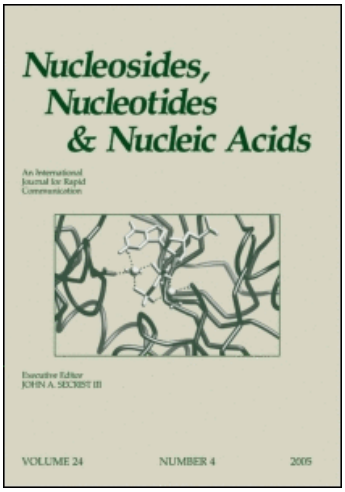


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# Determination of ATP Impurity in Adenine Dinucleotides

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# Determination of ATP Impurity in Adenine Dinucleotides

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## ABSTRACT

Adenine dinucleotides ($Ap_nA$) are extracellular signal molecules that are released from blood platelets, following stress, into the vascular system. The most abundant and best-characterized $Ap_nA$ ($Ap_4A$) interacts with a unique receptor on bovine aortic endothelial cells (BAEC) where it induces nitric oxide. $Ap_4A$ also interacts with P2 purinoceptors on BAEC to modulate $Ca^{2+}$ mobilization and prostacyclin release; this behavior can be equally well explained by $Ap_4A$ being either a partial agonist to these receptors, or an antagonist in the presence of ATP contamination. To discern between these two possibilities, we have investigated the presence of such contaminants in $Ap_nA$ preparations. The studies herein indicate that $Ap_nAs$ (n = 3–6) contain ATP impurities; thus, when characterizing the $Ap_nA$ interaction with ATP-binding sites, investigators must assure that the response elicited is not partly due to an ATP impurity. We here provide a means for detecting and estimating ATP impurities within $Ap_4A$ preparations while also eliminating them; the level of this contamination is estimated to be as low as 0.2%. We applied our method to distinguish the true effect of $Ap_4A$ at P2 purinoceptors; our findings are consistent with $Ap_4A$ acting as a partial

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agonist to these receptors. We also applied our method to characterizing the $Ap_nA$ interaction with luciferase, and found that decontaminated $Ap_nA$ (n = 4–6) are weak substrates for luciferase.

*Key Words:* Adenine dinucleotides; $Ap_4A$; ATP impurity; Firefly luciferase.

## INTRODUCTION

Adenine dinucleotides represent a diverse and interesting group of regulatory molecules controlling various physiological functions.[1–3] These molecules, consisting of two adenosine moieties linked via their 5′ positions by a chain of phosphates ($Ap_nA$; n = 2–7), are stored in blood platelets and co-released with mononucleotides in the bloodstream following stress.[4–6] It has been shown that $Ap_nAs$ interact with unique dinucleotides receptors on endothelial cells and brain synaptosomes and also with several ionotropic (P2X) and metabotropic (P2Y) purinoceptor subtypes.[7–16] In the rat brain, $Ap_4A$ and $Ap_5A$, but not mononucleotides, interact with a receptor-operated calcium channel on the surface of synaptic terminals.[8,9] $Ap_2A$ and $Ap_4A$, but not mononucleotides, interact with a dinucleotide receptor on bovine aortic endothelial cells (BAEC), triggering a signaling device that upregulates the synthesis of protein(s) required to generate a pore that delivers L-Arg to endothelial nitric oxide synthase.[11,12]

Besides the interaction with specific dinucleotide receptors, $Ap_nAs$, along with mononucleotides, were shown to interact with various P2 purinoceptors to mobilize $Ca^{2+}$.[13–16] We have found that in BAEC, $Ap_4A$ can both stimulate and negatively modulate P2 purinoceptors associated with $Ca^{2+}$ mobilization and prostacyclin ($PGI_2$) release.[13] Such complex interaction patterns as the ones displayed by $Ap_4A$ with BAEC could be attributed to a partial agonist behavior at the P2 receptors; however, the $EC_{50}$ value for the $Ap_4A$-induced $Ca^{2+}$ mobilization was three orders of magnitude higher than the corresponding value for ATP.[13] These data suggest that as little as 0.1% ATP contamination in $Ap_4A$ preparations could be sufficient to explain the $Ca^{2+}$ mobilization from P2 receptors on endothelial cells. To distinguish between these two possibilities, it is necessary to effectively eliminate the mononucleotide impurities from dinucleotide preparations. The presence of such contaminants in commercially available dinucleotide powders is a major concern in characterizing the interaction of $Ap_nAs$ with P2 purinoceptors. Since a small mononucleotide impurity may mimic an effect from $Ap_nAs$ at P2 receptors, elimination of such contaminants in the $Ap_nA$ samples is a requirement for the validity of the results.[16–18]

The aim of this study was to detect and remove ATP impurities within commercial preparations of $Ap_nA$. Due to its ATP specificity and sensitivity, we used the firefly luciferase assay—coupled with bovine alkaline phosphatase (BAP) treatment of the samples—to detect, estimate, and eliminate ATP impurities in the $Ap_nAs$. In this communication we demonstrate that $Ap_nAs$ (n = 3–6) all contain ATP impurities. We estimate that the ATP contaminant in commercial $Ap_4A$ can be as low as 0.2%. We also applied the above-mentioned decontamination method to correctly assess the effect of $Ap_4A$ on $PGI_2$ release from BAEC; our results demonstrate that eliminating the mononucleotide impurity decreases the $PGI_2$ response by only 29%. Thus, $Ap_4A$ appears to act as a partial agonist, as predicted by our previous paper.





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## MATERIALS AND METHODS

### Materials

ATP Bioluminescent Assay Kit, penicillin, streptomycin, BAP, $Ap_nAs$ and ATP were purchased from Sigma (St. Louis, MO). Minimal Essential Medium (MEM) was purchased from GIBCO (Gaithersburg, MD). Heat-inactivated fetal bovine calf serum (FBS) was purchased from HyClone (Logan, UT). En$^3$Hance Spray and [2,8-$^3$H]-ATP ([$^3$H]-ATP, 36.7 Ci/mmol) were from New England Nuclear (Boston, MA). [8-$^3$H(N)]-$Ap_4A$ ([$^3$H]-$Ap_4A$, 20 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). DC-Plastikfolien PEI-cellulose F plates were purchased from EM Science (Gibbstown, NJ). 6-keto prostaglandin $F_{1\alpha}$ acetylcholinesterase EIA Kit was purchased from Cayman Chemical (Ann Arbor, MI). All other reagents were of analytical grade or better.



