18O Labeling of Adenine Nucleotide α-Phosphoryls in Platelets

CONTRIBUTION BY PHOSPHODIESTERASE-CATALYZED HYDROLYSIS OF CAMP

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The kinetics of 18O incorporation into adenine nucleotide α-phosphoryls was examined in intact human platelets equilibrated with [18O]water to determine the extent to which this labeling process is related to phosphodiesterase-promoted hydrolysis of CAMP. 18O incorporation into nucleotide α-phosphoryls under basal conditions could be detected within 1 min and was found to proceed linearly for 20 min at a rate of 70 nmol of 18O/min/7.8 x 106 platelets (equivalent to 1 g wet weight). When platelet cAMP concentration was increased 10- to 40-fold by prostacyclin, the rate of total 18O incorporation increased 4- to 5-fold during the first 10 min of incubation. Between 10 and 20 min after exposure to prostacyclin, a spontaneous decline in platelet cAMP concentration attributable to "desensitization" is accompanied by a corresponding decrease in the rate of 18O labeling of α-phosphoryls. According to 18O-labeling rates of adenine nucleotide α-phosphoryls, an apparent value of Ks of 4 to 5 μM and Vmax of approximately 3 nmol/min/mg of protein can be calculated for the composite of operational phosphodiesterase activities in intact platelets. Equating 18O labeling of adenine nucleotide α-phosphoryls with cellular hydrolysis of cAMP, the t1/2 for turnover of the entire pool of this cyclic nucleotide is 200 ms under basal conditions and 1.3 s when the basal pool is increased 40-fold by prostacyclin.

Exposure of platelets to 1.5 mM 1-methyl-3-isobutylxanthine resulted in over 90% inhibition in the 18O-labeling rate at 1 min. However, there was a progressive accumulation of cAMP in platelets treated with 1-methyl-3-isobutylxanthine, which was accompanied by a progressive disappearance of inhibition. By 5 min when the new increased steady state level of cAMP was achieved, the rate of 18O labeling was the same in 1-methyl-3-isobutylxanthine-treated and untreated cells. This transient inhibition by 1-methyl-3-isobutylxanthine is behavior predictable of a dynamic metabolic system that can readjust levels of intermediates to overcome the influence of a competitive inhibitor and maintain metabolite flux.

A comparison of the rates and profiles of 18O incorporation into adenine nucleotide α-, β-, and γ-phosphoryls and orthophosphate indicated that 18O labeling of nucleotide α-phosphoryls does not derive from the transfer of 18O-labeled γ-phosphoryl of ATP or orthophosphate. It also revealed a broad potential for this approach to examine multiple aspects of adenine nucleotide metabolism and compartmentation in intact platelets.

The 18O-labeling kinetic data and observed ratios of adenine nucleotide α-phosphoryls containing one, two, or three atoms of 18O fit a model in which cAMP and related nucleotide metabolites are closely associated with a discrete compartment or complex of metabolic enzymes which prevents their dilution by larger pools of adenine nucleotides during metabolic flux but not the exchange of the nucleotide products with these pools.

The results obtained are consistent with 18O labeling of adenine nucleotide α-phosphoryls deriving primarily or solely from phosphodiesterase-promoted hydrolysis of cAMP in intact platelets. They also indicate that a means of monitoring cyclic nucleotide metabolism and compartmentation in intact cells may be possible by this 18O-labeling approach.

Over 2 decades ago, Sutherland and his co-workers (1) provided a conceptual framework and complement of experimental protocols for defining the biological meaningfulness of cyclic AMP as a regulatory "second messenger." These same basic principles and experimental approaches continue to guide the majority of current investigations in this field and serve as the basis for virtually all the information that has been gathered on the cellular metabolism of cyclic nucleotides. As a result, our knowledge on the subject derives almost exclusively from measurements of changes in cellular and tissue cyclic nucleotide steady state levels supplemented by in vitro assessments of adenylate cyclase and phosphodiesterase activities. Although considerable information in support of the intracellular mediator concept of CAMP action has been generated by these classical approaches, the dynamic aspects of cyclic nucleotide metabolism in the intracellular environment have not been defined.

We have explored the possibility of accomplishing the goal of monitoring the metabolism of cyclic nucleotides within intact cells by exploiting the property of phosphodiesterases to catalyze the hydrolysis of the 3'-phosphate bond of nucleoside 3',5'-monophosphates and to insert a hydroxyl ion from water in forming the 5'-nucleotide product. If carried out in a medium containing [18O]water, this reaction results in the incorporation of an atom of 18O into the 5'-nucleotide phosphoryl. The rate of appearance of this isotope in nucleotide α-phosphoryls could be envisaged to serve as a means for monitoring cyclic nucleotide hydrolytic rates. The feasibility...
of this approach was recently explored by examining certain aspects of the cyclic nucleotide phosphodiesterase reaction mechanism. From these studies it was established that cyclic nucleotide hydrolysis catalyzed by phosphodiesterases from several sources in the basal or activated states occurs by P-O bond cleavage and the incorporation of a single, nonexchangeable atom of $^{18}$O from $[^18]$O-water into one of three terminal, equivalent oxygens of the 5'-nucleoside monophosphate (2, 3). The $^{18}$O incorporated in conjunction with enzymic hydrolysis of a cyclic 3':5'-nucleotide is not removed by subsequent phosphorylation of the 5'-monophosphate to the triphosphate (3). Only upon pyrophosphorylytic cleavage as occurs, for example, in the conversion of the nucleoside triphosphate to its nucleoside 3':5'-cyclic monophosphate, is one of the terminal 5'-phosphoryls oxygen which becomes fixed in the $\alpha$-$\beta$ bridge position removed (3). This loss of precisely one-third of the $^{18}$O atoms originally incorporated into the nucleotide $\alpha$-phosphoryls presents no accounting problem because of its predictability. This information, combined with an appreciation of the reported catalytic capacities measured in vitro of cellular phosphodiesterase and adenylate cyclase activities, indicated that measurements of the rate of $^{18}$O incorporation into the phosphoryls of AMP and the 5'-phosphoryls of ADP and ATP may be a feasible and novel approach for monitoring the cellular metabolism of cAMP. In this report, the procedure for making these measurements is described (11, Miniprint) along with the results of experiments conducted with human platelets in which the metabolism of cAMP modified by adenylate cyclase stimulants or a phosphodiesterase inhibitor is assessed. The data obtained indicate that the metabolism of cyclic AMP in intact platelets even under basal conditions is a very dynamic process that exceeds previous estimates based on measurements of changes in cellular steady state levels and/or prelabeling of adenine nucleotides (4-7). The results also indicate that the insertion of $^{18}$O into 5'-nucleotide $\alpha$-phosphoryls by way of the phosphodiesterase pathway may represent a new means for studying in intact cells the kinetics, regulation, and compartmentation of enzymes involved in cyclic nucleotide as well as 5'-nucleotide metabolism.

**EXPERIMENTAL PROCEDURES**

**General Procedure**—The overall procedure is designed to measure the atom per cent excess of $^{18}$O in the $\alpha$-phosphoryls of adenine and guanine nucleotides. This is accomplished by the procedures outlined in the flow sheet shown in Fig. 2 of the Miniprint. The first step involves preparing acid extracts of cells equilibrated with $[^18]$O-water. The extracts are chromatographed on a phenylboronate resin to separate 5'-nucleotides from inorganic phosphate and cyclic nucleotides. The 5'-nucleotides are then chromatographed on DEAE-cellulose to fractionate nucleoside mono-, di-, and triphosphates. Next, the nucleoside di- and triphosphates are degraded enzymatically to their respective nucleoside monophosphates with aprase. The endogenous nucleoside monophosphates and the nucleoside monophosphates derived from the nucleoside di- and triphosphate fractions are purified by ion-pair reverse-phase HPLC employing a volatile solvent (8). Because of a contaminant in the AMP samples at this stage of isolation, it was found necessary to convert the AMP to IMP with adenylyl desaminase. The AMP (IMP) and GMP (GMP) are then subjected to a Smith degradation (9) to generate glycerol 3-phosphate, which is purified free of reactants and side products by anion exchange chromatography. The glycerol-3-P, which contains the $\alpha$-phosphoryls of the nucleotides of interest, is analyzed for atom per cent excess of $^{18}$O by gas chromatography/mass spectrometry after conversion to its trimethylsilyl derivative. The Me$_3$Si-glycerol-3-P is analyzed by selective ion monitoring, similar to the analysis of Me$_3$Si-P, respectively, was labeled with a single atom of $^{18}$O. In unstimulated platelets, the basal rate of appearance of [a-$^{18}$O]ADP and [a-$^{18}$O]ATP was linear throughout the incubation and by 10 min 7.5 and 9% of the total platelet ADP and ATP, respectively, were labeled with a single atom of $^{18}$O. In the basal state, less than 1% of the total ADP and ATP became labeled in the $\alpha$-phosphoryl position with two atoms of $^{18}$O although the appearance of this species increased appreciably during the second 5 min of the incubation. Species of nucleotide $\alpha$-phosphoryls containing three atoms of $^{18}$O did not achieve a detectable level during this 10-min period of incubation. After 10 min of incubation in $[^18]$O-water, when substantial labeling with two atoms of $^{18}$O was measurable, the ratio of $^{18}$O to $^{16}$O in the $\alpha$-phosphoryls of ADP and ATP ranged from 8 to 12. The sum of the fractions of each nucleotide pool labeled with one or two atoms of $^{18}$O (Fig. 1, C and F) indicates that the rate of total $^{18}$O incorporation into $\alpha$-phosphoryls of ADP and ATP is linear with respect to time of incubation in $[^18]$O-water under nonstimulated conditions. The

