Diadenosine polyphosphates and the control of cyclic AMP concentrations in isolated rat liver cells

Mandy Edgecombe, Alexander G. McLennan, Michael J. Fisher*

School of Biological Sciences, University of Liverpool, Liverpool L69 7ZB, UK

Received 8 July 1999; received in revised form 19 July 1999

Abstract Extracellular diadenosine polyphosphates (Ap_nA), through their interactions with appropriate P_2 receptors, influence a diverse range of intracellular activities. In particular, Ap_4A stimulates alterations in intracellular calcium homeostasis and subsequent activation of glycogen breakdown in isolated liver cells. Here we show that, like ATP, Ap_4A and other naturally occurring diadenosine polyphosphates attenuate glucagon-stimulated accumulation of cyclic AMP in isolated rat liver cells. The characteristics of Ap_4A- and ATP-dependent modulation of glucagon-stimulated cyclic AMP accumulation are similar. These results are discussed in the context of the repertoire of intracellular signalling processes modulated by extracellular nucleotides.

Key words: Diadenosine polyphosphate; Cyclic AMP; Glucagon; Liver cell

1. Introduction

Diadenosine polyphosphates (Ap_nA; where $n = 3$, 4, 5 or 6) have been identified in a wide variety of cells [1]. In mammalian cells they account for a significant proportion of the nucleotide content of a variety of storage granules including dense granules of platelets [2], secretory granules of chromaffin cells [3] and neuronal synaptic vesicles [4]. Liberation into the extracellular environment allows diadenosine polyphosphates to exert effects on a diverse range of target cells [5]. Compared to ATP, the diadenosine polyphosphates are characterised in vivo by relatively long half-lives and may therefore be suited to a role as long-range signalling molecules, exerting effects on target cells relatively distant from the site of their release into the extracellular space [2].

Diadenosine polyphosphates promote significant alterations in liver cell metabolism. Both Ap_3A and Ap_4A cause a transient release of CA^{2+} and stimulation of glucose output from the perfused rat liver [6]. In isolated rat liver cells, these effectors promote dose-dependent stimulation of glycogen phosphorylase activity [7] and oscillations in cytosolic free [Ca^{2+}] that resemble those seen in liver cells exposed to ATP or ADP [8]. An analysis of the characteristics of the binding of Ap_4A to rat liver cell membranes suggests that it exerts its effects through interaction with P_{2Y}-like purinoceptor population [9].

In addition to their well characterised effects on intracellular calcium metabolism, extracellular adenine mononucleotides (e.g. ATP), like many other Ca^{2+}-mobilising agents, influence cyclic AMP metabolism. Specifically, ATP attenuates the impact of both forskolin and glucagon on cyclic AMP accumulation in isolated liver cells [10,11]. These effects have been ascribed to mononucleotide-mediated stimulation of phosphodiesterase activity and/or inhibition of adenylate cyclase activity. Analogous effects of extracellular adenine mononucleotides have been observed in relation to agonist-stimulated cyclic AMP accumulation in a range of different cell types (e.g. isoproterenol-treated C6 glial cells [12]; PGE_1-treated NG108-15 and NIE-115 neural cells [13]). It has also been suggested that exposure of isolated liver cells to Ap_3A and Ap_4A may also result in a diminution of glucagon-mediated cyclic AMP accumulation [14]. In contrast, in other cell types extracellular ATP stimulates cyclic AMP accumulation (e.g. MDCK epithelial cells [15] and mouse C2C12 myotubes [16]).

Here we describe an analysis of the effects of extracellular diadenosine polyphosphates on intracellular cyclic AMP concentrations in isolated rat liver cells. The purpose of this investigation was to provide a better understanding of the overall impact of these unusual nucleotides on intracellular signalling processes and also to provide information on the nature of the relationship between diadenosine polyphosphate- and mononucleotide-mediated intracellular signalling processes in rat liver cells.

2. Materials and methods

2.1. Chemicals

All nucleotides and cyclic AMP-dependent protein kinase (from porcine heart) were obtained from Sigma, Poole, Dorset, UK. Collagenase and fatty acid-free bovine serum albumin were from Boehringer (London), Lewes, Sussex, UK. [8-^{3}H]Adenosine-3′,5′-cyclic phosphate ([^{3}H]cyclic AMP; 23 Ci/mmol) was obtained from Amersham International, Amersham, Bucks, UK. Optiphase Safe scintillation fluid was from Pharmacia-LKB Biotechnology, Milton Keynes, UK. All other chemicals were of the purest grade available from standard suppliers.

