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Research Article

Synthesis of dinucleoside tetraphosphates in transfected cells by a firefly luciferase reporter gene

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Abstract. The firefly luciferase gene is widely used as a reporter gene and its expression is generally considered to be non-toxic. In addition to its light-producing reaction, luciferase can synthesise dinucleoside polyphosphates, intracellular signalling molecules, in vitro. Here we show that COS-7 cells transfected with a luciferase expression vector accumulate up to 0.5 mM adenine-containing dinucleoside tetraphosphates (Ap_4N) during the 24 h following luciferin addition. The optimal external concentration of luciferin was 0.4–0.6 mM. In agreement with its poor ability to synthesise adenine-containing dinucleoside triphosphates in vitro, the level of these compounds did not increase after transfection. Consequently, the results of experiments involving luciferase-mediated light production by live cells should now be viewed in the light of the possible effects of an increased intracellular Ap_4N concentration on the properties of the system under investigation. This observation also points to a useful non-invasive procedure for the specific enhancement of intracellular Ap_4N for studies directed at understanding the functions of these compounds.

Key words. Firefly luciferase; reporter gene; diadenosine tetraphosphate synthesis; nucleotide.

The luciferase gene from the firefly *Photinus pyralis* is widely used as a reporter gene for the evaluation of gene transfer and for the non-invasive imaging of gene expression and promoter activity in single cells [1, 2]. Microinjected or plasmid-encoded luciferase has also been used to measure intracellular ATP [3, 4]. More recently, cells and organisms expressing recombinant luciferase have gained use as sensitive biosensors for cellular stress [5, 6]. Ideally, expression of a reporter gene should not alter the properties of the cell during the course of an experiment in such a way as to render the cell biochemically and physiologically different from the untransfected control. Expression of firefly luciferase is generally considered to be non-toxic; however, there is one aspect of luciferase biochemistry that has not been adequately considered in relation to its use as a reporter and which has the potential to disturb normal cellular metabolism.

In vitro, firefly luciferase can synthesise dinucleoside polyphosphates, predominantly those containing at least one adenine moiety and four or more phosphate groups, such as diadenosine 5′,5‴–P^1,P^4-tetraphosphate (Ap_4A) and adenosine(5′)tetraphospho(5′)guanosine (Ap_4G) [7, 8]. These compounds have been ascribed various intracellular signalling roles and are potentially toxic if allowed to accumulate [9–11]. In a mechanism analogous to that used by aminoacyl-tRNA synthetases, the enzymes believed to be the predominant source of Ap_4A in vivo, D-luciferin acts as an adenylate acceptor from ATP, forming luciferyl-AMP then dehydroluciferyl-AMP as intermediates, the latter then donating the AMP moiety to another ATP to yield Ap_4A [7, 12]. Reaction between dehydroluciferyl-AMP and various different nucleotide acceptors can give rise to a family of adenine-containing dinucleo-

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side polyphosphates (Ap_nN), where $n \geq 4$ [13, 14]. Compounds of the form Ap_3N are poorly synthesised. In vitro, Ap_nN synthesis is favoured by the addition of inorganic pyrophosphatase to the reaction mixture to prevent pyrophosphorolysis of the dehydroluciferyl-AMP and consequent regeneration of luciferin and ATP [7].

The ability of luciferase to synthesise Ap_nN in vivo has not been investigated. The possibility that this is a significant intracellular reaction in the firefly is suggested by the fact that extracts of firefly tails have normal levels of Ap_4N but a much higher specific activity of the enzyme diadenosine tetraphosphatase than other biological systems [15]. This member of the Nudix hydrolase family is primarily responsible for the catabolism of Ap_nN ($n \geq 4$) in vivo [16]. Ap_4Ns are likely to be of greatest biological significance, given the low intracellular levels of the p_4N and p_5N acceptors required to generate Ap_5N and Ap_6N, respectively [17]. In view of the widespread use of the firefly luciferase gene as a reporter, we investigated the ability of the enzyme to synthesise Ap_4N in COS-7 cells transiently transfected with a plasmid expressing the luciferase gene. The results show that cells expressing recombinant luciferase can accumulate substantial levels of Ap_4N.

