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Diadenosine Tetraphosphate-Gating of Recombinant Pancreatic ATP-Sensitive K^+ Channels

Sofija Jovanovic[1,2] and Aleksandar Jovanovic[1,2,3]

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Diadenosine tetraphosphate (Ap4A) has been recently discovered in the pancreatic β cells where targets ATP-sensitive K^+ (K_{ATP}) channels, depolarizes the cell membrane and induces insulin secretion. However, whether Ap4A inhibit pancreatic K_{ATP} channels by targeting protein channel complex itself was unknown. Therefore, we coexpressed pancreatic K_{ATP} channel subunits, Kir6.2 and SUR1, in COS-7 cells and examined the effect of Ap4A on the single channel behavior using the inside-out configuration of the patch-clamp technique. Ap4A inhibited channel opening in a concentration-dependent manner. Analysis of single channels demonstrated that Ap4A did not change intraburst kinetic behavior of K_{ATP} channels, but rather decreased burst duration and increased between-burst duration. It is concluded that Ap4A antagonizes K_{ATP} channel opening by targeting channel subunits themselves and by keeping channels longer in closed interburst states.

KEY WORDS: Diadenosine polyphosphates; diadenosine tetraphosphate; pancreas; K_{ATP} channels.

INTRODUCTION

Diadenosine polyphosphates (ApnA) are structurally "unusual" nucleotides with two adenosine moieties linked by a phosphate group chain [1]. A member of this group, diadenosine tetraphosphate (Ap4A), has been identified in the pancreas, and it has generated considerable interest as signaling molecule regulating insulin secretion [2, 3]. Specifically, it has been proposed that Ap4A levels increase in glucose-stimulated pancreatic β-cells, inhibit ATP-sensitive K^+ (K_{ATP}) channels, depolarizes the cell membrane leading to the activation of voltage-dependent Ca^{2+} channels, rise in the free intracellular concentration of Ca^{2+} and release of insulin [2–6]. However, it is still unknown whether Ap4A interacts with pancreatic K_{ATP} channel protein by targeting channel subunits themselves.

Therefore, the present study was undertaken to evaluate the interaction between Ap4A and recombinant pancreatic K_{ATP} channels expressed in a primate cell line.

[1]Tayside Institute of Child Health, Ninewells Hospital and Medical School, University of Dundee, Dundee, Scotland, UK.
[2]Department of Medicine, Mayo Clinic, Rochester, MN, USA.
[3]To whom correspondence should be addressed. Tel: +(01382) 496 269; Fax: +(01382) 632 597; E-mail: a.jovanovic@dundee.ac.uk

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We report that Ap4A directly targets pancreatic K_{ATP} channels and antagonizes their opening by keeping channels longer in closed interburst states. These data provide first direct evidence that ApnA is a ligand of pancreatic K_{ATP} channels.

MATERIALS AND METHODS

COS-7 Cells

Monkey kidney COS-7 cells (ATCC, Manassas, VA) lack K_{ATP} channels, and were cultured in a tissue flask (at 5% CO_2) containing Dulbecco's modified Eagle's medium (Gibco, Brooklyn, NY) supplemented with 10% fetal calf serum and 2 mM glutamine, as previously described [7, 8]. Cultured COS-7 cells were, then, trypsinized (for 5 min at 37°C), and plated ($2–6 \times 10^6$) on a culture dish, containing 12-mm glass coverslips. COS-7 cells were left untransfected or 24 hr later were transfected. COS-7 cells, at 40–60% confluence, were transfected using 8–24 μl lipofectamine (Gibco), according to the manufacturer's instructions, with 2–6 μg of total plasmid DNA (i.e., full length Kir6.2 subcloned into the expression vector pcDNA3.1$^+$ and/or SUR1 subcloned into the pcDNA1Amp expression vector) and with 0.2–0.6 μg of the reporter green fluorescent protein gene (Gibco) [9]. The DNA/lipofectamine mixture was incubated with cells for 3.5 hr in Opti-MEM I media (Gibco). Thereafter, cells were rinsed with phosphate-buffered saline (Gibco) and incubated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 2 mM glutamine for 36 hr prior to electrophysiological experiments. cDNA encoding Kir6.2 and SUR1 were kindly provided by Dr. S. Seino (Chiba University) and Drs. J. Bryan and L. Aguilar-Bryan (Baylor College of Medicine), respectively [10].