### General Methods

Prior to luminometric or chromatographic experiments, ATP, $Ap_nA$, and BAP samples were prepared at the indicated concentrations in the reaction buffer (Tris-HCl 10 mM, pH 7.8, EDTA 1 mM, $MgCl_2$ 1 mM), which was found neither to interfere with the luminescence assay nor with the BAP treatments (data not shown). The firefly luciferase was provided within the ATP Bioluminescent Assay Kit as an assay mix, also containing excess luciferin in a tricine buffer (pH 7.8). As recommended by the manufacturer, for the bioluminescent reactions the assay mix was diluted 500-fold in the same tricine buffer, to ensure assay sensitivity in our substrate concentration range. For chromatographic analysis of the luciferase-induced breakdown of substrates, the assay mix was used as provided by the manufacturer (undiluted). For 6-keto $PGF_{1\alpha}$ assays, ATP, $Ap_4$ and $Ap_4A$ samples were prepared in Krebs–Henseleit buffer (Hepes 10 mM, pH 7.4, NaCl 120 mM, KCl 4.6 mM, $CaCl_2$ 1.5 mM, $MgCl_2$ 0.5 mM, $NaH_2PO_4$ 1.5 mM, $Na_2HPO_4$ 0.7 mM, glucose 10 mM).

### Luminometric Assay

Luciferase assays were performed using the ATP Bioluminescent Assay Kit as per manufacturer's instructions. The light reaction was initiated by adding 100 µl sample to 100 µl assay mix. The signal was read (6 s integration time) in a Monolight 2010 Luminometer. Luminescence readings were taken as an estimate of the initial velocity of the reaction. Since the second substrate, luciferin, was present in high excess over the enzyme, the ATP and $Ap_4A$ interaction with the luciferase can be described as a single substrate (Michaelis–Menten) reaction. Kinetic data were fitted by nonlinear regression to a rectangular hyperbola to yield $K_m$ and $V_{max}$ values, and were then normalized to the $V_{max}$ of the control (100%). This compensates for experimental variations in the enzyme activity and allows a direct comparison of $V$ values obtained in different experiments. Where indicated, samples were pre-treated with the indicated concentration of BAP immediately before the measurement. All sample readings were compared to appropriate controls.



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## Thin Layer Chromatography (TLC)

[$^3$H]-ATP and [$^3$H]-$Ap_4A$ were prepared at the indicated concentrations in reaction buffer. For the assessment of the nucleotide breakdown by BAP, samples were incubated for 30 min at 37°C in the presence or absence of 100 units/ml BAP in reaction buffer. The final volume for the above reactions was 8 μl. After incubation, 4 μl sample aliquots were spotted on PEI-cellulose plates alongside 5 μM nonradiolabeled standards ($Ap_4A$, AMP, ADP and ATP). In experiments using BAP, 5 μM nonradiolabeled adenosine was added to the standards mixture. Separation by TLC was performed as previously described.[19] Briefly, the plates were first developed in deionized water up to 7 cm (to separate the salt component), followed by nucleotide separation in 1 M LiCl up to 15–20 cm height. After development, plates were dried and sprayed with En$^3$Hance Spray, and autoradiography was carried out at −80°C for 60–132 hours.

## BAP Treatment of ATP and $Ap_nAs$

Nucleotide samples and BAP at the indicated concentrations were incubated for 30 min at 37°C, and then either used in the bioluminescent assay or separated by ascending chromatography, as described above.

## BAP Inactivation

BAP samples (100–120 μl, 100 units/ml) were incubated for 30 min at 85°C and then submitted to two cycles of freeze-thawing (liquid nitrogen-room temperature). The efficacy of the inactivation procedure was tested by further incubation with ATP for 30 min at 37°C, prior to performing luminescence readings as described above. The stability of [$^3$H]-$Ap_4A$ during the inactivation procedure was assessed by TLC, after 30 min incubation at 85°C followed by 0, 1, or 2 freeze-thaw cycles.

## Cell Culture

BAEC were supplied by Dr. R. Auerbach of the University of Wisconsin. Cells were grown in MEM supplemented with 10% (v/v) FBS, 44 mM $NaHCO_3$, 100 units/ml penicillin and 100 μg/ml streptomycin, and maintained at 37°C in a humidified atmosphere of 95% air–5% $CO_2$. All experiments were performed with passage 10 cells.

## Measurement of 6-Keto $PGF_{1\alpha}$ Released by BAEC

The accumulation of 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) was used as an index of $PGI_2$ release. The 6-keto $PGF_{1\alpha}$ assays were performed as previously described.[13] Briefly, confluent BAEC monolayers were incubated overnight at 37°C in serum-free MEM; the cells were then washed and further incubated for 15 min with the indicated concentration of nucleotide sample in Krebs–Henseleit buffer. Where indicated, samples were treated (prior to the experiment) with 100 U/ml BAP and then subjected to the inactivation procedure as described above. Supernatant aliquots were then

removed, diluted 100-fold and assayed for 6-keto $PGF_{1\alpha}$ using the acetylcholinesterase immunoassay kit. Untreated nucleotide samples incubated with the cells in the presence and absence of inactivated BAP did not yield significant differences in $PGI_2$ synthesis (data not shown). Therefore, in all subsequent experiments untreated samples were incubated with the BAEC in the absence of BAP.

