 1992; 7:56–58.

Jacobson, KA. Adenosine (P1) and ATP (P2)-purinoceptors. In: Hansch, C.; Sammes, PG.; Taylor, JB., editors. Comprehensive Medicinal Chemistry. Pergamon; Oxford: 1990. p. 601-642.

Jacobson KA, Daly JW. Purine fiinctionalized congeners as molecular probes for adenosine receptors. Nucleos Nnclcot. 1991; 10:1029–1038.

Jacobson KA, Olah ME, Stiles GL. Trifunctional ligands: A radioiodinated high affinity acylating antagonist for the A_1 adenosine receptor. Pharmacol Res Commun. 1992; 2:145–154.

Kennedy C. P_1- and P_2-purinoceptor subtypes: An update. Arch Int Pharmacodyn Ther. 1990; 303:30–50. [PubMed: 2196860]

Kovacs T, Ötvös L. Simple synthesis of 5-vinyl- and 5-ethy-nyl-$2'$-deoxyuridine-$5'$-triphosphates. Tetrahedron Lctt. 1988; 29:4525–4528.

Lustig KD, Shiau AK, Brake AJ, Julius D. Expression cloning of an ATP receptor from mome neuroblastoma cells. Proc Natl Acad Sci USA. 1993; 90:5113–5117. [PubMed: 7685114]

Moffat JG. A general synthesis of nucleoside-$5'$-triphosphates. Can J Chem. 1964; 42:599.

Morr M, Kula M-R, Ernst L. Synthesis and ^{13}C NMR spectra of $3'$-amido analogues of adenosine $3'$, $5'$-cyclic monophosphate. Tetrahedron. 1975; 31:1619–1622.

O'Connor SE, Dainty IA, Leff P. Further subclassification of ATP receptors based on agonist studies. Trends Pharrnacol Sci. 1991; 12:137–141.

Ott DG, Kerr VN, Hansbury E, Hayes FN. Chemical synthesis of nucleoside triphosphates. Anal Biochem. 1967; 21:469. [PubMed: 5582133]

Pearson JD, Cordon JL. P_2 purinoceptors in the blood vessel wall. Biochem Pharmacol. 1989; 38:4157. [PubMed: 2512933]

Pirotton S, Raspe E, Demolle C, Erneux C, Boeynaems JM. Involvement of inositol 1,4,5-trisphosphate and calcium on the action of adenine nucleotides on aortic endothelial cells. J Biol Chem. 1987; 262:17461. [PubMed: 3500950]

Ralevic V, Burnstock G. Roles of P_2-purinoceptors in the cardiovascular system. Circulation. 1991; 84:1–14. [PubMed: 1647896]

Rubino A, Ralevic V, Burnstock G. Prejunctional modulation of sensory-motor nerve mediated vasodilation of the rat mescnteric arterial bed by adenosine. Eur J Pharmacol. 1992; 220:95–98. [PubMed: 1425985]

Satchell DG, Maguire MH. Inhibitory effects of adenine nucleotide analogues on the isolated guinea pig taenia coli. J Pharmacol Exp Ther. 1975; 195:540–548. [PubMed: 1195136]

Shinozuka K, Bjur R, Westfall DP. Characterization of prejunctional purinoceptors on adrenergic nerves of the rat caudal artery. Naunyn Schmiedebergs Arch Pharmacol. 1988; 338:221–227. [PubMed: 3194034]

Silinsky EM. Adenosine derivatives and neuronal functim. Semin Neurosci. 1989; 1:155–165.

von Kügelgen I, Starke K. Evidence for two separate vasoconstriction-mediating nucleotide receptors, both distinct from the P_{2X}-purinoceptor, in rabbit basilar artery: A receptor for pyrinidine nucleotides and a receptor for purine nucleotides. Naunyn Schmiedebergs Arch Pharinacol. 1990; 341:538–546.

Webb TE, Simon J, Krishek BJ, Bateson AN, Smart TG, King BF, Burnstock G, Barnard EA. Cloning and functional expression of a brain G-protein coupled ATP receptor. FEBS Lett. 1993; 324:219–225. [PubMed: 8508924]

Welford LA, Cusack NJ, Hourani SMO. The SAR of ectonucleotidase and of excitatory P2-purinoceptors: Evidence that dephosphorylation of ATP analogues reduces pharmacological potency. Eur J Pharinacol. 1987; 141:123–130.