**RESULTS**

The Intracellular Kinetics of Basal and PGI$_2$-Stimulated $^{18}$O Incorporation into Adenine Nucleotide $\alpha$-Phosphoryls—The relationship that $^{18}$O labeling of adenine nucleotide $\alpha$-phosphoryls may have to phosphodiesterase-catalyzed hydrolysis of cAMP intracellularly was explored by examining the kinetics of $^{18}$O incorporation in the basal state and after elevating the levels of cAMP by exposing the platelets to prostacyclin. The rate of $^{18}$O enrichment of the $\alpha$-phosphoryls of ADP and ATP during a 10-min incubation in platelets in $[^18]$O-water in the absence and presence of 25 $\mu$M PGI$_2$ is shown in Fig. 1. The analysis of AMP was omitted in this experiment because the precaution of converting AMP to IMP had not yet been adopted. This omission introduces an error of less than 2% in calculating the total $^{18}$O incorporated into adenine nucleotide $\alpha$-phosphoryls because of the small contribution of this nucleotide to the total adenine nucleotide pool in platelets (12, Fig. 2) and because of the apparently rapid rate of AMP conversion to ADP and ATP in the intact cell.

The rate of $^{18}$O enrichment of the $\alpha$-phosphoryls of ADP and ATP is shown in the upper two panels of panels in Fig. 1 as the increase in the percentage of the entire pool of each nucleotide labeled with one or two atoms of $^{18}$O. In unstimulated platelets, the basal rate of appearance of [a-$^{18}$O]ADP and [a-$^{18}$O]ATP was linear throughout the incubation and by 10 min 7.5 and 9% of the total platelet ADP and ATP, respectively, were labeled with a single atom of $^{18}$O. In the basal state, less than 1% of the total ADP and ATP became labeled in the $\alpha$-phosphoryl position with two atoms of $^{18}$O. The appearance of the species increased appreciably during the second 5 min of the incubation. Species of nucleotide $\alpha$-phosphoryls containing three atoms of $^{18}$O did not achieve a detectable level during this 10-min period of incubation. After 10 min of incubation in $[^18]$O-water, when substantial labeling with two atoms of $^{18}$O was measurable, the ratio of $^{18}$O to $^{16}$O in the $\alpha$-phosphoryls of ADP and ATP ranged from 8 to 12. The sum of the fractions of each nucleotide pool labeled with one or two atoms of $^{18}$O (Fig. 1, C and F) indicates that the rate of total $^{18}$O incorporation into $\alpha$-phosphoryls of ADP and ATP is linear with respect to time of incubation in $[^18]$O-water under nonstimulated conditions. The
linear nature of this $^{18}$O incorporation rate is also exhibited by the data presented in $H$ of Fig. 1 where the total nanomoles of $^{18}$O appearing in all species of the $\alpha$-phosphoryls of ADP and ATP combined are plotted with respect to time.

Exposure of platelets to PGI$_2$ (25 $\mu$M) resulted in a marked increase in the steady state level of cAMP (Fig. 1G) and a greatly enhanced rate of $^{18}$O incorporation into adenine nucleotide $\alpha$-phosphoryls (Fig. 1, A to F and $H$). In this experiment, platelet cAMP concentration was rapidly increased by PGI$_2$ from a basal level of 1.2 to 1.4 nmol/7.8 $\times$ 10$^8$ cells to a peak level at 5 min of 42 nmol/7.8 $\times$ 10$^8$ cells which declined to 22 nmol/7.8 $\times$ 10$^8$ cells by 10 min. With PGI$_2$ stimulation, an accelerated rate of $^{18}$O labeling was apparent in both ADP and ATP and in the species of $\alpha$-phosphoryl containing one or two atoms of $^{18}$O (Fig. 1, A to D). The enhanced rate of labeling of the species of $\alpha$-phosphoryl containing a single atom of $^{18}$O (Fig. 1, A and D) was linear for only the first 5 min of the incubation; an appreciable decline was apparent thereafter, although the rate of appearance remained increased. This decline in the accelerated rate of appearance of [\$^{\alpha-^{18}}$O]phosphoryl occurs coincident with and undoubtedly at the expense of an increasingly greater rate of appearance of the $^{18}$O-containing species of $\alpha$-phosphoryls of ADP and ATP. The ratio of the $^{18}$O/$^{18}$O$_2$ species of adenine nucleotide $\alpha$-phosphoryls in PGI$_2$-stimulated platelets was 6 to 8 in contrast to the ratio of 8 to 12 observed in the basal state. When the sum of the species of ADP or of ATP containing one and two atoms of $^{18}$O are plotted with respect to time of incubation, the rate of $\alpha$-phosphoryl $^{18}$O-labeling becomes linear for both adenine nucleotides (Fig. 1, C and F). After 10 min of PGI$_2$ stimulation, 30% of the total ADP and about 40% of the total ATP are labeled with one or two atoms of $^{18}$O. Because approximately 50% of the ADP and 10 to 20% of the ATP in platelets are considered to reside in a nonmetabolic storage granule pool (12), this translates into an equivalent of about 60% and 50% of the metabolic pools of ADP and ATP, respectively, becoming labeled with $^{18}$O in 10 min.

When the total nanomoles of $^{18}$O that are incorporated into the $\alpha$-phosphoryls of ADP and ATP are calculated for each of the time periods examined, a linear rate of $^{18}$O enrichment can be shown to occur during the 10-min incubation which is over 4-fold greater in platelets incubated with PGI$_2$ (Fig. 1H). A total of 677 and 2968 nmol of $^{18}$O/7.8 $\times$ 10$^8$ cells were incorporated in 10 min under basal and PGI$_2$-stimulated conditions, respectively. From this information regarding total nanomoles of $^{18}$O incorporated into adenine nucleotide $\alpha$-phosphoryls, it can be calculated that if the $^{18}$O incorporated results primarily or solely from phosphodiesterase-catalyzed hydrolysis of cAMP, the flux of cAMP through this pathway is equivalent to 68 nmol/min/g (wet weight) and 297 nmol/min/g (wet weight) under basal conditions and after PGI$_2$ stimulation, respectively. This corresponds to a half-life of the cAMP pool of approximately 200 ms under basal conditions and 1.3 s at the peak of the PGI$_2$-stimulated state when cAMP levels are elevated about 40-fold.

The results of an experiment employing a different protocol which provides confirmatory and supplementary information are shown in Fig. 2. In this experiment, platelets were preincubated for 10 min with $[\text{H}^{18}$O]water before a second 10-min incubation was conducted in the presence or absence of PGI$_2$. The PGI$_2$ concentration of 10 $\mu$M used in this experiment promoted an increase in platelet cAMP of 11- to 13-fold (Fig. 2D). In Fig. 2, the total nanomoles of $^{18}$O (i.e. $^{18}$O, plus $^{18}$O$_2$) detected in the $\alpha$-phosphoryls of each of the nucleotides are
incorporation into adenine nucleotide α-phosphoryls after preincubation of platelets in medium containing [15O]water. Washed human platelets (5 × 10^9) were preincubated for 10 min in 2 ml of HBSS containing 48 atom % enriched [15O]water. An additional 10 min of incubation in this medium was conducted in the absence or presence of 10 μM PGI2. A to C, total nanomoles of 15O (15O + 18O) in the α-phosphoryls of AMP, ADP, and ATP. D, increase in platelet cAMP induced by PGI2. Species of nucleotide containing two atoms of 15O in the α-phosphoryl were corrected for a 33% loss of 15O. Vertical arrows indicate time of PGI2 addition.

shown with respect to time of incubation. The incorporation rate of 15O into each of the three nucleotides under basal conditions is linear with respect to time from the onset of the preincubation through the second 10-min period of incubation (Fig. 2, A to C). The effect of the PGI2 stimulus is manifest as an increase in 15O incorporation into the α-phosphoryls of each of the three nucleotides. The extent of the increase varies with each of the nucleotides from 2- (i.e. AMP) to 4.5- (i.e. ATP) fold. These differences may stem from the velocity of enzyme-catalyzed conversions of these nucleotides, the size of the nucleotide pools, and the rates of equilibration between different pools of a particular nucleotide. Overall, there is a 4.2-fold increase in 15O incorporation comparing the total nanomoles incorporated under basal and PGI2-stimulated states during the second 10 min of the incubation (Fig. 2E).

