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REGULATORY EFFECTS OF PURINE NUCLEOTIDE ANALOGS WITH LIVER GLUTAMATE DEHYDROGENASE

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Summary

A total of 26 different purine nucleotides with specific modifications in the base moiety and/or in the polyphosphate chain as well as various combinations of nucleotides were tested as allosteric effectors of beef liver glutamate dehydrogenase (L-glutamate : $NAD(P)^+$ oxidoreductase (deaminating), EC 1.4.1.3).

The capacity of these nucleotide analogs to activate or to inhibit the glutamate dehydrogenase activity is expressed quantitatively and scaled between the extreme effects of ADP and GTP, respectively. The significance of distinct structural elements for the enzyme-effector interaction is discussed.

While the inhibitory GTP site is less specific, accepting many natural and most modified nucleoside triphosphates as inhibitors, the activating ADP site shows a much higher specificity for nucleotides as activators.

Introduction

Mammalian glutamate dehydrogenase (L-glutamate : $NAD(P)^+$ oxidoreductase (deaminating), EC 1.4.1.3) which catalyzes the interconversion of α-ketoglutarate and L-glumatic acid, is a mitochondrial enzyme known to use a wide variety of modifiers. The reactive residues of this enzyme have been

Abbreviations: o^1AMP, o^1ADP and o^1ATP: adenosine-N^1-oxide 5′-mono-, 5′-di-, and 5′-triphosphate; ϵADP, ϵATP: 1,N^6-ethenoadenosine 5′-di-, and 5′-triphosphate; 8-BrADP, 8-BrATP: 8-bromoadenosine 5′-di-, and 5′-triphosphate; iGDP, iGTP: isoguanosine 5′-di, and 5′-triphosphate; o^1IDP, o^1ITP: inosine-N^1-oxide 5′-di-, and 5′-triphosphate; AOPCP: adenosine 5′-methylene diphosphonate; XDP, XTP: xanthosine 5′-di-, and 5′-triphosphate; AMP-P(CH_2)P: 5′-adenylyl-(β,γ-methylene)diphosphonate; AMP-P(NH)P: 5′-adenylyl-(β,γ-imido)diphosphate; GMP-P(CH_2)P: guanosyl-(β,γ-methylene)diphosphonate; GMP-P(NH)P: guanosyl-(β,γ-imido)diphosphate; ATP-(γF): adenosine 5′-(3-fluoro)triphosphate; ATP(γCH_3): adenosine 5′-(3-*O*-methyl)triphosphate; ATP(γPh): adenosine 5′-(3-*O*-phenyl)triphosphate.

probed by a large number of reagents and methods and the complex and often conflicting results reviewed by Goldin and Frieden [1], Fisher [2], Dalziel [3] and Smith et al. [4]. We were particularly interested in the allosteric control of glutamate dehydrogenase activity by purine nucleotides. Ever since Frieden [5] showed that GTP inhibits and ADP activates the enzyme, attempts have been made to clarify the mechanism and to determine the sites of these effects. However, due to the unusually complex behaviour of the extensively investigated beef liver enzyme, this has not yet been achieved and the metabolic significance of the enzyme inhibition by GTP and /or its activation by ADP is not yet fully understood.

In the present investigation we tested various purine nucleotides with specific modifications in the base moiety and/or in the polyphosphate chain with the aim of identifying possible correlations between certain structural elements of these nucleotides and their capability to activate or to inhibit beef liver glutamate dehydrogenase.

