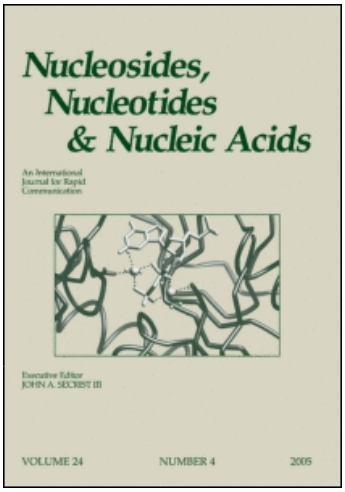

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Design, Syntheses and Pharmacology of ATP Analogues Selective for Subtypes of P_2-Purinoceptors

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DESIGN, SYNTHESES AND PHARMACOLOGY OF ATP ANALOGUES SELECTIVE FOR SUBTYPES OF P_2-PURINOCEPTORS

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Abstract: A series of analogues of adenine nucleotides have been synthesized and tested for pharmacological potency and resistance to dephosphorylation at a variety of isolated tissue preparations where ATP is active. Structure-activity studies defined four subtypeso f purinoceptors, and enabled the design of specific agonists for P_{2X} and for P_{2Y} purinoceptors to be undertaken. L-Adenosine 5'-ß,γ-methylenetriphosphonate (L-AMP-PCP) is a specific P_{2X} purinoceptor agonist, and adenosine 5'-ß-fluorodiphosphate (ADP-ß-F) is a specific P_{2Y} agonist.

INTRODUCTION

The activity of extracellular adenine nucleotides, ATP (Fig. 1), ADP, and AMP, are mediated by P_2-purinoceptors.[1] Differences in responses of various tissue preparations towards ATP, ADP, and AMP, and some analogues of ATP, lead to a division into four subtypes, the P_{2X}-, P_{2Y}-, P_{2T}-, and P_{2Z}-purinoceptors.[2] We synthesized and tested the pharmacological actions of a variety of analogues of adenine nucleotides in order to examine the structure-activity relationships for each subtype of P_2-purinoceptor. Rat mast cells, where ATP induces permeabilization, were used to study the P_{2Z} subtype; human platelets, where ADP induces aggregation, were used to study the P_{2T} subtype; guinea-pig taenia coli, where ATP induces relaxation, was used to study the P_{2Y} subtype; and guinea-pig urinary bladder, where ATP induces contraction, was used to study the P_{2X} subtype of P_2-purinoceptors.

SYNTHESES OF ANALOGUES OF ADENINE NUCLEOTIDES

Analogues of AMP[3] were synthesized from the parent nucleosides by direct phosphorylation with phosphoryl chloride in trimethyl orthophosphate.[4] In this way, L-AMP,[5] 2-azido-AMP,[6] 2-azido-L-AMP,[7] 2-chloro-AMP,[8] 2-chloro-L-AMP,[7] 2-methylthio-

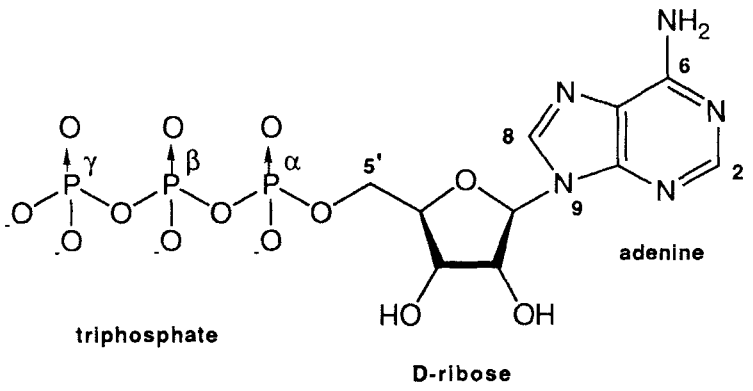

FIG. 1. Structure of ATP

AMP,[8] 2-methylthio-L-AMP,[9] N^6-phenyl-AMP[10] and 8 bromo-AMP[11] can be obtained from the corresponding adenosine analogues. The monophosphorothioates, AMPS,[12] 2-chloro-AMPS,[13] and 2-ethylthio-AMPS[13] are prepared similarly by using thiophosphoryl chloride. However, homo-AMP,[14] where the oxygen linking 5'phosphate to the ribose sugar has been replaced by methylene to generate a phosphonate analogue of AMP, requires oxidation of 2',3'-O-isopropylideneadenosine to the 5'-aldehyde,[15] reaction of this with diphenyl triphenylphosphoranylidenemethylphosphonate,[16] followed by reduction of the unsaturated product and deprotection.