The basal rate of 15O incorporation calculated from the total nanomoles detected in the three nucleotide pools (Fig. 2E) is 67.5 nmol/min/7.8 × 10^9 platelets which is virtually identical with the value determined in the previous experiment. The PGI2-stimulated rates in this and the experiment shown in Fig. 1 are also very similar: 303 and 297 nmol/min/7.8 × 10^9 platelets in Figs. 1 and 2, respectively. The results in Fig. 2 demonstrate that 15O labeling of the AMP pool contributes only 1.9% and 1% to the total nanomoles of 15O determined to be incorporated in adenine nucleotide α-phosphoryls in the basal and stimulated states, respectively. If the 15O flux being detected is primarily related to the velocities of the different forms of cellular phosphodiesterase activities, the combined results from experiments shown in Figs. 1 and 2 indicate that a near maximum velocity of the composite of these activities is approached at a concentration of cAMP equivalent to 10 μM.

The apparent relationship between cellular cAMP concentration and the activity of the functional cellular phosphodiesterase system as indicated by 15O labeling is also shown in the results of an experiment presented in Table I. This experiment was conducted with a density of platelets in suspension that more closely approximates that of plasma (2 × 10^9/ml) and the incubation in [15O]water in the presence or absence of PGI2 (10 μM) was extended for 20 min. The rate of 15O incorporation in nonstimulated platelets was relatively constant ranging from 89 to 99 nmol of 15O/min/7.8 × 10^9 platelets at the 3-, 10-, and 20-min intervals of incubation in [15O]water. Under basal conditions, cAMP ranged from levels equivalent to 1.6 to 1.8 μM. With PGI2 stimulation, cAMP increased to concentrations equivalent to 89 and 49 μM at 3 and 10 min, respectively. This increased rate of 15O labeling represented an approximate 4-fold increase over the control rate. However, during the final 10 min of exposure to PGI2 there was a spontaneous decrease in the concentration of cAMP to a level equivalent to 3.3 μM. There was also a corresponding decline in 15O labeling rate to 125 nmol of 15O/min/7.8 × 10^9 cells. The combined results of the experiments in Figs. 1 and 2 and Table I indicate that there is a relationship between cAMP levels and rate of 15O incorporation into adenine nucleotide α-phosphoryls whereby a near maximum rate of 1547

Fig. 2. The effect of PGI2 on 15O incorporation into adenine nucleotide α-phosphoryls after preincubation of platelets in medium containing [15O]water. Washed human platelets (5 × 10^9) were preincubated for 10 min in 2 ml of HBSS containing 48 atom % enriched [15O]water. An additional 10 min of incubation in this medium was conducted in the absence or presence of 10 μM PGI2. A to C, total nanomoles of 15O (15O + 18O) in the α-phosphoryls of AMP, ADP, and ATP. D, increase in platelet cAMP induced by PGI2. Species of nucleotide containing two atoms of 15O in the α-phosphoryl were corrected for a 33% loss of 15O. Vertical arrows indicate time of PGI2 addition.
can be estimated to lie between 4 and 5 μM and the \( V_{\text{max}} \) to be 3.0 to 3.4 nmol/min/mg of protein. From these calculated kinetic parameters, the \( t_{1/2} \) for turnover of the cAMP pool would be approximated at from 120 to 170 ms and 333 to 1300 ms under basal and PGI-stimulated conditions, respectively. These kinetic values based on \(^{18}O\) incorporation into adenine nucleotide α-phosphoryls in the intact cell compare very closely with those determined by conventional in vitro assessment of this enzyme activity in cell-free extracts of platelets reported by Pichard and Cheung (13). For the phosphodiesterase activity in a 30,000 × g soluble fraction from human platelets exhibiting "high affinity" for cAMP, these investigators reported an apparent \( K_{\text{m}} \) value of 3 μM and a \( V_{\text{max}} \) of 4 nmol/min/mg of protein. A precaution in comparing the in vitro kinetic data with results obtained in the intact platelets is that only the phosphodiesterase activity in the 30,000 × g supernatant fraction but not the activity associated with the particulate fraction was dealt with in the former.

**Effect of 1-Methyl-3-isobutylxanthine on \(^{18}O\) Incorporation into α-Phosphoryls of Adenine Nucleotides**—The contribution by phosphodiesterase catalysis to the observed \(^{18}O\) labeling was examined further by determining the effect that a recognized inhibitor of this enzyme activity has on \(^{18}O\) incorporation into adenine nucleotide α-phosphoryls. The effectiveness of 1-methyl-3-isobutylxanthine and other methylxanthine derivatives in acting as competitive inhibitors of cAMP resulting in reduced phosphodiesterase activities in cell-free systems is well documented (14). The extent to which cyclic nucleotide hydrolysis is inhibited by such agents in intact cells has not, however, been established. In the experiments shown in Fig. 3, changes in the steady state levels of cAMP and in the rate of \(^{18}O\) incorporation into adenine nucleotide α-phosphoryls were monitored in high (2.5 × 10⁹/ml) and low (2 × 10⁹/ml) density suspensions of platelets incubated in \(^{18}O\) water in the presence or absence of 1.5 mM MIX. The effects of this methylxanthine derivative on the parameters examined were similar in the high and low density suspensions but there were some quantitative differences at the two different densities.

In the high density suspension (Fig. 3, upper panels), exposure to MIX led to a progressive rise in cAMP concentration throughout the 10-min incubation to a level equivalent to 4.8 μM from a relatively stable basal level of 1.2 to 1.4 μM in the nontreated cells. The rate of α-phosphoryl \(^{18}O\) incorporation in nontreated platelets was relatively linear throughout the 10-min incubation. In the MIX-treated cells, \(^{18}O\) incorporation was diminished during the first 5 min of the incubation but proceeded at a rate that paralleled that of the nontreated cells after this initial period of apparently suppressed incorporation (Fig. 3, upper left panel). When the absolute rates of \(^{18}O\) incorporation (nanomoles of \(^{18}O\)/min) were determined at each time interval that samples were analyzed (Fig. 3, upper right panel), the initial rate of \(^{18}O\) incorporation in the MIX-treated cells measured at 2 min was only about 40% of the \(^{18}O\) labeling rate in nontreated platelets. However, the rate of \(^{18}O\) incorporation in MIX-treated platelets increased progressively so that by 5 min the rate was approximately 70% of the control rate and during the final 5-min period, the rates in control and MIX-treated platelets were nearly comparable. These data indicate that in the presence of MIX, there appears to be an initial suppression of \(^{18}O\) incorporation which corresponds to an inhibition of phosphodiesterase-promoted hydrolysis of cAMP, but that this initial reduced rate of \(^{18}O\) incorporation is progressively overcome in association with a continuously increasing cellular level of cAMP.

The experiment shown in the lower portion of Fig. 3 was conducted with a lower density platelet suspension with the intention of minimizing nonspecific protein binding of the methylxanthine derivative in order to maximize the effective inhibitory concentration directed at phosphodiesterase activity. The greater increases induced in cAMP steady state levels by MIX in the lower density suspension (Fig. 3, lower left panel)


**Comparison of $^{18}$O Enrichment of α-, β-, and γ-Phosphoryls of Adenine Nucleotides and Inorganic Phosphate**—The possible contribution to the $^{18}$O labeling of adenine nucleotide α-phosphoryls by metabolic pathways other than cyclic nucleotide phosphodiesterase was examined by comparing the rate and extent of α-phosphoryl labeling with that of the β- and γ-phosphoryls of adenine nucleotides and of inorganic phosphate. The rationale for making these comparisons stems from the consideration that nucleoside kinase and adenine phosphoribosyltransferase represent two metabolic routes that could indirectly lead to $^{18}$O labeling of AMP and adenine nucleotide α-phosphoryls. In both cases, secondary pathways resulting in $^{18}$O labeling of inorganic phosphate and the γ-phosphoryl of ATP would also be required. This is accomplished within the cell by the sequential actions of enzymes such as adenosine triphosphatases and nucleoside diphosphate kinases or oxidative phosphorylation. The labeling of the β'-phosphoryl of phosphoribosylpyrophosphate could result from alternate metabolic routes (i.e., from hexose monophosphate shunt metabolism of $^{18}$O-glucose-6-P or phosphorylation of ribose by purine nucleoside phosphorylase plus the action of isomerase). If metabolic pathways such as these combine to contribute significantly to the $^{18}$O detected in the α-phosphoryls of the adenine nucleotides, this phosphoryl moiety would exhibit labeling rates and profiles of $^{18}$O-containing species similar to those of the γ-phosphoryl of ATP and possibly the β-phosphoryl of ADP as well as orthophosphate with which these nucleotide phosphoryls are likely to be in equilibrium.

