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Stimulatory effect of exogenous diadenosine tetraphosphate on insulin and glucagon secretion in the perfused rat pancreas

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1 Diadenosine triphosphate (AP3A) and diadenosine tetraphosphate (AP4A) are released by various cells (e.g. platelets and chromaffin cells), and may act as extracellular messengers. In pancreatic B-cells, AP3A and AP4A are inhibitors of the ATP-regulated K^+ channels, and glucose increases intracellular levels of both substances.

2 We have studied the effect of exogenous AP3A and AP4A on insulin and glucagon secretion by the perfused rat pancreas.

3 AP3A did not significantly modify insulin or glucagon release, whereas AP4A induced a prompt, short-lived insulin response ($\approx$4 fold higher than basal value; $P<0.05$) in pancreases perfused at different glucose concentrations (3.2, 5.5 or 9 mM). AP4A-induced insulin release was abolished by somatostatin and by diazoxide. These two substances share the capacity to activate ATP-dependent K^+ channels, suggesting that these channels are a potential target for AP4A in the B-cell.

4 AP4A stimulated glucagon release at both 3.2 and 5.5 mM glucose. This effect was abolished by somatostatin.

5 The results suggest that extracellular AP4A may play a physiological role in the control of insulin and glucagon secretion.

Keywords: Diadenosine triphosphate; diadenosine tetraphosphate; insulin; glucagon; rat pancreas

Abbreviations: AMP, adenosine monophosphate; APnA, diadenosine polyphosphates; AP3A, diadenosine triphosphate; AP4A, diadenosine tetraphosphate; ATP, adenosine triphosphate

Introduction

Diadenosine polyphosphates (APnA) are ubiquitous members of a group of dinucleotide polyphosphates that have received considerable attention in recent years (Rapaport & Zamecnik, 1976; Lee *et al*., 1983; Olgilvie *et al*., 1996). These substances have been considered putative 'alarmones' (Lee *et al*., 1983; Ogilvie *et al*., 1996), a term coined by Ames and coworkers to describe low molecular weight compounds that are synthesized by bacterial cells in response to specific metabolic stress and serve to regulate mechanisms that enhance cell survival (Lee *et al*., 1983).

Almost all cells that have been investigated (bacterial to mammalian cells) seem to contain at least low concentrations of APnA (Rapaport & Zamecnik, 1976; Ogilvie, 1992). Furthermore, diadenosine tetraphosphate (AP4A) and diadenosine triphosphate (AP3A) are released by several cells, e.g. platelets (Ogilvie, 1992) and chromaffin cells (Castillo *et al*., 1992), thus indicating that besides being intracellular modulators, these nucleotides may act as extracellular messengers. They have been reported to affect platelet function (Luthje *et al*., 1985), vasomotor control (Ralevic *et al*., 1995) and hepatic glucose metabolism (Busshardt *et al*., 1989; Keppens, 1996).

APnA have been demonstrated to inhibit the ATP-dependent K^+ channel (Jovanovic *et al*., 1997) and electrophysiological studies have revealed that AP3A and AP4A behave as effective inhibitors of ATP-dependent K^+ channels in the pancreatic B-cell (Ripoll *et al*., 1996). These authors also found that glucose, at concentrations that induce insulin secretion, evokes an increase in the intracellular levels of AP3A and AP4A in cultured pancreatic islet cells from adult mice. In view of these findings, Ripoll *et al*. (1996) suggested that these adenosine polyphosphates act as intracellular molecules linking glucose metabolism and insulin secretion.

To gain further insight into the extracellular effect of APnA, in the present study we have investigated the effect of exogenous AP3A and AP4A on insulin and glucagon secretion in the isolated perfused rat pancreas.

Methods

Male Wistar rats (200–225 g body weight) fed *ad libitum* were maintained in accordance with the guidelines established by the European Union (86/609). After anaesthesia with pentobarbital sodium (50 mg kg^{-1}, i.p.), the pancreas was dissected and perfused *in situ* at 37°C according to the procedure of Leclercq-Meyer *et al*. (1976) as adapted in our laboratory (Silvestre *et al*., 1986). Effluent samples were collected from the portal vein, without recycling, at 1 min intervals (flow rate, 2 ml min^{-1}) and frozen at −20°C until the time of assay. The perfusion medium consisted of a Krebs-Henseleit buffer (mM): NaCl 115, KCl 4.7, $CaCl_2$ 2.6, H_2KPO_4 1.19, $MgSO_4.7H_2O$ 1.19 and $HNaCO_3$ 24.9 (gas phase 95:5, O_2:CO_2; pH 7.4) , supplemented with 4% (w v^{-1}) dextran T-70 (Pharmacia LKB Biotechnology, Uppsala, Sweden), 0.5% (w v^{-1}) Cohn Fraction V bovine albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.) and glucose (mM): 3.2, 5.5 or 9 (Sigma). Solutions of AP3A, AP4A, ATP (adenosine triphosphate) and AMP (adenosine monophosphate) (Sigma) in 0.9% NaCl, containing 0.1% bovine albumin (Cohn Fraction V), were prepared daily, immediately before experiments. When added to the perfusate, the final concentration was 75 or 15 μM, as indicated in the corresponding figures. After a 35-min equilibration period,