Wiklund NP, Gustaffson LE. Agonist and antagonist characterization of the P_2-purinceptors in the guinea-pig ileum. Acta Physiol Scand. 1988; 148:361–370.

van Galen PJM, Melman N, IJzerman AP, Jacobson KA. Characterization of [^{35}S]ADPβS binding to bovine brain mernhranes. Pharmacol Comm. 1992; 1:279–290.

Ziminet J, Järlcbark L, van Galen PJM, Jacobson KA, Heilbronn E. Synthesis and biological activity of novel 2-thio derivatives of ATP. Nucleos Nucleot. 1993; 12:l–20.
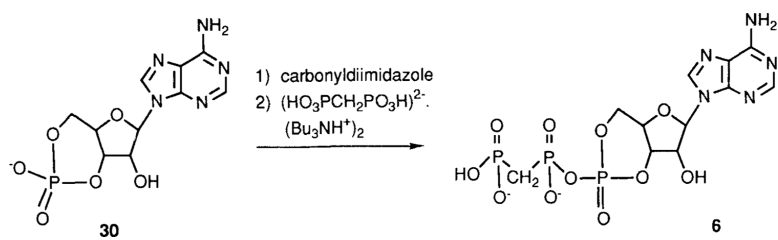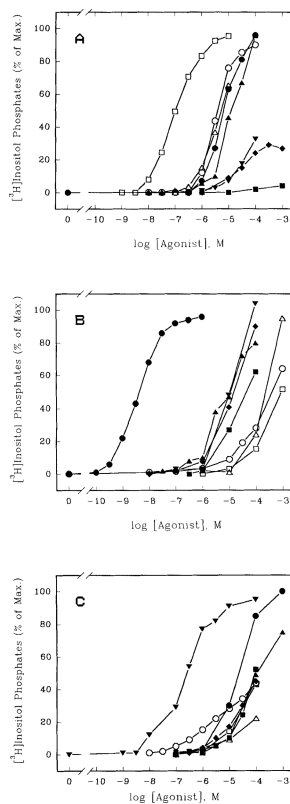
Figure 1.
Synthesis of a 3′,5′-cyclic analogue of α,β-rnethylene ATP.
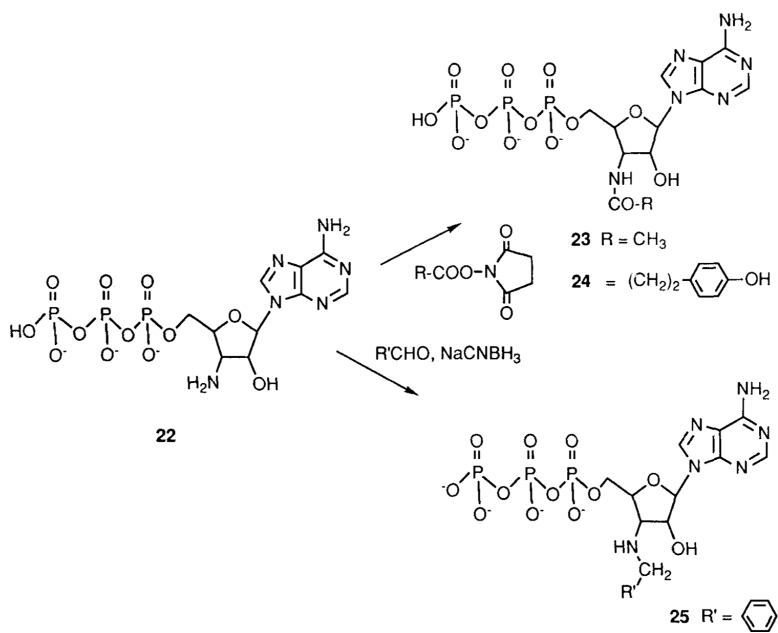
Figure 2.
Synthesis of ribose 3′-position-modified ATP analogues.