2.2. Animals

Male Wistar rats (University of Liverpool breeding colony) weighing 180–220 g were used throughout. Animals were fed ad libitum (Labsure Animal diet (CRM); C. Hill Group, Poole, Dorset, UK).

2.3. Preparation and incubation of isolated rat liver cells

Liver cells were prepared as described previously [17]. Cells were incubated at 37°C in Krebs-Henseleit medium [18]. In all cases, the incubation medium was supplemented with 2.5 mM $CaCl_2$, 2% (w/v) fatty acid-free bovine serum albumin, lactate/pyruvate (9:1; final conc. 10 mM) and 10 mM glucose, Incubation volumes were 0.4 ml throughout. Incubations were terminated by addition of 0.04 ml of

*Corresponding author. Fax: (44) (151) 794-4349.
E-mail: fishermj@liv.ac.uk

Abbreviations: Ap_2A, diadenosine 5′,5‴-P^1,P^2-diphosphate; Ap_3A, diadenosine 5′,5‴-P^1,P^3-triphosphate; Ap_4A, diadenosine 5′,5‴-P^1,P^4-tetraphosphate; Ap_5A, diadenosine 5′,5‴-P^1,P^5-pentaphosphate; Ap_6A, diadenosine 5′,5‴-P^1,P^6-hexaphosphate; Ap_4, adenosine 5′-tetraphosphate; 2MeSATP, 2-methylthioadenosine 5′-triphosphate

PII: S0014-5793(99)01099-6

2 M $HClO_4$. Acidified suspensions were centrifuged at 12 000 × *g* for 2 min at 4°C to remove denatured protein. Portions of supernatant were then neutralised with 0.5 M triethanolamine/HCl containing 2 M KOH. After storage on ice, neutralised samples were centrifuged at 12 000 × *g* for 2 min at 4°C (to remove $KClO_4$) and supernatants were retained for determination of cyclic AMP concentrations. In all experiments, metabolic integrity was assessed by measurement of the cellular ATP content [19].

2.4. Measurement of cyclic AMP concentrations

Samples from isolated liver cell incubations, prepared as described above, were utilised for assay of cyclic AMP concentrations essentially as described previously [20]. Briefly, the assay depended upon the displacement, by unlabelled cyclic AMP, of [^{3}H]cyclic AMP from the cyclic AMP-dependent protein kinase. Construction of a standard curve, prepared by addition of known amounts of unlabelled cyclic AMP (0–25 pmol) to neutralised, perchloric acid-treated incubation buffer (see above), allowed the estimation of the cyclic AMP content of neutralised liver cell extracts.

3. Results

Incubation of isolated liver cells in the presence of extracellular ATP or Ap_4A did not result in significant alterations in the basal intracellular cyclic AMP concentration (Table 1). In contrast, when the intracellular cyclic AMP concentration was elevated, by exposure of liver cells to a physiological concentration of glucagon, both ATP and Ap_4A were able to significantly attenuate this change. Fig. 1 shows time courses for glucagon-induced cyclic AMP concentration changes in the absence and presence of these nucleotides. It is clear from these data that ATP and Ap_4A are similarly effective in attenuating the glucagon-mediated increase in cyclic AMP concentration. Both effectors act rapidly (<1 min) to decrease the change in glucagon-stimulated cyclic AMP accumulation. It should be noted that over this time course, under these incubation conditions, significant degradation of the Ap_4A does not occur [7] implying that it is the intact nucleotide that is directly responsible for the observed effects.

Fig. 2 shows the effect of variation in nucleotide concentration on the accumulation of cyclic AMP observed in response to a physiological concentration of glucagon. Attenuation of cyclic AMP accumulation was apparent at concentrations of Ap_4A (and ATP) <100 μM. Given that Ap_4A concentrations in vivo have been estimated to reach 10–100 μM [2], it would appear that the attenuation of hormone-stimulated increases in liver cell cyclic AMP concentration is, potentially, a significant aspect of the intracellular signalling processes triggered by this agent. Fig. 3 shows the impact of Ap_4A and ATP on the accumulation of cyclic AMP brought about by different concentrations of glucagon. These data indicate that both nucleotides are potent attenuators of the cyclic AMP accumulation induced by concentrations of glucagon within the physiological range. Taken together the data in Figs. 2 and 3 suggest that the ability of Ap_4A to diminish glucagon-stimulated increases in cyclic AMP concentrations is of physiological significance; additionally, the characteristics of this effect are essentially similar for both ATP and Ap_4A. However, in view of the relative stabilities of ATP and Ap_4A in the extracellular environment (see Section 1), it might be anticipated that Ap_4A is the more effective agent, if released from a site relatively distant from the target tissue (i.e. liver).

