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ORIGINAL ARTICLE

Evidence for the possible involvement of the $P2Y_6$ receptor in Ca^{2+} mobilization and insulin secretion in mouse pancreatic islets

Masahiro Ohtani · Jun-ichiro Suzuki · Kenneth A. Jacobson · Takami Oka

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Abstract Subtypes of purinergic receptors involved in modulation of cytoplasmic calcium ion concentration ($[Ca^{2+}]_i$) and insulin release in mouse pancreatic β-cells were examined in two systems, pancreatic islets in primary culture and beta-TC6 insulinoma cells. Both systems exhibited some physiological responses such as acetylcholine-stimulated $[Ca^{2+}]_i$ rise via cytoplasmic Ca^{2+} mobilization. Addition of ATP, ADP, and 2-MeSADP (each 100 μM) transiently increased $[Ca^{2+}]_i$ in single islets cultured in the presence of 5.5 mM (normal) glucose. The potent $P2Y_1$ receptor agonist 2-MeSADP reduced insulin secretion significantly in islets cultured in the presence of high glucose (16.7 mM), whereas a slight stimulation occurred at 5.5 mM glucose. The selective $P2Y_6$ receptor agonist UDP (200 μM) transiently increased $[Ca^{2+}]_i$ and reduced insulin secretion at high glucose, whereas the $P2Y_{2/4}$ receptor agonist UTP and adenosine receptor agonist NECA were inactive. $[Ca^{2+}]_i$ transients induced by 2-MeSADP and UDP were antagonized by suramin (100 μM), U73122 (2 μM, PLC inhibitor), and 2-APB (10 or 30 μM, IP_3 receptor antagonist), but neither by staurosporine (1 μM, PKC inhibitor) nor depletion of extracellular Ca^{2+}. The effect of 2-MeSADP on $[Ca^{2+}]_i$ was also significantly inhibited by MRS2500, a $P2Y_1$ receptor antagonist. These results suggested that $P2Y_1$ and $P2Y_6$ receptor subtypes are involved in Ca^{2+} mobilization from intracellular stores and insulin release in mouse islets. In beta-TC6 cells, ATP, ADP, 2-MeSADP, and UDP transiently elevated $[Ca^{2+}]_i$ and slightly decreased insulin secretion at normal glucose, while UTP and NECA were inactive. RT-PCR analysis detected mRNAs of $P2Y_1$ and $P2Y_6$, but not $P2Y_2$ and $P2Y_4$ receptors.

Keywords Pancreatic islets · $P2Y_1$ nucleotide receptor · Purines · Pyrimidines · Insulin · Calcium

Abbreviations

ADP	adenosine 5′-diphosphate
2-APB	2-aminoethyl diphenylborinate
2-MeSADP	2-methylthioadenosine 5′-diphosphate
FBS	fetal bovine serum
HEPES	*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid
IP_3	inositol 1,4,5-trisphosphate
MRS2500	2-iodo-N^6-methyl-(*N*)-methanocarba-2′-deoxyadenosine-3′,5′-bisphosphate
NECA	5′-*N*-ethylcarboxamide adenosine
PBS	phosphate-buffered saline
PKC	protein kinase C
PLC	phospholipase C
RT-PCR	reverse transcription polymerase chain reaction
U73122	1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione
U73343	1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]- 2,5-pyrrolidinedione

M. Ohtani · J.-i. Suzuki · T. Oka (✉)
Research Institute of Pharmaceutical Sciences,
Musashino University,
1–1–20 Shinmachi,
Nishi-Tokyo, Tokyo 202–8585, Japan
e-mail: toka@musashino-u.ac.jp

K. A. Jacobson
Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institutes of Diabetes, Digestive and Kidney Diseases, National Institutes of Health,
Bethesda, MD 20892, USA