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baseline samples were collected for 5 min and, at zero time, normal saline with or without AP3A or AP4A was infused through a sidearm cannula. The effect of these APnA on insulin release was examined at different constant glucose concentrations and during B-cell stimulation, e.g. by increasing the perfusate glucose concentration from 5.5–9 mM. In addition, AP4A was tested in the presence of known inhibitors or B-cell secretion. In these experiments, 75 μM AP4A, with or without 10 nM 14-somatostatin (Peninsula Laboratories, Inc., Belmont, CA, U.S.A.) or 300 μM diazoxide (Sigma), were infused into pancreases perfused at 5.5 mM glucose. The effect of 15 μM ATP on insulin release, both at a constant glucose concentration (5.5 mM) and during B-cell stimulation, achieved by increasing the glucose level from 5.5–11 mM, as well as the effect of 75 μM AMP on insulin and glucagon secretion at 5.5 mM glucose were also examined. Finally, we studied the effect of APnA on glucagon secretion. AP3A (75 μM) was infused at a constant glucose level (5.5 mM). AP4A (75 μM) was infused at two glucose levels, 3.2 mM and 5.5 mM. In addition, AP4A infusions were performed in the presence of somatostatin (10 nM). Insulin and glucagon were analysed by radioimmunoassay (Yalow & Berson, 1960; Faloona & Unger, 1974). Anti-pig insulin serum (I8510, Sigma) and rat insulin standards (Novo, Nordisk, Denmark) were employed. Anti-glucagon serum (O4A) was kindly donated by R.H. Unger (University of Texas, Health Sciences Center, Dallas, TX, U.S.A.). All samples for each series of experiments were analysed within the same assay. Results are expressed as the mean ± s.e.mean. Hormone response was calculated as the integrated area of the curve above the mean preinfusion level (average of all the baseline levels for each perfusion) using the trapezoidal method. The normal distribution of our data was demonstrated by the Kolmogorov–Smirnov test (Siegel, 1956). Differences between values were tested for significance by analysis of variance and by the Student's *t*-test for unpaired samples.

Results

In a first series of experiments, we examined the effect of 75 μM AP3A (Figure 1, upper panel) and AP4A (Figure 1, lower panel) on insulin secretion at 5.5 mM glucose, and on the insulin response to increasing the perfusate glucose concentra-

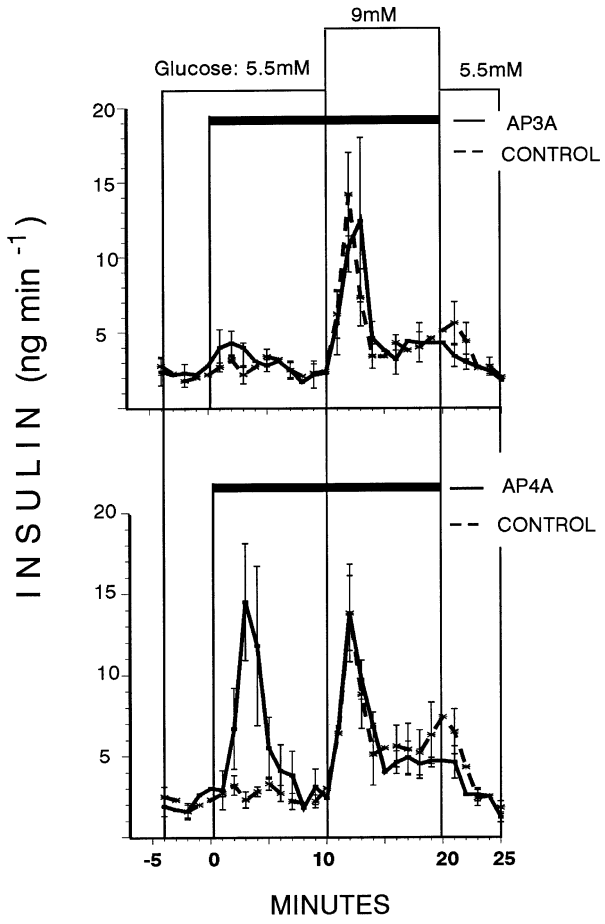

Figure 1 Effects of 75 μM AP3A (upper panel) and 75 μM AP4A (lower panel) on insulin secretion at 5.5 mM glucose, and on the insulin response to an increase in the perfusate glucose concentration (from 5.5 mM–9 mM) in the perfused rat pancreas. Broken lines corresponds to control experiments ($n=5$): pancreases were perfused at 5.5 mM glucose until 10 min; from 10–20 min, the glucose concentration was increased to 9 mM. Solid line corresponds to diadenosine polyphosphate experiments: from 0–10 min, AP3A ($n=4$) or AP4A ($n=4$) infusion at 5.5 mM glucose; from 10–20 min, AP3A or AP4A at 9 mM glucose. Means ± s.e.mean.