Materials and Methods

Chemicals

Commercially available nucleotides were obtained from Boehringer, Mannheim (ATP, ADP, AMP, GTP, GDP, AMP-P(CH_2)P, AMP-P(NH)P, GMP-P(CH_2)-P, GMP-P(NH)P, NADH and NADPH) and from Calbiochem (AOPCP). Beef liver L-glutamate dehydrogenase was purchased from Fluka, Switzerland. o^1ATP, o^1ADP and o^1AMP were prepared by a gentle oxidation of the natural nucleotides with permaleic acid in aqueous solutions at neutral pH [6]. iGTP and iGDP were obtained by a photoisomerisation reaction of the corresponding adenine-1-oxide nucleotides [7]. ITP, IDP, o^1ITP, o^1IDP, XTP and XDP were prepared by oxidative deamination of ATP, ADP, o^1ATP, o^1ADP, GTP and GDP, respectively. 8-BrATP and 8-BrADP were obtained by bromination of ATP and ADP in aqueous solutions [8]. ϵATP and ϵADP were synthesized according to the method of Secrist et al. [9] and were a gift of Professor Klingenberg, München, Germany. ATP(γF) was synthesized essentially as described by Haley and Yount [10]. ATP(γCH_3) and ATP(γPh) were synthesized according to the method of Eckstein et al. [11] and were a gift of Professor Eckstein, Göttingen, Germany. The modified nucleotides were purified by ion exchange column chromatography on Dowex-1-carbonate (mesh 200—400) and eluted with a linear gradient of ammonium bicarbonate (0.01—1 M). The purity of each of the nucleotides was checked by their spectroscopic properties and by thin layer chromatography on PEI-cellulose [12]. The following millimolar extinction coefficients (at neutral pH) were used for the evaluation of the nucleotide concentrations: 15.4 at 260 nm for ATP, ADP, AMP, AMP-P(CH_2)P, AOPCP, AMP-P(NH)P, ATP(γF), ATP(γCH_3) and ATP-(γPh); 14.5 at 265 nm for 8-BrATP and 8-BrADP; 5.7 at 265 nm for ϵATP and ϵADP; 40.8 at 233 nm for o^1ATP and o^1ADP; 44.8 at 228 nm for o^1ITP and o^1IDP; 11.2 at 293 for iGTP and iGDP; 13.7 at 253 nm for GTP, GDP, GMP-P-(CH_2)P and GMP-P(NH)P; 12.7 at 249 nm for ITP and IDP; 8.9 at 278 nm for XTP and XDP. Most triphosphates and diphosphates are substrates for phosphofructokinase and pyruvate kinase respectively [13,14], so that their con-

centration could also be determined enzymatically in the presence of excess enzyme and fructose 6-phosphate or phospho*enol*pyruvate, respectively. There was good agreement between these two methods (±3%).

Assay of glutamate dehydrogenase activity

Unless otherwise stated, the reaction medium contained in a final volume of 1 ml at 24°C: 80 mM Tris/acetate buffer, pH 8, 100 mM NH_4Cl, 50 mM α-ketoglutarate, 0.08 mM NADH, 0.01 mM EDTA, and the corresponding nucleotides. The reaction was triggered by addition of pure beef liver L-glutamate dehydrogenase. All enzymatic reaction rates were determined at 366 nm with an Eppendorf 1101 photometer, equipped with a W + W type 4410 recorder (full scale deflection 0.25 absorbance units).

Results

Regulatory effects of natural and modified purine nucleotide triphosphates

The natural inhibitor of glutamate dehydrogenase is GTP which is by far the strongest and the most specific inhibitor of this enzyme. As shown in Table I, the other natural purine triphosphates, as well as the investigated triphosphate analogs, have not completely lost the property of regulation, although the inhibitory effect is much reduced and varies over three orders of magnitude. One exception, the phosphate-modified ATP analogs, will be discussed separately. The natural triphosphates ITP and XTP, which like GTP have a keto group at C_6 and a hydrogen at N-1, show an inhibitory effect on glutamate dehydrogenase of 4.5 and 3.3% of that of GTP, while under comparative conditions the effect of ATP, the most abundant natural triphosphate, is only 0.15%. The effect of ATP on glutamate dehydrogenase had been shown to depend on a

TABLE I

ALLOSTERIC INHIBITION OF BEEF LIVER GLUTAMATE DEHYDROGENASE BY NATURAL AND MODIFIED PURINE NUCLEOSIDE TRIPHOSPHATES

I_{50} represents the concentration required for 50% inhibition while I_{max} is the maximal inhibition obtained. The actual glutamate dehydrogenase activity in the absence of any purine nucleotide was 72 μmol/min per mg protein and was considered as 100%. k and k^* represent the relative I_{max}/I_{50} ratios for GTP and the corresponding analog. The $-k^*/k$ ratio is a better measure than the corresponding I_{50} value for defining the inhibitory efficiency of substrate analogs since it includes also the maximal inhibition. The negative sign indicates a negative allosteric effect.