UDP uridine 5′-diphosphate
UTP uridine 5′-triphosphate

Introduction

Extracellular ATP and other purine nucleotides serve important functions in regulating metabolism, energy supply, and various cellular activities in many types of cells and tissues [5, 40]. The effects of these agents are mediated through specific P2 receptors that are subdivided into P2X and P2Y families [23, 40]. The P2X receptors are ionotropic, ligand-gated cation channels, whereas P2Y receptors are G protein-coupled receptors [19, 23, 40]. At present, seven P2X and eight P2Y receptor subtypes have been cloned [23]. The role of nucleotides in the endocrine function of the pancreas has been studied. Immunohistochemical studies indicated the presence of $P2X_1$, $P2X_4$, $P2X_7$, $P2Y_1$, and $P2Y_4$ receptors in rat and mouse pancreatic islets [8, 9]. Exogenous application of ATP and other nucleotide derivatives has been reported to stimulate insulin secretion in the rat pancreas and isolated rat islets [3, 4, 6, 7, 11, 13, 26, 31, 32]. In addition, stimulation of a P2Y receptor by ATP in rat pancreatic islets has been shown to mobilize Ca^{2+} from internal calcium sources via phospholipase C (PLC) activation and the production of inositol 1,4,5-trisphosphate (IP_3) [4, 35]. The rise in cytosolic Ca^{2+} is a key event in the regulation of insulin secretion in rat pancreatic islets [16]. In addition, stimulation of insulin secretion through a P2Y receptor in rat islets was suggested to be associated with the cAMP/PKA pathway [7]. Enhancement of insulin release by ATP also has been shown in the dog pancreas [35], human islets [12], and several insulinoma cell lines [2, 15, 25, 37]. In addition, ATP caused a transient increase in $[Ca^{2+}]_i$ in mouse islet β-cells [16, 41], which was suggested to involve internal calcium stores [41]. In contrast, ATP and its related nucleotide analogues have been shown to inhibit insulin secretion in mouse pancreatic islets [30, 33]. Poulsen et al. [33] have shown that a $P2Y_1$ receptor and a serine/threonine protein phosphatase calcineurin mediate inhibition of exocytosis of insulin-containing vesicles in the mouse β-cells. Recently, Zhao et al. showed that Ca^{2+} mobilization induced by ATP differed in duration between rat and mouse islet β-cells and suggested that the opposite effect of ATP on insulin release in these two species is due to the difference in the type of calcium ion channel being affected [41]. These results suggest that a P2 receptor subtype and $[Ca^{2+}]_i$ are closely associated with control of insulin release. In the present study, we used selective agonists and an antagonist of P2Y receptor subtypes [20, 21] to identify the particular receptor(s) involved in Ca^{2+} mobilization and insulin release in mouse primary pancreatic islets in culture and the mouse β-cell line, beta-TC6. The effect of various inhibitors of signaling molecules was also examined in order to gain insight into the mechanism whereby a P2Y receptor regulates insulin secretion in mouse islet β-cells. The results suggest that P2Y receptor subtypes, $P2Y_1$ and $P2Y_6$, are involved in the elevation of $[Ca^{2+}]_i$ via Ca^{2+} mobilization from intracellular stores and inhibition of insulin secretion in mouse islet β-cells.

Experimental procedures

Reagents

ATP, UTP, ADP, UDP, NECA, suramin, U73122, U73343, 2-APB, and staurosporine were obtained from the Sigma Chemical Company (St. Louis, MO, USA). 2-MeSADP and MRS2500 were from Tocris Bioscience (Bristol, UK). RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM), and penicillin/streptomycin were from Invitrogen Corp. (Carlsbad, CA, USA). Fetal bovine serum (FBS) was from BioWest (Nuaill, France). U73122, U73343, 2-APB, and staurosporine were dissolved in dimethyl sulfoxide (DMSO).

Islet isolation

A pancreas from a male C57 BL/6 mouse (7–12 weeks old) was inflated by injecting 1–2 ml Hank's balanced salt solution (HBSS, pH 7.4, Invitrogen, Carlsbad, CA, USA) containing 20 mM HEPES, 5 mM $NaHCO_3$, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 1 mg/ml collagenase type XI (Sigma, St. Louis, MO, USA) and digested for 15–20 min in a water bath at 37°C. The digested tissues were filtered through several metal and nylon meshes with repeated washing with HBSS. Islets were collected on a 40-μm mesh cell strainer (BD Biosciences, Bedford, MA, USA). Pancreatic islets were placed on a 60-mm tissue culture dish (BD Biosciences, Bedford, MA, USA) and cultured for approximately 1 day in RPMI 1640 medium containing 10% FBS, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 5.5 mM glucose. After initial culture, adequate numbers of islets (100~400) were picked by hand with a micropipette under a stereomicroscope and used for the indicated experiments.

Cell culture

Beta-TC6 cells purchased from ATCC (Manassas, VA, USA) were cultured in DMEM containing 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 25 mM glucose in a CO_2 incubator (5% CO_2) at 37°C. The cells were subcultured every week. Cells from passage 10–30 were used for all experiments.

Measurement of $[Ca^{2+}]_i$ changes in single mouse islet and beta-TC6 cells

Thirty islets were transferred into a glass-based dish coated with collagen I (Asahi Techno Glass Corp., Tokyo, Japan) and cultured for 6–10 days in RPMI 1640 medium containing 5.5 mM glucose in 5% CO_2 at 37°C. After removal of the medium, islets were washed once with Krebs/HEPES buffer (NaCl: 129 mM, $NaHCO_3$: 5 mM, KCl: 4.7 mM, $MgSO_4$: 1.2 mM, KH_2PO_4: 1.2 mM, $CaCl_2$: 2.56 mM, and HEPES: 10 mM, adjusted with 5N NaOH to pH 7.4) without glucose and the islets were incubated for 20–30 min at 37°C in 5% CO_2 in 1 ml of the buffer containing 5.5 mM glucose and 3 μM of fura-2 acetoxymethyl ester (Invitrogen, Carlsbad, CA, USA). After one wash with glucose-free buffer, 1 ml of the buffer containing the desired glucose concentrations was added to the dish. Reagent solutions were applied using a pipette. In single islets the temporal change in $[Ca^{2+}]_i$ was monitored using a microscope (Nikon, Tokyo, Japan) equipped with a fluorescence ratio analyzer (Hamamatsu Photonics, Hamamatsu, Japan). The data were expressed as a ratio (340/380 nm) of the fluorescence intensity (emission wavelength at 510 nm) obtained when excited at a 340 or 380 nm wavelength. In Figs. 3 and 5 and Table 1, the $[Ca^{2+}]_i$ response induced by the nucleotide in the absence or presence of an inhibitor was expressed as a change in the ratio (340/380 nm), which was calculated by subtraction of a baseline value from a peak height value. Inhibitors were dissolved in DMSO and mixed well in assay buffer prior to application to the cells. The volume of DMSO per 1 ml buffer was less than 3 μl since 5 μl of DMSO itself increased the baseline of the ratio.