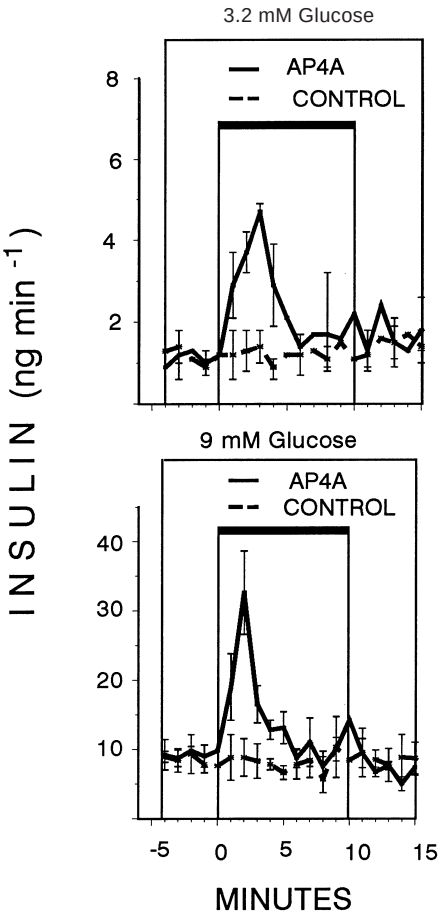

Figure 2 Effect of 75 μM AP4A on insulin secretion in pancreases perfused at different constant glucose concentrations. Upper panel: broken line corresponds to control experiments: infusion of 3.2 mM glucose alone ($n=5$). Solid line corresponds to AP4A experiments: infusion of 3.2 mM glucose plus infusion of AP4A (from 0–10 min), ($n=3$); Lower panel: broken line corresponds to control experiments: infusion of 9 mM glucose alone ($n=3$). Solid line corresponds to AP4A experiments: infusion of 9 mM glucose plus infusion of AP4A (from 0–10 min), ($n=4$). Means ± s.e.mean.

tion from 5.5 to 9 mM. Infusion of AP3A had no effect on either insulin release at 5.5 mM glucose or on the insulin response to 9 mM glucose. Infusion of AP4A markedly stimulated insulin secretion at 5.5 mM glucose ($F_{10,30,}=7.39$; $P<0.01$; peak at 3 min: 14.5 ± 3.6 ng min^{-1}; control: 2.3 ± 0.5 ng min^{-1} $P<0.05$), although it did not significantly modify glucose-stimulated insulin release (incremental area: 41.5 ± 2.5 ng 10 min^{-1}, vs 46 ± 14 ng 10 min^{-1} in control experiments; $P=0.83$).

Figure 2 illustrates the effect of 75 μM AP4A on insulin secretion in pancreases perfused at different glucose concentrations. AP4A induced a prompt, short-lived insulin response at constant low glucose concentration (3.2 mM) (Figure 2, upper panel; peak at 3 min: 4.7 ± 0.2 ng; zero value: 1.4 ± 0.4 ng; $P<0.01$) and at constant high glucose concentration (9 mM) (Figure 2, lower panel; peak at 2 min: 32.6 ± 6 ng; zero value: 8.8 ± 2.7 ng; $P<0.05$).

As shown in the upper panel of Figure 3, the stimulatory effect of AP4A (75 μM) at 5.5 mM glucose was virtually abolished when somatostatin (10 nM) was simultaneously infused (incremental area: 52 ± 11 ng 10 min^{-1} vs -3.3 ± 1.7 ng 10 min^{-1} in somatostatin experiments; $P<0.01$). Diazoxide (300 μM) (Figure 3, lower panel) completely blocked AP4A-induced insulin release (incremental area: 39 ± 16 ng 10 min^{-1} vs -10 ± 3 ng 10 min^{-1} in diazoxide experiments; $P<0.025$).

Figure 4 (upper panel) shows that AP4A, at a lower concentration (15 μM), similar to that found in the B-cell after glucose stimulation, also displayed insulinotropic activity (peak at 2 min: 8.5 ± 1.8 ng; zero value: 4 ± 0.5 ng; $P<0.05$). A similar increase in insulin secretion was elicited by 15 μM ATP (Figure 4, lower panel) peak at 2 min: 14.5 ± 3.8 ng; zero value: 4 ± 0.5 ng; $P<0.05$). However, as depicted in Figure 5, ATP potentiated the insulin response to an increase in the perfusate glucose level from 5.5–11 mM (incremental area: 177 ± 37 ng 20 min^{-1} vs 57 ± 12.4 ng 20 min^{-1} in control experiments; $P<0.05$), while AP4A did not.

Infusion of AMP (100 μM) at a constant 5.5 mM glucose level did not modify insulin secretion (data not shown).

Finally, we studied the effect of APnA on glucagon secretion. As shown in Figure 6, AP4A (75 μM) induced a prompt elevation of glucagon release at 5.5 mM glucose (incremental area: 1307 ± 234 vs -985 ± 196 pg 10 min^{-1} in control experiments; $P<0.05$). This glucagon response was