## RESULTS

### Bioluminescent Interaction Between Luciferase and $Ap_4A$

To determine whether luciferase could use $Ap_4A$ as a substrate, we compared the effect of varying concentrations of ATP and $Ap_4A$ on their interaction with luciferase. As shown in Fig. 1, light emission from the luciferase interaction with both ATP and $Ap_4A$ displays typical hyperbolic behavior. However, the amount of luminescence generated by $Ap_4A$ is significantly less than that generated by ATP (the $V_{max}$ value for $Ap_4A$ is 1.8% of the $V_{max}$ obtained for ATP). Data analyzed by nonlinear least-square regression generated calculated $K_m$ values of 322 ± 23 and 519 ± 54 μM for ATP and $Ap_4A$, respectively. These findings are consistent with $Ap_4A$ being a weak substrate for luciferase, which—despite a relatively good affinity ($K_m$)—does not induce efficient light output ($V_{max}$). Light emission from the luciferase-$Ap_4A$ interaction was accompanied by $Ap_4A$ hydrolysis, as assessed by TLC separation (unpublished observation). This finding, consistent with $Ap_4A$'s role as a substrate for luciferase, suggested that the low levels of light obtained from $Ap_4A$ preparations might not be due only to a minor ATP contaminant. Nevertheless, the luminescent signal obtained

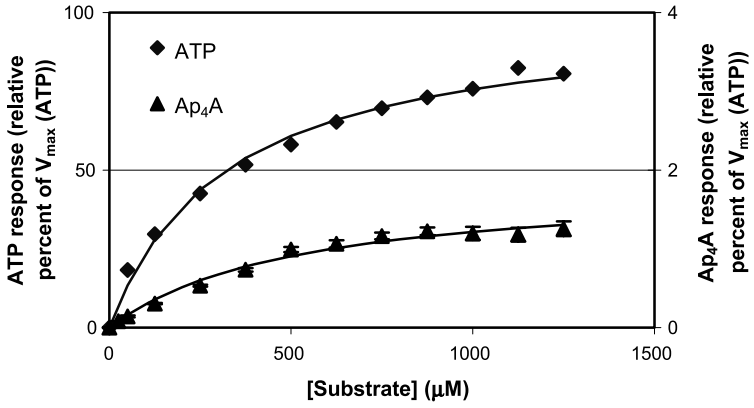


***Figure 1.*** $Ap_4A$ is a weak substrate for the luciferase enzyme. Varying concentrations of ATP and $Ap_4A$ were added to the bioluminescent assay mix and luminescence was determined as described in the Methods. Light intensity data were fitted to a rectangular hyperbola to yield $K_m$ and $V_{max}$ values and were plotted as relative percent of the $V_{max}$ obtained for ATP (mean ± SEM from at least three experiments performed in at least two replicates). The left and right ordinate axes refer to luminescence data induced from ATP and $Ap_4A$, respectively. (*View this art in color at www.dekker.com.*)

from contaminated $Ap_4A$ could be a combination of responses obtained from both pure $Ap_4A$ and ATP (present as an impurity).

### BAP Interaction with ATP and $Ap_4A$

Our first series of experiments was to determine the concentration of BAP necessary for the effective hydrolysis of 100 μM ATP. Varying concentrations of BAP were incubated with 100 μM ATP for 30 min at 37°C prior to performing luminescence assays. As shown in Fig. 2A, treatment with 50 units/ml of BAP inhibited essentially 100% of the ATP-induced luminescence. All subsequent experiments were performed using 100 units/ml of BAP. At this concentration, BAP did not interfere with the firefly luciferase enzymatic activity (data not shown).

To ensure that $Ap_4A$ can be safely treated with BAP and will not be hydrolyzed during the treatment, we incubated 5 μM [$^3$H]-$Ap_4A$ with 100 units/ml BAP for 30 min at 37°C, prior to performing PEI-cellulose chromatography and autoradiography. As shown in Fig. 2B, BAP completely hydrolyzes ATP to adenosine (lane 2), but does not break down $Ap_4A$ (lane 4); these data are consistent with $Ap_4A$ not being actively degraded by BAP. The ability of BAP to hydrolyze 100 μM ATP, assessed by TLC, was not impaired by the presence of up to 100 fold excess of either $Ap_4A$ or adenosine, consistent with BAP's specificity for the free phosphate groups and not for the nucleoside portion of a nucleotide (data not shown). Moreover, our radiolabeled $Ap_4A$ samples have several small contaminants, of which one co-migrates with the ATP standard and disappears after incubation with either BAP (compare lanes 3 and 4 in Fig. 2B) or luciferase (unpublished observation), suggesting the presence of an ATP impurity. The other contaminants, still present after BAP treatment (Fig. 2B), are most likely not of the mononucleotide type, as BAP would have degraded those. These results indicate that, while safe for $Ap_4A$, BAP treatment can effectively degrade [$^3$H]-ATP in the presence or absence of $Ap_4A$.

### The Effect of BAP on $Ap_4A$-Induced Luciferase Response

Since the $Ap_4A$-induced luminescent response could be partly due to the presence of a minor ATP impurity, we assessed the presence of such contaminants in our nonradiolabeled $Ap_4A$ samples. Varying concentrations of $Ap_4A$ were incubated in the presence and absence of BAP for 30 min at 37°C, prior to performing the luminescence assay. As shown in Fig. 3, light emission from the luciferase interaction with $Ap_4A$ displays hyperbolic behavior in both the presence and absence of BAP. Data analyzed by nonlinear regression generated calculated $K_m$ values of 519 ± 54 and 781 ± 63 μM for $Ap_4A$ in the absence and the presence of BAP, respectively. The $V_{max}$ for $Ap_4A$ after BAP treatment is 64.4% of the $V_{max}$ obtained for $Ap_4A$ in absence of BAP. These data are consistent with $Ap_4A$ containing a BAP-sensitive impurity that also acts as a substrate for luciferase. The decreased luminescence obtained for BAP-treated $Ap_4A$ samples could be also caused by the product of the BAP reaction, adenosine, which may interfere with the luciferase activity. However, this product would be present in minor concentration as compared to the substrate, and even 10-fold excess adenosine did not inhibit the light response induced by 100 μM ATP (data not shown). Since the luciferase is highly specific for ATP, and ATP is known to be BAP-degradable, our