Single Channel Recordings

To monitor activity of single channel molecules, the giagaohm seal patch-clamp technique was applied in the inside-out configuration, as we previously described [6, 11]. Cells were superfused with (in mM): KCl 140, $MgCl_2$ 1, EGTA 5, HEPES-KOH 5 (pH 7.4). Fire-polished pipettes, coated with Sylgard (resistance 5–7 MΩl), were filled with (in mM): KCl 140, $CaCl_2$ 1, $MgCl_2$ 1, HEPES-KOH 5 (pH 7.3). Recordings were made at room temperature (22°C), using a patch-clamp amplifier (Axopatch-1C). Single-channel activity was monitored on-line on a high-gain digital storage oscilloscope (VC-6025; Hitachi) and stored on tape with the aid of a PCM converter system (VR-10, Instrutech). Data were reproduced, low-pass filtered at 4 KHz (–3 dB) by a Bessel filter (Frequency Devices 902), sampled at 50 μgs rate, and further analyzed using the "pClamp6" software. The threshold for judging the open state was set at half of the single channel amplitude. Channel activity, assayed by digitizing segments of current recordings and forming histograms of base line and open level data points, were expressed as NPo (N, number of channels in the patch; Po, probability of each channel to be open). To determine the efficacy and potency of Ap4A on K_{ATP} channels, concentration-response curve was constructed and data fitted by the Hill equation: $y = 1/\{1 + ([ApnA]/EC_{50})^H\}$, where y is the

relative NPo at each tested concentration of ApnA, EC_{50} the concentration of ApnA at half-maximal channel inhibition, and H the Hill coefficient [11]. EC_{50} is presented as pEC_{50} ($-\log EC_{50}$). For analysis of intraburst channel behavior, periods of channel silence that exceeded 3 msec were omitted, and amplitude, open- and closed-dwell-time histogram was constructed and fitted by a single exponent. For burst analysis, a burst in channel activity was defined as a set of opening and closures terminated by a close event with a duration that exceeded the critical time (interburst interval) determined by "test interburst interval" using software pClamp 6. Burst duration and between burst duration histogram was constructed and fitted by the sum of 1–2 exponents.

Statistical Analysis

Data are presented as mean ± S.E.M, with n representing the number of patched cells. Mean values were compared by the Student's t-test. $p < 0.05$ was considered statistically significant.

RESULTS

The excision of a membrane patch from cotransfected COS-7 cells in ATP-free environment was associated with vigorous opening of K_{ATP} channels (Fig. 1). Ap4A (15 μM) inhibited the opening of K_{ATP} channels (Fig. 1). The NP_0 was estimated to be 2.6 ± 0.5 in the absence and 1.1 ± 10.3 in the presence of Ap4A ($n = 5$, $p < 0.01$). The inhibitory effect of Ap4A was concentration-dependent (Fig. 2), and pEC_{50} and Hill coefficient values were calculated to be 4.95 ± 10.04 and of 1.14, respectively ($n =$ 4–6). Kinetic analysis of single channels (Fig. 3) revealed that Ap4A did not change intraburst kinetic behavior of K_{ATP} channels, but rather decreased burst duration and increased between-burst duration (Fig. 4, Table 1).

DISCUSSION

This study demonstrates that Ap4A regulates pancreatic K_{ATP} channel activity by keeping the channel longer in closed interburst states. This is the first evidence that ApnA directly target K_{ATP} channels.

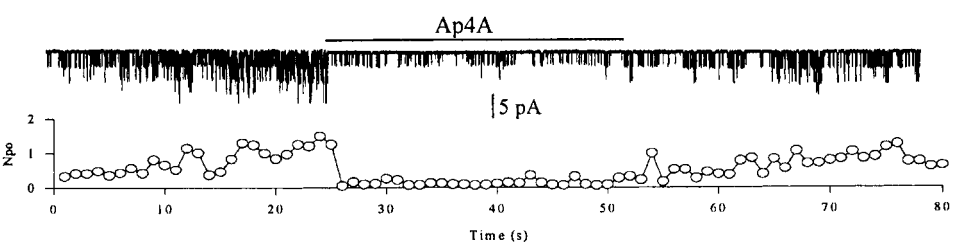

Fig. 1. The effect of Ap4A on recombinant pancreatic K_{ATP} channels in excised membrane patches. *Upper trace*: continuous recording of K_{ATP} channel activity in the absence and presence of Ap4A (15 μM). *Lower trace*: corresponding NPo values calculated over 2.5-s long intervals. Dotted line represents the zero current level. Holding potential: −60 mV.