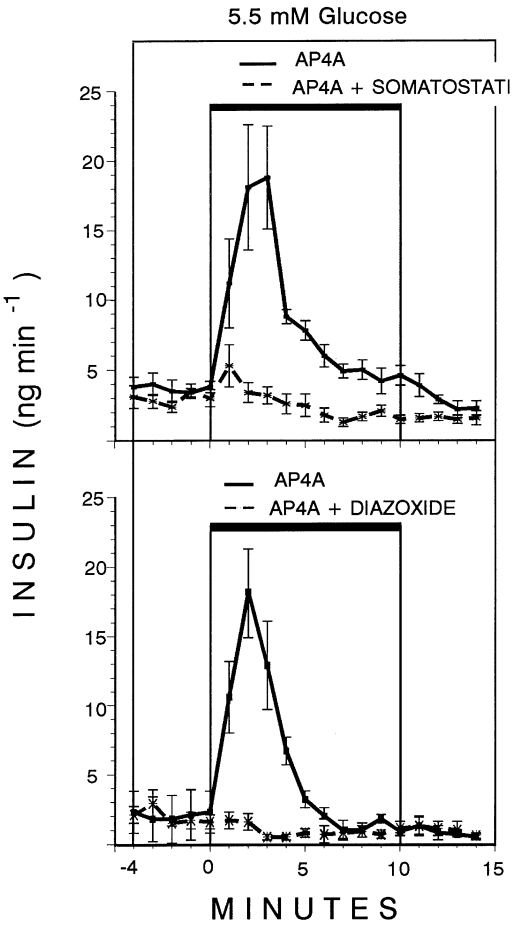

Figure 3 Upper panel: effect of 10 nM 14-somatostatin on the insulin response to 75 μM AP4A in the rat pancreas perfused at 5.5 mM glucose. Solid line corresponds to AP4A experiments: from 0–10 min, AP4A infusion ($n=5$). Broken line corresponds to somatostatin experiments: from 0–10 min, AP4A plus somatostatin infusion ($n=6$). Means$\pm$s.e.mean. Lower panel: effect of 300 μM diazoxide on the insulin response to 75 μM AP4A in the rat pancreas perfused at 5.5 mM glucose. Solid line corresponds to AP4A experiments: from 0–10 min, AP4A infusion ($n=3$). Broken line corresponds to diazoxide experiments: from 0–10 min, AP4A plus diazoxide infusion ($n=4$). Means$\pm$s.e.mean.

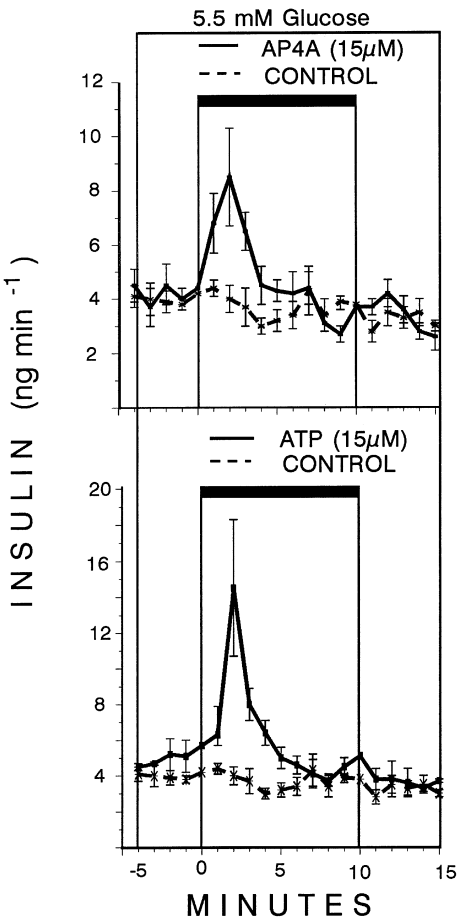

Figure 4 Upper panel: effect of 15 μM AP4A on insulin secretion at 5.5 mM glucose. Broken line corresponds to control experiments: from 0–10 min, saline infusion ($n=4$). Solid line corresponds to AP4A experiments: from 0–10 min, AP4A infusion ($n=4$). Means $\pm$s.e.mean. Lower panel: effect of 15 μM ATP on insulin secretion at 5.5 mM glucose. Broken line corresponds to control experiments: from 0–10 min, saline infusion ($n=4$). Solid line corresponds to ATP experiments: from 0–10 min, ATP infusion ($n=3$). Means$\pm$ s.e.mean.

abolished by 10 nM somatostatin. AP4A (Figure 7) also stimulated glucagon release at a low (3.2 mM) glucose level (incremental area: 3558 ± 664 pg 10 min^{-1} vs -130 ± 83 pg 10 min^{-1} in control experiments; $P < 0.025$).

In contrast to AP4A, infusion of AP3A (75 μM) did not modify glucagon release in pancreases perfused at a constant glucose level ($F_{10,30} = 0.61$; N.S) (Figure 8, upper panel). However, AMP (Figure 8, lower panel), at a similar concentration, markedly stimulated glucagon output (incremental area: 7400 ± 1290 pg 10 min^{-1} vs 150 ± 390 pg 10 min^{-1} in control experiments; $P < 0.05$).

Discussion

The foregoing results demonstrate that, in the pancreas isolated from normal rat, infusion of AP4A induces a short-lived monophasic insulin response, comparable to the typical first-phase elicited by glucose. The insulinotropic effect of AP4A takes place at low, normal and high glucose concentrations, indicating that it is independent of the metabolic state of the B-cell. However, AP4A did not augment the insulin response to an abrupt increase in the perfusate glucose level, e.g. it did not potentiate the triggering effect of glucose on insulin release. Given that glucose alone induces an elevation of AP4A in B-cells (Ripoll *et al*., 1996), it might be thought that such an increment represents the maximal insulinotropic activity of this nucleotide, and that a further increase in its concentration does not enhance the response. In agreement with our findings, Verspohl & Johannwille (1998) recently reported the stimulation of insulin output by AP4A in a preparation of perfused rat INS-1 cells.

Furthermore, Ripoll *et al*. (1996) have shown that AP4A increases the bioelectrical activity of the B-cell according to an excitatory pattern, and that when applied to the inner face of B-cell membrane patches, AP4A strongly inhibits ATP-dependent K^+ channel activity with an estimated half-maximal