Figure 3.
Guanine nucleotide-dependent activation of turkey erythrocyte phospholipase C by ATP analogues. Substitution was on the phosphate chain **(A)**, on the purine base **(B)**, or on the ribose moiety **(C).** Turkey erythrocyte ghosts were incubated for 5 min at 30°C in the presence of 1 μM GTPγS and the indicated concentrations of ATP analogues, as described in Methods. The formation of total [^{3}H]inositol phosphates stimulated by ATP analogues is shown. The ATP analogues tested in A were: ATP, **1** (●); ADP, **2** (▲); AMP, 3 (■); α,β-Me-ATP, **4** (▼); β,γ-Me-ATP, 5 (●); AppNHp, **7** (○); ATP-α-S (*S*-isomer), 8 (△); and ADP-β-S, **10** (□). For B: N^6-Me-ATP, 12 (▲); 8-Br-ATP, 13 (■); 8-(6-aminohexyl-amino)-ATP, **14** (▼); 2-(6-cyanohexylthio)-ATP, 11b (●); adeno-sine-N1-oxide-5′-triphosphate, **15** (◆); N1,N^6-etheno-ATP, 16 (○); UTP, **17** (△); and 5-F-UTP, **18**(□). For C: 2′-deoxy-ATP, **19** (●); 3′-deoxy-ATP, **20** (▲); 2′,3′-dideoxy-ATP, **21** (■); 3′-amino-3′-deoxy-ATP, **22** (▼); 3′-acetylamino-3′-deoxy-ATP, **23** (◆); 3′-[3-(4-hydroxyphenyl)propionylaminol-3′-deoxy-ATP, **24** (○); 3′-ben zylamino-3′-deoxy-ATP, **25** (△); and 2′,3′-isopropylidene-AMP, **27**(□).
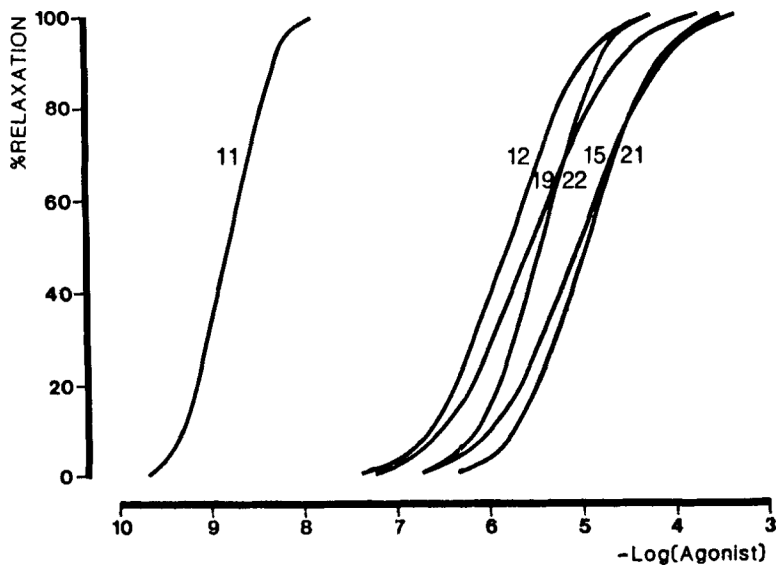
Figure 4.
Concentration-response relationships for ATP analogues causing relaxation of the carbachol-contracted guinea pig taenia coli (P_{2Y}-purinoceptor). Each curve is the mean of 2–5 determinations. Ordinate axis shows % relaxation of the carbachol (50 nM)-induced contraction; abscissa axis shows -log concentration of applied agonists, which are (from left to right at the 50% level): **11b**, 2-(6-cyanohexyl)thio-ATP; 12, N^6methyl-ATP; $_{19}$, 2′-deoxy-ATP; **22**, 3′-amino-3′-deoxy-ATP; **15**, adenosine-N-oxide-5′-triphosphate; 21, 2′, 3′-dideoxy-ATP.
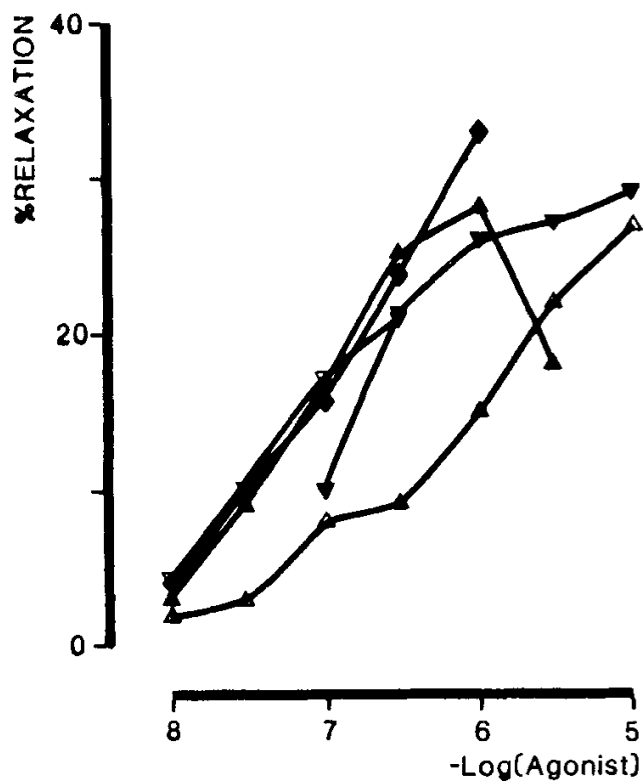
Figure 5.
Concentration-response relationships for ATP analogues causing relaxation of the rabbit aorta (endothelial P_{2Y}-purinoceptor). Ordinate axis shows % relaxaiion of the noradrenaline (1 μM)-induced contraction; abscissa axis shows -log concentration of applied agonist; **11b**, 2-(6-cyanohexyl)thio-ATP (▲); **15**, adenosine-N1-oxide-5′-triphosphate (△); **14**, 8-(6-amino-hexylamino)-ATP (▽); **18**, 5-fluoro-UTP (◆); **22**, 3′-deoxy-3′-amino-ATP (▼). Each point is the mean of 2–4 determinations.
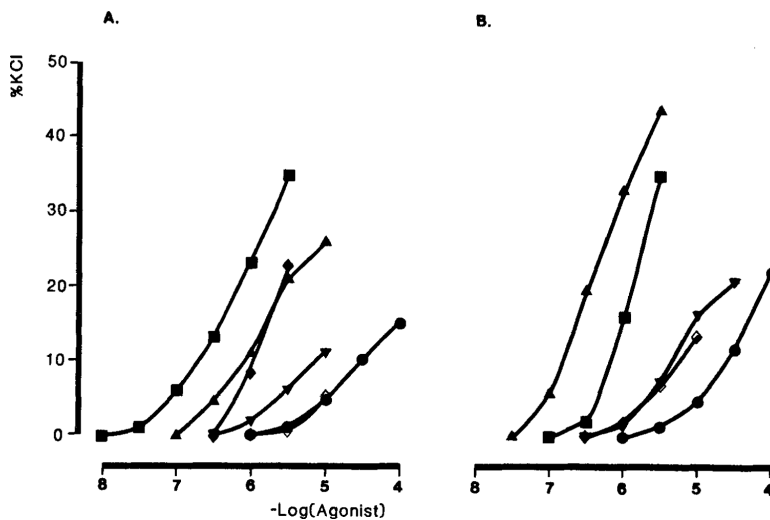
Figure 6.
Concentration-response relationships for ATP analogues causing contraction of (**A**) guinea pig isolated urinary bladder detrusor muscle and (**B**) guinea pig isolated vas deferens (both possess P_{2X}-purinoceptors). Agonists: **25**, 3′-benzylamino-3′-deoxy-ATP (■); **11b**, 2-(6-cyanohexyl)thio-ATP (▲); **18**, 5-fluoro-UTP (◆); 22, 3′-deoxy-3′-arnino-ATP (▼); 1, ATP (●); **21**, 2′, 3′-dideoxy-ATP (◇). Each curve is the mean of two determinations. Ordinate axis shows % contraction relative to a standard dose of KCl (60 mM); abscissa axis shows -log concentration of applied agonist.