Materials and methods

Plasmids

pMW41 is a 7-kb vector based on pBR322. It has an SV40 replication origin and expresses native, peroxisomally targeted *P. pyralis* luciferase under the control of the immediate early human cytomegalovirus promoter/enhancer [18]. It was kindly provided by Dr. M. R. H. White (University of Liverpool, U. K.). The control plasmid, pMW41Δ*luc*, was generated by excising the major portion of the *luc* gene between the two *Xba*I sites.

Transfection

COS-7 African green monkey kidney cells were grown in 35-mm^2 dishes in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum under 5% CO_2. Cells were seeded 16–24 h before transfection then transfected at 60–70% confluence with 1 µg plasmid DNA per dish (in triplicate) using 2 µl FuGENE 6 transfection reagent (Roche) per microgram DNA, following the manufacturer's instructions. Cell counts were performed in triplicate, mock-transfected dishes.

Luciferase assay

Cell lysates were prepared at different times after transfection in Cell Culture Lysis Reagent (Promega). Luciferase activity was determined luminometrically using the Bright-Glo Luciferase Assay System (Promega) following the manufacturer's instructions.

Dinucleoside polyphosphate assay

Cell extracts were prepared and nucleotides extracted, purified and assayed as previously described [19] except that, for 35-mm^2 dishes, a total of 2 ml 0.4 M TCA was used and subsequent volumes of reagents were adjusted accordingly. Briefly, neutralised acid extracts were digested with alkaline phosphatase, the resistant nucleotide fraction isolated on DEAE-Sephacel, then eluted, freeze-dried and split into two portions. Adenine-containing dinucleoside tetraphosphates (Ap_4N) were measured in one portion by treating with snake venom phosphodiesterase and measuring the ATP released luminometrically. Adenine-containing dinucleoside triphosphates (Ap_3N) were similarly measured in the second portion by including PEP and pyruvate kinase to generate additional ATP from the ADP released by the phosphodiesterase. The amounts of Ap_3N and Ap_4N were then estimated making two assumptions. The first is that Ap_3A and Ap_4A are each 50% of the Ap_3N and Ap_4N pools, respectively. This is supported by measurements of individual Ap_3N and Ap_4N nucleotides in *Escherichia coli* [20]. The second is that the phosphodiesterase used in the assay cleaves equally efficiently at either end of each Ap_nN. So, for example, 1 mol Ap_4A yields 1 mol ATP + 1 mol AMP while 1 mol Ap_4N yields 0.5 mol ATP, 0.5 mol NTP, 0.5 mol AMP and 0.5 mol NMP. A lack of base specificity when cleaving dinucleoside polyphosphates has frequently been observed with this enzyme. These assumptions require the figure for Ap_nN concentration to be multiplied by 1.33 to compensate for the 50% efficiency in ATP production from Ap_nN ($N \neq A$).

Results and discussion

Transfection of COS-7 African green monkey kidney cells with the luciferase expression vector pMW41 led to a time-dependent increase in luciferase activity in cell extracts. Activity reached a maximum 24 h after transfection and this steady-state value was maintained for a further 30 h (fig. 1). The control plasmid pMW41Δ*luc* produced no measurable activity. To determine whether this recombinant luciferase could synthesise dinucleoside tetraphosphates of the form Ap_4N under intracellular conditions, luciferin was added to cells 24 h after transfection with pMW41 and the cells incubated for up to a further 24 h. Cells were then extracted and assayed luminometrically using an assay that specifically detects Ap_4N [19]. Figure 2 shows the time-dependent accumulation of Ap_4N from a basal value of 0.4 pmol/10^6 cells at the time of addition of luciferin to a mean value of 444 pmol/10^6 cells after 24 h, a massive 1000-fold increase. The levels of Ap_4N in untransfected cells and cells transfected with pMW41Δ*luc* were 0.35 and 0.6 pmol/10^6 cells respectively, 24 h after addition of luciferin. The dependence of Ap_4N accumula-