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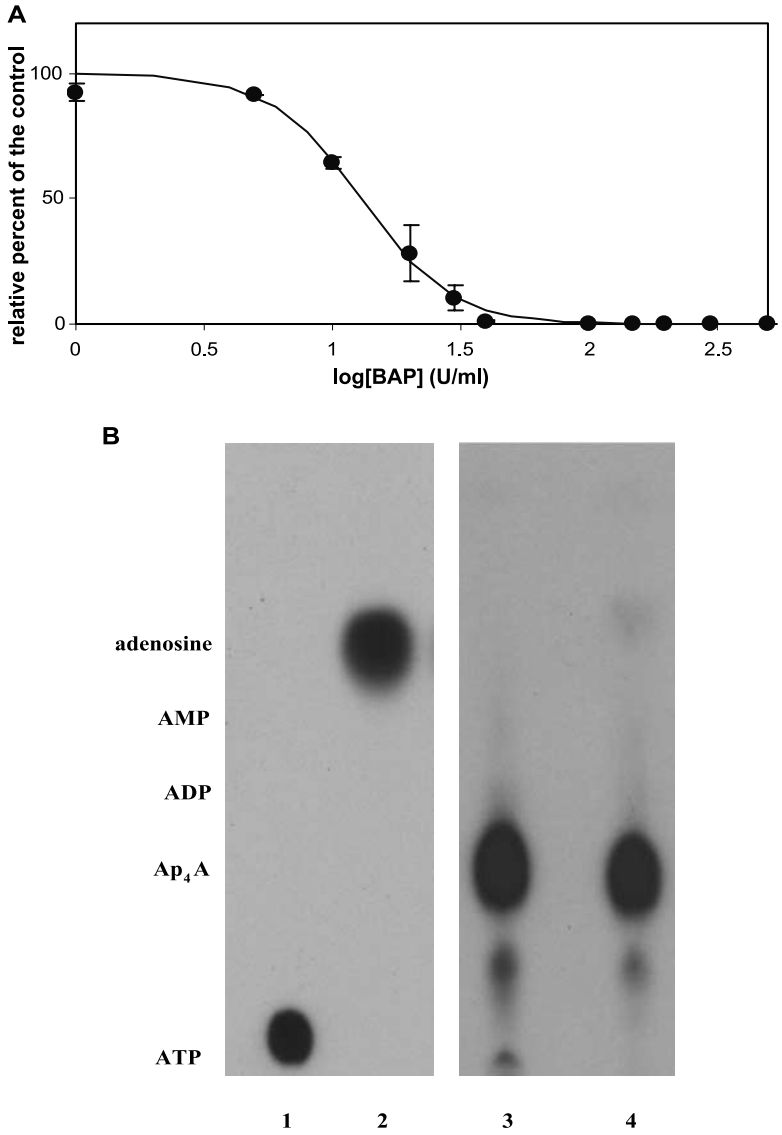


***Figure 2.*** Degradation of ATP and $Ap_4A$ by BAP. A. 100 μM ATP was incubated with varying concentrations of BAP for 30 min at 37°C prior to performing luminescence assays as described in the Methods. Data are an average of three experiments performed in duplicate. B. One hundred micromolar [$^3$H]-ATP ($\sim 0.2 \times 10^4$ cpm/pmol) or 5 μM [$^3$H]-$Ap_4A$ ( $\sim 1 \times 10^4$ cpm/pmol) were incubated in the presence and absence of BAP (100 units/ml) for 30 min at 37°C, prior to performing PEI-cellulose chromatography (one development in 1 M LiCl) and autoradiography as described in the Methods. Lane 1, 100 μM ATP, in absence of BAP; Lane 2, 100 μM ATP, in presence of BAP; Lane 3, 5 μM $Ap_4A$, in absence of BAP; Lane 4, 5 μM $Ap_4A$, in presence of BAP. Position of the standards is indicated on the left. Similar intensities for the ATP and $Ap_4A$ spots, despite the difference in specific activities, were achieved by modulating the respective concentrations and exposure times. (*View this art in color at www.dekker.com.*)





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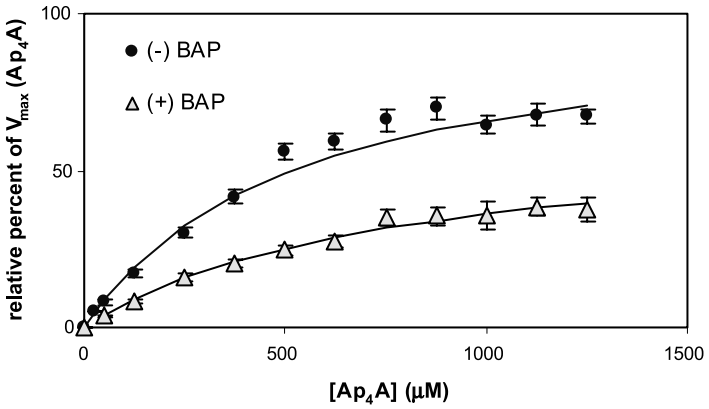


***Figure 3.*** The effect of BAP on $Ap_4A$ induced luciferase response. Varying concentrations of $Ap_4A$ were incubated in the absence and presence of 100 units/ml of BAP for 30 min at 37°C prior to performing the bioluminescence assay as described in the Methods. Light intensity data were fitted to rectangular hyperbolae to yield $K_m$ and $V_{max}$ values for each curve, and were plotted as relative percent of the $V_{max}$ obtained for the untreated $Ap_4A$ control (mean ± SEM from at least three experiments performed in at least two replicates). (*View this art in color at www.dekker.com.*)



data suggest that $Ap_4A$ is contaminated with an ATP impurity. Alternatively, $Ap_4$ may also be a contaminant, in view of the fact that this mononucleotide too is susceptible to BAP degradation and also acts as luciferase substrate.[20]