Beta-TC6 cells (2×10^5 cells) were seeded in the glass-based dish and cultured for 1 day in DMEM containing 25 mM glucose in 5% CO_2 at 37°C. After reaching 40–50% confluency, cells were washed with glucose-free Krebs/HEPES buffer once and loaded with 3 μM fura-2 acetoxymethyl ester for 30–40 min at 37°C in darkness. Then the incubation buffer was removed and replaced with fresh Krebs/HEPES buffer (5.5 mM glucose). Measurement of temporal changes in $[Ca^{2+}]_i$ in an islet-like cluster composed of a few or several beta-TC6 cells was performed with the same method as those mentioned above.

Insulin secretion assay

Isolated islets on a culture dish were preincubated for 60 min at 37°C in 5% CO_2 in Krebs/HEPES buffer containing 3.3 mM glucose. Groups of seven islets were manually transferred to a centrifuge tube and incubated in 1 ml of solution for 60 min at 37°C in the presence of indicated concentrations of glucose, appropriate test agents, and bovine serum albumin (1 mg/ml). After incubation, each tube was centrifuged and the supernatant was stored frozen at −80°C prior to the enzyme immunosorbent assay (EIA) of insulin.

Beta-TC6 cells were seeded in a 24-well plate at a density of 1×10^5 cells/well and were grown at 37°C in the CO_2 incubator (5% CO_2) for 4 days until the 40–50% confluency was reached ($2–4\times10^5$ cells/ml). Cells were washed once with Krebs/HEPES buffer and preincubated for 30 min in glucose-free buffer at 37°C. Then the indicated concentration of glucose was added to each well along with other reagents, and the plate was incubated for 90 min at 37˚C in 5% CO_2. At the end of the assay, a supernatant was collected for insulin determination.

Insulin enzyme immunosorbent assay (EIA)

The concentration of insulin released into the assay buffer from the mouse islets and beta-TC6 cells was measured using

Table 1 Effect of a $P2Y_1$ receptor antagonist MRS2500, a P2 receptor antagonist suramin, a PKC inhibitor staurosporine, and depletion of extracellular Ca^{2+} on (**a**) 2-MeSADP- or (**b**) UDP-induced transient $[Ca^{2+}]_i$ rise in the mouse islet and beta-TC6 cells

	+MRS2500		+Suramin	+Staurosporine	No extracellular Ca^{2+}
	10 μM	30 μM	100 μM	1 μM	
a					
	% of control (100 μM 2-MeSADP alone)				
Mouse islet	49±11 (n=6)*	23±8 (n=5)*	15±7 (n=5)*	80±13 (n=3)	80±11 (n=7)
Beta-TC6 cells	35±4 (n=14)*	24±3 (n=16)*	49±10 (n=31)*	113±11 (n=24)	51±12 (n=30)*
b					
	% of control (200 μM UDP alone)				
Mouse islet			11±6 (n =4)*	120±28 (n=5)	112±23 (n=6)
Beta-TC6 cells			51±6 (n=15)*	82±9 (n=32)	65±9 (n=24)*

MRS2500 and suramin were added with the nucleotides simultaneously to the culture of the islet or to beta-TC6 cells in the presence of 5.5 mM glucose. The islet and beta-TC6 cells were preincubated with staurosporine for 20 min (mean±SE)
*P<0.05

a rat insulin EIA kit (Morinaga Institute of Biological Science, Yokohama, Japan), according to instructions of the company. Each assay was run in duplicate and closely agreeing values (less than 5% variation) were consistently obtained.

Reverse transcription-polymerase chain reaction (RT-PCR) in islets and beta-TC6 cells