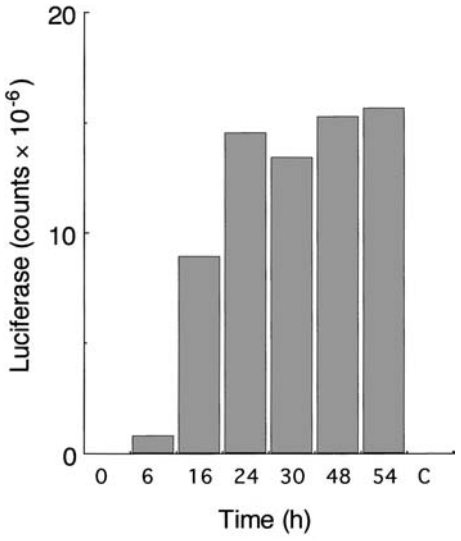

Figure 1. Luciferase expression in COS-7 cells. Cells were transfected with pMW41 and cell lysates assayed for luciferase activity at various times after transfection (0–54 h). Cells were also transfected with pMW41Δ*luc* and assayed after 24 h (C). Values are means of triplicate determinations.

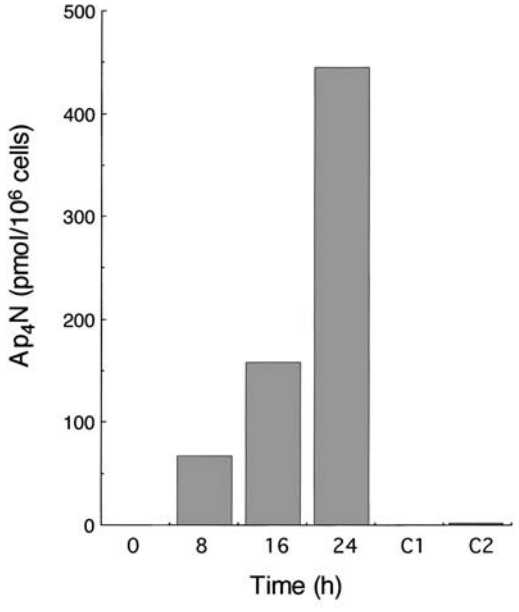

Figure 2. Dinucleoside tetraphosphate (Ap_4N) synthesis in transfected COS-7 cells. Cells were transfected with pMW41. After 24 h, beetle D-luciferin (Promega) was added to 0.5 mM and Ap_4N measured in cell extracts at various times thereafter (0–24 h). Control cells were either not transfected (C1) or transfected with pMW41Δ*luc* (C2). Values are means of triplicate determinations.

tion on luciferin concentration is shown in figure 3. Luciferin was absolutely required, and maximum synthesis was obtained when the external luciferin concentration was between 0.4 and 0.6 mM. Although the mammalian plasma membrane is not freely permeable to luciferin at physiological pH, an external concentration of 0.5 mM luciferin would be expected to yield an intracellular concentration of 25–50 µM [21]. Given the reported K_m for Ap_4N synthesis of 2–3 µM [13], this should support close to the maximum achievable rate of Ap_4N synthesis. Higher concentrations led to a reduction in Ap_4N. This may reflect the accumulation of an inactive, luciferase-oxyluciferin complex due to the limiting level of intracellular inorganic pyrophosphatase that normally prevents complex formation at lower luciferin levels [21]. Figure 3 also shows that, in agreement with its properties in vitro, luciferase did not lead to an increase in dinucleoside triphosphates of the form Ap_3N. Values for Ap_3N were 5.3 pmol/10^6 cells in the absence of luciferin, declining gradually to 2.1 pmol/10^6 cells at 1 mM external luciferin.