The percentage of the total ADP and ATP pools labeled in the α, β, or γ positions with one, two, or three atoms of $^{18}$O at different times during a 20-min incubation in $^{18}$O water in the

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**Fig. 4.** The effect of pretreatment with MIX on basal and PGJ$_2$-stimulated $^{18}$O incorporation into the α-phosphoryls of AMP, ADP, and ATP. Washed human platelets (5 x 10$^7$) were preincubated for 10 min in 2 ml of HBSS containing 88 atom % enriched $^{18}$O water with or without 1.5 mM MIX. During a subsequent 10-min period, which is the interval of the total incubation shown, the platelets were incubated with or without 10 μM PGJ$_2$. The zero time period shown represents the end of the 10min preincubation. Left panel, cAMP concentration; middle and right panels, total nanomoles of $^{18}$O in the α-phosphoryls of AMP, ADP, and ATP. Other considerations were as described in Fig. 1.
presence or absence of PGI₂ or MIX is shown in Fig. 5. The overall conclusion that can be drawn from these results is that there is no similarity whatever between the various parameters of ¹⁸O incorporation into the α-phosphoryl of ADP compared to ¹⁸O labeling of β-ADP, γ-ATP, or orthophosphate. The striking dissimilarities in ¹⁸O labeling of α-ADP (which was very similar to the labeling of α-ATP, not shown) compared to the β- and γ-phosphoryls and orthophosphate is apparent with regard to the rate and extent of ¹⁸O appearance in the three different isotope-containing species of these phosphates as well as from the markedly different effects of PGI₂ and MIX. The rate of appearance of the ¹⁸O-containing species of α-ADP is linear with respect to time throughout the incubation and does not reach equilibrium with [¹⁸O]water even after 20 min when only 12% of the total ADP pool exists as the species containing a single atom of ¹⁸O. In contrast, the ¹⁸O₁ species of γ-ATP and β-ADP in nontreated platelets are over 95% equilibrated with [¹⁸O]water by 3 min; over 60% of the total ATP and 30% of the ADP pools are labeled with one atom of ¹⁸O at that time compared to only 12% in α-ADP. The ¹⁸O₂ and ¹⁸O₃-containing species of γ-ATP and β-ADP in the untreated platelets reach virtually total equilibration with the isotopic water at 6 and 10 min, respectively, whereas there is no appearance of significant amounts of [α-¹⁸O]ADP from the untreated platelets until 6 min and no species of [α-¹⁸O]ADP appears in the nontreated platelets even after 20 min of incubation; only in platelets exposed to PGI₂ is any [α-¹⁸O] ADP detectable and not until 20 min.

The manifestations of the PGI₂ or MIX treatment on ¹⁸O labeling of α-ADP are also not reflected in β-ADP, γ-ATP, or orthophosphate labeling. For example, the marked increase in the appearance of all species of [α-¹⁸O]ADP with PGI₂ treatment contrasts with a detectable inhibition of ¹⁸O appearance in all species of β-ADP and a no change or small inhibition of γ-ATP labeling in PGI₂-treated platelets. The effect of MIX to produce a lag in α-ADP labeling with ¹⁸O followed by a rate that parallels that of nontreated cells compares with an effect of the methylxanthine to diminish the rate of appearance of all species of [α-¹⁸O]ADP and to produce a small suppression of ¹⁸O labeling of γ-ATP which is most evident in the species of the γ-ATP containing two and three atoms of ¹⁸O. The effect of PGI₂ or MIX on ¹⁸O labeling of orthophosphate is very similar to the effects manifest in β-ADP labeling both of which are very unlike the influence each has on the incorporation of ¹⁸O into α-ADP. These results clearly argue against the possibility of a direct or indirect transfer of ¹⁸O-labeled γ-phosphoryl of ATP or of orthophosphate to account for the presence of any significant amount of ¹⁸O in the α-phosphoryl of adenine nucleotides.

In addition to the utility of these measurements for assessing alternate routes of labeling adenine nucleotide α-phosphoryls, they provide considerable information regarding nucleotide metabolism and compartmentation in platelets. They also indicate that βcAMP may have effects on certain aspects of adenine nucleotide metabolism not previously appreciated. For example, the fraction of total platelet ADP localized in storage granules, which is considered to be a nonmetabolic pool because of the slow rate of exchange of cytosolic and storage granule ADP, can be determined from the percentage of the total ADP pool that does not undergo β-phosphoryl labeling with ¹⁸O. Since at steady state the fraction of the ADP pool represented by the three species of [β-¹⁸O]ADP is

**Fig. 5.** Comparison of ¹⁸O labeling of α-, β-, and γ-phosphoryls of adenine nucleotides and inorganic phosphate. Washed human platelets (2 × 10⁷/ml) were incubated in HBSS containing 38 atom % enriched [¹⁸O]water for the times indicated in the absence (C——O) or presence (Δ——Δ) of PGI₂ or 1.5 mM MIX (□——□). The vertical columns of panels from left to right show the percentage of the total pool of nucleotide or inorganic phosphate labeled with one, two, or three atoms of ¹⁸O. Top row, α-phosphoryls of ADP; second row, β-phosphoryls of ADP; third row, γ-phosphoryls of ATP; and bottom row, inorganic phosphate. Vertical line interruptions indicate absence of data for that point on curve.
50.8% of the total ADP present, this is the percentage of the total ADP in the metabolic pool and the remainder is equivalent to the fraction in the storage granules. This is in good agreement with the most recent estimates of the relative sizes of these two pools (12). It is generally accepted that 10 to 20% of the total platelet ATP is also sequestered in the nonmetabolic storage granules (12). However, according to the results obtained with respect to the 18O labeling of the 2-phosphoryl of ATP, at equilibrium which is apparently achieved after 10 min of incubation, virtually 100% of the ATP is present as one of the three 18O species of 2-ATP. These results indicate that all of the platelet ATP is in an active metabolic pool or that the fraction of ATP in the storage granules is actively involved metabolically at least with regard to hydrolytic activity related to the 2-phosphoryl. One useful piece of information obtained from these studies is that the entire pool of cellular ATP is turned over in about 2 min.

The apparently very low percentage of the total orthophosphate labeled with 18O resulted from the relatively large pool of orthophosphate represented by the 2 mM of medium containing 1 mM P, which was included in the analysis. Nevertheless, the results indicate a potential for analysis of this type to provide valuable information regarding the rate of exchange of cellular and medium orthophosphate as well as the possibility for determining the activities of cellular adenosine triphosphatases in intact cells. The data obtained in the experiments shown suggest that PGI2 and MIX may influence the rate of 18O incorporation into orthophosphate which could reflect an inhibition of a specific ATPase activity and/or a suppression of platelet and medium exchange of orthophosphate. These and other aspects of nucleotide metabolism and transport can be examined more directly in future experiments with 18O labeling in intact cells.

One of the most prominent effects of PGI2 and MIX was an apparent suppression of 18O labeling of beta-ADP. Since PGI2 and MIX produced markedly different effects on the rate of 18O incorporation into alpha-ADP, it is unlikely that this reduced rate of beta-ADP labeling stemmed from differences in AMP generation from cAMP. However, it is likely that the similarities in the effects of PGI2 and MIX on 18O labeling of beta-ADP derive from the effect common to both agents which is to elevate cAMP levels by different mechanisms. What influence cAMP may have directly or indirectly to diminish the rate of 18O incorporation into the beta-ADP of ADP would seem worthwhile to define in the future.

**DISCUSSION**

The experiments described in this report were conducted to explore the relationship between 18O labeling of adenine nucleotide alpha-phosphoryls and phosphodiesterase-catalyzed hydrolysis of cAMP in intact human platelets. The first approximations represented by the results of the different experimental approaches suggest a major contribution by cellular phosphodiesterase(s) to the 18O incorporated into adenine nucleotide alpha-phosphoryls. The experimental evidence in support of this view is discussed below and modeling of the alpha-phosphoryl 18O-labeling data has been carried out to gain some insight into the cAMP metabolic system of intact platelets.

