Diadenosine Polyphosphates

A Novel Class of Glucose-Induced Intracellular Messengers in the Pancreatic β-Cell

Cristina Ripoll, Franz Martin, Juan Manuel Rovira, Jesús Pintor, María Teresa Miras-Portugal, and Bernat Soria

Diadenosine polyphosphates are a group of low-weight compounds that increase after exposure to a wide variety of oxidants and have been suggested to act as "alarmones," alerting the cell to the onset of metabolic stress. We demonstrate here that glucose at concentrations that induce insulin release produce a 30- to 70-fold increase in the concentration of diadenosine triphosphate (Ap_3A) and tetraphosphate (Ap_4A) in β-cells. Furthermore, Ap_3A and Ap_4A, at the concentrations found in glucose-stimulated cells, are effective inhibitors of the ATP-regulated K^+ channels when applied to the intracellular side of excised membrane patches from cultured β-cells. We suggest that Ap_3A and Ap_4A act as second messengers mediating a glucose-induced blockade of the pancreatic β-cell ATP-regulated potassium channel. *Diabetes* 45:1431–1434, 1996

In response to stimulatory glucose concentrations, the β-cell membrane depolarizes, initiating a characteristic glucose-induced pattern of electrical activity and insulin release (1). There is considerable evidence that this depolarization results from a decrease in the resting K^+ permeability of the β-cell as a consequence of glucose metabolism (2,3). The ATP-regulated K^+ (K_{ATP}) channel constitutes the major contribution to the resting conductance in this preparation. Glucose metabolism is believed to exert its inhibitory action on K_{ATP} channels by increasing the cytoplasmic ATP/ADP ratio (4). It has been proposed that the regulatory action of mononucleotides on K_{ATP} channels is determined by the operational state of the channel (5); this hypothesis considers K_{ATP} channels as dynamic targets, which response to ligands depends on the operational channel condition in a particular moment: spontaneous or nucleotide induced. This picture of the interaction between K_{ATP} channels and nucleotides is completed with the observation that other nucleotides, such as GDP, GTP, and some nonhydrolyzable analogs, can mimic the effect of ADP (6).

It has been recently shown that some diadenosine polyphosphates (Ap_4A, Ap_5A, and Ap_6A) inhibit the activity of the K_{ATP} channel from cardiac muscle (7–9). Diadenosine polyphosphates (DPs) constitute a group of ubiquitous compounds formed by two adenosine molecules bridged by three to six phosphates, which are present at very low intracellular concentrations (<1 μmol/l) under resting conditions and which increase after exposure to a wide variety of oxidants or during metabolic stress. Based on this, DPs have been suggested to act as "alarmones," alerting the cell to the onset of metabolic stress (10).

DPs are widespread in living cells, they are very effective inhibitors of cardiac K_{ATP} conductance, and their intracellular concentrations are altered under different circumstances. These features strongly suggest the possibility that DPs could be implicated in β-cell regulation. Therefore, the aim of this study was to determine the effects of DPs on pancreatic K_{ATP} channels and their role as potential mediators in β-cell signaling. We demonstrate here that glucose, at concentrations that induce insulin release, produces a 30- to 70-fold increase in the concentration of diadenosine triphosphate and tetraphosphate. Whereas resting levels of Ap_3A and Ap_4A (similar to those found in other cell types) do not modify significantly the activity of the K_{ATP} channel, Ap_3A, and Ap_4A at the concentrations found in glucose-stimulated cells, they are effective inhibitors of the K_{ATP} channel when applied to the intracellular side of excised membrane patches from cultured β-cells. We conclude that Ap_3A and Ap_4A may act as second messengers mediating the glucose-induced blockade of the ATP-regulated K^+ channel in the pancreatic β-cell.

RESEARCH DESIGN AND METHODS

Cell isolation and culture. Islets from adult (8- to 10-week-old) Swiss mice (OF1) were isolated, dispersed into single cells, and cultured as previously described (11). Briefly, islets were isolated by collagenase digestion, separated by centrifugation, and hand picked under a stereomicroscope. Once isolated, islets were disaggregated into single cells in a low-calcium medium. Cells were then centrifuged; resuspended in culture medium RPMI 1640 supplemented with 10% fetal calf serum, penicillin (100 U/ml), strepto-

From the Department of Physiology and Institute of Neurosciences (C.R., F.M., J.M.R., B.S.), University of Alicante, School of Medicine, Alicante; and the Department of Biochemistry (J.P., M.T.M.-P.), School of Veterinary, University Complutense, Madrid, Spain.