Nucleotide	I_{50} (μM)	I_{max} (%)	$-100 \cdot k^*/k$
GTP	0.12	100	100
GMP-P(NH)P	0.16	89.7	67.28
GMP-P(CH_2)P	0.83	88.5	12.80
iGTP	2.6	100	4.62
ITP	2.5	93	4.46
XTP	3.6	100	3.33
o^1ITP	20	92	0.55
εATP	30	91	0.36
8-BrATP	46	60	0.15
ATP	46	57	0.15
o^1ATP	164	66	0.05

great number of factors, such as the source of the enzyme, the pH, the concentration of NH_4^+ or the nature and concentration of the pyridine nucleotides used [15—17], but it is always at least two orders of magnitude less than that of GTP.

Interesting results are provided by the chemically modified purine nucleotides shown in Table I. The GTP analogs GMP-P(NH)P and GMP-P(CH_2)P where the terminal P-O-P moiety has been replaced respectively by a P-NH-P and a P-CH_2-P group, are both good allosteric effectors of glutamate dehydrogenase. A first conclusion is that the specificity of glutamate dehydrogenase for GTP seems to be localized in the base and/or ribose moiety. However, in spite of the similarities of the P-O-P, P-NH-P and P-CH_2-P fragments, there are evident quantitative differences. If the inhibitory effect of GTP is taken as 100% (expressed as the $100 \cdot k^*/k$ ratio in Table I), the efficiency of GMP-P(NH)P and GMP-P-(CH_2)P under identical conditions is 67.3% and 12.8%, respectively. The structural difference among these analogs is due to the different geometry of the terminal P-X-P fragment. The bond angles and distances for the P-O-P, P-N-P and P-C-P moieties are 130.2° and 1.61 Å, 127.2° and 1.68 Å and 117° and 1.79 Å, respectively [18,19]. As shown by these structural GTP-analogs, a more acute P-X-P bond angle and a larger P-X bond distance of the terminal phosphate gradually reduces the interaction with the enzyme. Consequently the terminal phosphate also must be implicated in the binding to the GTP-sensitive site. We have shown recently [14] in the case of rabbit muscle phosphofructokinase that for a similar reason AMP-P(CH_2)P is a much weaker competitive inhibitor than AMP-P(NH)P.

The ITP-analog o^{1}ITP where the N-1/C-6 amide structure of ITP has been replaced by that of a cyclic hydroxamic acid, has an efficiency as effector which is about 8 times smaller than that of ITP. ATP and base-modified ATP-analogs are all at the bottom in Table I, being very weak inhibitors as compared to GTP. There are also distinctions among these; the N_1-oxide analog o^{1}ATP, which structurally is similar to the etheno-derivative ϵATP, is almost 8 times weaker as an inhibitor than the latter. It is also interesting that the effect of 8-BrATP on glutamate dehydrogenase is almost identical to that of ATP. Due to its bulky substituent in position 8 and its unusual syn conformation 8-BrATP is generally an extremely poor ATP substrate substitute and has completely lost its regulatory properties with phosphofructokinase [14].

Interesting is the behaviour of the newly synthesized analog iGTP [7] which is both a structural analog of ATP with an additional ketonic group at C_2, and a positional isomer of GTP. Indeed, iGTP shows the highest efficiency for a base modified GTP-analog, which represents actually only 4.62% that of GTP, but 3080% that of ATP. This shows that for this enzymatic system iGTP behaves much more like a GTP-analog than an ATP-analog.

Allosteric effects of ATP analogs with modifications in the polyphosphate chain

It is surprising that, as shown in Table II, all investigated ATP-analogs with a modified polyphosphate chain increase the glutamate dehydrogenase activity at pH 8. This activation effect is rather interesting in view of the fact that ATP

TABLE II

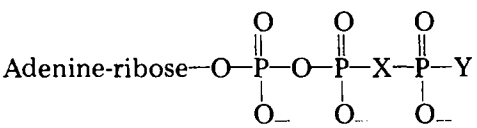
ALLOSTERIC ACTIVATION OF BEEF LIVER GLUTAMATE DEHYDROGENASE BY PHOSPHATE-MODIFIED ATP ANALOGS

K_a is the half maximal activation and A_{max} the maximal achieved activation. k and k^* represent the relative A_{max}/K_a ratios for ADP (the natural activator) and the corresponding analog. The positive sign of the k^*/k ratio indicates a positive allosteric effect.