## Estimation of the Mononucleotide Contamination in $Ap_4A$ Preparations

Calculations were made under the assumption that the luciferase response to ''impure'' $Ap_4A$ is due to the additive responses to: 1) the ATP contaminant and 2) the ATP-free (BAP treated) $Ap_4A$. Thus, the difference between the untreated $Ap_4A$ and BAP-treated $Ap_4A$ signals from Fig. 3 should correspond to an ATP curve described by:

$$V = \frac{V_{\max} f[Ap_4A]}{K_m + f[Ap_4A]}$$

where $V$ is the velocity of the reaction, $f$ is the fraction of ATP contaminant in the $Ap_4A$ samples, and $V_{max}$ and $K_m$ are the previously calculated values for the luciferase interaction with ATP. Fitting this theoretical curve to the experimental data (response lost after BAP treatment) will then give an estimate for the value of $f$. The calculated $f$ value for the $Ap_4A$ samples was 0.002, consistent with $Ap_4A$ containing 0.2% ATP impurities.

Previous studies have established that $Ap_4$ as well can act as a substrate for luciferase;[20] since this mononucleotide is also BAP-degradable, it can therefore be considered as a potential contaminant in our $Ap_4A$ samples. However, $Ap_4$ has the ability to trigger only 2.2% of the response induced by the same concentrations of



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ATP.[20] This behaviour is consistent with a ~50 times decrease in the maximum response induced by ATP ($V_{max}' = 2.2\%\ V_{max}$, where $V_{max}'$ is the extrapolated maximum velocity of the luciferase-$Ap_4$ interaction) and with no change in the $K_m$ value ($K_m' = K_m$). Based on these assumptions, we used the calculation procedure described above to determine the $f'$ fraction of the $Ap_4$ contaminant, if it were the only impurity in our $Ap_4A$ samples. The calculated $f'$ value was 0.093, consistent with $Ap_4A$ potentially containing 9.3% $Ap_4$ impurities. Though commercially available $Ap_4A$ powders are not likely to contain as much impurities, this fairly large value only refers to the case where $Ap_4$ is the sole contaminant. Therefore, we cannot eliminate the possibility that $Ap_4$ be partially present, alongside ATP, as an impurity in the $Ap_4A$ samples, but BAP treatment of the dinucleotides will effectively eliminate either or both mononucleotide contaminants.

## The Effect of BAP Treatment on the $Ap_4A$-Induced $PGI_2$ Release

We have previously shown that $Ap_4A$ induces $Ca^{2+}$ mobilization and $PGI_2$ release from BAEC with an affinity several orders of magnitude smaller than the one for ATP.[13] Those results, together with the negative modulation profile displayed by the dinucleotide, could be interpreted as $Ap_4A$ interacting with P2 receptors on BAEC as either a partial agonist, or an antagonist—if the stimulation was due only to a minor ATP contaminant. The data presented so far in this communication demonstrate that $Ap_4A$ is contaminated with either ATP or $Ap_4$ impurities; therefore, we need to discern between partial agonistic and antagonistic behaviors by determining how much of the previously detected $Ap_4A$-induced stimulation via P2 receptors[13] is due to such impurities and how much to $Ap_4A$ itself. First, we determined the $PGI_2$ release triggered by $Ap_4$ in BAEC and compared the response to those obtained after ATP and $Ap_4A$ stimulation of the cells. The results, presented in Table 1, show that—while ATP has an effect much stronger than $Ap_4A$, $Ap_4$ induces $PGI_2$ release values similar to those triggered by the same concentration of $Ap_4A$. Therefore, in order to induce a response similar to that triggered by a minute ATP impurity in the $Ap_4A$ samples, an $Ap_4$ contaminant should be present in concentrations much higher than ATP. However,

***Table 1.*** Agonist-induced $PGI_2$ release from BAEC.

| | $PGI_2$ release (pg/$10^5$ cells) | | |
|---|---|---|---|
| | Agonist | | |
| Concentration | $Ap_4$ | $Ap_4A$ | ATP |
| 10 μM | 395.3 ± 104.9 | 370.2 ± 82.6 | 2572 ± 109.3* |
| 100 μM | 632.9 ± 171.8 | 773.5 ± 49.5 | 2811 ± 132.4* |

Experiments were performed as described in Materials and Methods. In each experiment, $PGI_2$ release from unstimulated cells (average: 908 ± 58.5 pg/$10^5$ cells, N = 26) was subtracted from the respective nucleotide induced $PGI_2$ synthesis values. Data are an average of three different experiments performed in triplicate.
*$p < 0.05$ versus response obtained with the same concentration of $Ap_4A$.





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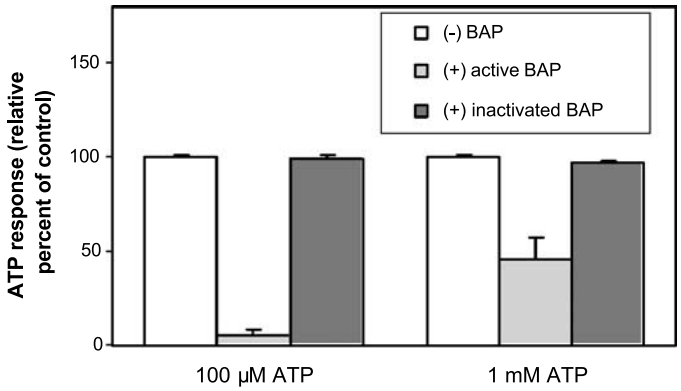


***Figure 4.*** The effect of inactivated BAP on ATP-induced luciferase response. ATP samples (100 μM and 1 mM) were incubated in the presence and absence of 100 units/ml BAP (active or inactivated), prior to performing the luminescence assay as described in the Methods. Data are an average of three experiments performed in duplicate and are reported as relative percent of the controls (obtained for ATP in the absence of BAP).



it is conceivable that either or both of these mononucleotides be present as impurities in the dinucleotide preparations. To determine the degree in which such contaminants may influence the $Ap_4A$-induced P2 receptor stimulation, we compared $PGI_2$ release from BAEC upon stimulation with untreated and BAP-treated $Ap_4A$ samples. To avoid the presence of active BAP during these measurements, we devised a procedure to inactivate BAP in the BAP-treated ATP and $Ap_4A$ samples. Since incubation for 30 min at 85°C did not completely inactivate BAP (data not shown), the heat treatment was followed by two cycles of freeze-thawing. This procedure totally inactivated the activity of 100 units/ml BAP, as assessed by luminescence readings for 100 μM and 1 mM ATP (Fig. 4). Furthermore, $Ap_4A$ stability was not affected by the inactivation procedure, as assessed by TLC (data not shown). Therefore, BAP treatment of the $Ap_4A$ samples was followed by BAP inactivation before incubation with the cells for $PGI_2$ assessment.

