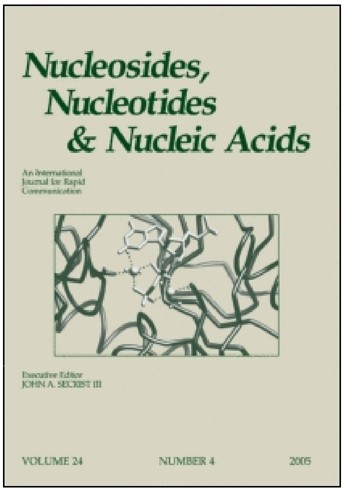

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THE SYNTHESIS OF CYCLONUCLEOTIDES WITH FIXED GLYCOSIDIC BOND LINKAGES AS PUTATIVE AGONISTS FOR P2-PURINERGIC RECEPTORS

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Abstract: Cyclonucleotides with fixed glycosidic bond linkages were investigated as possible ligands for purinoceptors in PC12 cells. $P2Y_2$-purinoceptors were not activated by the ATP analogue, 8,2'-thioanhydroadenosine-5'-triphosphate (**4**) and only weakly by the UTP analogue, 2,2' -anhydrouridine-5'-triphosphate (**6**). However, both analogues were agonists for $P2X_2$-purinoceptors although the potencies were approximately 30-fold less than that of the parent nucleotides.

Adenine nucleotides are ubiquitous signaling molecules that target a variety of cell types through the interaction with a heterogeneous group of cell surface P2-purinoceptors. The physiological agonist for the P2-purinoceptor is usually ATP, although some subtypes can be activated by uridine nucleotides (UTP and UDP) or ADP. The P2-purinoceptors comprise two large superfamilies, the G-protein-coupled metabotropic receptors ($P2Y_{1-n}$) and the ligand-gated cation channels ($P2X_{1-n}$)[1]. Many cell types express multiple P2-purinoceptor subtypes and it would be of great advantage to selectively target individual receptor classes. To this end, many ATP analogues with substitutions on the base or polyphosphate tail have now been shown to display receptor specificity[2]. Most of these are agonists with varying degrees of potencies; few receptor-specific antagonists have been identified to date.

Nucleotides, by virtue of free rotation around the glycosidic bond are conformationally flexible and can adopt either the *anti* or *syn* conformation. Nonetheless, one confomer may be preferred in solution or even within a ligand binding site in a protein[3, 4]. ATP in solution prefers the *anti*-conformation[5] with the plane of the base perpendicular and bisecting the sugar residue. In this paper we describe the synthesis of

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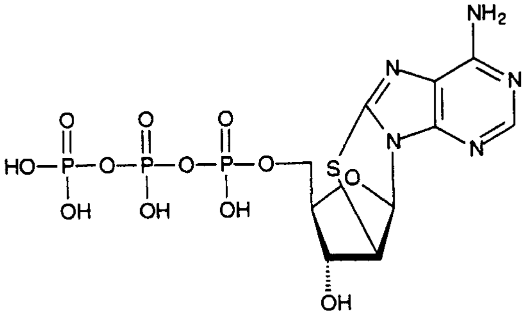
two conformationally locked "cyclonucleotides" with fixed glycosidic bond linkages, 8,2'-thioanhydroadenosine-5'-triphosphate (**4**) and 2,2'-anhydrouridine-5'-triphosphate (**6**) (FIG.1). The purpose of this study was to attempt to determine if purinoceptors display conformation-based selectivity of the ligand. By using the preferred conformer of the nucleotide as a template, it may be possible to design more selective and potent ligands for these receptors.