In addition Ap_4A, both higher and lower homologues are also present in mammalian tissues. The ability of a range of diadenosine polyphosphates to modulate glucagon-stimulated

Table 1
The effect of different nucleotides on intracellular cyclic AMP concentration in isolated liver cells

Incubation condition	[cyclic AMP] after 1 min incubation (pmol/mg dry weight)
Basal	1.59 ± 0.38
1 mM ATP	2.97 ± 0.90
1 mM Ap_4A	1.67 ± 0.48
1 nM Glucagon	13.61 ± 1.46*
1 nM Glucagon+1 mM ATP	6.25 ± 0.74*†
1 nM Glucagon+1 mM Ap_4A	5.36 ± 0.99*†

Results are expressed as means ± S.E.M. for at least three independent liver cell incubations. The significance of difference between means was assessed by Student's *t*-test. *$P<0.05$, versus basal condition; †$P<0.05$, for glucagon and the indicated nucleotide versus glucagon only. Other differences were not significant.

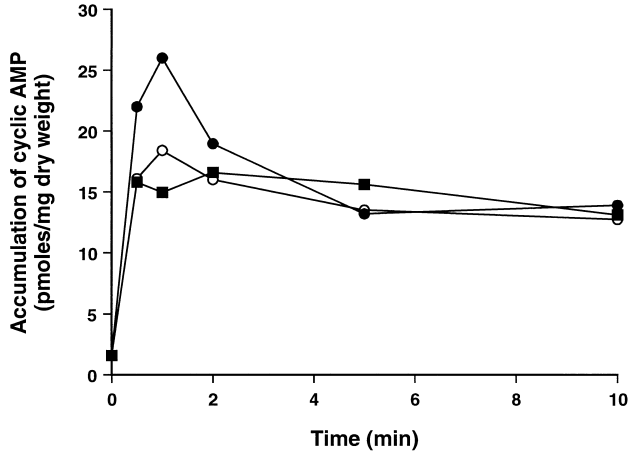

Fig. 1. Time course of cyclic AMP accumulation following exposure of isolated liver cells to glucagon (10 nM), either alone (●) or in the presence of 100 μM ATP (■) or 100 μM Ap_4A (○). Results are the means of triplicate values from a single representative experiment.

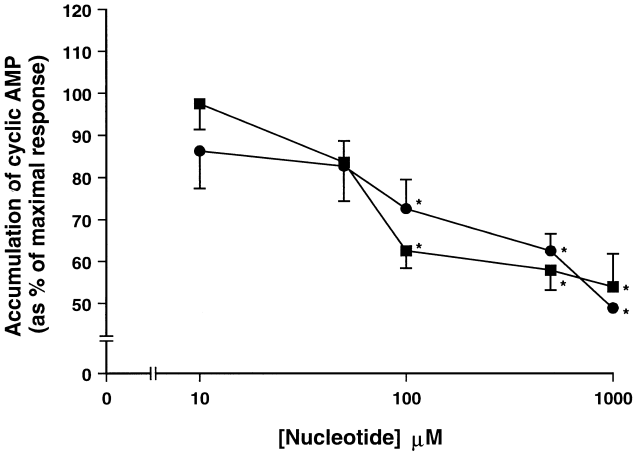

Fig. 2. Dose-dependent effect of ATP (■) or Ap_4A (●) on 10 nM glucagon-stimulated cyclic AMP accumulation in isolated liver cells, after 1 min incubation. Results are expressed as means ± S.E.M. for at least three independent liver cell incubations. The significance of difference between means was assessed by Student's *t*-test. *$P<0.05$, for glucagon and the indicated nucleotide versus glucagon only. Other differences were not significant.