The observation that 18O labeling of adenine nucleotide alpha-phosphoryls proceeds by the sequential incorporation of as many as three atoms of 18O over a span of time covering up to 20 min indicates that the process(es) by which this occurs is probably ordered and proceeds with a fixed stoichiometry. This labeling behavior, in the intact cell, parallels that observed for the phosphodiesterase catalysis and the related adenine nucleotide metabolic enzymes in vitro (3) and contrasts with the behavior of enzyme-catalyzed reactions that result in scrambling and/or rapid exchange of phosphate with water oxygens due to multiple reversals (15, 16). The linearization of the 18O-incorporation rate data by the mathematical correction based on the prediction that a specific fraction of the original 18O inserted via phosphodiesterase catalysis is removed upon recycling of alpha-18O-beta and alpha-18O-alpha ATP through the adenylate cyclase step indicates that the pathway in question is one with very similar mechanistic and kinetic characteristics underlies this 18O labeling of nucleotide alpha-phosphoryls. It also indicates that the pool of adenine nucleotides related to the metabolism of cAMP may be relatively discrete and distinguishable from a larger pool(s) of adenine nucleotides with which it may slowly exchange. This implication of compartmentation is discussed in more detail below in the section on modeling of the alpha-phosphoryl 18O-labeling data.

The comparison that was made of the 18O-labeling rate of adenine nucleotide alpha-phosphoryls with that of beta- and gamma-phosphoryls and orthophosphate clearly distinguishes the alpha-phosphoryl labeling pathway(s) from the processes that lead to the labeling of these three other phosphate moieties. In addition to the strikingly different rate and profile of 18O labeling of alpha-phosphoryls, the contrasting effects of PGI2 and MIX on 18O incorporation into alpha- versus beta- and gamma-phosphoryls and orthophosphate minimize the likelihood of any significant contribution to alpha-phosphoryl labeling by pathways involving transfer of the gamma-phosphoryl of ATP (i.e. via nucleoside kinase) or orthophosphate (i.e. via a sequence of steps involving purine nucleoside phosphorylase, ribose phosphate isomerase, phosphoribosylpyrophosphate synthase, and adenosine phosphoribosyltransferase).

Other metabolic pathways besides phosphodiesterase that could theoretically lead to 18O labeling of adenine nucleotide alpha-phosphoryls have also been given serious attention and experiments have been conducted or are planned to assess the extent, if any, of their contribution. The hydrolytic cleavage of mRNA by ribonuclease represents such a pathway (17). Since platelets are nucleic blood elements, it is improbable that significant amounts of mRNA turn over at a sufficiently rapid rate to constitute a source of interference. The probability of significant incorporation of 18O into alpha-phosphoryls by way of the enzymic hydrolysis of mRNA would appear remote even in nucleated cells since the most active species of RNA in eukaryotic cells (i.e. hnRNA) which represents a relatively small pool of total cellular RNA exhibits a half-life of 7 to 18 to 20 (59 min. This compares to a half-life of less than 200 ms for the cyclic nucleotide pool if the 18O incorporation into alpha-phosphoryls is assumed to be representative of cAMP hydrolysis. Nevertheless, since hydrolytic degradation of RNA would be expected to generate proportionate amounts of other 18O-labeled 5'-nucleotides, an assessment was made of 18O present in GMP and the alpha-phosphoryls of GDP and GTP which represent the only other 5'-nucleotides besides IMP that are detectable in the platelet acid extracts. Although some incorporation was detectable in the alpha-phosphoryls of the guanine nucleotides, the percentage of the total monophosphate, di-, and triphosphate pools labeled with 18O never exceeded 1.5%, even after a 20-min incubation in the absence or presence of MIX or PGI2. Since the concentration of the total pool of guanine nucleotides is no more than 17% of the total pool of adenine nucleotides, the maximum contribution to 18O labeling of adenine nucleotide alpha-phosphoryls would be less than 1%, even if RNA hydrolysis were to represent the sole source of this 18O in guanine nucleotide alpha-phosphoryls (data not shown). These results could also be interpreted to indicate that phosphodiesterase-promoted hydrolysis of cGMP appears to proceed much more slowly than that of cAMP in
platelets if guanine and adenine nucleotide α-phosphoryl labeling is considered to arise primarily from phosphodiesterase-promoted hydrolysis of their respective cyclic nucleotides. The metabolism of cGMP has subsequently been found to be profoundly influenced by the density of platelets in the suspensions and to be markedly suppressed at the high density at which the experiments described above were conducted. (The relationship between ¹⁸O labeling of guanine nucleotide α-phosphoryls which is readily detectable in several cell systems we have examined and phosphodiesterase-promoted hydrolysis of cGMP will be the subject of a separate report.)

Synthetic processes involving activation of amino acids and fatty acids in which ATP undergoes pyrophosphorylytic cleavage represent routes by which ¹⁸O that may be inserted via phosphodiesterase catalysis could be removed. Insertion of ¹⁸O into adenine nucleotide α-phosphoryls could also be envisaged if the carboxyl oxygens of amino acids or fatty acids liberated by proteases or by acyl hydrolase(s), respectively, were to undergo labeling and to exchange carboxyl-¹⁸O with phosphoryl oxygens of AMP in the formation of aminooacyl adenylate and fatty acyl adenylate intermediates. Although these pathways for ¹⁸O exchange will require rigorous assessment in future experiments, it would seem unlikely from the information on hand that they would present a serious problem in the system under study. Net protein synthesis, although observed to occur in platelets by some investigators, has been reported to proceed at minimal rates (19). This slow rate of protein synthesis implies that there is a slow rate of protein turnover and commensurately minimal rates of proteolysis and aminooacyl tRNA synthesis. Considering that the platelet is a terminally differentiated nucleic blood element, this would not be unexpected. The activities of acid CoA ligases as in the case of aminooacyl-tRNA synthetases has not been reported in platelets and there is no quantitative information regarding the rate of turnover (i.e. acylation/deacylation) of phospholipids, in spite of the considerable literature on the metabolism of radiolabeled arachidonic acid in platelets. It is well documented, however, that deacylation of phospholipids (which should be accompanied to a significant extent by reacylation) is markedly suppressed in platelets by agents such as PGI₂ that increase cAMP accumulation (20). This influence of cAMP would be expected to result in a diminished rate of fatty acid turnover and diminished ¹⁸O incorporation into nucleotide α-phosphoryls that may occur by way of fatty acid activation. This, of course, is the opposite of what was observed; PGI₂ treatment of platelets resulted in maximal ¹⁸O-labeling rates. Also, with collagen stimulation of platelet aggregation, which is known to increase the rate of arachidonic acid release (21), no change or a slightly diminished rate of ¹⁸O labeling of adenine nucleotide α-phosphoryls was observed (data not shown). In a single experiment conducted to determine the possible contribution of fatty acid turnover to the phosphorlyl labeling in question, the effect of mepacrine (500 μM), an inhibitor of cellular phospholipase activities (22), was examined and was found to produce no significant effect on ¹⁸O labeling of adenine nucleotide α-phosphoryls during a 3-min incubation with ¹⁸O/water (data not shown). Although the potential exists for interference by these pathways involving amino acid and fatty acid activation, there is no indication from the information available or from the results obtained that they contribute significantly to the ¹⁸O labeling of nucleotide α-phosphoryls observed in these experiments.

The results of the experiments conducted with PGI₂ favor a direct relationship between ¹⁸O labeling of α-phosphoryls and intracellular phosphodiesterase catalysis. It is conceivable that PGI₂ may have multiple effects on adenine nucleotide metabolism that have not as yet been uncovered. It is reasonable to expect, however, that the recognized action of PGI₂ as one of the most potent activators of platelet and other cellular adenylate cyclases is related to the altered rate of ¹⁸O labeling of adenine nucleotide α-phosphoryls observed in these experiments. The increases in platelet cAMP concentration resulting from PGI₂-induced activation of membrane adenylate cyclase were consistently found to be associated with 4- to 5-fold increased rates of ¹⁸O labeling of adenine nucleotide α-phosphoryls. Virtually identical results were also obtained with prostaglandin E₂ (not shown), which is also a relatively potent activator of platelet adenylate cyclase (23). Prostaglandin E₂ produced increases in both platelet cAMP levels and ¹⁸O incorporation into nucleotide α-phosphoryls, comparable to those seen with PGI₂. The most straightforward explanation for this greater rate of ¹⁸O incorporation with these activators of platelet adenylate cyclase is that it stems from an increased rate of phosphodiesterase-catalyzed hydrolysis of cAMP by virtue of a concentration-dependent increase in the velocity of this enzyme activity in the intact cell. Under basal conditions, it would appear that adenylate cyclase activity limits flux through the system by maintaining a steady state level of cAMP of approximately 1 μM, which is subsaturating with respect to the substrate requirement for phosphodiesterase(s).