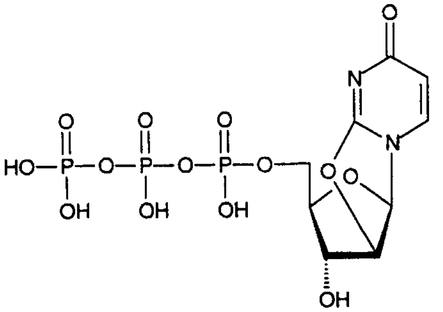
The bioactivity of these two nucleotide analogues was determined in PC12 cells, a cell line that expresses multiple P2-purinoceptors[6-8] that can be distinguished experimentally. The $P2X_2$-purinoceptor has been cloned from PC12 cells[9], and $P2Y_2$-purinoceptors have been identified in PC12 cells by Northern analysis using a $P2Y_2$ clone[10].

EXPERIMENTAL

Synthesis of cyclonucleotides: 8,2'-Thioanhydroadenosine-5'-triphosphate (**4**) was prepared by phosphorylation of the parent nucleoside, 8,2'-thioanhydroadenosine (**3**). The synthesis of 8,2'-thioanhydroadenosine (**3**) was based on a procedure described by Ogilvie et al.[11] with modifications for larger scale preparation which allowed all intermediates to be identified. The procedure involved four steps; bromination of adenosine to obtain 8-bromoadenosine (**1**), formation of the 2',3'-cyclic carbonate, 8-bromoadenosine-2',3'-carbonate (**2**) which upon treatment with thiourea and refluxing, cyclizes to yield the sulfur-bridged nucleoside, 8,2'-thioanhydroadenosine (**3**). All intermediates were obtained in high purity and characterized by ^{1}H-NMR, HPLC, FT-IR and FAB-MS. The 2', 3'-substitution in (**2**) was confirmed by a downfield shift in the ^{1}H-NMR signals (2'H, 6.26 ppm and 3'H, 5.60 ppm) compared to adenosine. The procedure for the formation of the triphosphate product was adapted from Zimmet et al.[12] The unprotected nucleoside (**3**) (1.1 mmole, 0.390 g) was reacted with $POCl_3$, (2.2 mmole, 205 μL) under anhydrous conditions in the presence of 1,8-bis(dimethylamino) naphthalene (proton sponge, 1.7 mmole, 0.234 g, 1.5 equiv. Aldrich) and trimethyphosphate (15 mL). After 2 hours at 0°C an excess (5 equiv.) of 0.5 M tri-n-butylammonium pyrophosphate in DMF was added quickly and allowed to react for 1 min. The reaction was quenched with 0.2 M aqueous triethylammonium bicarbonate (TEAB) (100 mL) and agitated gently for 30 min at room temperature to destroy metaphosphates. The selective phosphorylation of the 5'-hydroxyl was achieved by maintaining low temperatures. The nucleoside phosphate products were separated by DEAE-Sephadex-25 eluted with a linear gradient of TEAB (0-700 mM). The fractions containing the triphosphate were pooled and lyophilized to obtain 8,2'-thioanhydroATP (**4**) as the triethylammonium salt (translucent glass). The triethylammonium salt was



8,2'-ThioanhydroATP (**4**)



2,2'-AnhydroUTP (**6**)

FIG. 1.

dissolved in methanol (0.05 M) and five volumes of sodium perchlorate (15 equiv. in acetone) were added. The precipitated sodium salt was collected by centrifugation, washed with acetone (4 x 5 mL), and dried over P_2O_5 under vacuum. This afforded the sodium salt of the nucleoside-5'-triphosphate (**4**), (102.2 mg, 0.17 mmole, yield 15%) : ^{1}H-NMR (200 MHz, D_2O) δ 3.94 (m, 2H, 5'-H,5"-H, $J_{H5'/5'',P}$ = 6.6 Hz), 4.39 (q, 1H, 4'-H, $J_{H4',5'/5''}$ = ^{a} 5.2 Hz), 4.71 (t, 1H, 3'-H, $J_{H3',4'}$ = 3.7 Hz), 4.97 (dd, 1H, 2'-H, $J_{H2',3'}$ = 3.0 Hz), 6.53 (d, 1H, 1'-H, $J_{H1',2'}$ = 7.4 Hz), 8.00 (s, 1H, 2-H adenine) ; ^{31}P-NMR (200 MHz, D_2O, pH 7) δ -22.48 (br s, 1P, βP), -10.87 (d, 1P, αP, $J_{\alpha\beta}$ = 20.2 Hz), -9.79 (d, 1P, γP, $J_{\gamma\beta}$= 18.3 Hz) ; FAB-MS calculated for $C_{10}H_{10}O_{12}N_5P_3^{4-}$ of the free acid [M^{4-}] = 517.2, found 541.9 [M^{4-} + $2H^{+}$ + Na^{+}] ; HPLC : reverse-phase C18 column (4.6 x 250 mm), eluted isocratically for 40 min (0.1 M K_2HPO_4, 0.2 mM $(Bu)_4NH^{+}SO_4^{-}$, 2.5% MeOH, pH 6.5, 1 ml/min) purity > 99%, retention = 33.6 min.