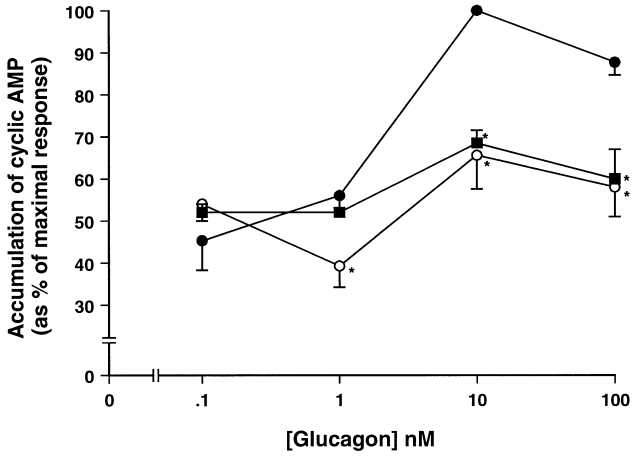

Fig. 3. Accumulation of cyclic AMP in isolated liver cells following addition of glucagon, either alone (●), in the presence of 100 μM ATP (○) or 100 μM Ap_4A (■), after 1 min incubation. Results are expressed as means ± S.E.M. for at least three independent liver cell incubations. The significance of difference between means was assessed by Student's *t*-test. $^*P < 0.05$, for glucagon and the indicated nucleotide versus glucagon only. Other differences were not significant.

cyclic AMP accumulation in isolated liver cells is illustrated in Table 2. It is clear from these data that the other diadenosine polyphosphates are also effective attenuators of glucagon-stimulated cyclic AMP accumulation. The only exception is the synthetic nucleotide Ap_2A which appears to be without effect. This parallels the inability of Ap_2A to displace [^{3}H]Ap_4A from hepatic purinoceptors [9]. It is also of interest that, whereas UTP is an effective attenuator of cyclic AMP accumulation [11], 2-methylthioadenosine 5′-triphosphate (2MeSATP) is ineffective [21]. We have previously observed that both UTP and 2MeSATP displace [^{3}H]-Ap_4A from hepatic purinoceptors [9], indicative of the presence of a mixed population of P_{2Y}-purinergic receptors responsible for binding Ap_4A (i.e. P_{2Y1} and P_{2Y2}). Consequently, it would appear that the ability of the diadenosine polyphosphates to attenuate glucagon-induced cyclic AMP accumulation may be mediated by UTP-(P_{2Y2}) rather than 2MeSATP-(P_{2Y1}) recognising receptors.

Table 2
Effect of different nucleotides on glucagon-stimulated cyclic AMP accumulation in isolated liver cells

Nucleotide (100 μM)	10 nM glucagon-stimulated cyclic AMP accumulation after 1 min incubation (as % of maximum)
Ap_6A	71 ± 4*
Ap_5A	72 ± 11*
Ap_4A	73 ± 7*
Ap_3A	72 ± 3*
Ap_2A	115 ± 6
Ap_4	70 ± 8*
ATP	63 ± 4*
UTP	57 ± 10*
2MeSATP	92 ± 8

Results are expressed as means ± S.E.M. for at least three independent liver cell incubations. The significance of difference between means was assessed by Student's *t*-test. $^*P < 0.05$, for glucagon and the indicated nucleotide versus glucagon only. Other differences were not significant.

4. Discussion

Here we have presented evidence that the diadenosine polyphosphates are capable of modulation of cyclic AMP accumulation mediated by physiological concentrations of glucagon. The characteristics of Ap_4A-mediated attenuation of cyclic AMP accumulation are essentially similar to those exhibited by ATP. It is not clear from the present data if this attenuation is a consequence of inhibition of adenylate cyclase activity and/or stimulation of cyclic AMP phosphodiesterase activity. It has been suggested that ATP-dependent inhibition of adenylate cyclase activity may be mediated by phosphatidic acid, arising from the hydrolysis of phosphatidyl-choline by phospholipase D [22,23]. In this context it is of interest that we have recently demonstrated that the diadenosine polyphosphates are capable of activating phospholipase D in isolated rat liver cells [24]; however, it should also be pointed out that UTP, an effective attenuator of agonist-stimulated cyclic AMP accumulation (see Table 2), is a relatively weak stimulator of phospholipase D activation [24].