The total RNA of the mouse islets (500–1,000 islets) was isolated with RNeasy Mini Kit (Qiagen, Germantown, MD, USA) after genomic DNA was truncated using a QIAshredder (Qiagen, Germantown, MD, USA). Beta-TC6 cells were homogenized in a glass homogenizer on ice and the total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the method described previously [28]. The total RNAs were then treated with DNase I (Ambion, Austin, TX, USA) for 30 min at 37°C to remove genomic DNA. The RNA (islets: 0.1 μg, beta-TC6 cells: 0.5 μg) was reverse transcribed into cDNA using a GeneAmp RNA PCR kit (Applied Biosystems, Branchburg, NJ, USA) and then amplified by PCR with 40 or 30 cycles in the case of islets and beta-TC6 cells, respectively. The oligo primers for mouse $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptor subtypes and β-actin (internal control) were synthesized commercially (Operon Biotechnology, Tokyo, Japan), according to the sequences for $P2Y_{1,2,4,6}$ receptors [38] and β-actin [22]. These primer sequences were as follows: $P2Y_1$: sense 5′-TGGCGTGGTGTACCC TCTCAAGTC-3′, antisense 5′-ACCGTGCTCGCAAATT CATCGTT-3′, $P2Y_2$: sense 5′-ACCAGCGTGCGGG GAACC-3′, antisense 5′-GCATCTCGGGCAAAGCGGA CAAGT-3′, $P2Y_4$: sense 5′-TGCCTCGTGCCCAAC CTCTTCTTT-3′, antisense 5′-CAGTTGTTCGGCGCT TAGGTGTGC-3′, $P2Y_6$: sense 5′-CCTGGCACTGGCG GACCTGAT-3′, antisense 5′-GGCGGGCCATGCGA CAATAAC-3′, and β-actin: sense 5′-GATGACGA TATCGCTGCGCTGGTCGTC-3′, antisense 5′-GACCCT CAGGGCATCGGAACCGCTCG-3′. Annealing temperatures for $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and β-actin in PCR were 57°C, 59°C, 60°C, 59°C and 65°C, respectively. Contamination of genomic DNA in the total RNAs was examined by performing RT-PCR in the absence of a reverse transcriptase. Electrophoresis of the amplified cDNA was performed on a 1.5% agarose gel containing ethidium bromide and the cDNA was visualized using a UV transilluminator at 302 nm.

Statistics

Data were expressed as mean ± SE Statistical significance was evaluated by Student's t test. A P value < 0.05 was statistically significant.

Results

Effect of purinergic agonists on $[Ca^{2+}]_i$ in mouse single islets and beta-TC6 cells

We first examined the effect of high concentrations of glucose and acetylcholine (ACh), which are well-known potent stimulators of $[Ca^{2+}]_i$ in the primary islet in culture [10, 14]. Both the increase of the glucose concentration from 5.5 mM to16.7 mM (Fig. 1a) and ACh at 100 μM (Fig. 1b) induced an increase in $[Ca^{2+}]_i$ over a control that was incubated in the presence of 5.5 mM glucose without ACh. These results were consistent with previous findings [14].

We next examined the effect of various purinergic compounds on $[Ca^{2+}]_i$ in mouse single islets in culture. As shown in Fig. 2a, ATP at 100 μM induced a transient increase in $[Ca^{2+}]_i$, but it had little effect at 1 and 10 μM (data not shown). In addition, both a synthetic analogue of ADP, 2-MeSADP and ADP at 100 μM (data not shown), which are $P2Y_{1/12}$ receptor agonists, and a $P2Y_6$ receptor-selective agonist UDP at 200 μM stimulated $[Ca^{2+}]_i$ (Fig. 2b,c). We found that UDP had little effect at 100 μM and that UTP, which has a high affinity for $P2Y_2$ and $P2Y_4$ receptors, did not elevate $[Ca^{2+}]_i$ even at 200 μM (data not shown). A selective adenosine receptor agonist, NECA (200 μM) [34],

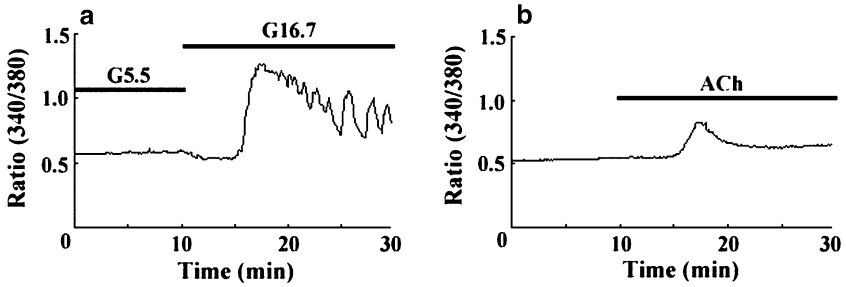

Fig. 1 Effect of a high concentration of glucose and acetylcholine (*ACh*) on $[Ca^{2+}]_i$ in a single mouse islet in culture. Glucose at 16.7 mM (*G16.7*, **a**) and ACh at 100 μM (**b**) were added to the mouse islet in the presence of 5.5 mM glucose (*G5.5*) at the time indicated by the *bar*. The change in $[Ca^{2+}]_i$ was monitored for 30 min using excitation at 340 and 380 nm and emission at 510 nm, and the *bar* indicates exposure to the indicated nucleotide. Each experiment was repeated 3 times