Conversion to the corresponding ADP analogues[3] can often be accomplished most easily by activation of the AMP analogues with excess carbonyldiimidazole followed by reaction of the formed AMP-phosphoroimidazolate with excess orthophosphate.[17] In this way, L-ADP,[5] 2-azido-ADP,[6] 2-azido-L-ADP,[7] 2-chloro-ADP,[8] 2-chloro-L-ADP,[7] 2-methylthio-ADP,[8] 2-methylthio-L-ADP,[7] N^6-phenyl-ADP,[10] 8-bromo-ADP,[18] and homo-ADP are obtained. However, ADP-α-S has to be made by activation[19] of AMPS with diphenyl phosphochloridate, and displacement of diphenyl phosphate by orthophosphate.[20] The mixture of Rp and Sp diastereoisomers of ADP-α-S is easily separated by reverse-phase, high-pressure, liquid chromatography (HPLC).[21] ADP-ß-S requires to be synthesized by displacement of diphenyl phosphate from activated AMP by S-carbamoylethyl derivatized thiophosphate, followed by removal of the carbamoylethyl group.[22] Displacement of tosylate from 5'-tosyladenosine by analogues of diphosphate is a convenient way to prepare otherwise difficult to obtain ADP analogues.[23] In this way, displacement by

methylenediphophonate, difluoromethylenediphosphonate, generates α,ß-methylene-ADP,[23] α,ß-difluoromethylene-ADP,[24] α,ß-dichloromethylene-ADP,[24] and α,ß-imido-ADP,[24] respectively.

ATP analogues[3] are conveniently prepared by reaction of the corresponding AMP-imidazolate with diphosphate,[17] and L-ATP,[5] 2-azido-ATP,[25] 2-azido-L-ATP,[25] 2-chloro-ATP,[26] 2-chloro-L-ATP,[9] 2-methylthio-ATP,[26] 2-methylthio-L-ATP,[9] N^6-phenyl-ATP,[10] 8-bromo-ATP,[11] and homo-ATP[10,14] are made in this fashion. Similarly, reaction with methylendiphosphonate, difluoromethylenediphosphonate dichloromethylene-diphosphonate, and imidodiphosphate, generates ß,γ-methylene-ATP,[27] ß,γ-difluoromethylene-ATP,[28] ß,γ-dichloromethylene-ATP,[28] and ß,γ-imido-ATP,[29] respectively. Activation of orthophosphate with excess carboxyldiimidazole[30] followed by addition of α,ß-methylene-ADP affords α,ß-methylene-ATP.[10] Displacement by diphosphate of diphenylphosphate from AMPS that has been activated with diphenyl phosphochloridate affords ATP-α-S,[20] whose Rp and Sp diastereoisomers are easily separable by reverse-phase HPLC.[31] The Rp and Sp diastereoisomers of ATP-ß-S are best synthesized enzymically from ADP-ß-S,[20,32] by incubation with pyruvate kinase, and acetate kinase, respectively. ATP-γ-S can be made by addition of ADP to 5-carbamoylethyl thiophosphate, followed by removal of the carbamoylethyl group.[22]