Address correspondence and reprint requests to Bernat Soria, Department of Physiology and Institute of Neurosciences, University of Alicante, School of Medicine, Aptdo. 374, Alicante 03080, Spain.

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Ap_3A, diadenosine triphosphate; Ap_4A, diadenosine tetraphosphate; DP, diadenosine polyphosphate; HPLC, high-performance liquid chromatography; K_{ATP}, ATP-regulated K^+ channel; K_i, concentration of Ap_4A causing half maximal inhibition of channel activity; mfs, millivolts full scale.

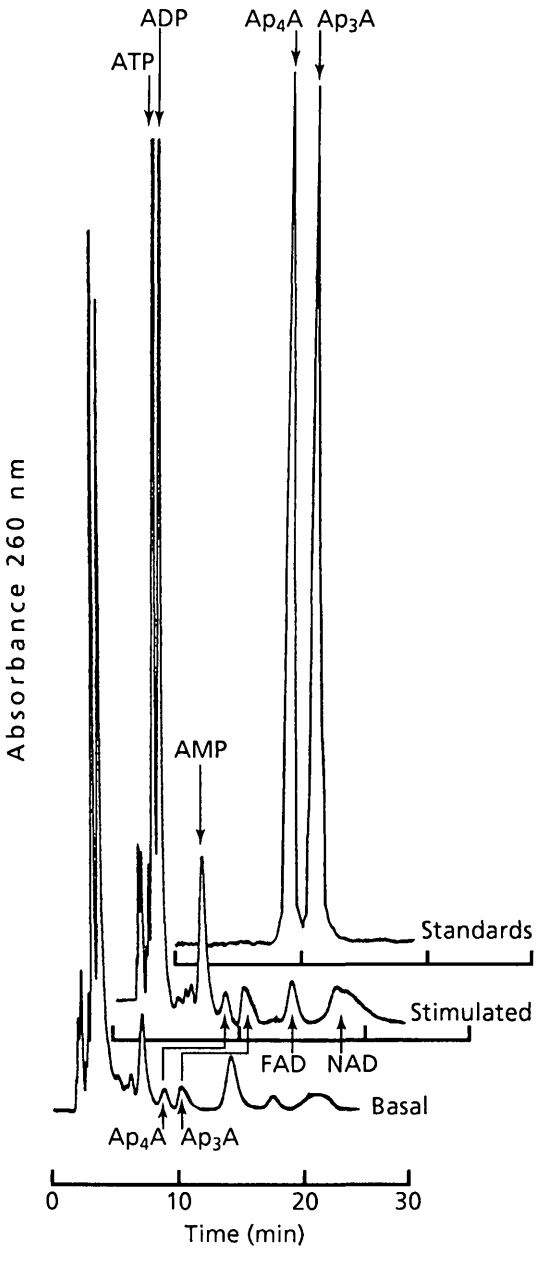

FIG. 1. HPLC elution profile of pancreatic islet cells cytosolic fraction. Samples of pancreatic islet cell were analyzed by HPLC using the protocol described in METHODS. Fractions showing different DP quantities were compared with 10 µl of 100 mmol/l standards. From 1,200 islets, 200 µl was injected for the basal elution profile, and 10 µl from 600 islets for the stimulated elution profile. Adenine mononucleotides eluted in the following order: ATP (3.1 min), ADP (3.4 min), and AMP (6.4 min). Adenine dinucleotides eluted as follows: Ap_4A (8.3 min), Ap_3A (10.1 min), FAD (17 min), and NAD (21.2 min). Adenine dinucleotide concentrations were calculated by comparing the areas with their corresponding commercial standards. Integrator attenuation sensitivity: 16 millivolts full scale (mfs).

mycin (0.1 mg/ml), and 5.6 mmol/l glucose; and plated on glass coverslips. Cells were kept at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2 and used within 1–3 days of culture.