To determine whether the mononucleotide contaminants in the $Ap_4A$ samples affect the $PGI_2$ response from BAEC, we incubated confluent cell monolayers with

***Table 2.*** Effect of BAP treatment on the ATP- and $Ap_4A$-induced $PGI_2$ release by BAEC.

| | $PGI_2$ release | |
|---|---|---|
| Agonist | (−) BAP treatment | (+) BAP treatment |
| ATP (10 μM) | 2572 ± 109.3 pg/$10^5$ cells (100%) | 44.9 ± 99.8 pg/$10^5$ cells (1.7%) |
| $Ap_4A$ (100 μM) | 773.5 ± 49.5 pg/$10^5$ cells (100%) | 550 ± 142 pg/$10^5$ cells (71%) |

Experiments were performed as described in Materials and Methods and in the legend of Table 1. Data are an average of three different experiments performed in at least two replicates. Values in parentheses are percentages from the corresponding controls (untreated agonists).



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untreated and BAP-treated ATP (10 μM) and $Ap_4A$ (100 μM) samples, prior to determining the $PGI_2$ release. As shown in Table 2, untreated ATP induced a significantly higher response than untreated $Ap_4A$ samples, consistent with our previous findings.[13] Removal of mononucleotide contaminants from $Ap_4A$ by BAP treatment decreased the $PGI_2$ release by only 29%, as compared to the response to untreated $Ap_4A$. The remaining response could not be due to unreacted ATP in the dinucleotide sample, since 10 μM ATP (a concentration well exceeding the possible amount of impurity in 100 μM $Ap_4A$) were almost completely annihilated (> 98%) by BAP treatment (Table 2). We have previously determined that the $PGI_2$ release from BAEC originates from P2 stimulation;[13] therefore the product of the BAP reaction (adenosine) is not likely to be responsible for the remaining response from the treated $Ap_4A$ samples. Moreover, 10 μM adenosine failed to induce either $Ca^{2+}$ mobilization or $PGI_2$ release from BAEC (data not shown), consistent with the lack of involvement of P1 receptors in this response. These data suggest that, besides the partial stimulation due to the presence of mononucleotide contaminants, $Ap_4A$ has an intrinsic agonistic effect on $PGI_2$ release from BAEC.

## Bioluminescent Interaction Between Luciferase and Other $Ap_nAs$ (n = 3–6)

To determine whether some other $Ap_nAs$ (n = 3–6) were contaminated with ATP, we compared luminescence values obtained from BAP-treated and untreated dinucleotides (final concentration 500 μM) (Fig. 5). The $Ap_nAs$ tested induced luminescence responses at levels comparable to the one obtained for $Ap_4A$, with the exception of $Ap_2A$, which did not induce any effect above the baseline, even at higher concentrations (data not shown). As seen from Fig. 5, BAP inhibits the luciferase

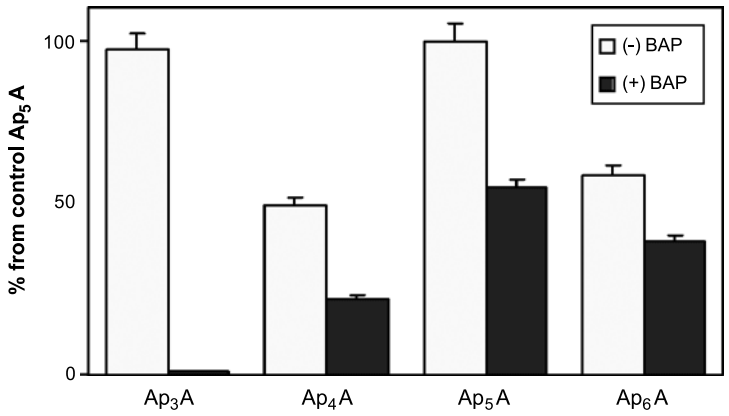


***Figure 5.*** The effect of BAP on $Ap_nA$-induced luciferase response. 500 μM $Ap_nAs$ (n = 3, 5, 6) were incubated in the absence and presence of BAP (100 units/ml) for 30 min at 37°C prior to addition to the bioluminescent assay mixture and luminescence was determined as described in the Methods. Data are an average of three experiments performed in duplicate and are reported as relative percent of the maximum (obtained for untreated $Ap_5A$). The values for $Ap_4A$ (500 μM) were obtained from the curves in Fig. 3 and are shown only for comparison purposes.





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response from all the dinucleotides tested. The inhibition was greater than 99% for $Ap_3A$, suggesting that the initial response (obtained from untreated samples) was entirely due to a light producing and BAP-sensitive contaminant, most likely ATP. BAP inhibited 44 and 34% of the $Ap_5A$ and $Ap_6A$ luminescence, respectively; this behavior is consistent with these two dinucleotides containing ATP impurities, but also having an inherent activity as weak luciferase substrates. Our data suggest that among the $Ap_nAs$ that appear to induce a bioluminescent response ($n = 3-6$), only those with more than four phosphates may be actually used as weak substrates by the luciferase enzyme, while all of them contain mononucleotide impurities.