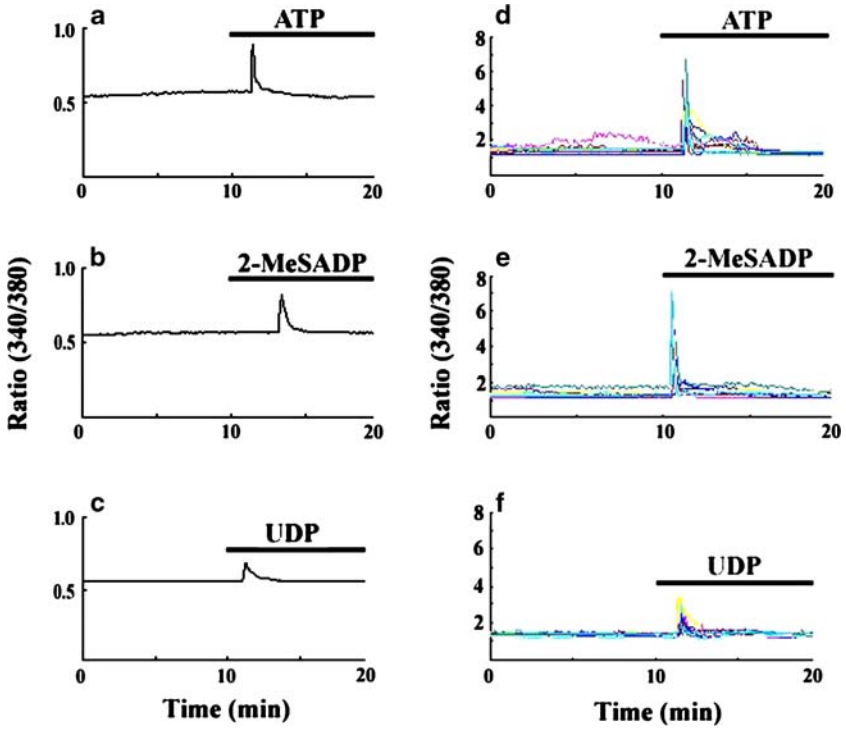

Fig. 2 Effect of ATP, 2-MeSADP, and UDP on $[Ca^{2+}]_i$ in a single mouse islet and beta-TC6 cells in culture. ATP at 100 μM (**a**, **d**), 2-MeSADP at 100 μM (**b**, **e**), and UDP at 200 μM (**c**, **f**) were added to the mouse single islet (**a**–**c**) and beta-TC6 cells (**d**–**f**) in the presence of 5.5 mM glucose at the time indicated by the *bar*. In beta-TC6 cells, $[Ca^{2+}]_i$ rise occurring in each islet-like cluster composed of a few or several cells (9–10 clusters) is shown with overlapping different colors. The change in $[Ca^{2+}]_i$ was monitored for 20 min using excitation at 340 and 380 nm and emission at 510 nm, and the *bar* indicates exposure to the indicated nucleotide. Each experiment was repeated 7–15 times

also showed no effect (data not shown), suggesting that ATP or ADP did not raise $[Ca^{2+}]_i$ via adenosine receptors through metabolic conversion to adenosine. To determine whether $[Ca^{2+}]_i$ rises induced by the nucleotides were mediated through purinergic receptors, we examined the effect of a nonspecific P2 receptor antagonist, suramin, and a highly specific $P2Y_1$ receptor antagonist, MRS2500 [39], in the mouse islet. Suramin (100 μM) significantly blocked both 2-MeSADP- (100 μM) and UDP (200 μM)-induced transient $[Ca^{2+}]_i$ elevations, suggesting that these responses were mediated via the P2 receptor (Table 1). MRS2500 at 30 μM significantly inhibited the $[Ca^{2+}]_i$ rise induced by 2-MeSADP (100 μM) in the islet, whereas it had a smaller inhibitory effect at 10 μM (Table 1). The above results indicated that $P2Y_1$ and $P2Y_6$ receptors were functional in mouse pancreatic islets in culture.

ATP, 2-MeSADP, and ADP (data not shown) each at 100 μM and UDP at 200 μM also induced an increase in

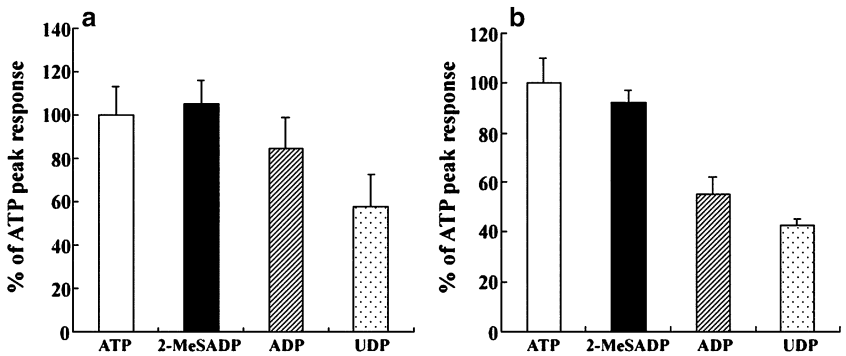

Fig. 3 The effect of purinergic compounds on $[Ca^{2+}]_i$ in a mouse single islet in culture and beta-TC6 cells. The following compounds were tested: ATP (100 μM), 2-MeSADP (100 μM), ADP (100 μM), and UDP (200 μM). The effect of the nucleotides on $[Ca^{2+}]_i$ is shown as % of the peak response induced by ATP (100 μM) in the mouse single islet in culture (**a**) and beta-TC6 cells (**b**) in the presence of 5.5 mM glucose. Each *bar* represents the mean±SE (n=7–15 in **a** and n=24–54 in **b**)