Given the widespread occurrence of 'crosstalk' between intracellular signalling pathways, it is perhaps not surprising that the diadenosine polyphosphates, like other nucleotides, are capable of modulating the accumulation in cyclic AMP mediated by agonists such as glucagon. Likewise, it would not be surprising if alterations in cyclic AMP metabolism led to changes in the ability of the diadenosine polyphosphates to stimulate oscillations in liver cell cytosolic free [Ca^{2+}]. In this respect, recent work has confirmed that this is the case for cytosolic free [Ca^{2+}] oscillations induced by ATP and ADP [25] and also for diadenosine polyphosphate-stimulated oscillations in cytosolic free [Ca^{2+}] [26].

Acknowledgements: We thank the Wellcome Trust for financial support.

References

[1] Garrison, P.N. and Barnes, L.D. (1992) in: Ap_4A and Other Dinucleoside Polyphosphates (McLennan, A.G., Ed.), pp. 29–61, CRC Press, Boca Raton, FL.
[2] Ogilvie, A. (1992) in: Ap_4A and Other Dinucleoside Polyphosphates (McLennan, A.G., Ed.), pp. 229–273, CRC Press, Boca Raton, FL.
[3] Günther Sillero, M.A., Del Valle, M., Zaere, E., Michelena, P., García, A.G. and Sillero, A. (1994) Biochimie 76, 404–409.
[4] Pintor, J., Diaz-Rey, M.A., Torres, M. and Miras-Portugal, M.T. (1992) Neurosci. Lett. 136, 141–144.
[5] Baxi, M.D. and Vishwanatha, J.K. (1995) J. Pharmacol. Toxicol. Methods 33, 121–128.
[6] Busshardt, E., Gerok, W. and Haüssinger, D. (1989) Biochim. Biophys. Acta 1010, 151–159.
[7] Craik, K.M., McLennan, A.G. and Fisher, M.J. (1993) Cell. Signal. 5, 89–96.
[8] Green, A.K., Dixon, C.J., McLennan, A.G., Cobbold, P.H. and Fisher, M.J. (1993) FEBS Lett. 322, 197–200.
[9] Edgecombe, M., McLennan, A.G. and Fisher, M.J. (1996) Biochem. J. 314, 687–693.
[10] Okajima, F., Tokumitsu, Y., Kondo, Y. and Ui, M. (1987) J. Biol. Chem. 262, 13483–13490.
[11] Keppens, S., Vandekerckhove, A. and De Wulf, H. (1992) Br. J. Pharmacol. 105, 475–479.
[12] Pianet, I., Merle, M. and Labouesse, J. (1989) Biochem. Biophys. Res. Commun. 163, 1150–1157.
[13] Ehrlich, Y.H., Snider, R.M., Kornecki, E., Garfield, M.G. and Lenox, R.H. (1988) J. Neurochem. 50, 295–301.
[14] Keppens, S. (1996) Biochem. Pharmacol. 52, 441–445.

[15] Post, S.R., Jacobson, J.P. and Insel, P.A. (1996) J. Biol. Chem. 271, 2029–2032.
[16] Henning, R.H., Duin, M., den Hertog, A. and Nelemans, A. (1993) Br. J. Pharmacol. 110, 133–138.
[17] Elliott, K.R.F., Ash, R., Pogson, C.I., Smith, S.A. and Crisp, D.M. (1976) in: Use of Isolated Liver Cells and Kidney Tubules in Metabolic Studies (Tager, J.M., Soling, H.-D. and Williamson, J.R., Eds.), pp. 139–143.
[18] Krebs, H.A. and Henseleit, K. (1932) Hoppe-Seyler's Z. Physiol. Chem. 210, 33–66.
[19] Dickson, A.J. and Pogson, C.I. (1977) FEBS Lett. 83, 27–32.
[20] Munirathinam, G. and Yoburn, B.C. (1994) Pharmacol. Biochem. Behav. 48, 813–816.
[21] Keppens, S. and De Wulf, H. (1991) Br. J. Pharmacol. 104, 301–304.
[22] Murayama, T. and Ui, M. (1987) J. Biol. Chem. 262, 5522–5529.
[23] Tyagi, S.R., Olson, S.C., Burnham, D.N. and Lambeth, J.D. (1991) J. Biol. Chem. 266, 3498–3504.
[24] Edgecombe, M., Eckersley, S.P., McLennan, A.G. and Fisher, M.J. (1998) Cell. Signal. 10, 505–509.
[25] Green, A.K., Cobbold, P.H. and Dixon, C.J. (1994) Biochem. J. 302, 949–955.
[26] Green, A.K., Cobbold, P.H. and Dixon, C.J. (1995) Biochem. J. 310, 629–635.