Diadenosine polyphosphates determination. Pancreatic islets from 15 mice were obtained as explained above and incubated 90 min in either 3 mmol/l (1,200 islets) or 22 mmol/l glucose (600 islets). The higher number of islets used in basal glucose ensured a correct high-performance liquid chromatography (HPLC) determination of DPs, even at the low levels expected in these conditions. Both batches were sonicated in 50% water/ethanol. The samples were centrifuged for 30 min at 65,000 rpm and 4°C. Proteins in the supernatant were precipitated in acetone. The final suspension was lyophilized. DPs were then analyzed by HPLC. The equipment consisted of a Spectraphysics SP8800 delivery system, a reodyne injector, a Spectra 100 spectrophotometer, and a Chromjet integrator. Analysis was performed equilibrating the chromatographic system with the following mobile phase: 0.1 mmol/l KH_2PO_4 and 3% methanol, pH 6.0, as described by Pintor et al. (12). The column was a RSiL C18 HL (25-cm length, 0.4-cm inner diameter) from Biorad (Belgium). Detection was monitored at 260 nm wavelength.

Electrophysiology. Patch pipettes were pulled from Clark Electromedical glass capillaries (Reading, U.K.) using a two-stage puller (Mecanex BB-CH, Geneva, Switzerland), with resistances in the range of 3–12 MΩ when filled with a standard solution (in mmol/l): 5 KCl, 135 NaCl, 10 HEPES, 2.5 $CaCl_2$, 1.1 $MgCl_2$, pH 7.4. Bath solution contained (in mmol/l): 140 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 1 EGTA, pH 7.2. Ap_4A, Ap_3A, and ATP were purchased from Sigma (St. Louis, MO). Solutions containing DPs and ATP were applied through a RSC-100 rapid solution changer (Biologic, Claix, France). K_{ATP} channel unitary currents were registered from excised membrane patches in the inside-out configuration (13). Currents were measured using an Axopatch 200 amplifier (Axon Instruments, Foster City, CA) and stored in a tape recorder (DAT, DTR-1202, Biologic, Claix, France) for subsequent analysis with a homemade program. Experiments were replayed through an eight-pole Bessel filter (Frequency Devices, Haverhill, MA) at 1 kHz and sampled at 10 kHz. Pipette potential was held at 0 mV throughout the record. The experiments were carried out at room temperature (20–24°C). Concentration-response curves were obtained by integrating the area corresponding to channel activity in the presence of different DP concentrations and fitting this data to the Hill equation. Data are expressed as mean ± SE.

RESULTS

Evidence of DPs presence in β-cell cytosol. Identification of adenine dinucleotides present in the cytosolic fraction of pancreatic β-cell was carried out, in a first approach, by comparing their retention times with those of commercial standards (Fig. 1). Two peaks present were identified in the samples as presumable Ap_4A and Ap_3A because of their retention times (8.3 and 10.1 min, respectively) when compared with standards. Enrichment of the samples with diadenosine polyphosphates resulted in the co-elution of both the putative polyphosphates and the standards (data not shown). To definitely verify the nature of the substances tentatively identified as Ap_4A and Ap_3A, peaks were collected and submitted to digestion with phosphodiesterase from *Crotalus durissus* (EC 1.5.15.1; Pharmacia, Sweden) (12). This enzyme cleaves dinucleotides producing a nucleotide monophosphate plus another mononucleotide with a phosphate chain length, depending on the original dinucleotide. Treatment carried out with putative Ap_4A (Fig. 2*A*) yielded the presence of AMP and ATP (Fig. 2*C*), thus confirming the presence of the dinucleotide in the samples. The same protocol was applied to putative Ap_3A (Fig. 2*B*). After digestion with the phosphodiesterase, the appearance of AMP and ADP was detected by HPLC (Fig. 2*D*). Quantification of both adenine dinucleotides present in the samples was performed by comparing the areas of the peaks with commercial standards (Fig. 1). Islets incubated in basal conditions (3 mmol/l glucose, 90 min) showed intracellular dinucleotide values of 0.29 ± 0.01 µmol/l for Ap_3A and 0.18 ± 0.01 µmol/l for Ap_4A (n = 4). Under stimulatory conditions (22 mmol/l glucose, 90 min), intracellular concentrations of adenine dinucleotides increased significantly, giving concentration values of 11.24 ± 0.06 µmol/l and 13.68 ± 0.88 µmol/l for Ap_3A and Ap_4A, respectively (n = 4). Noteworthy, these increments of DPs were comparatively much greater than those found for ATP (2.5-fold increase) and ADP (2.6-fold decrease).