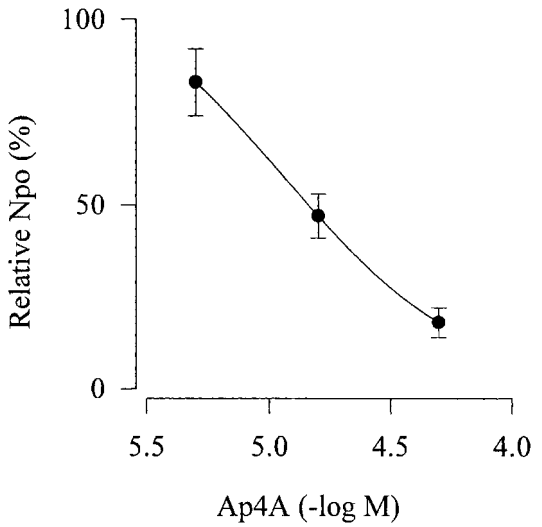

Fig. 2. The effect of different concentrations of Ap4A on recombinant pancreatic K_{ATP} channels in excised membrane patches. Concentration-response curve for Ap4A on K_{ATP} channels ($pEC_{50} = 4.95 \pm 0.04$; Hill coefficient = 1.14). Each point represents the mean ± S.E.M. ($n = 4$–6).

Proteins constituting the K_{ATP} channel complex have been cloned and complementary DNAs (cDNAs) for individual channel subunits constructed [10, 12]. Pancreatic K_{ATP} channel is composed of a K^+ channel, Kir6.2, and an ATP-binding cassette (ABC) protein, SUR1 [10, 12]. Coexpression of Kir6.2 and SUR1 has been

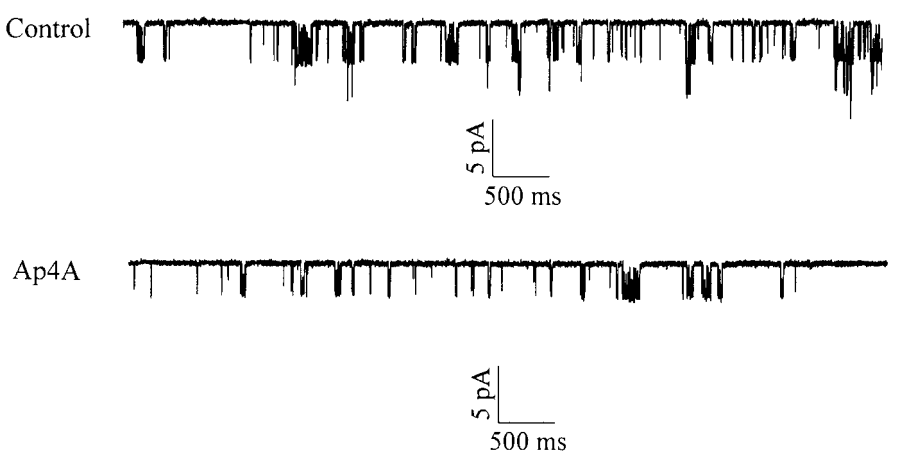

Fig. 3. Typical segments of channel recordings used to analyze kinetic behavior. *Upper trace*: continuous channel recording in the absence of Ap4A. *Lower trace*: continuous channel recording in the presence of Ap4A (15 μM).