The ¹⁸O-labeling rates that appeared to approach maximal (i.e. 300 to 340 nmol of ¹⁸O/min/g of cells), when platelet concentrations of cAMP were elevated by PGI₂ to a level equivalent to 10 μM or greater, suggests that the phosphodiesterase-catalyzed hydrolysis of cAMP at these higher concentrations of cAMP. The apparent Vₐ₅₀ value calculated to be 3 to 3.4 nmol/min/mg for protein for this ¹⁸O flux compares very closely with the reported value of 4 nmol/min/mg for the soluble form of the low K₉₀ cAMP phosphodiesterase activity in platelets (13). This concentration versus velocity relationship between cAMP levels and ¹⁸O-labeling rates would be best demonstrated with graded increases in platelet cAMP from 1 to 10 μM. We have not yet been successful in achieving gradations in this range because of the extreme sensitivity of platelets to PGI₂, which, even at the lowest concentrations tested, produced increases in cAMP of no less than 10-fold, but at higher concentrations raised them as much as 100-fold. The relationship between cAMP concentration and ¹⁸O-labeling rates may be more easily examined by exploiting the "desensitization" of platelets to PGI₂. The spontaneous decrease in PGI₂-elevated platelet cAMP concentration that occurred in spite of the continuing presence of the adenylate cyclase stimulator in the medium undoubtedly resulted from the commonly observed phenomenon referred to as "desensitization" (24). The concentration versus velocity relationship in question was strongly indicated by the decline in the rate of ¹⁸O incorporation from 323 to 125 nmol of ¹⁸O/min/g of platelets in close association with a spontaneous decrease in cAMP concentration from approximately 50 to 3 μM. Michaelis-Menten formulation of the ¹⁸O-labeling rates observed at different platelet levels of cAMP (i.e. basal states and upon desensitization to PGI₂) until the upper limit of the ¹⁸O-labeling rate is approached (i.e. estimated at 330 nmol of ¹⁸O/min/g of platelets) obtained an apparent value of K₉₀ for cAMP of 4 to 5 μM. The close correspondence of this value with reported K₉₀ values determined by conventional assay in cell-free systems of 3 μM for the soluble phosphodiesterase activity from platelets (13) and 2 to 6 μM for this activity from numerous other sources (25) is consistent with the identity of the pathway for ¹⁸O labeling of α-phosphoryls as that deriving from phosphodiesterase-catalyzed hydrolysis of cAMP. The results of one experiment in which cAMP levels were increased to an equivalent of 50 to 90 μM with PGI₂ with a lower
density platelet suspension indicated that a secondary "high" apparent $K_v$ activity for $^1$O labeling may also be demonstrable in the intact platelet, which may correspond with the "high" $K_v$ phosphodiesterase activity detected in platelets in vitro (13). At platelet cAMP levels equivalent to 50 and 90 $\mu$M, $^1$O-labeling rates of 323 and 343 nmol/min/g of platelets, respectively, were observed while an apparent plateau in the $^1$O-labeling velocity of 300 nmol/min/g was seen at concentrations of cAMP equivalent to 10 and 40 $\mu$M in two earlier experiments with higher density platelet suspensions. Whether this greater rate of $^1$O labeling is appreciable and is related to a "higher" $K_v$ enzyme activity or the activity expressed in the lower density platelet suspension remains to be determined.

An alternate approach to determine the contribution of cellular phosphodiesterase activity to $^1$O labeling of adenine nucleotide a-phosphoryls was to examine the effect of an inhibitor of this enzyme activity. It is tacitly assumed that methylxanthine derivatives such as MIX, when added to intact cells, more or less abolish this pathway of hydrolytic activity because of the proven effectiveness of these agents to inhibit phosphodiesterase in disrupted cell systems. A different apparent behavior of the putative inhibitor was seen with regard to $^1$O labeling of adenine nucleotide a-phosphoryls. The increased steady state levels of cAMP following treatment with MIX are consistent with numerous previous observations made in intact cells for which no adequate kinetic explanations have been provided in the past. There are two characteristics of the conditions in situ in comparison to those that ordinarily prevail in vitro that probably account for the different but predictable effectiveness of this competitive inhibitor in the intact cell. The effective concentration of the inhibitor achievable in the intact cell depends upon its entry into the cell which may be limited because of nonspecific interactions of the methylxanthine with other extra- and intracellular components. The latter is probably reflected by the greater apparent effectiveness of a given concentration of MIX as an inhibitor in a suspension containing $2 \times 10^7$ platelets/ml, as indicated by the greater increase in cAMP accumulation in the former. Most likely, the effective intracellular concentration of the inhibitor directed at phosphodiesterase activity is much less than the 1.5 mM concentration present in the total suspension. This probably accounts for why an elevated new steady state level, rather than a continuously increasing level of cAMP that never reaches steady state, is seen with MIB treatment. Virtually total blockade of phosphodiesterase activity in situ would be expected if a concentration of 1.5 mM MIX was actually achieved intracellularly; the $K_v$ value for this inhibitor with this enzyme activity from platelets is in the low micromolar range (14).

In the intact cell, unlike the circumstances in vitro, the dynamic flow of metabolites has the potential to adjust steady state levels of reactants to maintain flux in the system. The establishment of the new steady state level of cAMP in the presence of MIX indicates that adenylate cyclase activity increases the concentration of cAMP more rapidly than it can be hydrolyzed by the suppressed activity of phosphodiesterase until the cAMP concentration increases sufficiently to sustain a rate of hydrolysis equal to the rate of synthesis. This would be predicted to occur when the cAMP rises to a level that can compete with the concentration of MIX that is present (i.e. the denominator term of the Michaelis-Menten equation for the inhibited and noninhibited reactions becomes the same). This interpretation of the consequences of MIX action on cAMP metabolism are entirely consistent with the incremental increases in cAMP concentration that precede attaining the new steady state. It is also consistent with the corresponding increases in the rate of $^1$O incorporation and cAMP concentration observed for the system which was initially inhibited by more than 90% by MIX but returns to a rate equivalent to that seen in the absence of MIX when the new steady state level of cAMP is achieved. If this interpretation is correct, the increasingly greater rate of $^1$O incorporation that coincides with the progressive increments in the concentration of platelet cAMP represents a concentration (i.e. cAMP) versus velocity (i.e. $^1$O incorporation) relationship in an intact cell. The fact that over 90% of the basal $^1$O incorporation into adenine nucleotide a-phosphoryls could be inhibited by MIX (i.e. 1 min after MIX addition to a low density platelet suspension), supports the argument that over 90% of the $^1$O incorporation seen in the basal state derives from phosphodiesterase-promoted hydrolysis of cAMP. The very similar effect of MIX on $^1$O incorporation into a-phosphoryls and in elevating cAMP levels, even in PGL-stimulated cells, indicates that the pathway for $^1$O labeling of a-phosphoryls is the same in the PGL-stimulated and the basal states. These results also indicate that the effectiveness of methylxanthine derivatives to inhibit phosphodiesterases in intact cell and tissue preparations may require redefinition. If the foregoing interpretation is correct, the methylxanthine inhibitor suppresses phosphodiesterase activity only transiently because of the potential in the intact cell to readjust cAMP steady state levels and overcome the inhibitory influence. If this were not the case, the dynamics of the flux through the adenylate cyclase/phosphodiesterase system predicts that virtually all of the cellular ATP would exist in the form of cAMP in a matter of several min if phosphodiesterase activity were to have "shut off."

If the evidence favoring phosphodiesterase catalysis as the major or sole pathway for the $^1$O labeling of adenine nucleotide a-phosphoryls is borne out by additional experimentation, considerable new information about the dynamics of cyclic nucleotide metabolism in intact cells should be obtainable by the approach described in this report. One of the basic concepts that emerges from the work reported here is that the metabolism of cAMP in an intact platelet is a much more dynamic process, even under basal conditions, than had been appreciated previously. From the observed rate of $^1$O labeling of adenine nucleotide a-phosphoryls, the half-life of the 1 $\mu$M cAMP pool in the basal state was determined to be in the range of 200 ms and to increase to just over a second when the cAMP pool had been increased 40-fold by PGL. This information obtained by what may represent a more or less direct measurement of cAMP hydrolysis within the cell differs by as much as three orders of magnitude from indirect measurements of cAMP turnover in other cell types. The turnover of cAMP has been estimated in dog thyroid slices (6), chicken erythrocytes (5), cultured fibroblasts (4), and lymphoma cells (7). The $t_{1/2}$ value in dog thyroid slices, chicken erythrocytes, and cultured fibroblasts was reported to be in the region of from 1.8 to 6 min and 8 min in cultured lymphoma cells.

In the most recent and sophisticated effort reported to measure cellular cAMP turnover indirectly, Barber and Goka (7) derived a relationship between the increase in specific radioactivities of ATP and cAMP using $[\text{H}]$adenine to label the cellular nucleotides. One problem in such an approach may stem from compartmentation of metabolic pools of adenine nucleotides. It is also not apparent that the data met the assumption made in these experiments, that the rate of hydrolysis of cAMP is equivalent to $k_2$ [cAMP] where $k_2$ is the

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fractional turnover constant of cAMP. This assumption is valid only under conditions in which the rate of hydrolysis of cAMP is linearly related to cAMP concentration. Furthermore, the formulation used to estimate the turnover of cAMP is based only on the exchange of ATP into the cAMP-metabolizing system and underestimates the turnover rate of any fraction of ATP which is directly recycled in the adenylate cyclase/phosphodiesterase complex. Whether the differences in cAMP flux and turnover rates reported here for platelets and those previously reported for other cell types cited are related to different metabolic characteristics of the systems or stem from the different analytical approaches is a question worthy of further experimental consideration.