TABLE 1

Activity of Nucleotide Analogues in Various Biochemical and Pharmacological Models

	P2Y-purinoceptors	Relaxation, potency relative to ATP (pD2) [b]			P2X-purinoceptors		
	Phosphatidyl inositol metabolism ($K_{0.5}$, nM) [a]				Contraction, potency relative to ATP (pD2) [b]		
Compound	Erythrocyte	Taenia coli	Aorta	Mesenteric artery	Saphenous artery [c]	Vas deferens	Bladder
ATP and triphosphate modifications							
1. ATP	2,800 ± 700	= (6.2)[d]	= (4.5)	= (6.0)	= [5%]	= (3.5)[e]	=[e]
2. ADP	8,000 ± 2,000	=[h]	= (5.2)	= (5.2)	na	_[e]	=e[e]
3. AMP	4 ± 2% at 10^{-4}M	_[h]	= (4.8)	= (5.0)	na	—	na[e]
4. α,β-Me-ATP	>100,000	= (5.6)[e]	f	f	= [5.9%]	+ (5.3)[e]	+ + (5.7)[e]
5. β,γ-Me-ATP	>100,000	_[e]	f	g	+ + [89%]	+[e]	+ +[e]
6. β,γ-Me-Adenosine-3′,5′-cyclic triphosphate	na		na	na	na	na	na
7. AppNHp	4,450 ± 1,150	_[e]	+ (5.5)	+ (6.6)	na	+[b]	+[b]
8. ATP-α-S (S-isomer)	8,930 ± 4,440	+[e]	f	+ (6.6)	na	=[i]	=[i]
9. ATP-γ-S	1,260 ± 380	=[e]	+ (5.7)	+ (5.8)	na	+ +[i]	+
10. ADP-β-S	96 ± 27	=	+ (5.8)	+ (5.8)	na	=[i]	=[i]
Base modifications							
11a. 2-MeS-ATP	8 ± 2	+ + (8.0)	+ + (6.8)[e]	+ + (6.5)	na	=[e]	=[e]
11 b. 2-(cyanohexylthio)-ATP	10 ± 5	+ + (8.8)	+ + (6,9)	+ + (7.0)	= [9.2%]	+ +	h
12. [i] N6-Me-ATP	19,000 ± 6,000	+ (5.8)	—	na	na	na	na[e]
13. 8-Br-ATP	47,400	—	na	na	na	na	–
14. 8-(6-aminohexylamino)-ATP	8,200 ± 1,200	—	+ + (7.3, sl. <max)	na	na	na	na
15. Adenosine N1-oxide 5′-triphosphate	16,900 ± 4,900	– (4.9)	+ + (6.7, >max)	na	na	na	na
16. N1,N6-etheno-ATP	>100,000	—	na	na	na	na	–
17. UTP	143,000 ± 44,000	– (3.5)	= (4.8)	+ (6.7)	na	na	=[e]
18. 5-F-UTP	>100,000	—	+ (6.0, ≈max)	na	na	na	+ +