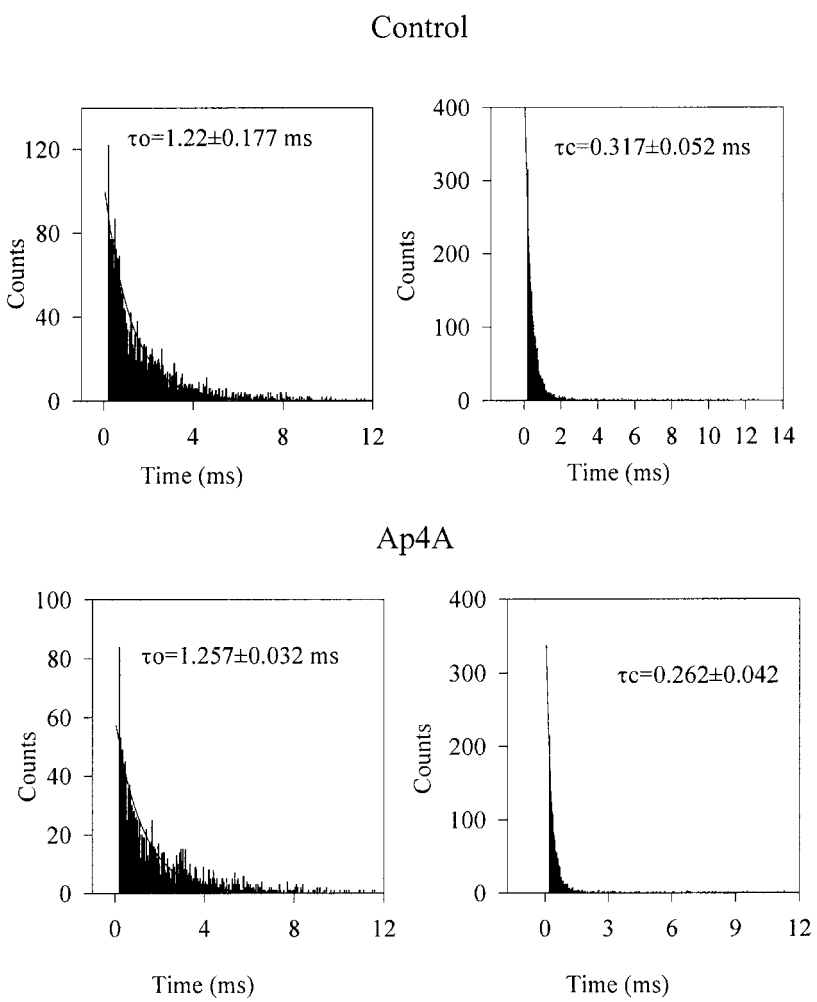

Fig. 4. Mean open-time and closed-time histograms plotted with a bin width size of 50 μs and fitted by one exponential function. Results of data fitting are plotted as *solid lines*.

Table 1. Parameters of Single Recombinant Pancreatic ATP-Sensitive K^+ Channel Activity in the Absence (Control) and Presence of Ap4A (15 μM)

	Control	Ap4A
Amplitude (pA)	3.050 ± 0.022	2.774 ± 0.052
Mean open time (ms)	1.227 ± 0.118	1.243 ± 0.021
Mean closed time (ms)	0.311 ± 0.032	0.293 ± 0.034
Burst duration (ms)	231.872 ± 0.160	60.684 ± 0.445
Between burst duration (ms)	80.309 ± 0.594	177.927 ± 0.379

found to reconstitute functional pancreatic K_{ATP} channels, including its gating property [10]. It has been previously suggested that Ap4A may inhibit pancreatic K_{ATP} channels [2]. To elucidate whether Ap4A directly interact with channel protein complex, we coexpressed pancreatic K_{ATP} channel subunits, Kir6.2 and SUR1, in COS-7 cells, and examined the effect of Ap4A on single channels. The present findings that Ap4A inhibits the opening of recombinant K_{ATP} channels in a concentration-dependent manner with EC_{50} and Hill coefficient values similar to those described on native pancreatic K_{ATP} channels suggests that Ap4A directly target channel protein complex.

What is the mechanism of Ap4A-induced decrease in K_{ATP} channels opening has remained unknown. Here, we demonstrate that Ap4A decreases the probability of K_{ATP} channel opening by prolonging the duration of intervals the channel spends between bursts without affecting the single channel amplitude nor the mean open or closed time. This suggests that Ap4A does not directly disrupt K^+ flow by altering the channel pore. In fact, Ap4A-mediated increase in the duration the K_{ATP} channel spends between bursts may rather suggest that Ap4A acts through binding sites on the channel protein complex outside the pore itself. Thus, Ap4A is a genuine novel regulator of K_{ATP} channel activity, with properties similar to those found in conventional K_{ATP} channel ligands [13, 14].

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