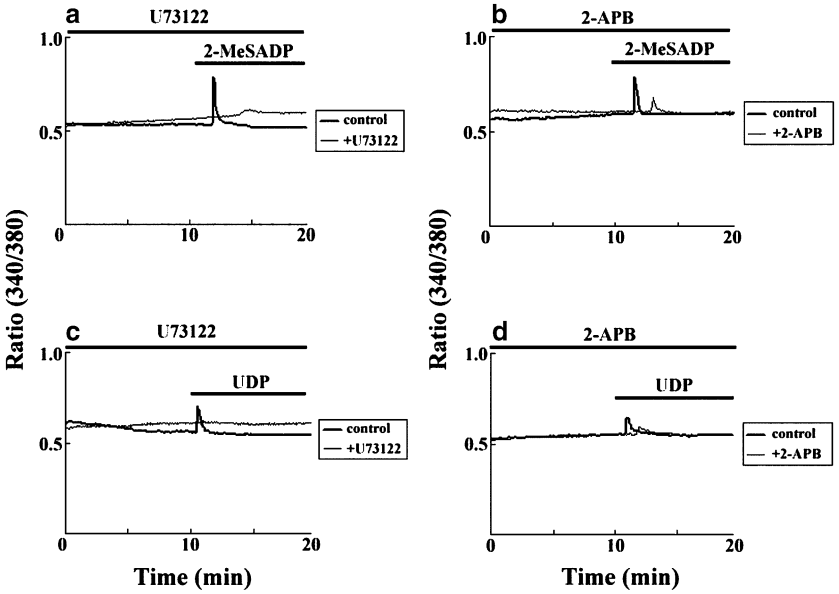

Fig. 4 Effect of the PLC inhibitor U73122 and the IP_3 receptor antagonist 2-APB on 2-MeSADP- or UDP-induced $[Ca^{2+}]_i$ rise in a single mouse islet in culture. U73122 at 2 μM (**a**, **c**) and 2-APB at 30 μM (**b**) or 10 μM (**d**) were added to the islet 20 min prior to the addition of the nucleotides. The change in $[Ca^{2+}]_i$ was monitored for 20 min using excitation at both 340 and 380 nm and emission at 510 nm, and the *bar* indicates exposure to the indicated nucleotide or the inhibitor. Each experiment was repeated 3–4 times

$[Ca^{2+}]_i$ in mouse insulinoma beta-TC6 cells (Fig. 2d–f), suggesting that the nucleotide-induced $[Ca^{2+}]_i$ increase in pancreatic islets was attributable, at least in part, to the response of β-cells in the islet. As shown in Table 1, these effects both by 2-MeSADP and UDP were significantly blocked by suramin (100 μM). The effect of 2-MeSADP was also significantly inhibited by MRS2500 (10 or 30 μM).

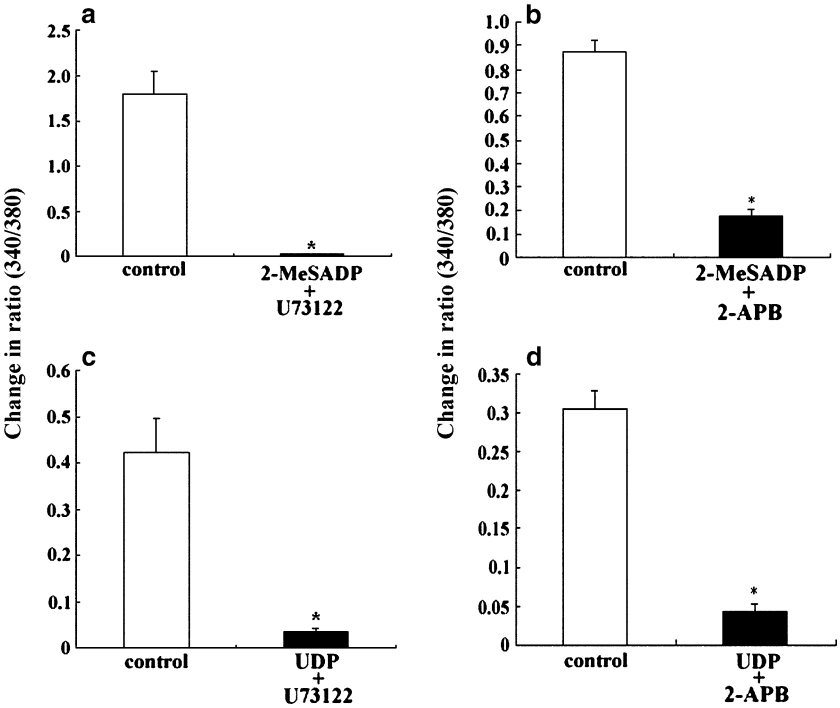

Fig. 5 Effect of the PLC blocker U73122 and the IP_3 receptor antagonist 2-APB on 2-MeSADP- or UDP-induced $[Ca^{2+}]_i$ rise in beta-TC6 cells. Cells were incubated in the presence of either 2 μM U73122 (**a**, **c**) or 30 μM 2-APB (**b**, **d**) for 20 min prior to addition of the nucleotides. The results are expressed as a change in ratio (calculated by subtraction of a baseline value from a peak height value). Each *bar* represents the mean±SE (n=16–32). *P<0.05 in comparison to control in the absence of nucleotides