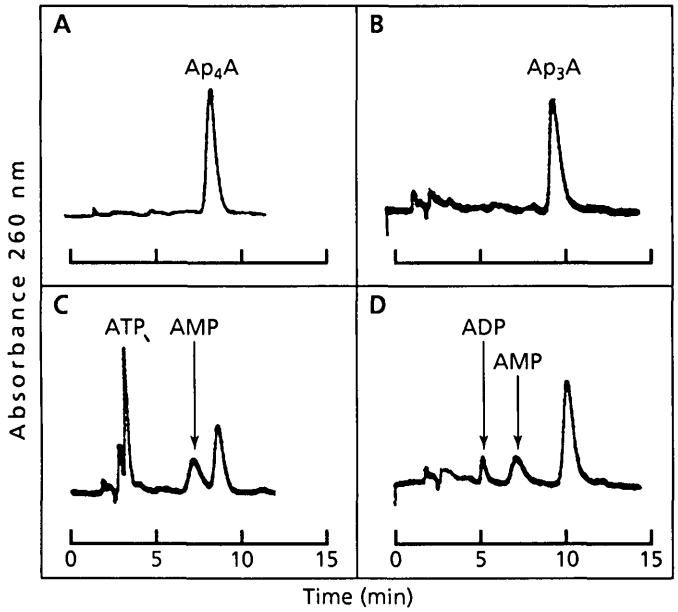

FIG. 2. Rechromatography and phosphodiesterase treatment for Ap_4A and Ap_3A. *A*: rechromatography of Ap_4A from the stimulated cytosolic fraction sample shown in Fig. 1. *B*: rechromatography of Ap_3A from the stimulated cytosolic fraction sample shown in Fig. 1. *C*: elution profile after digestion with phosphodiesterase of the Ap_4A peak shown in *A* (see RESULTS). *D*: elution profile after digestion with phosphodiesterase of the Ap_3A peak shown in panel *C*. Integrator attenuation sensitivity was 8 mfs.

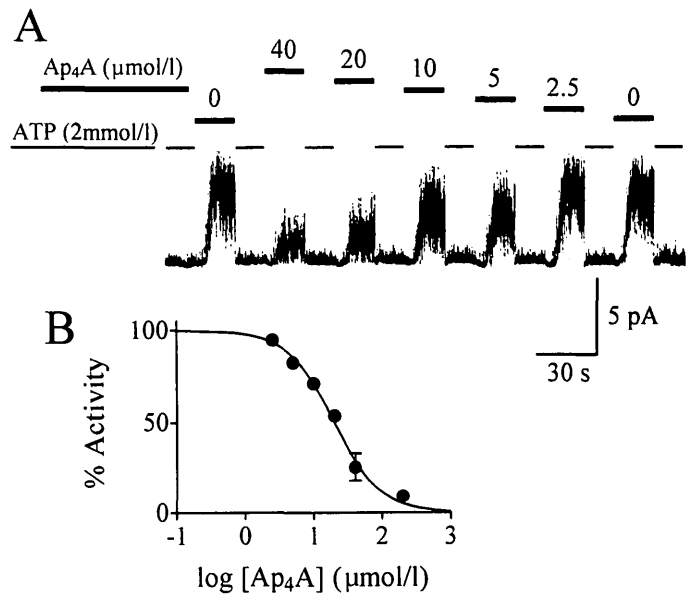

FIG. 3. Effect of Ap_4A on K_{ATP} channel activity in inside-out membrane patches from pancreatic β-cells. *A*: representative recording of channel activity in a patch exposed sequentially to decreasing concentrations of Ap_4A, as indicated above the record. *B*: concentration-response relationship for Ap_4A inhibition of channel activity. In this figure, the ordinate represents patch current relative to that in 0 mmol/l ATP(I_{max}). The points show mean ± SE for n = 3.