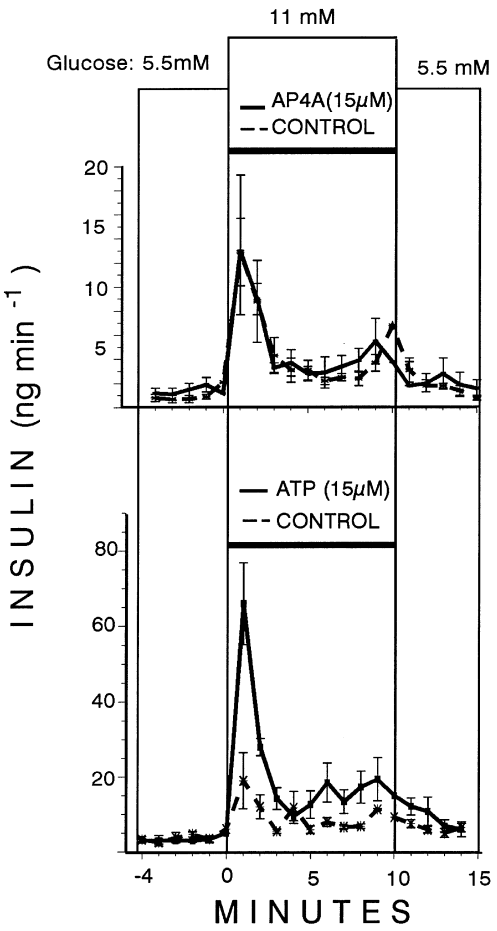

Figure 5 Upper panel: effect of 15 μM AP4A on the insulin response to increasing glucose concentrations (from 5.5–11 mM). Broken line corresponds to control experiments: from 0–10 min, 11 mM glucose ($n = 4$). Solid line corresponds to AP4A experiments: from 0–10 min, 11 mM glucose plus AP4A infusion ($n = 6$). Means ± s.e.mean. Lower panel: effect of 15 μM ATP on the insulin response to increasing glucose concentrations (from 5.5–11 mM). Broken line corresponds to control experiments: from 0–10 min, 11 mM glucose ($n = 3$). Solid line corresponds to ATP experiments: from 0–10 min, 11 mM glucose plus ATP infusion ($n = 3$). Means ± s.e.mean.

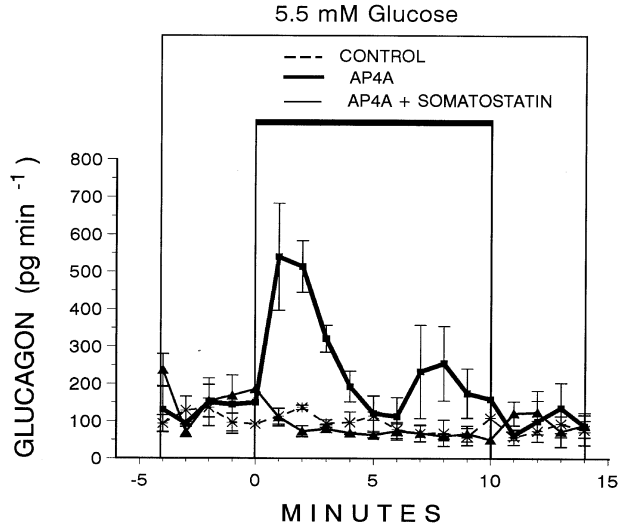

Figure 6 Effect of 75 μM on glucagon secretion at 5.5 mM glucose with or without 10 nM 14-somatostatin. Broken line corresponds to control experiments: from 0–10 min, saline infusion ($n = 3$). Thick solid line corresponds to AP4A experiments in the absence of somatostatin: from 0–10 min, AP4A infusion ($n = 4$). Thin solid line corresponds to AP4A experiments in the presence of somatostatin: from 0–10 min, AP4 plus somatostatin infusion ($n = 4$). Means ± s.e.mean.

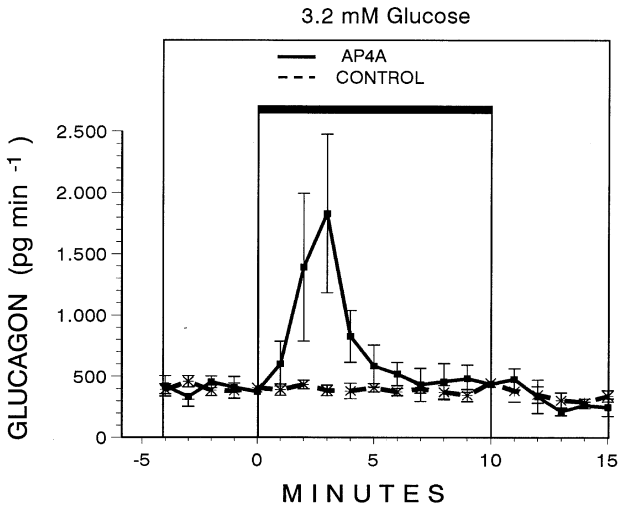

Figure 7 Effect of 75 μM AP4A on glucagon secretion at 3.2 mM glucose. Broken line corresponds to control experiments: from 0–10 min, saline infusion ($n = 4$). Solid line corresponds to AP4A experiments: from 0–10 min, AP4A infusion ($n = 4$). Means ± s.e.mean.