2,2'-Anhydrouridine-5'-triphosphate (**6**) was prepared by phosphorylation of 2,2'-anhydrouridine (**5**) (1.51 mmoles, 0.342 g) as outlined above. 2,2'-Anhydrouridine (**5**) was prepared from uridine by treatment with diphenylcarbonate in DMF[13]. Since the cyclonucleoside (**5**) is susceptible to acid hydrolysis at the bridging oxygen, an excess of proton sponge (1.7 equiv.) was required to protect the nucleoside during phosphorylation. Identical work-up and DEAE-Sephadex-25 purification yielded the triethylammonium triphosphate salt, which was converted to the sodium salt, to afford 2,2'-anhydroUTP (**6**) as an off-white powder (148 mg, 0.27 mmoles, yield 18%) : ^{1}H-NMR (200 MHz, D_2O) δ 4.05 (m, 2H, 5'-H,5"-H, $J_{H5'/5'',P}$ = 5.6 Hz), 4.59 (m, 1H, 4'-H, $J_{H4',5'/5''}$ = [a] 2.3 Hz), 4.85 (t, 1 H, 3'-H, $J_{H3',4'}$ = 0.8 Hz), 5.58 (dd, 1H, 2'-H, $J_{H2',3'}$ = 0.8 Hz), 6.29 (d, 1H, 5-H uracil, $J_{H5,6}$ = 7.4 Hz), 6.60 (d, 1H, 1'-H, $J_{H1',2'}$ = 5.8 Hz), 8.00 (d, 1H, 6-H uracil) ; ^{31}P-NMR (200 MHz, D_2O, pH 7) δ -22.51 (t, 1P, βP), -11.08 (d, 1P, αP, $J_{\alpha\beta}$ = 19.4 Hz), -9.67 (d, 1P, γP, $J_{\gamma\beta}$= 19.8 Hz) ; FAB-MS calculated for $C_{10}H_{10}O_{12}N_5P_3^{4-}$ of the free acid [M^{4-}] = 462.0, found 486.9 [M^{4-} + $2H^+$ + Na^+] ; HPLC : purity > 95%, retention = 11.4 min (contaminate UTP absent and analogue was stable under assay conditions).

[a] Due to the near equivalence of the 5' and 5" protons only an average J_H is reported.

Biological assays: PC12 cells were cultured in RPMI media supplemented with 5% fetal calf serum, 5% heat-inactivated horse serum and 1% penicillin-streptomycin. $P2Y_2$ activity was determined by measuring the agonist-induced increase in intracellular Ca^{2+} using fura-2[14] as described by Raha et al.[15]. $P2X_2$ activity was monitored using the fluorescent potential-sensitive anionic dye, bisoxonol (bis[1,3-diethylthiobarbiturate]-trimethine oxonol)[16] as outlined previously[6].

RESULTS AND DISCUSSION

The major conformational variations in nucleotides are associated with modes of puckering of the ribofuranose ring and with rotations around the glycosidic and the 4',5' bonds. Based on X-ray crystallography, computer simulations and NMR spectroscopy of nucleotide conformations,[5, 17-18] it has been suggested that there is a low glycosidic rotational barrier with the *anti* rotomer in large excess over the *syn* rotomer.