Nucleotide	K_a (μM)	A_{max} (%)	$100 \cdot k^*/k$
AMP-P(NH)P	36	200	46.30
ATP(γF)	48	167	29.00
AMP-P(CH_2)P	74	200	22.52
ATP(γCH_3)	66	160	20.20
ATP(γPh)	81	190	19.55

and the base modified ATP-analogs are all negative and not positive effectors of glutamate dehydrogenase activity.

As shown below, the common feature of these ATP-analogs is a modified terminal phosphate group:



X=O	Y=OH	ATP
X=O	Y=F	ATP(γF)
X=O	Y=OCH_3	ATP(γCH_3)
X=O	Y=OC_6H_5	ATP(γPh)
X=CH_2	Y=OH	AMP-P(CH_2)P
X=NH	Y=OH	AMP-P(NH)P

The simplest explanation for this behaviour, which is unusual for triphosphates, could be that regardless of the particular modification, the altered terminal phosphate group behaves like an "inert" substituent so that these analogs actually mimic ADP as an allosteric modifier. Again, there are quantitative differences due perhaps to structural details of the "inactivated" third phosphate group. While AMP-P(NH)P still has an effect of activation which is 46.3% that of ADP, this effect is reduced to 22.5% in AMP-P(CH_2)P. As shown in the preceeding section for GMP-P(NH)P and GMP-P(CH_2)P, the P-CH_2-P analog is the weaker effector of the two, due to a larger deviation in the geometry of the P-X-P fragment. This difference must be due entirely to a difference in binding strength, since the maximal activation of these two analogs is the same. The replacement of a terminal OH group in ATP by fluorine (which has almost the same size as an OH group) leads to an ATP-analog which is used by glutamate dehydrogenase as an ADP-substitute with an efficiency of 29%. Bulkier substituents, such as OCH_3 or OC_6H_5 groups, reduce the efficiency to 20% but even these act as ADP-substitutes and not as ATP-analogs.

Regulatory effects of natural and modified purine nucleoside diphosphates

ADP, the natural activator of glutamate dehydrogenase activity, has been

shown to possess its own site for allosteric regulation, which is different from that of GTP [15,20]. As in the case of modified GTP analogs, we were interested in correlating the sensitivity of the ADP-specific site with certain structural modifications of this purine nucleotide. Again, the natural nucleotide ADP is so far the strongest known activator of glutamate dehydrogenase. Interesting enough, the other natural nucleotide diphosphates and most chemically modified diphosphates are all inhibitors and not activators of glutamate dehydrogenase. (Table III). Among the investigated derivatives, the only other positive effectors besides ADP, are 8-BrADP and AOPCP. A bromine substituted imidazole ring as in 8-BrADP reduces the efficiency of ADP to 4.4% while a replacement of the P-O-P fragment by P-CH_2-P as in AOPCP yields an effect which is less than 2.2%.

The inhibitory effect of nucleoside diphosphates is also structurally dependent and varies as shown in Table III from the extremely low effect of o^{1}ADP to the relatively high effect shown by GDP, which is 3.2% that of GTP. iGDP which again is both a structural ADP analog and a positional isomer of GDP, behaves much more like GDP. Actually the more the diphosphate analogs resemble GTP, the higher their inhibitory effect. GDP, which is only lacking the third phosphate group, comes closest in inhibitory effect to GTP, indicating that the severest structural restriction is localized in the base moiety and particularly in the substituted pyrimidine ring of the base.

The structural characteristics of the investigated diphosphates show that the ADP site does not accept other natural diphosphates besides ADP while the GTP site is able to use besides GTP all other natural purine triphosphates. Since we have shown earlier that the phosphate modified ATP analogs are, like ADP, positive allosteric effectors, we must assume that the ATP analogs in Table II have switched sites and do not bind to the GTP site, but to the ADP site. Similarly, the other diphosphates in Table III, which become inhibitors, appear to have switched to the inhibitory GTP-specific site. This actually would mean that the ADP site is activating only, while the GTP site is inhibitory only.