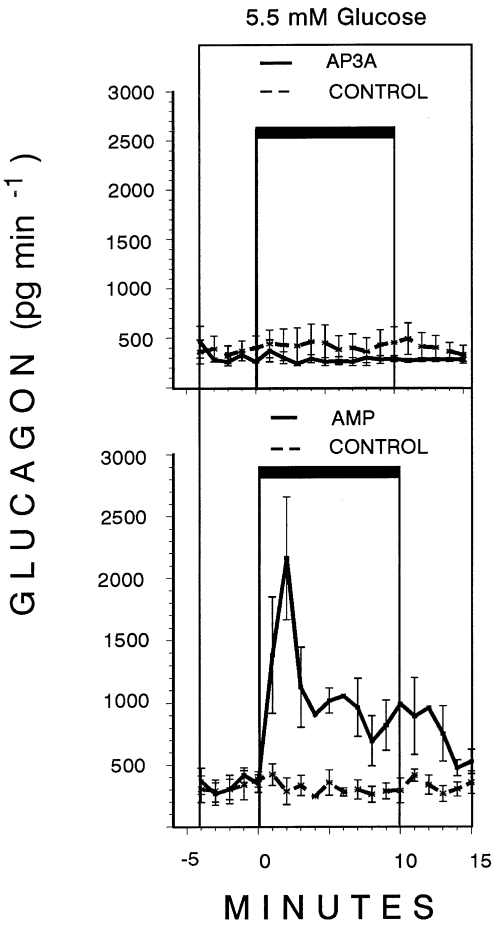

Figure 8 Upper panel: effect of 75 μM AP3A on glucagon secretion at 5.5 mM glucose. Broken line corresponds to control experiments: from 0–10 min, saline infusion ($n=4$). Solid line corresponds to AP3A experiments: from 0–10 min, AP3A infusion ($n=4$). Means ±s.e.mean. Lower panel: effect of 75 μM AMP on glucagon secretion at 5.5 mM glucose. Broken line corresponds to control experiments: from 0–10 min, saline infusion ($n=3$). Solid line corresponds to AMP experiments: from 0–10 min, AMP infusion ($n=4$). Means ±s.e.mean.

inhibition at 17 μM. This concentration is similar to the lower AP4A concentration we tested, showing its insulinotropic activity. Thus, AP4A-induced insulin output might be accounted for by inhibition of this channel activity.

The insulinotropic effect of AP4A is suppressed by both somatostatin and diazoxide. The fact that these two substances have been shown to activate ATP-dependent K^+ channels (De Weille *et al*., 1989; Trube *et al*., 1986) supports the concept that these channels might be a potential target of AP4A in the B-cell, as has been proposed to explain the *in vivo* vasodilatory effect of AP4A on coronary circulation (Nakae *et al*., 1996). An effect distal to K^+ channels cannot, however, be excluded.

Ectoenzymes capable of cleaving AP4A, yielding ATP plus AMP, have been reported in the vascular endothelium (Ogilvie *et al*., 1996; Guranowski & Sillero, 1992); therefore, it cannot be ruled out that the stimulatory effect of exogenous AP4A is mediated by these monoadenosine compounds. Indeed, in our system, AMP was without effect on insulin secretion, an observation that has been previously documented (Loubatières-Mariani & Chapal, 1988). ATP, when infused at a constant glucose level (5.5 mM), induced a monophasic increase in insulin release comparable to that observed for AP4A. However, in contrast to AP4A, ATP potentiated the insulin response to an abrupt increase in the perfusate glucose concentration, a finding described by other laboratories (Chapal *et al*., 1983). These distinct actions point to a direct effect of AP4A on the B-cell. Interestingly, Verspohl & Johannwille (1998) demonstrated that in rat INS-1 cells, adenosine is the final product of AP4A degradation, and adenosine behaves as an inhibitor of insulin release (Hillaire-Buys *et al*., 1994). Taken together, these observations indicate that the stimulatory effect of AP4A on insulin secretion reported herein represents a direct effect of this substance on the B-cell.

The stimulation of insulin release by exogenous AP4A strongly suggests that this nucleotide may play a role in the control of B-cell secretion at the extracellular level. This insulinotropic effect was observed at 15 μM AP4A, a concentration comparable to the intracellular level of this substance recorded in glucose-stimulated B-cells (Ripoll *et al*., 1996). Interestingly, a higher AP4A concentration (75 μM) induced a 2 fold insulin response. As already mentioned, AP4A is released by a number of cells (platelets, chromaffin cells) (Ogilvie, 1992; Castillo *et al*., 1992) and, thus, this nucleotide may reach the islet through the systemic circulation. On the other hand, AP4A is one of the soluble mass constituents released by cholinergic synaptic vesicles (Zimmerman *et al*., 1993), a fact that poses the concept of AP4A as a putative neurotransmitter.

Although, so far, no specific receptor for AP4A has been identified in the B-cell, AP4A binding has been observed in membranes from different mammalian tissues (Ogilvie *et al*., 1996; Hilderman *et al*., 1991) including B-cell membranes (Verspohl & Johanwille, 1998). Pharmacological studies indicate that AP4A interacts with a subclass of P2 purinoceptors (Hoyle *et al*., 1996; Pintor *et al*., 1991; Edgecombe *et al*., 1996) and the presence of purinoceptors in the B-cell has been widely documented (review in Loubatières-Mariani & Chapal, 1988). Moreover, activation of type P2 induces insulin release in the rat pancreas (Loubatières-Mariani & Chapal, 1988; Hillaire-Buys *et al*., 1994). Thus the insulinotropic effect of AP4A we observed might be mediated by the binding of this compound to P2 purinoceptors of the B-cell.

AP4A consistently stimulated glucagon release at low and normal glucose concentrations, an observation that makes the role of this polyphosphate in the hormonal control of glucose metabolism more complex. Indeed, a number of molecules containing an adenosine moiety stimulate the secretion of both insulin and glucagon (Loubatières-Mariani & Chapal, 1988; Weir *et al*., 1975).

At present, no studies on AP4A binding to A-cells have been reported. However, it is well known that A-cells are endowed with P1 purinoceptors (adenosine receptors), and activation of these purinoceptors is related to an increase in glucagon release (Loubatières-Mariani & Chapal, 1988; Chapal *et al*., 1985). Thus, the stimulatory effect of AP4A on glucagon output might be mediated by its binding to A1 purinergic receptors located in the A-cell membranes.