The effect of DPs on the K_{ATP} channel activity. Figure 3*A* shows a typical current record and illustrates the protocol adopted to measure the effect of DPs on K_{ATP} channel activity. Patches were initially exposed to 2 mmol/l ATP, then to 0 mmol/l ATP, then to decreasing concentrations of Ap_4A, and then back to 0 mmol/l ATP. ATP (2 mmol/l) was applied after every Ap_4A concentration to minimize rundown. Current in 2 mmol/l ATP was subtracted from the current measured in each test solution. The mean of the two determinations in 0 mmol/l ATP, at the beginning and the end of the protocol, was taken as I_{max}. The average current in each Ap_4A exposure was expressed as a fraction of I_{max}. Figure 3*B* shows the concentration-response relationship for inhibition of K_{ATP} channel in the presence of Ap_4A. The curve was a least-squares fit to the data. The fitted K_i (concentration of Ap_4A causing half maximal inhibition of channel activity) was 17 μmol/l and the slope factor was 1.2. The decrease in channel activity resulted from decreased channel open probability, with no effect on unitary current magnitude. A similar set of experiments was performed for Ap_3A, giving a K_i of 74 μmol/l.

Additionally, we performed experiments to examine the effect of DPs on K_{ATP} current rundown, a phenomenon that consists in the spontaneous decline of channel activity after excision of membrane patches into solutions with no ATP (14). On exposure to Ap_4A or Ap_3A, we did not observe any significant recovery from rundown, even at the highest doses examined (200 μmol/l, data not shown).

DISCUSSION

The present study examines the physiological role of several members of the diadenosine polyphosphate family in the pancreatic β-cell and their possible action on K_{ATP} channels. Using HPLC, we have detected the presence of Ap_4A and Ap_3A in the cytosolic fraction of β-cells. From the peak areas analysis, we have estimated the concentrations of both agents in two different samples: islets incubated in basal glucose (3 mmol/l) and high glucose (22 mmol/l). Nonstimulated cells (cells incubated in Krebs with 3 mmol/l glucose) showed intracellular dinucleotide levels in the submicromolar range. After islet incubation in high glucose, we detected a substantial concentration increase of Ap_4A (70-fold) and Ap_3A (30-fold). Increase in DP synthesis has been measured in different cell preparations in response to stress conditions (10). However, to our knowledge, this is the first report showing an increase in intracellular levels of DPs induced by a physiologic secretagogue like glucose. Interestingly, these changes in DP intracellular concentrations in response to glucose are much more pronounced that those measured for ATP (2.5-fold increase) and ADP (2.6-fold decrease), or even the ATP/ADP ratio, which augments 6- to 7-fold.

Besides, we have investigated the effect of Ap_4A and Ap_3A on K_{ATP} channel activity. When applied to the inner face of β-cell membrane patches, Ap_3A and Ap_4A strongly inhibit K_{ATP} channel activity. The estimated Ap_4A half-maximal inhibition (17 μmol/l) closely resembles those found for DPs in cardiac K_{ATP} channel (14–17 μmol/l) (7–9) and the K_i measured for ATP channel inhibition (2). Affinity to Ap_3A is comparatively lower but still within the range of measured Ap_3A intracellular concentrations. Taking together the findings on DP levels in different glucose concentrations and K_i for K_{ATP} channels, a striking conclusion arises: DPs may only effectively inhibit K_{ATP} channel in the presence of glucose.

Until recently, it has practically been a dogma that ATP, or the ATP/ADP ratio, is the best candidate for the physiological second messenger linking metabolism and channel inhibition. The evidences favoring this hypothesis are as follows: *1*) ATP inhibits K_{ATP} channels in inside-out patches, *2*) those substances that raise intracellular ATP reduce channel activity and vice versa, and *3*) the ATP/ADP ratio increases in the presence of glucose. The present findings show that DPs accomplish these conditions and in some aspects, like concentration changes in response to glucose, might even be more suitable than ATP as a linking molecule between nutrient metabolism and insulin secretion.

We are aware that there is a need for a more detailed understanding of the complex inhibitory regulation of K_{ATP} channels, and in particular, the role that DPs play in this process. We have shown the absence of recovery from channel rundown after exposure to DPs. In a recent study, Jovanovic et al. (15) show that the inhibitory action of DPs on ventricular K_{ATP} channels depends on the operative condition of the channel as it happens for ATP (15).

The present work can be summarized as follows: *1*) concentrations of DPs in β-cells noticeably increase under stimulation with glucose, and *2*) the measured DP concentrations necessary to effectively alter K_{ATP} channel activity are in the range of the Ap_4A and Ap_3A cytosolic levels found after cell stimulation with glucose. These results imply that DPs are likely to be involved in K_{ATP} channel activity regulation in β-cells. Moreover, the degree of involvement of DPs in channel modulation seems to rely critically on the metabolic condition of the cell.

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