The Metabolism of Platelet cAMP Determined from Modeling Kinetic Data of $^{15}O$ Labeling of Nucleotide $\alpha$-Phosphoryls—The kinetics of $^{15}O$ labeling of adenine nucleotide $\alpha$-phosphoryls has been examined with the view of providing an explanation for the labeling behavior observed and some information regarding the metabolism of cAMP in intact platelets. The approach taken was to develop models to which the data obtained could be fit. The kinetic data and conceptual background for development of these models are as follows.

Under basal conditions, $^{15}O$ is incorporated into the $\alpha$-phosphoryl residues of the adenine nucleotides at a rate of approximately 70 nmol/min in a volume of platelets equivalent to 1 ml (7.8 x 10$^9$ platelets). Assuming that all $^{15}O$ incorporated into the nucleotides results from the action of cAMP phosphodiesterase, 1 nmol of cAMP, a quantity equivalent to the cAMP pool, turns over in 0.85 s. The rate of incorporation of $^{15}O$ into the nucleotides to form $[\alpha-^{15}O]ADP$ and $[\alpha-^{15}O]ATP$ is 22 and 41 nmol/min, respectively, and that for $[\alpha-^{15}O]ADP$ and $[\alpha-^{15}O]ATP$ is 2.7 and 3.3 nmol/min, respectively (Fig. 2). The rate of accumulation of the $\alpha-^{15}O$ and $\alpha-^{15}O_2$ species is approximately linear over a span of 20 min and ratios of $\alpha-^{15}O_2/\alpha-^{15}O$ for ADP and ATP of 8.1 and 12.4, respectively, were obtained. Although the rate of incorporation of $^{15}O$ into the $\beta$- and $\gamma$-phosphoryls of ADP and ATP, respectively, is much more rapid than that of $^{15}O$ into the $\alpha$-phosphoryl of AMP, approximately 2 to 3 min are required for complete equilibration of the $\beta$- and $\gamma$-phosphoryls in the ADP and ATP pools (viz. Fig. 5). The extent of equilibration of the $\beta$- and $\gamma$-phosphoryls during the 0.85 s required to turn over the entire pool of cAMP is estimated to be less than 1%. Any model describing this system must reconcile the observation that $^{15}O$ appears at the onset to accumulate linearly in the $\alpha$-phosphoryls of ADP and ATP with the fact that a triple dilution of the $[\alpha-^{15}O]AMP$ formed will occur as only a small fraction of the AMP, ADP, and ATP pools is converted to ADP, ATP, and cAMP, respectively, during the time required to turn over the entire cAMP pool.

Three models were considered as first approximations of the cAMP-metabolizing system and a fit of the data to these models was attempted. All models consider the fraction of the water represented by $[^{15}O]H$ and the probability of incorporating $^{15}O$ from water into AMP by the action of phosphodiesterase on cAMP, $[\alpha-^{15}O]cAMP$, and $[\alpha-^{15}O]cAMP$. The probability of loss of an atom of $^{15}O$ from the $\alpha$-phosphoryl of the three possible $^{15}O$-labeled species of ATP by the action of adenylate cyclase was also taken into consideration. The first model assumes no compartmentation of the nucleotides associated with metabolism of cAMP. In this system, AMP formed from cAMP at a rate of about 1 nmol/s becomes diluted 400-fold when reappeared in the pool of AMP containing 400 nmol followed by a dilution of 1500-fold when 1 nmol of AMP is converted to ADP (metabolically active), which in turn is diluted 4500-fold when ADP is converted to ATP. This would represent a maximum dilution of 2.7 x 10$^4$-fold. In this model, $^{15}O$ would accumulate most rapidly into AMP and least rapidly into ATP; the increase in $^{15}O$ in ATP would be curvilinear with respect to time and the $\alpha-^{15}O/\alpha-^{15}O$ ratios in the nucleotides would be greater than 1000. Little $[\alpha-^{15}O]$ ATP would form during the first 10 to 20 min. None of the data is consistent with this model.

A second model assumes that cAMP is metabolized in a discrete compartment which does not exchange adenine nucleotides with the other compartments. If the size of each of the adenine nucleotide pools in this compartment is relatively small, the accumulation of $[\alpha-^{15}O]cAMP$ and $[\alpha-^{15}O]cAMP$ will occur much more rapidly than is predicted by the first model. However, total labeling of nucleotides can be no greater than the size of the nucleotide pool in this compartment. Small nucleotide pools of AMP, ADP, and ATP are required to account for the rapid and linear rate of labeling of ATP. Far too much incorporation of $^{15}O$ into too large an apparent pool of ATP and of ADP and too little $\alpha-^{15}O_2$ and $\alpha-^{15}O_2$-nucleotide occurs for the data to be consistent with this model.

A third model assumes a discrete compartment in which cAMP is metabolized and assumes further that this compartment recycles only a fraction of its nucleotides and that it exchanges the remainder for nucleotides in other compartments. This system should have properties of both the models described above. This model was simulated with different dilutions of the nucleotides within the discrete cAMP-metabolizing system and with different fractions of the nucleotides exchanging for nucleotides outside of the discrete pool as variables. Simulation of this model predicts dilution of less than 2-fold within the cAMP-containing adenine nucleotide compartment and exchange of 80 to 90% of the nucleotides within this compartment for those outside of it to occur in the platelet system (Fig. 6) within the time frame investigated. All of the simulation studies suggest that dilution within the cAMP-metabolizing compartment is very limited. That is, the concentrations of AMP, ADP, and ATP within the system are of the same order of magnitude as cAMP, resulting in minimal dilution of the $^{15}O$-labeled metabolites as they traverse from AMP to ATP. Because dilution is minimal and $^{15}O$ incorporation into ATP is linear and rapid, it seems likely that most of the $\alpha-^{15}O$-labeled ADP is derived primarily from the metabolism of $[\alpha-^{15}O]ATP$ outside of the cAMP compartment. The majority of the $[\alpha-^{15}O]AMP$ found in the cell may also result from transphosphorylations occurring outside of this compartment.

The fraction of the nucleotides that exchange out of the cAMP-metabolizing compartment per unit of time influences the rate of accumulation of $[\alpha-^{15}O]$-adenine nucleotides. Initially, most of the $[\alpha-^{15}O]$-adenine nucleotides will be derived from $[\alpha-^{15}O]$-adenine nucleotides recycled within the cAMP-metabolizing compartment because the probability that an $[\alpha-^{15}O]AMP$ from the large exchangeable pool of ATP will be among the ATP molecules transferred back into the metabolizing compartment is much less than 0.01. It follows that an estimate of the fraction of adenine nucleotides which are exchanged out of the cAMP-metabolizing compartment is obtained by measuring the accumulation of $[\alpha-^{15}O]$-adenine nucleotides in the system and that the fraction recycled within the pool will be a function of the $[\alpha-^{15}O]cAMP$ and $[\alpha-^{15}O]cAMP$ nucleotides. However, in 50% $^{15}O$-water in the metabolizing compartment, only one-third of the $[\alpha-^{15}O]AMP$, which is converted to cAMP will be converted to $[\alpha-^{15}O]AMP$, $[\alpha-^{15}O]ADP$, and $[\alpha-^{15}O]ATP$. This contrasts to the one-half of the unlabeled molecules of ATP which will be converted to $[\alpha-^{15}O]AMP$. By similar reasoning, only one-sixth of the $[\alpha-^{15}O]ATP$ is converted to $[\alpha-^{15}O]AMP$. Therefore, to measure the fraction recycled, it is necessary to adjust the concentration of $[\alpha-^{15}O]cAMP$.
**Fig. 6.** Simulated time course of accumulation of $\alpha$-$^15$O$_2$-nucleotides as a function of the fraction of the adenine nucleotides exchanged out of the cAMP-containing compartment. The simulated system was assumed to be in water containing 50 atom % excess $^15$O. It was also assumed that: 1) the total nucleotide pool contained 400 nmol: 400, 1500, and 4500 nmol of AMP, ADP, and ATP, respectively; 2) no dilution in percentage of $^15$O in the $\alpha$-$^15$O$_2$, $\alpha$-$^15$O$_3$, and $\alpha$-$^15$O$_4$-labeled phosphoryl residues occurred in the course of the conversion of cAMP to ATP within the cAMP-containing compartment; and 3) the distribution of $^15$O atoms within the $\alpha$-phosphoryl group of ATP was random; that is, one-third of the $^15$O in $[\alpha-^15$O$_2]$-ATP was located in the atom bridging between the $\alpha$- and $\beta$-phosphoryl phosphorus atoms. One cycle was set as the time to convert 1 nmol of cAMP to ATP and result in one complete metabolic turnover of the platelet cAMP pool. The size of AMP, ADP, and ATP pools within the cAMP-containing compartment was assumed to be equivalent to that of cAMP, otherwise dilution would have occurred and the second assumption above would have been violated. This model is consistent with the sequential conversion of ATP through cAMP, AMP, ADP, and back to ATP without exchange of the AMP and ADP generated with adenine nucleotides in the larger pools. The fraction of ATP formed in each cycle was assumed to exchange with ATP in the larger pool(s) and the species of ATP bound to adenylate cyclase were, therefore, expected to comprise a fraction that was recycled and the remainder deriving from the larger ATP pool, as indicated. The simulation considered the probable loss of $^15$O from the $\alpha$-$\beta$ bridge oxygen for ATP labeled with one, two, or three atoms of $^15$O. The simulated lines $a$ through $f$ represent those for the exchange of 0.95, 0.90, 0.85, 0.75, and 0.55, respectively, of the ATP formed in one turnover cycle for ATP in the larger pool(s). Solid circles indicate experimental data points.