Where the base and sugar are free to rotate, the *syn* and *anti* designations reflect a range of possible glycosidic bond angles as illustrated in FIG. 2 and a more accurate description of the glycosidic conformation requires defining the dihedral angle χ_{CN}. For purines, a χ_{CN} of 0° is when O(1') of the sugar and C(8) of the base are eclipsed[19]. For the pyrimidines, a χ_{CN} of 0° is when the O(1') of the sugar and C(6) of the base are eclipsed[20]. From X-ray crystallography data of the cAMP analogue, 8,2'-thioanhydroadenosine-3',5'-cyclic monophosphate[21], the adenine base is fixed with an χ_{CN} of 122° (high *anti* region) compared to the χ_{CN} for ATP of 39 and 69° [17] still in the designated anti range.

Several enzymes have been shown to require the nucleoside or nucleotide substrate in the favoured *anti* or high *anti* conformation. NMR studies on the

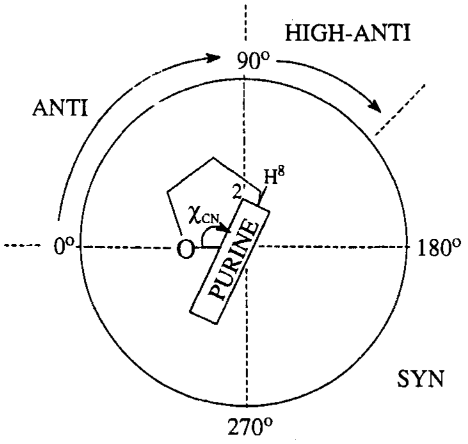


FIG. 2. Schematic representation of the position of the base in ATP. For 8,2'-thioanhydroATP the χ_{CN} is 122° (high-*anti* region).

conformation of ATP bound to rabbit muscle creatine kinase suggest that ATP binds preferentially in the *anti* conformation[3]. Adenosine deaminase favours adenosine analogues that possess high *anti* character as substrates[4]. Adenosine analogues favouring the high *anti* conformation have been shown to activate adenosine receptors in lymphocytes and cardiac cells[22]. With these studies in mind, the conformational specificity of the P2-purinoceptors in PC12 cells was assessed using 8,2'-thioanhydroATP (**4**), an *anti*-locked rotomer of ATP and 2,2'-anhydroUTP (**6**) a *syn*-locked rotomer of UTP. Although *syn* rotomers are not highly favoured in solution there are examples of enzymes preferring *syn*. For example, potent inhibitors of uridine phosphorylase, such as 2,2'-anhydro-5-substituted uridines, are fixed rotomers of uridine with high *syn* character[23]. The X-ray crystal structure of an ATP bound-phosphoenolpyruvate carboxykinase (PCK) complex stabilizes a *syn* conformation of the base[24]. An investigation of the energy profiles of the *syn* and *anti* conformers of nucleosides by quantum mechanical methods suggests the difference in energy between *syn* and *anti* conformers is less than 1 kcal•mole^{-1}.[22] Thus the small energetic cost incurred by binding ATP when it is in a *syn* conformation appears to be outweighed by the favourable interactions it promotes with the enzyme.

Activation of $P2Y_2$-purinoceptors in PC12 cells elicits a transient increase in $[Ca^{2+}]_i$ triggered by intracellular release from the endoplasmic reticulum as well as from Ca^{2+} influx. FIG. 3 shows a series of typical calcium transients; ATP and UTP at concentrations as low as 10 μM produced appreciable responses, while the addition of