The glucagonotropic effect of AP4P was blocked by somatostatin. At present, any interpretation of such an effect is highly speculative. Activation of A1-purinergic receptors is generally associated with an increase in adenylate cyclase activity (Londos & Wolff, 1977), and somatostatin has been shown to block cyclic AMP production (Sharp, 1996). A distal effect, however, cannot be excluded (Wolheim *et al*., 1977).

In contrast to AP4A, AP3A is devoid of beta- or alpha-cytotropic activity. To interpret this lack of insulin or glucagon response, it should be pointed out that AP3A and AP4A show

divergent biological effects on a variety of target cells and organs (Luthje *et al*., 1985; Busshardt *et al*., 1989; Keppens, 1996). Furthermore, AP4A and AP3A may activate different purinoceptor subtypes, a difference that critically depends on the number of phosphate groups (Van der Giet *et al*., 1997).

As previously mentioned, the presence in the endothelium of ectoenzymes capable of cleaving AP3A, yielding ADP plus AMP, has been reported (Guranowski & Sillero, 1992). The distinct effects of AP3A (no effect) and AMP (stimulatory effect) on glucagon output observed in our perfused pancreas system do not support the possibility that the reported pancreatic effects of APnA are mediated by their degradation products.

In summary, in the isolated perfused rat pancreas, exogenous AP4A induces the release of insulin and glucagon at different glucose concentrations. This observation favours the concept of extracellular AP4A as a stimulatory agent of both B- and A-cell function.

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References

BUSSHARDT, E., GEROK, W. & HAUSSINGER, D. (1989). Regulation of hepatic parenchymal and non-parenchymal cell function by the diadenine nucleotides Ap3A and Ap4A. *Biochem. Biophys. Acta,* **1010,** 151 – 159.

CASTILLO, C.J.F., MORO, M.A., DEL VALLE, M., SILLERO, A., GARCÍA, A.G. & SILLERO, M.A.G. (1992). Diadenosine tetraphosphate is co-released with ATP and catecholamines from bovine adrenal medulla. *J. Neurochem.,* **59,** 723 – 732.

CHAPAL, J., BERTRAND, G., HILLAIRE-BUYS, D., GROSS, R. & LOUBATIÈRES-MARIANI, M.M. (1983). Prior glucose deprivation increases the first phase of glucose-induced insulin response: possible involvement of endogenous ATP and (or) ADP. *Can. J. Physiol. Pharmacol.,* **71,** 611 – 614.

CHAPAL, J., LOUBATIÈRES-MARIANI, M.M., PETIT, P. & ROYE, M. (1985). Evidence for an A subtype adenosine receptor on pancreatic glucagon secreting cells. *Br. J. Pharmacol.,* **86,** 565 – 569.

DE WEILLE, J.R., SCHMID-ANTOMARCHI, H., FOSSET, M. & LAZDUNSKI, M. (1989). Regulation of ATP-sensitive K^+ channels in insulinoma cells: Activation by somatostatin and protein kinase C and the role of cAMP. *Proc. Natl. Acad. Sci. U.S.A.,* **86,** 2971 – 2975.

EDGECOMBE, M., MCLENNAN, A.G. & FISHER, M.J. (1996). Characterization of the binding of diadenosine 5′-5″-P1,P4-tetraphosphate (Ap4A) to rat liver cell membranes. *Biochem. J.,* **314,** 687 – 693.

FALOONA, G.R. & UNGER, R.H. (1974). Glucagon. In: Jaffe, B.M. & Behrman, H.R. (eds). *Methods of Hormone Radioimmunoassay*. Academic Press: New York, pp 317 – 330.

GURANOWSKI, A. & SILLERO, A. (1992). Enzymes cleaving dinucleoside polyphosphates. In: McLennan, A.G. (ed). *AP4A and other Dinucleoside Polyphosphates*. CRC Press Inc: USA, pp 81 – 133.

HILDERMAN, R.H., MARTIN, M., ZIMMERMAN, J.K. & PIVORUN, E.B. (1991). Identification of a unique membrane receptor for adenosine 5′-5‴-P1-P4-tetraphosphate. *J. Biol. Chem.,* **266,** 6915 – 6918.

HILLAIRE-BUYS, D., CHAPAL, J., BERTRAND, G., PETIT, P. & LOUBATIÈRES-MARIANI, M.M. (1994). Purinergic receptors on insulin secreting cells. *Fundam. Clin. Pharmacol.,* **8,** 117 – 127.

HOYLE, C.H., ZIGANSHIN, A.U., PINTOR, J. & BURNSTOCK, G. (1996). The activation of P1- and P2-purinoceptors in the guinea-pig left atrium by adenosine polyphosphates. *Br. J. Pharmacol.,* **118,** 1294 – 1300.

JOVANOVIC, A., ALEKSEEV, A.E. & TERZIC, A. (1997). Intracellular diadenosine polyphosphates. A novel family of inhibitory ligands of the ATP-sensitive K^+ channel. *Biochem. Pharmacol.,* **54,** 219 – 225.

KEPPENS, S. (1996). Effects of diadenosine triphosphate and diadenosine tetraphosphate on rat liver cells. Differences and similarities with ADP and ATP. *Biochem. Pharmacol.,* **52,** 441 – 445.

LECLERCQ-MEYER, V., MARCHAND, J., LECLERCQ, R. & MALAISSE, W.J. (1976). Glucagon and insulin release by the in vitro perfused rat pancreas. *Diabete. Metab.,* **2,** 57 – 65.