P_{2Z}- PURINOCEPTOR ON MAST CELLS

Rat mast cells are susceptible to permeabilization by ATP, the effective agonist at the P_{2Z}-purinoceptor being ATP^{4-}.[33] Permeabilization is followed conveniently by monitoring the increase in fluorescence that occurs after uptake of the normally impermeant dye cation, ethidium.[33] The P_{2Z}-purinoceptor on mast cells that mediates permeabilization of the plasma membrane is extremely sensitive to alterations to the ATP molecule. Of the naturally occurring nucleotides, only ATP itself induces permeabilization, while AMP, ADP, GTP, CTP and UTP are inactive.[33] Substitutions on the N^6 or C^8 position of the adenine base lead to loss of activity, but C^2 substitution is allowed as 2-chloro-ATP, 2-methylthio-ATP, and 2-ethylthio-ATP, are as active as ATP. The ß-D-ribofuranose sugar is an absolute requirement, as the unnatural enantiomer L-ATP, is inactive, as are 2-chloro-L-ATP and 2-methylthio-L-ATP.[34] Replacement by sulphur of an ionized oxygen on the innermost phosphate, or middle phosphate of the 5′-triphosphate chain is allowed, and ATPα-S and ATPß-S have enhanced permeabilizing activities relative to ATP, but the receptor does not distinguish between the Rp and Sp diastereoisomers.[34] Other alterations to the triphosphate chain, as in homo-ATP, α,ß-methylene-ATP and ß,γ-methylene-ATP, generate inactive

analogues and no competitive antagonists of the mast cell P_2 purinoceptor are known. However, 2-methylthio-L-ATP inhibited weakly ATP-induced permeabilization, and may point to the design of more potent inhibitors.[34]

P_{2T}-PURINOCEPTOR ON BLOOD PLATELETS

The human platelet P_{2T}-purinoceptor is unique among P_2 purinoceptors in that ADP is an agonist, inducing platelet aggregation, whereas AMP and ATP are specific competitive antagonists of ADP.[35] The human platelet P_{2T}-purinoceptor is very sensitive to modifications to the structure of ADP molecule. The adenine base is an absolute requirement, CDP, GDP, and UDP being inactive, and substitutions on the C^8 or N^6 position lead to a severe reduction of agonist potency, but a variety of substituents may be placed at the C^2 position with no loss of potency. 2-Azido-ADP, 2-chloro-ADP, and 2-methylthio-ADP, are up to 10-fold more potent than ADP at inducing platelet aggregation[6,8] and up to 300-fold more potent than ADP at inhibiting stimulated adenylate cyclase in intact human platelets.[36] Since very large substituents are tolerated at the C^2 position,[37] this region of the adenine base is probably orientated away from the ADP receptor. The ß-D-ribofuranose sugar is essential for agonist activity, and cannot be replaced by other pentose or hexose sugars. The P_{2T}-purinoceptor exhibits absolute stereoselectivity for ADP as the unnatural ß-L-ribofuranose enantiomers, L-ADP, 2-chloro-L-ADP, and 2-azido-L-ADP are completely inactive as agonists.[7] The unmodified 5′-diphosphate chain of ADP is required for full agonist potency. Replacement of an ionized oxygen of the inner or outer phosphate by ionized sulphur is allowed, but ADP-α-S and ADP-ß-S, are both less potent than ADP at inducing platelet aggregation, and are partial agonists.[21,38] The diastereoisomers of ADP-α-S are distinguished by the ADP receptor, since Sp-ADP-α-S is 5-fold more potent than Rp-ADP-α-S at inducing platelet aggregation.[21] ADP-ß-S also inhibits stimulated adenylate cyclase as a partial agonist,[38] but ADP-α-S does not and instead antagonizes competitively this action of ADP, and Sp-ADP-α-S is 5-fold more potent than Rp-ADP-α-S.[21] Replacement of the oxygen linking the inner and outer phosphates by imido is barely tolerated as α,ß-imido-ADP is 100-fold less potent than ADP at inducing platelet aggregation and it does not inhibit stimulated adenylate cyclase.[24] Replacement of the oxygen joining the ribose to the diphosphate chain by methylene to produce homo-ADP, or a terminal ionized oxygen by fluorine to produce ADP-ß-F, generates inactive analogues.[39]