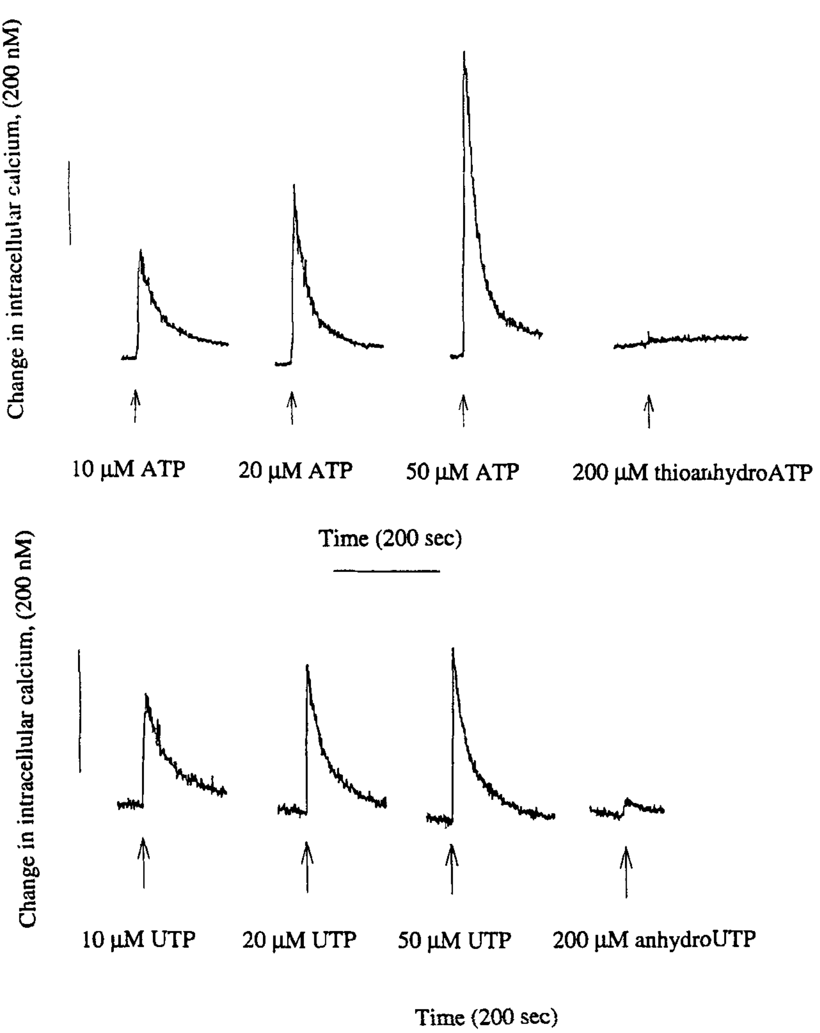


FIG. 3. Activation of P2Y$_2$-purinoceptors in PC12 cells leads to a transient increase in intracellular calcium as measured using fura-2.

200 µM 8,2'-thioanhydroATP (**4**) was without effect. In separate experiments, concentrations as high as 500 µM also produced no response (data not shown). 200 µM 2,2'-anhydroUTP (**6**) only produced a greatly reduced although reproducible transient. Neither analogue (200 µM) antagonized the response elicited by ATP or UTP (20 µM) (data not shown).

Activation of P2X$_2$-purinoceptors leads to membrane depolarization and in intact PC12 cells this can be measured using potential sensitive lipophilic anionic dyes such as