## DISCUSSION

The work presented here arose from the observations that $Ap_nAs$, along with mononucleotides, interact with various P2 purinoceptors in different tissues to induce $Ca^{2+}$ mobilization.[2,3] In BAEC, we found that $Ca^{2+}$ is mobilized from P2 receptors with significantly lower affinity and efficiency by $Ap_4A$, as compared to ATP.[13] These data could be explained equally well by $Ap_4A$ interacting as a partial agonist to P2 purinoceptors,[13,21] and/or by the presence of a minor ATP impurity (< 1%) in the $Ap_4A$ preparations. Other investigators have reported nucleotide contaminants in various mono- and dinucleotide preparations,[16–18,22–30] and have shown that even minor concentrations of such contaminants were responsible for significant alterations of the responses obtained at various P2X and P2Y receptors.[16,23–28]

The studies herein clearly indicate that when characterizing the physiological effect of $Ap_nAs$ on P2 purinoceptors or other ATP-binding proteins, investigators must assure that the response elicited is not due to an ATP impurity. In an attempt to correctly establish the $Ap_4A$ profile at the P2 receptors on BAEC and potentially at other purinergic targets, we devised a simple method that enables one to detect, estimate, and eliminate ATP impurities in $Ap_4A$. The novelty of the method presented here consists in an enhanced detection capability achieved by coupling two simple techniques that are readily available to most laboratories (BAP treatment and firefly luciferase assay) followed by the use of an uncomplicated formula. Three lines of evidence support the validity of this method. First, we have found that treatment with BAP is a simple, safe, and efficient method for eliminating mononucleotide impurities in $Ap_4A$ samples. Our data show that 100 units/ml BAP do not hydrolyze $Ap_4A$, while effectively breaking down ATP (Figs. 2B and C). Second, we have demonstrated that the coupled assay can detect minute ATP impurities in $Ap_4A$ preparations. By incubating $Ap_4A$ samples in the presence and absence of BAP, prior to luminescence measurents, we have found that the dinucleotide is indeed contaminated with a small amount of ATP (Fig. 3). Third, the straightforward formula described in the Results enables one to estimate the degree of ATP contamination in $Ap_4A$ samples. By using our formula, we have found that the $Ap_4A$ preparations contained at least 0.2% ATP impurity. This estimate is in fact a lower limit of the contamination range, because $Ap_4A$ actually exerts an inhibitory action on the ATP induced response (unpublished observations), so that the ''impure'' $Ap_4A$ response will be less than the predicted sum of the ''pure'' $Ap_4A$ and contaminant curves. Similar calculations revealed the presence of at least 0.3% ATP impurity in $Ap_5A$ preparations (data not shown). Our results are comparable with





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previous data obtained by other laboratories with HPLC and capillary electrophoresis, which detected 0.1–0.5% ATP contaminants within dinucleotide samples;[29,30] therefore our method could be used as a more affordable alternative by laboratories that are not equipped with such devices. This pertains especially to situations where complete purification of the $Ap_nA$ samples is not a requirement, i.e. when the breakdown products of the targeted contaminant are not interfering with the experimental setup (in our case, the study of the $Ap_nA$ interaction with P2 receptors in BAEC). Though $Ap_4$ could also be present in the $Ap_4A$ samples, much higher concentrations of this contaminant would be required to explain both the luminescence and the $PGI_2$ response data (see ''Estimation of the Mononucleotide Contamination in $Ap_4A$ Preparations'' and Tables 1 and 2). It is however conceivable that both ATP and $Ap_4$ be present, in different degrees, within the $Ap_4A$ preparations, with a smaller concentration of ATP exerting more interference than a higher concentration of $Ap_4$. Though our procedure cannot distinguish between these two mononucleotide impurities, the BAP-treatment of the dinucleotide samples can effectively eliminate either or both contaminants.

Our procedure can also be used to detect ATP impurities in other $Ap_nAs$. We have found that $Ap_nAs$ (n = 3, 5 and 6) also appear to be contaminated with varying amounts of ATP (Fig. 5). Since BAP treatment was safe and efficient for $Ap_4A$, and in view of the fact that BAP only recognizes free phosphate residues and it is not inhibited by the nucleoside portion of the nucleotides, we believe that the security and effectiveness of the procedure apply to all $Ap_nAs$ (n = 2–6). While our method could be used for decontamination purposes on all $Ap_nAs$, its use for detecting and estimating the ATP impurities is restricted for samples that induce a luminescent response before being treated with BAP. Since untreated $Ap_2A$ did not induce a light reaction, it was not possible to estimate the degree of contamination, although the absence of a luminescent signal suggested that this dinucleotide was devoid of ATP impurities (data not shown). The applicability of our method may not be restricted to the adenine dinucleotides tested herein ($Ap_{3-6}A$), but could also comprise other dinucleotides ('$Xp_nX$').

Previous findings from our laboratory have led to the conclusion that $Ap_4A$ interacts with two populations of binding sites on the BAEC surface: one specific for dinucleotides—possibly P4 receptors, as well as a population of mononucleotide-specific sites—P2 purinoceptors,[11] which are involved in the $Ap_nA$-induced $Ca^{2+}$ mobilization and $PGI_2$ release.[13] The increased detection capability of the method presented herein could be very useful when analyzing the $Ap_4A$ interaction with targets that are specific for ATP and not for the dinucleotides. In support of this statement, we have demonstrated that the stimulated $PGI_2$ release from BAEC in response to $Ap_4A$[13] is mostly due to the agonistic action of $Ap_4A$ itself, with only partial influence from the mononucleotide impurities (Table 2). Since the BAP-resistant impurities still present in Fig. 2B could not belong to the mononucleotide class, they are not likely to have interfered with the $PGI_2$ release from P2 purinoceptors. It is however possible that $PGI_2$ can be induced by such contaminants by stimulation of a different receptor category, provided that the impurities fall in the class of extremely potent agonists and reach a significant effect at extremely low doses (as is the case for ATP); such issues should therefore be addressed by investigators concerned with the study of the respective receptors. By using our method, the true effect of $Ap_4A$ on P2 receptors was easily