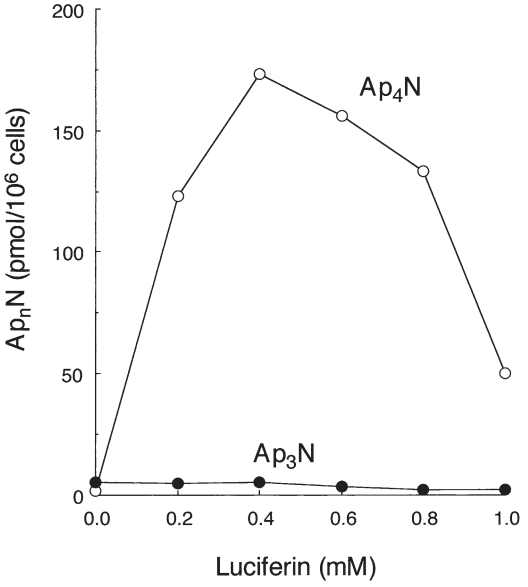

Figure 3. Effect of luciferin concentration on dinucleoside polyphosphate (Ap_nN) synthesis. COS-7 cells were transfected with pMW41. After 24 h, D-luciferin was added and dinucleoside triphosphate (Ap_3N) and tetraphosphate (Ap_4N) pools measured in cell extracts after a further 16 h. Values are means of triplicate determinations.

The level of Ap_4N in untransfected cells is within the range previously measured for several mammalian cell lines [19, 22] and is in very close agreement with the figure of 0.45 pmol/10^6 cells we previously reported for Vero green monkey kidney cells [23]. Using a figure of 1.75 pl for the intracellular volume of a COS-7 cell (based on a diameter of 15 µm) and assuming that a maximum of 50% of the cells were transfected, then a figure of 444 pmol/10^6 cells for Ap_4N equates to an intracellular concentration of 0.5 mM, compared to the control level of 0.5 µM. Such a high concentration almost certainly reflects the high level of luciferase expression directed by pMW41 through the combined effects of the SV40 replication origin and the strong cytomegalovirus promoter. Since high levels of luciferase have been used to measure intracellular ATP [3, 4], possible perturbation to nu-

cleotide pools caused by Ap_4N synthesis should be considered, particularly if exposure to luciferin is prolonged. Although most experiments involving cloned luciferase do not lead to such high-level expression, the accumulation of lower levels of Ap_4N may also have significant effects. Ap_4A has in the past been linked to the regulation of DNA replication and repair and to the modulation of stress responses, although none of these has been fully substantiated in mammalian cells [10, 11]. More recently, intracellular Ap_3A and Ap_4A have been shown to mediate the glucose-induced blockade of the pancreatic β cell ATP-regulated potassium channel [24, 25], while addition of Ap_4A at 10 µM to certain reversibly permeabilised cells has been reported to induce cell cycle arrest and apoptosis [26]. Ap_4A and Ap_3A are also important, possibly competitive, ligands of Fhit, a tumour suppressor protein involved in a pro-apoptotic signalling pathway [27, 28]. Elevated Ap_nN may also interact with signalling systems involving diphosphoinositol polyphosphates [29], while in bacteria, elevated Ap_nN can have profound effects on transcription and phenotype, causing filamentation [30, 31]. Finally, Ap_4A and other Ap_4Ns can bind to and inhibit a variety of enzymes, including nucleotide kinases and protein kinases and so are potentially toxic if allowed to accumulate [10, 32].

Thus, Ap_nNs are biologically active molecules and, while their biological properties remain to be fully defined, the potential effects of increased Ap_4N in experiments where luciferase is used as a real-time reporter gene or biosensor in the presence of luciferin should be considered when interpreting the results of such experiments. The effect will almost certainly depend on the cell type, the level of luciferase expression and the organelle to which it is targeted. The construct used in the experiments reported here targeted luciferase to its normal location, the peroxisomes, so much of the Ap_4N in our transfected COS cells may be confined to these organelles, where effects may only be noticed under certain conditions. In view of these findings, we recommend that investigators measure the intracellular Ap_4N content of their transfected cells to ensure that substantial levels have not accumulated.

On a different note, cells transfected with a luciferase construct, particularly if under the control of a tightly regulatable promoter, should offer a useful system with which to study the biological effects of increased Ap_4N and an increased Ap_4N/Ap_3N ratio. Luciferase has the advantage over most aminoacyl-tRNA synthetases in its specificity for Ap_nN synthesis (where $n \geq 4$) while transfection is also to be preferred over permeabilisation procedures, which can lead to unwanted cellular stress.

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