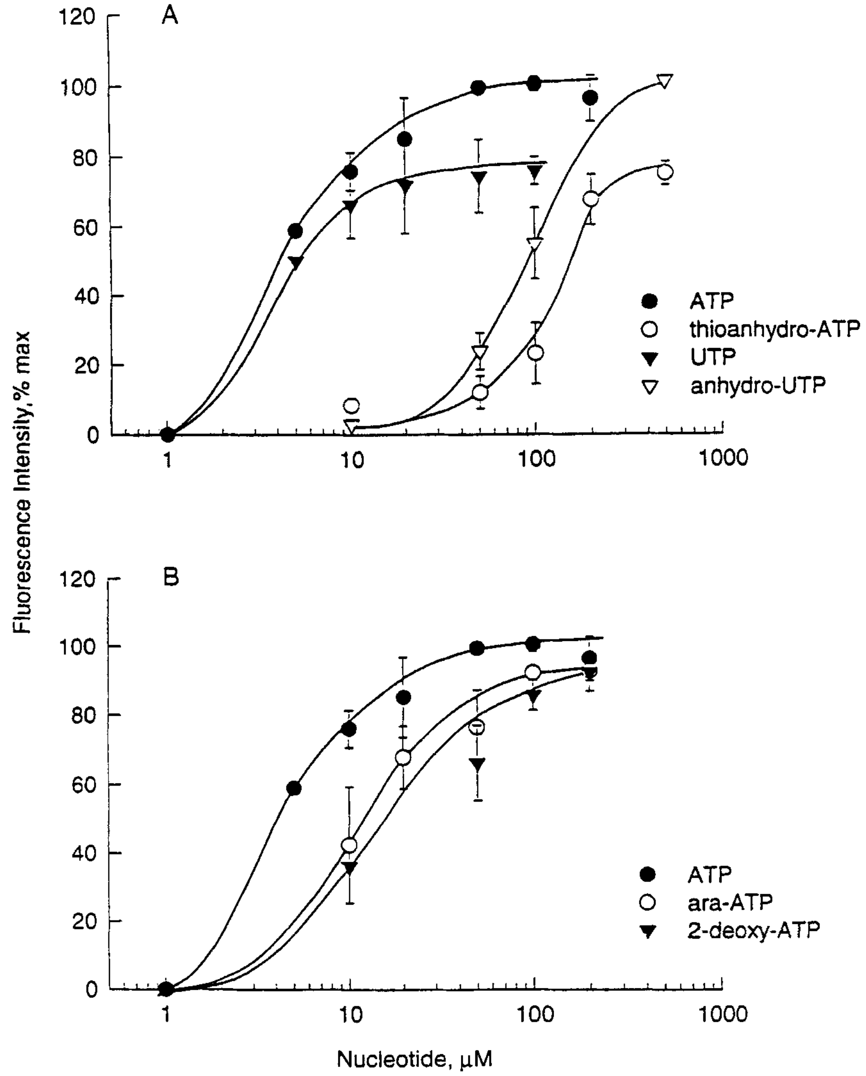


FIG. 4. Agonist-induced depolarization of PC12 cells measured by an increase in bisoxonol fluorescence (value ± SEM)

bisoxonol. FIG. 4 shows the dose response profiles for ATP, UTP, the locked nucleotide analogues and the two 2'-modified nucleotides araATP and 2'-deoxyATP. Both 8,2'-thioanhydroATP (**4**) (EC_{50} ~150 μM) and 2,2'-anhydroUTP (**6**) (EC_{50} ~100 μM) are agonists for $P2X_2$-purinoceptors in PC12 cells although both are significantly weaker than the parent nucleotides (EC50 ~4 μM). Both araATP and 2'-deoxyATP were clearly good agonists (EC_{50} ~12-15 μM) suggesting that the reduced efficacy of the conformationally locked analogues is not due to loss of the 2'-hydroxyl and possible H-bonding capacity nor can it be attributed solely to a loss of stereochemistry at that position.

The lack of P2Y$_2$-purinoceptor agonist activity of either locked nucleotide suggests that the ligand recognition site in these receptors may not accommodate the molecule in either the constrained high *anti* or even a smaller but inflexible *syn* conformation. Alternatively, the constraints imposed by either the sulfur or oxygen bridge between the base and C(2') may distort the molecule especially the sugar pucker. The observation that both molecules are significantly more potent with P2X$_2$-purinoceptors may not be altogether surprising given that the membrane topography of these receptors is completely different from that of the metabotropic receptors. Indeed it would appear that the ligand recognition site in P2X$_2$-purinoceptors may accommodate far more base flexibility than that of the P2Y$_2$-purinoceptor. Whether this selectivity holds for other members of the P2 superfamilies remains to be determined.

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