| | P2Y-purinoceptors | | | | P2X-purinoceptors | | |
| | Phosphatidyl inositol metabolism (K$_{0.5}$, nM) [a] | Relaxation, potency relative to ATP (pD$_2$) [b] | | | Contraction, potency relative to ATP (pD$_2$) [b] | | |
Compound	Erythrocyte	Taenia coli	Aorta	Mesenteric artery	Saphenous artery [c]	Vas deferens	Bladder [b]
Ribose modifications							
19. 2′-deoxy-ATP	19,200 ± 6,200	= 5.6	na	na	na	na [e]	na
20. 3′-deoxy-ATP	75,500 ± 14,800	—	na	na	na	=	−
21. 2′,3′-dideoxy-ATP	70,800	= (5.0)	na	na	na	=	+
22. [j] 3′-amino-3′-deoxy-ATP	193	= (5.4)	++ (6.4, ≈max)	na	na	+	=
23. [j] 3′-acetylamino-3′-deoxy-ATP	>100,000	na	na	+ (<max)	na	=	+
24. [j] 3′(4-hydroxyphenylpropionylamino)-3′-deoxy-ATP	>100,000	na	++ (<max)	na	na	=	+
25. 3′-benzylamino3′-deoxyATP	>100,000	—	na	na	na	++	++
26. Isopropylidene-ATP	201,000 ± 63,000	—	na	—	= [7.7%]	na	−
27. [j] Isopropylidene-AMP	>100,000	na	++ (<max)	na	na	na	na [f]

[a] pK$_{0.5}$ (nM) for stimulation of production of inositol phosphates in turkey erythrocyte membranes, expressed as the mean ± s.e.m. for at least 2–5 determinations, or % stimulation at concentration indicated, na, not active at 10^{-5} M.

[b] ++, significantly more potent than ATP; +, more potent than or equal to ATP; =, equal to ATP; −, less potent than or equal to ATP; −−, significantly less potent than ATP; na, not active at the highest concentration tested (usually around 10^{-5} M). Numerical value, if given, is pD$_2$ in −log molar units, and for some compounds maximum relaxation relative to 2-methylthioATP (<, ≈, or >) is indicated in parentheses. The taenia coli, vas deferens, and bladder were from guinea pig. The aorta, mesenteric artery, and saphenous artery were from rabbit.

[c] For the saphenous artery, the percentages in brackets are responses at 10 μM relative to the contraction produced by 1 μM α,β-Me-ATP (~half maximal effect). ATP in this assay is very weak (EC50 = 1.78 mM). The highest concentrations of the analogues tested were 3–10 μM.

[d] 6.2 ± 0.08 (n = 38).

[e] Data from literature reports (Burnstock et al., 1983; Jacobson, 1990; Cooper et al., 1989; Cusack and Hourani, 1990).

[f] Contraction, not relaxation.

[g] Relaxation observed, but pD2 not calculable.

[h] Compound **11b** was approximately 100 times more potent than ATP in the bladder, but it produced tonic contractions rather than the phasic contractions of ATP. In the presence of indomethacin (1 μM), it was much less potent than ATP under the same conditions.

[j] Data from Burnstock et al., 1983.

[j]Data from Burnstock et al., 1984.