The structure-activity relationships for antagonists are much more relaxed than for agonists, and a wide variety of analogues of AMP, ADP and ATP are competitive inhibitors of both ADP-induced aggregation and ADP-induced inhibition of stimulated adenylate

cyclase. Analogues of AMP and of ATP, shown by Schild analysis to be competitive inhibitors of both these actions of ADP, were used to show that one type of ADP receptor, rather than two different subtypes of ADP receptor, mediated both effects of ADP.[31] Replacement of the oxygen linking the inner and outer phosphates by methylene, difluoromethylene or by dichloromethylene is tolerated and α,ß-methylene-ADP, α,ß-difluoromethylene-ADP, and α,ß-dichloromethylene-ADP, are antagonists, but Schild analysis showed that only α,ß-dichloromethylene-ADP had competitive kinetics for inhibition of aggregation, and only α,ß-difluoromethylene-ADP and dichloromethylene-ADP antagonized inhibition by ADP of stimulated adenylate cyclase.[24] Substitution of ADP at the C^8 position by bromine is allowed and 8-Br-ADP is a specific inhibitor of ADP induced aggregation.[18] ADP receptor antagonism by 2-alkylthio analogues of AMP and of ATP is anomalous since, although they do not inhibit aggregation induced by other agents, they antagonize completely ADP-induced inhibition of stimulated adenylate cyclase, but only 60% of ADP-induced aggregation.[39,40] One of these analogues, 2-methylthio-ß,γ-methylene-ATP, inhibits aggregation induced by ADP-ß-S, which antagonizes stimulated adenylate cyclase, but not aggregation induced by ADP-α-S, which does not antagonize adenylate cyclase, which suggests that 2-alkylthio analogues are selective for one component of aggregation.[41] ß-[^{32}P]-2-azido-ADP binds to 200 - 500 sites per platelet, with a K_D of 60-125 nM.[42] Binding is displaced by ATP but only weakly by AMP and not by adenosine, in accord with their pharmacology. Photolysis of platelets in the presence of 2-azido-ADP, however, fails to label covalently proteins relevant to the adenine nucleotide receptor. Radioligand binding of ß-[^{32}P]-2-methylthio-ADP to intact platelets in plasma detects one class of binding site, with 400-1200 ADP receptors per platelet, a K_D of 5-20 nM , and displacements of binding by agonists and by antagonists in accord with their pharmacology.[43] Radioligand binding of 2-[^{3}H]-ADP to formaldehyde-fixed intact platelets detects both high affinity and low affinity ADP binding sites, with dissociation constants of 0.35 μM and 7.9 μM respectively,[18] and displacement by agonists and antagonists was in some accord with their pharmacology.[44]

P_{2Y}-PURINOCEPTOR ON TAENIA COLI

ATP relaxes the guinea-pig taenia coli, and this tissue preparation has been used extensively in structure-activity relationships for the inhibitory P_{2Y} purinoceptor.[45] The adenine base is required for maximal activity, ADP is equipotent with ATP, and GTP, CTP, and UTP are also active, but AMP, GDP, CDP and UDP are much less active than ATP at inducing relaxation.[11] Substitutions on the adenine base at N^6 abolish activity, but those

at C^8 are approximately as potent as ATP, while C^2 substitution enhances potency up to 200-fold that of ATP.[26,9] Replacement of the ribose by other sugars generates analogues less potent than ATP,[11] and L-ATP is 3- to 6-fold less potent than ATP.[25] Stereoselectivity towards the enantiomers increases greatly with the C^2 substituted analogues, and 2-methylthio-ATP is 24-fold more potent than 2-methylthio-L-ATP.[9,25] The effect of modifications of the 5′-triphosphate chain depends on where they take place, so that homo-ATP is 70-fold more potent than ATP, α,ß-methylene-ATP is equipotent with ATP, and ß,γ-methylene-ATP is 10-fold less potent than ATP.[46] The isopolar versions, ß,γ-difluoromethylene-ATP and ß,γ-dichloromethylene-ATP are more potent than ß,γ-methylene-ATP but still less potent than ATP itself, but 2-methylthio-ß,γ-difluoromethylene-ATP was twice as potent as ATP though again much less potent than 2-methylthio-ATP itself.[47] Of the phosphorothioate analogues of ATP, stereoselectivity is exhibited only towards the diastereoisomers of ATP-α-S, the Rp isomer being 50-fold and the Sp isomer 9-fold more potent than ATP, while ATP-ß-S and ATP-γ-S are about as potent as ATP.[48] No competitive antagonists of the inhibitory P_{2Y} purinoceptor are known. Adenosine 5′-[2-fluorodiphosphate] ADP-ß-F appeared to be a suitable candidate for a specific P_{2Y} agonist, because it has the same number of negative charges as AMP, which is inactive at P_{2X}, P_{2Z} and P_{2T} purinoceptors and, having a similar size to ADP, is active at P_{2Y}-purinoceptors. ADP-ß-F, synthesized by reaction of AMP with excess carbonyldiimidazole-activated monofluorophosphate, is a specific agonist for P_{2Y} purinoceptors, with no agonist or antagonist activity at P_{2X} receptors, platelet P_{2T} receptors or mast cell P_{2Z} receptor.[49]