For metabolic compartment may be diffusion-controlled and not a formal compartment in the sense of having a discrete barrier.

The time course of accumulation of $[\alpha-^15$O$_2]$-adenine nucleotides in a simulated system with an $^15$O incorporation rate of 70 nmol/min was determined as a function of the fraction of the adenine nucleotides exchanged into the compartments outside of the cAMP-metabolizing compartment (Fig. 6). The time course of $^15$O incorporation into $[\alpha-^15$O$]_2$-adenine nucleotides increased linearly. However, the rate of increase in concentration of $[\alpha-^15$O$_2]$-adenine nucleotides decreased as the fraction of the nucleotides in the cAMP-metabolizing compartment which exchanged for those in other pools was decreased. The experimental data (Fig. 6) in which approximately 9.1% of the ATP pool was in the form of $^15$O was equivalent to that obtained in the simulated system (Fig. 6) in which 85% of the adenine nucleotides of the cAMP-metabolizing compartment exchange each second. The simulated system predicts that 6.8, 4.7, and 2.6% of the nucleotides would be in the $^15$O form with fraction exchange of 0.65, 0.43, and 0.25, respectively, and no dilution in the cAMP-metabolizing compartment. The data suggest that in the platelet system approximately 85% of the adenine nucleotides are exchanged under nonstimulated conditions.

The influence of the extent of dilution by nucleotides within the cAMP-metabolizing compartment and the fraction of these nucleotides exchanged for those in the larger pool(s) on the ratio $\alpha$-$^15$O$_2$/$\alpha$-$^15$O$_3$ found in the adenine nucleotides was determined. These data were compared with those predicted by the simulated system described by the third model. The simulated system predicts a value of 15 at 10 min; experimental values of 19 and 15 were obtained. The data and the simulated system are in agreement. The PGI$_2$-stimulated system had a ratio of 7 and 8 for ADP and ATP, respectively,
The simulation analysis predicted a value of less than 100 for tide species will accumulate slowly and then only if dilution control platelets. The experimental data are in qualitative metabolites that remain tightly associated with a complex of is turning over rapidly, along with related adenine nucleotide the simplest one which is in general agreement with the data. other, more complex and sophisticated models which fit the ratio was obtained in a few experiments. In these experi-ments, values ranging from 50 to 90 were obtained. Unlike the relationship of the \( \alpha^3\mathrm{O}/\alpha^\mathrm{15}\mathrm{O} \) ratio to cycle number, assuming 85% of the nu- cleotides is exchanged and that negligible dilution occurs. The simulation analysis predicted a value of less than 100 for a system like the PGL-stimulated platelets in which the activity is of the order of 5-fold greater than occurs in the control platelets. The experimental data are in qualitative agreement with the values generated by the simulation model. The \( \alpha^3\mathrm{O}/\alpha^\mathrm{15}\mathrm{O} \)-nucleotide ratios are most sensitive to both the dilution that occurs in the CAMP-metabolizing compartment and the fraction of the nucleotides which exchange from this compartment with those outside of it. There may be other, more complex and sophisticated models which fit the data but, for the present, the third model described above is the simplest one which is in general agreement with the data. This conceptual model with which the results correspond implies that in the basal state there is a pool in which CAMP is turning over rapidly, along with related adenine nucleotide metabolites that remain tightly associated with a complex of metabolic enzymes. From this model of cellular metabolism, a majority of the molecules of CAMP generated under basal conditions could be envisaged to be so rapidly hydrolyzed and recycled that it may not leave this metabolic complex of enzymes in the form of CAMP. If this were the case, very little if any CAMP would dissociate from the complex to activate protein kinases, unless the latter were one of the components comprising this complex of enzymes. Because the larger pool of CAMP that arises from hormone stimulation would traverse the metabolizing complex of enzymes more slowly, there is a greater probability of a larger number of CAMP molecules diffusing from this pool to other sites in the cell to activate protein kinases in those locales. The implications, therefore, may be that a large proportion of the basal CAMP pool is not available for protein kinase activation because it never leaves the adenylyl cyclase/phosphodiesterase complex. Such a metabolic arrangement could explain why CAMP which is in a concentration range well above the \( K_c \) value for protein kinase activation in the nonstimulated cell (29) has little, if any, influence on this enzyme activity. One question that can be raised about such a metabolic arrangement within the cell is whether the rapidly turning over CAMP tightly associated with the coupled cyclase/phosphodiesterase complex contributes to any specific cellular function. Alternatively, it could be viewed as a characteristic that renders the system highly responsive by virtue of its very active state of flux and nearly saturated capacity which can be rapidly overloaded to provide increased steady state levels of allosteric effector.

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Supplemental Material for

**O Incorporation into Nucleotide α-Phosphoryls**

**Platelia, L.**

**Concentration of Nucleotide Monophosphates by Sep-Pak C18 Cartridges**

The nucleotide monophosphates from BE were subjected to preparative thin-layer chromatography (TLC) using silica gel plates and visualized by UV light. After purification, the nucleotide monophosphates were eluted from the Sep-Pak C18 cartridges using 2 ml of 100 mM ammonium formate, pH 4.0, followed by 2 ml of 300 ml NH₄OH and 500 ml of 3M sodium acetate, pH 4.0. The material was then added to the reaction mixture with ADP and ATP to achieve a final concentration of 1 mM. The reaction mixture was then incubated for 60 min at 30°C. The reaction was then quenched by adding 5 ml of 10% trichloroacetic acid (TCA) to the reaction mixture. The reaction mixture was then centrifuged at 12,000 rpm for 10 min. The supernatant was then added to the Sep-Pak C18 cartridge and washed with 2 ml of 100 mM ammonium formate, pH 4.0. The nucleotide monophosphates were then eluted from the Sep-Pak C18 cartridge with 2 ml of 1 M ammonium formate, pH 4.0, followed by 2 ml of 3 M sodium acetate, pH 4.0. The nucleotide monophosphates were then concentrated under reduced pressure and stored at -20°C until further use.

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**18O Incorporation into Nucleotide α-Phosphoryls**

**EXPERIMENTAL.** The orthophosphate recovered from the chromatographic purification of the nucleotide extract in PBA-60 was further purified at 55°C to 56°C. The eluting effluent was mixed with 2 ml of a chloroform containing 500 μl of trichloroacetic acid (TCA) (1:1 ratios). The mixture was then neutralized with 1 N Na2CO3, and the supernatant was applied to a 1.5 x 15 cm column of AG50-X2 (H+) resin. The orthophosphate was eluted by washing with 20 ml of 10 M HCl. The effluent was evaporated to dryness under reduced pressure, and the orthophosphate was converted to the trimethylsilyl derivative by the method described above for 3-3P-PDE (see above). GC/MS analysis of the effluent was performed on a 3.5 ft glass column containing 30% OV-121 on 8/100 mesh Supelcoport, 0.2 mm inner diameter. The temperature program was from 110°C to 140°C at 10°C per min was employed. Two ion fragments were obtained by selective ion monitoring. These fragments were m/z 299, 311, 303, 305, and 307, and m/z 314, 316, 318, 320, and 322.

**Fig. 2.** Gel filtration of platelet cellular water. Upper panel: time course of equilibration of platelet cellular water at various densities of platelets in the suspension. The time points labeled with the arrows indicate the time points at which the platelet suspension was removed and used for the experiment. Lower panel: time course of equilibration of platelet cellular water at various densities of platelets in the suspension. The time point labeled with the arrow indicates the time point at which the platelet suspension was removed and used for the experiment.