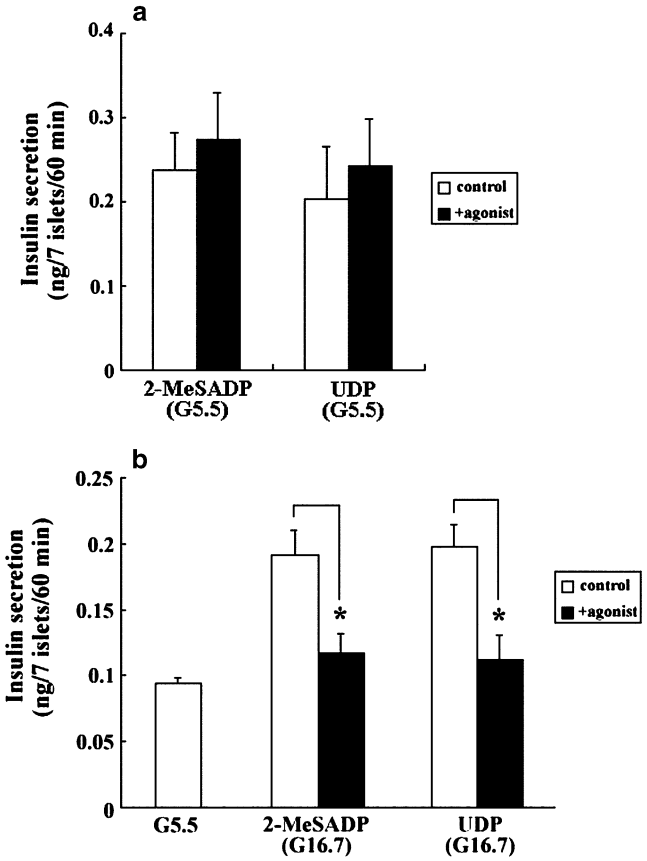

Fig. 6 Effect of 2-MeSADP and UDP on insulin secretion in mouse islets in culture. The islets were incubated in Krebs/HEPES buffer (pH 7.4) containing 5.5 mM (*G5.5*, **a**) or 16.7 mM glucose (*G16.7*, **b**) in the presence of 100 μM 2-MeSADP or 200 μM UDP for 60 min at 37°C. Each *bar* represents the mean±SE (n=8 in **a** and n=10 in **b**). *P<0.05 in comparison to control in the absence of nucleotides

In order to determine quantitatively the effect of the nucleotides on $[Ca^{2+}]_i$, a peak height induced by each nucleotide was expressed as % of that induced by 100 μM ATP. The effect of 2-MeSADP on $[Ca^{2+}]_i$ appeared to be almost the same as that of ATP in both the islet and beta-TC6 cells, while the effect of UDP even at 200 μM was smaller than that of ATP (Fig. 3a,b).

$[Ca^{2+}]_i$ transients induced by $P2Y_1$ and $P2Y_6$ receptor activations are mediated through intracellular Ca^{2+} mobilization

It has been demonstrated that both $P2Y_1$ and $P2Y_6$ receptors are Gq protein-coupled receptors and their stimulation results in the activation of PLC, which produces the second messengers IP_3 and diacylglycerol (DG) [23, 40]. Then IP_3 induces Ca^{2+} mobilization from intracellular stores in the endoplasmic reticulum and protein kinase C (PKC) is activated by DG. In order to gain insight into the mechanism of Ca^{2+} mobilization induced by the nucleotides in islets, we examined the effect of U73122, a PLC inhibitor and an IP_3 receptor inhibitor 2-APB. As shown in Fig. 4a and c, U73122 at 2 μM significantly inhibited 2-MeSADP- (100 μM) and UDP (200 μM)-induced $[Ca^{2+}]_i$ increases, whereas U73343, an inactive analogue of U73122, did not (data not shown). 2-APB also significantly inhibited $[Ca^{2+}]_i$ rises induced by 2-MeSADP at 30 μM (Fig. 4b) and UDP at 10 μM (Fig. 4d). The transient $[Ca^{2+}]_i$ rises induced by both the nucleotides in the islet were still observed when the extracellular Ca^{2+} was depleted (Table 1). By contrast, a PKC inhibitor staurosporine showed no significant inhibitory effect. These results suggested the involvement of intracellular Ca^{2+} stores in the $[Ca^{2+}]_i$ elevation induced by the $P2Y_1$ and $P2Y_6$ receptor activation, whereas the PKC did not appear to play a role. Similar inhibitory effects were observed in beta-TC6 cells (Fig. 5a–d and Table 1). However, $[Ca^{2+}]_i$ responses induced by both 2-MeSADP and UDP were significantly inhibited in the absence of extracellular Ca^{2+} in beta-TC6 cells (Table 1). It was possible that Ca^{2+} influx through a Ca^{2+} channel partly contributed to the $[Ca^{2+}]_i$ elevation induced by stimulation of $P2Y_1$ or $P2Y_6$ receptor in beta-TC6 cells.