P_{2X}-PURINOCEPTOR ON URINARY BLADDER

ATP contracts rapidly the guinea-pig urinary bladder, and this excitatory P_{2X} purinoceptor has the most promiscuity in its structure-activity relationships.[45] GTP, CTP and UTP are nearly as active as ATP and ADP, but GDP, CDP and AMP are inactive.[50] Substitutions on the adenine base at N^6 as in N^6-phenyl-ATP abolishes activity, at C^8 as in 8-Bromo-ATP do not affect activity, and in contrast to P_{2Y} receptors, C^2 substitutions do not improve potency relative to ATP.[9,51] Again, in contrast to P_{2Y} receptors, L-ATP is equipotent with ATP and this lack of stereoselectivity by the P_{2X} receptor is not altered by C^2 substitution. The effects of modifications of the 5′-triphosphate chain depends on where they take place. Homo-ATP is equipotent with ATP, but α-ß-methylene-ATP and ß,γ-methylene-ATP are much more potent than ATP.[50,51,47] Again in contrast to the P_{2Y} receptor, the isopolar analogues ß,γ-difluoromethylene-ATP and ß,γ-dichloromethylene-ATP are no

more potent than ß,γ-methylene-ATP itself. Of the phosphorothioate analogues of ATP, no stereoselectivity is exhibited towards the diastereoisomers, and ATP-ß-S is more potent than ATP, while ATP-α-S is considerably less potent than ATP,[48] again in contrast to the P_{2Y} receptor. No competitive antagonists of the excitatory P_{2X} purinoceptor are known. The unnatural enantiomer of ß,γ-methylene-ATP, ß,γ-methylene-L-ATP (L-AMP-PCP)[52] seemed likely to be a candidate for a specific P_{2X}-purinoceptor agonist because is combines the lesser potencies relative to ATP of L-ATP and of ß,γ-methylene-ATP, and cannot be dephosphorylated to an active nucleoside. L-AMP-PCP is a very potent specific agonist for P_{2X}-purinoceptors, with no agonist or antagonist activity at P_{2Y} inhibitory receptors, platelet ADP receptors, or mast cell ATP receptors.[34,52,53,54,55] L-AMP-PCP is synthesized by activation of L-AMP with carbonyldiimidazole, and reaction of the imidazolate with methylenediphsophonate.[52] L-AMP-PCP provides a framework for the design of other potent specific agonists for P_{2X} receptors, including the ß,γ-dihalomethylene versions, L-AMP-PCF_2P and L-AMP-$PCCl_2P$,[45,47] all of which, like L-AMP-PCP itself, are resistant to dephosphorylation by the ectonucleotidases present on smooth muscle.[10,51,56]

SUMMARY AND CONCLUSION

Analogues of adenine nucleotides have been used to study the pharmacology of P_2-purinoceptors, and structure-activity relationships obtained support a division into the P_{2Z}-, P_{2T}-, P_{2X}-, and P_{2Y}-subtypes. Specific agonists for P_{2X}- and P_{2Y}-purinoceptors have been described, and the challenge lies now in the design of inhibitors of ectonucleotidases that dephosphorylate adenine nucleotides, and of specific competitive antagonists for especially the P_{2Z}-, P_{2X}- and P_{2Y}-subtypes of P_2-purinoceptors, so as to provide pharmacological tools to help further an understanding of the purinergic regulation of cell function.[57]

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