LEE, P.C., BOCHNER, B.R. & AMES, B.N. (1983). AppppA, heat-shock stress, and cell oxidation. *Proc. Natl. Acad. Sci. U.S.A.,* **80,** 7496 – 7500.

LONDOS, C. & WOLFF, J. (1977). Two distinct adenosine sensitive sites on adenylate cyclase. *Proc. Natl. Acad. Sci. U.S.A.,* **74,** 5482 – 5486.

LOUBATIÈRES-MARIANI, M.M. & CHAPAL, J. (1988). Purinergic receptors involved in the stimulation of insulin and glucagon secretion. *Diabete. Metab.,* **14,** 119 – 126.

LUTHJE, J., BARINGER, J. & OGILVIE, A. (1985). Effects of diadenosine triphosphate (Ap3A) and diadenosine tetraphosphate (Ap4A) on platelet aggregation in unfractionated human blood. *Blut,* **51,** 405 – 413.

NAKAE, I., TAKAHASHI, M., TAKAOKA, A., LIU, Q., MATSUMOTO, T., AMANO, M., SEKINE, A., NAKAJIMA, H. & KINOSHITA, M. (1996). Coronary effects of diadenosine tetraphosphate resemble those of adenosine in anesthetized pigs: involvement of ATP-sensitive potassium channels. *J. Cardiovasc. Pharmacol.,* **28,** 124 – 133.

OGILVIE, A. (1992). Extracellular functions for ApnA. In: McLennan, A.G. (ed). *AP4A and other Dinucleoside Polyphosphates*. CRC Press Inc: England, pp 229 – 273.

OGILVIE, A., BLASIUS, R., SCHULZE-LOHOFF, E. & STERZEL, R.B. (1996). Adenine dinucleotides: a novel class of signalling molecules. *J. Auton. Pharmacol.,* **16,** 325 – 328.

PINTOR, J., TORRES, M., CASTRO, E. & MIRAS-PORTUGAL, M.T. (1991). Characterization of diadenosine tetraphosphate binding sites in cultured chromaffin cells: evidence for a P2y site. *Br. J. Pharmacol.,* **103,** 1980 – 1984.

RALEVIC, V., HOYLE, C.H. & BURNSTOCK, G. (1995). Pivotal role of phosphate chain length in vasoconstrictor versus vasodilator actions of adenine dinucleotides in rat mesenteric arteries. *J. Physiol. (Lond),* **483,** 703 – 713.

RAPAPORT, E. & ZAMECNIK, P.C. (1976). Presence of diadenosine 5′,5‴-P1,P4-tetraphosphate (Ap4A) in mammalian cells in levels varying widely with profilerative activity of the tissue: a possible positive 'pleiotypic activator'. *Proc. Natl. Acad. Sci. U.S.A.,* **73,** 3984 – 3988.

RIPOLL, C., MARTÍN, F., ROVIRA, J.M., PINTOR, J., MIRAS-PORTUGAL, M.T. & SORIA, B. (1996). Diadenosine polyphosphates. A novel class of glucose-induced intracellular messengers in the pancreatic β-cell. *Diabetes,* **45,** 1431 – 1434.

SHARP, G.W. (1996). Mechanism of inhibition of insulin release. *Am. J. Physiol.,* **271,** C1781 – C1799.

SIEGELS, S. (1956). Non parametric statistic for the behavioural changes. McGraw-Hill Book Company, New York, U.S.A. (Spanish Edition, 1978), pp 56-83.

SILVESTRE, R.A., MIRALLES, P., MORENO, P., VILLANUEVA, M.L. & MARCO, J. (1986). Somatostatin, insulin and glucagon secretion by the perfused pancreas from the cysteamine-treated rats. *Biochem. Biophys. Res. Commun.,* **134,** 1291 – 1297.

TRUBE, G., RORSMAN, P. & OHNO-SHOSAKU, T. (1986). Opposite effects of tolbutamide and diazoxide on the ATP-dependent K^+ channel in mouse pancreatic beta-cells. *Pflugers Arch.,* **407,** 493 – 499.

VAN DER GIET, M., KHATTAB, M., BORGEL, J., SCHLUTER, H. & ZIDEK, W. (1997). Differential effects of diadenosine phosphates on purinoceptors in the rat isolated perfused kidney. *Br. J. Pharmacol.,* **120,** 1453 – 1460.

VERSPOHL, E.J. & JOHANWILLE, B. (1998). Diadenosine polyphosphates in insulin-secreting cells: interaction with specific receptors and degradation. *Diabetes,* **47,** 1727–1734.

WEIR, G.C., KNOWLTON, S.D. & MARTIN, D.B. (1975). Nucleotide and nucleoside stimulation of glucagon secretion. *Endocrinol.,* **97,** 932–936.

WOLHEIM, C.B., BLONDEL, B., RENOLD, A.E. & SHARP, G.W. (1977). Somatostatin inhibition of pancreatic glucagon release from monolayer cultures and interactions with calcium. *Endocrinol.,* **101,** 911–919.

YALOW, R.S. & BERSON, S.A. (1960). Immunoassay of endogenous plasma insulin in man. *J. Clin. Invest.,* **39,** 1157–1175.

ZIMMERMAN, H., VOLKANDT, W., WITTICH, B. & HAUSINGER, A. (1993). Synaptic vesicle life cycle and synaptic turnover. *J. Physiol. (Paris),* **87,** 159–170.

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