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discernible from the effect of contaminating mononucleotides. Thus, in view of the negative modulation clearly exerted by $Ap_4A$ on endothelial P2 receptors,[13] the data herein are consistent with $Ap_4A$ acting as a partial agonist to these receptors. Contrary to our results, other investigators have shown that pre-treatment of $Ap_nAs$ (n = 4–6) with phosphatase enzymes precluded responses from native P2Y1 and P2Y2 receptors in human endothelial cells.[17,18] The reason for this discrepancy could lie in the different origin of the cells used (bovine versus human), as well as in the possibility that $Ap_4A$ interact with other P2 receptors subtypes present on BAEC surface. Alternatively, the use of a higher enzyme concentration and the presence of active phosphatase enzymes in the experimental cell system could have interfered with the measurements reported in Ref. [17,18]. To avoid prolonged incubation of the cells with active BAP in our experiments, we developed an effective BAP inactivation procedure that does not affect $Ap_4A$'s stability; this extends the applicability of our method to situations where an active BAP could interfere with the experimental design.

We have also applied our method to investigating the luciferase interaction with $Ap_nAs$ as potential substrates. We have demonstrated that BAP-treated $Ap_4A$ is a substrate for the luminescent reaction catalyzed by luciferase, with a relatively strong binding to the enzyme, but without the ability of inducing efficient light output (Figs. 1 and 3). Similar light levels were obtained when BAP-treated $Ap_{5-6}A$ were used as substrates in the luminescent reaction (Fig. 5). Our data are consistent with previous reports noticing the firely luciferase interaction with $Ap_4A$ and $Ap_5A$ to induce low levels of light;[20,31] however, in those reports the mononucleotide contaminants had not been eliminated from the dinucleotide preparations. The decreased efficacy of $Ap_{4-6}A$ as compared to ATP is likely associated with steric interferences of the bulkier dinucleotides with the luciferase environment, which may significantly restrict conformational changes required for efficient light production. Since in our experiments the $K_m$ and $V_{max}$ values were determined from luminescence measurements, they give information on the aggregate result of the light reaction; thus, $Ap_4A$ steric interferences may occur at any of the steps involved in bioluminescence. The $Ap_4A$ cleavage shown herein seems to be the reverse reaction of the demonstrated dinucleotide synthesis activity of the luciferase.[32] The high $K_m$ for ATP (4 mM) obtained by Sillero and collaborators for the $Ap_4A$ production, as well as the prolonged incubation times needed for their determinations,[33] suggest that, at pH 7.8, the use of $Ap_4A$ as a substrate ($K_m < 1$ mM) may be favored energetically over its synthesis. Interestingly, $Ap_nAs$ with two and three phosphates do not function as substrates for the light production reaction, although untreated $Ap_3A$ seemed to induce luminescence. In agreement with our findings, it has been shown that the synthetic activity of luciferase preferentially yields $Ap_nAs$ ($n \geq 4$),[32] consistent with the favored use of the same dinucleotide substrates in the reverse reaction. A more complete analysis of the $Ap_nA$ interaction with luciferase is beyond the purpose of this paper and will be presented elsewhere.

Besides BAP, other phosphate-cleaving enzymes like the apyrase and the creatine phosphokinase (CPK) have been previously used to eliminate various mononucleotide impurities within $Ap_nA$ samples.[16–18] Our method can take such treatments—by coupling them with the luciferase assay—one step further: from the level of elimination to that of estimation of the ATP contaminants. However, the use of CPK or apyrase may have several limitations. Unlike apyrase that degrades ATP and ADP down to





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AMP, CPK is not suitable for general purpose $Ap_nA$ decontamination, but rather for cases where investigators specifically seek either the ATP degradation into ADP, or the regeneration of ATP from ADP. Since either of these compounds are active at several P2 receptors, the interest of using CPK would be limited to those P2 receptor systems where one (ATP) or the other (ADP) is inactive. Therefore, the use of BAP presents the advantage of degrading adenine mononucleotide contaminants down to adenosine, which does not interact with P2 receptors and neither does it interfere with the luciferase assay (data not shown), thus allowing an efficient coupling. Similarly, the product of the apyrase reaction (AMP) does not interact with P2 receptors, which allows an efficient decontamination of $Ap_nA$ samples; however, both AMP and ADP are likely to inhibit the luciferase activity,[34] thus possibly precluding an attempt to couple the treatment by apyrase or CPK with the luciferase assay. Such an inquiry was not within the aim of our paper, but would be fully justified if attempted by other laboratories, together with the assessment of $Ap_nA$ stability in the presence of such enzymes.



## CONCLUSION

The data from this paper demonstrate that $Ap_nAs$ are contaminated with minute amounts of ATP and thus emphasizes the importance of decontamination when investigating the effects of $Ap_nAs$ on P2 receptors or any ATP-binding target. The method we propose to estimate and remove ATP impurities from $Ap_nA$ preparations is characterized by an enhanced detection capability, achieved by coupling two simple techniques (BAP treatment and luciferase assay) that are both specific for ATP and not for dinucleotides. Thus, dinucleotides treatment with BAP or possibly other phosphate-cleaving enzymes, together with the use of appropriate controls, could provide a means for correct interpretation of the results.

## ABBREVIATIONS

| | |
|---|---|
| 6-keto $PGF_{1\alpha}$ | 6-keto prostaglandin $F_{1\alpha}$ |
| ADP | adenosine 5′-diphosphate |
| AMP | adenosine 5′-monophosphate |
| $Ap_4$ | adenosine 5′-tetraphosphate |
| $Ap_nA$ | diadenosine polyphosphates |
| ATP | adenosine 5′-triphosphate |
| BAEC | bovine aortic endothelial cells |
| BAP | bovine alkaline phosphatase |
| $Ca^{2+}$ | intracellular calcium |
| CPK | creatine phosphokinase |
| EIA | enzyme immunometric assay |
| FBS | fetal bovine serum |
| L-Arg | L-arginine |
| MEM | minimal essential medium |



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| $PGI_2$ | prostacyclin |
| TLC | thin layer chromatography |

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