TABLE III

REGULATORY CONSTANTS OF BEEF LIVER GLUTAMATE DEHYDROGENASE FOR NATURAL AND MODIFIED PURINE NUCLEOSIDE DIPHOSPHATES

K_a, I_{50}, A_{max} and I_{max} are the half maximal activation, half maximal inhibition, maximal activation and maximal inhibition respectively. The k^*/k values represent the A_{max}/K_a ratios for the corresponding nucleotides relative to ADP or the I_{max}/I_{50} ratios for the corresponding nucleotides relative to GTP. A positive sign indicates an activating effect and a negative sign an inhibitory effect.

Nucleotide	K_a (μM)	I_{50} (μM)	A_{max} (%)	I_{max} (%)	$100 \cdot k^*/k$
ADP	25		300		100
8-BrADP	380		200		4.39
AOPCP	>500		140		<2.23
o^{1}ADP		>100		8	<−0.01
o^{1}IDP		80		82	−0.12
cADP		60		90	−0.18
IDP		46		85	−0.22
XDP		48		100	−0.25
iGDP		20		96	−0.58
GDP		3.7		100	−3.24

Effect of nucleotide combinations on the glutamate dehydrogenase activity

The complex behaviour of base- and phosphate-modified ATP analogs as allosteric effectors of beef liver glutamate dehydrogenase prompted us to investigate also the effect of various nucleotide combinations. These results are summarized in Table IV. In trying to discriminate between the binding of individual nucleotides to either the GTP site or the ADP site, it is important to bear in mind that while an observable allosteric effect always implies a binding interaction, a nucleotide could also bind to the enzyme without producing any apparent effect of activation or inhibition.

As shown in Table IV, GTP produces a drastic reduction of the enzyme activity both at pH 8 and pH 7. Although ATP alone inhibits the enzyme at pH 8, it diminishes the inhibitory effect of GTP. This effect of reactivation is more pronounced at pH 7 where even ATP can act as activator [21], which implies that under special conditions it can bind also to the ADP site. Addition of ADP to the GTP-inhibited enzyme can annihilate this inhibition. ATP(γF), with an "inert" terminal phosphate, behaves very similarly to ADP not only because it is an activator rather than an inhibitor at both pH values, but like ADP it is also able to reactivate the GTP-inhibited enzyme. Interesting is the effect of the nucleotide diphosphates o^1ADP and ϵADP on the GTP-inhibited enzyme. Although these two analogs have very similar structural and enzymatic properties [7,9], their role as effectors seems to differ greatly. o^1ADP, which is

TABLE IV

EFFECT OF NUCLEOTIDE COMBINATIONS ON BEEF LIVER GLUTAMATE DEHYDROGENASE AT pH 8 AND pH 7

Experimental conditions are given in the text. The enzyme activity is expressed in percentages and has been normalized for the enzyme activity in the absence of any nucleotide effectors at pH 8 and pH 7, respectively. The actual specific activities at pH 8 and pH 7, which were considered as 100% activity, were respectively 70 and 22 μmol NADH oxidized/min per mg protein.

Added nucleotides or combinations of nucleotides (μM)	Enzyme activity (%)	
	pH 8	pH 7
No additions	100	100
GTP (0.1)	56	84
GTP (0.3)	22	59
ATP (300)	60	194
GTP (0.1) + ATP (300)	55	184
GTP (0.3) + ATP (300)	46	148
ADP (100)	237	200
GTP (0.3) + ADP (100)	106	176
ATP(γF) (100)	158	245
GTP (0.3) + ATP(γF) (100)	77	200
o^1ADP (100)	96	
GTP (0.1) + o^1ADP (100)	54	
GTP (0.3) + o^1ADP (100)	20	
ADP (100) + o^1ADP (100)	229	
ϵADP (40)	42	
GTP (0.1) + ϵADP (40)	31	
GTP (0.3) + ϵADP (40)	13	
iGTP (3)	39	
GTP (0.1) + iGTP (3)	35	
GTP (0.3) + iGTP (3)	30	

practically without any effect on glutamate dehydrogenase, also does not modify the enzyme response to either GTP-inhibited or to ADP-activated enzyme, which must indicate a very weak binding of this analog to the enzyme. ϵADP on the other hand acts synergistic with GTP (also with GMP-P(NH)P), the inhibition of these two nucleotides being additive. Assuming that there is only one inhibitory site for nucleotides, this means that both the triphosphate GTP and the diphosphate analog ϵADP must bind to the same site. iGTP finally, which by itself is a much stronger inhibitor than ϵADP or ϵATP, does not show an additive inhibitory effect with GTP, but like ATP diminishes the GTP-inhibition at higher concentrations. This raises the question whether iGTP, which structurally is both an ATP analog and/or a positional isomer of GTP, can interact with either site, depending on the conditions.

Discussion

While purine nucleotides are known to play a major metabolic role both in the phosphoryl group transfer and as modulators of many key enzymes, it is interesting that their specificity not as substrates but as allosteric effectors has been investigated much less extensively. The exploration of this aspect of the metabolic role of nucleotides could, however, lead to a much better understanding of the in vivo effect of many structural nucleotide analogs. Since each of the six polypeptide subunits which make up the hexameric structure of mammalian glutamate dehydrogenase has one distinct binding site for ADP and one for GTP, this enzyme provides a very interesting model for investigating the binding forces involved in the regulatory behaviour of an allosteric site by means of structurally modified nucleotide analogs.

By now it seems well established (see ref. 4 and references therein) that the two natural modifiers GTP and ADP not only have opposite and mutually exclusive effects, but also distinct allosteric binding sites, although various lines of evidence indicate that the two sites are close to one another.

In order to have one scale only for measuring both activating and inhibitory effects, in Tables I—III we use the k^*/k ratios which not only include half-maximal (K_a, I_{50}) and maximal effects (A_{max}, I_{max}) but also scale these between the extreme effects of ADP (100) and GTP (—100). The larger the absolute value of this ratio, the higher the efficiency as effector, a positive value indicating a positive allosteric effect and a negative value a negative allosteric effect.

As shown in Tables I—IV, the inhibitory GTP site seems to be less specific, accepting many natural and most modified nucleoside triphosphates as inhibitors (even if the efficiency is lower), while the activating ADP site shows a much higher specificity, accepting only the natural nucleoside diphosphate ADP as activator. All other naturally occuring diphosphates are inhibitors. The binding to the inhibitory GTP site seems to be mostly due to the purine base and is localized primarily in the C-6/N-1 amide structure of the pyrimidine ring. Less important for this binding is the imidazole part of the purine base. Pyrimidine nucleotides such as CTP and UTP do not bind at all. A modified ribose structure such as in the 2'-deoxy analog dGTP has been shown by Frieden [5] to reduce the inhibitory effect of GTP to about 10%. Modifications in the phosphate chain such as in GTP-P-X-P derivatives also reduce the activity by an

order of magnitude. A much larger decrease is observed if the base itself is modified. For the analogs iGTP, ITP and XTP, which still possess a C-6/N-1 amide structure, the effect is reduced by 2 orders of magnitude with the same extent of inhibition at saturation. For ATP and its analogs the effect is reduced by 3 orders of magnitude.

The above results show that despite the tight binding of GTP (I_{50} = 0.12 μM) many other nucleoside triphosphates also are able to bind to this site. This would explain why even diphosphates (such as GDP, iGDP, IPD, XDP) could bind to this site, perhaps via their base part alone. On the other hand, the binding of ADP to the ADP site is weaker, (K_a = 25 μM), but much more specific for ADP and especially for its adenine moiety. As we have postulated elsewhere [7,13], we believe that for adenine nucleotides, the base-stacking interaction, with suitably located heteroaromatic aminoacid-residues such as that of histidine, plays an important role in their reversible interaction with allosteric enzymes such as phosphofructokinase [14]. The somewhat looser but more specific binding of ADP could be explained partly by such base-stacking interactions since all other natural nucleotides are much poorer "stackers". This would also explain why ATP analogs with an "inactivated" third phosphate group but with an intact adenine moiety can bind to this site too.

Although in the context of our structure-function correlations we have been able to identify certain sites of the purine nucleotides which are essential for the allosteric interaction with glutamate dehydrogenase, if the observed regulatory effect becomes very small, it is difficult to distinguish accurately by kinetic means whether a given nucleotide is binding to the GTP site or the ADP site. Therefore we have turned also to binding studies with the native and chemically modified enzyme [22]. Our preliminary studies using photoaffinity labels (Bârzu, O. et al., unpublished), show that glutamate dehydrogenase which has been previously irradiated in the presence of azidoadenine nucleotides loses its sensitivity to ADP-activation.

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