Inhibitory effect of nucleotides on insulin secretion in mouse islets and beta-TC6 cells

Both 2-MeSADP (100 μM) and UDP (200 μM) enhanced glucose (5.5 mM)-induced insulin secretion in mouse islets by 15 and 19%, respectively (Fig. 6a), however, these effects were not statistically significant. In the presence of a high concentration of glucose (16.7 mM glucose), however, 2-MeSADP and UDP produced significant inhibitions of insulin secretion (P<0.05) by 39 and 43%, respectively (Fig. 6b). In beta-TC6 cells cultured in the presence of 5.5 mM glucose insulin secretion was also reduced by 2-MeSADP at 100 μM and UDP at 200 μM, but these effects were smaller (2-MeSADP: 81±4% compared with 5.5 mM glucose alone, n=8; UDP: 84±9%, n=8). Among the nucleotides tested (ATP, 2-MeSADP, ADP, and UDP), only the effect of 2-MeSADP was statistically significant.

$P2Y_1$ and $P2Y_6$ receptor mRNA expression in mouse islets and beta-TC6 cells

The above pharmacological studies suggested that $P2Y_1$ and $P2Y_6$ receptors were present and functional in both the mouse islets and beta-TC6 cells. We next examined whether $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptor mRNAs were expressed by RT-PCR. As shown in Fig. 7a and b, the $P2Y_1$ and $P2Y_6$ receptor mRNAs were present, whereas expression of $P2Y_2$ and $P2Y_4$ receptor mRNAs was not detectable in both the mouse islets and beta-TC6 cells. As a

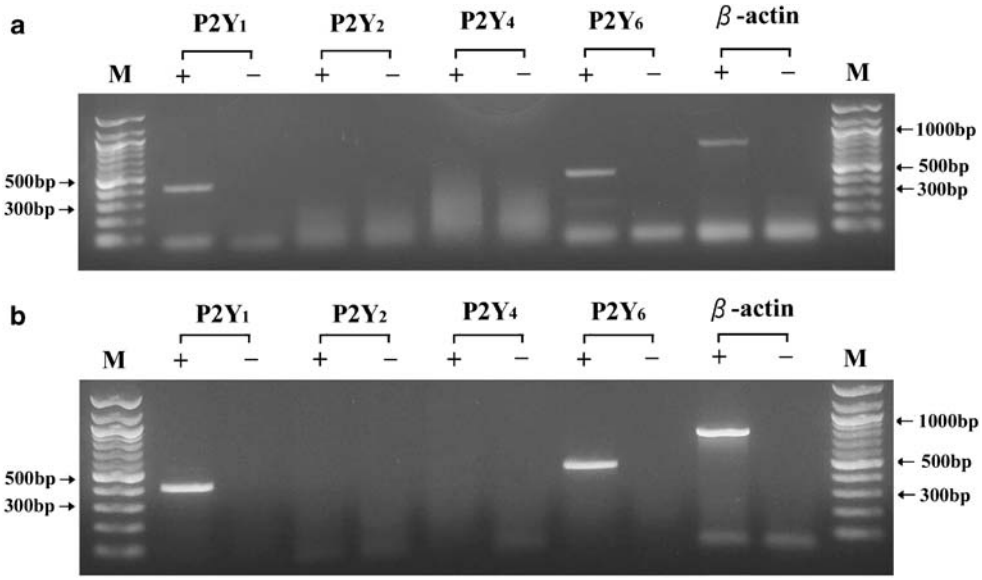

Fig. 7 RT-PCR analysis of P2Y receptor subtype mRNAs in mouse islets and beta-TC6 cells. PCR reactions were performed by 40 cycles in the islets (**a**) and by 30 cycles in beta-TC6 cells (**b**) with specific primers for each P2Y receptor subtype. The expected PCR product lengths for $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and β-actin were 410, 440, 499, 452, and 778 bp, respectively. Reverse transcription was performed with (+) or without (−) a reverse transcriptase to assess genomic DNA contamination. *M* shows a 100-bp DNA ladder

positive control, expression of $P2Y_2$ and $P2Y_4$ mRNAs in the mouse brain was confirmed with the specific primers (data not shown), indicating that failure to detect these receptor mRNAs in β-cells was not due to the primer sequences. The estimated sizes of the PCR products of $P2Y_1$ and $P2Y_6$ receptors were the same in both the islets and beta-TC6 cells.

Discussion

In the rat and mouse pancreas, $P2X_1$, $P2X_4$, $P2X_7$, $P2Y_1$, $P2Y_2$, and $P2Y_4$ receptors were detected by immunohistochemical studies [8, 9]. Recently, Garcia et al. reported by RT-RCR and the Western blot analyses that $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{12}$ receptors were expressed in rat insulinoma INS-1 cells [27]. In the present study we found that several P2Y receptor agonists elevated $[Ca^{2+}]_i$ in mouse islets in culture in the presence of 5.5 mM glucose. The findings that ATP, ADP, 2-MeSADP, and UDP but not UTP induced $[Ca^{2+}]_i$ rises suggested the involvement of the P2Y receptor, especially $P2Y_1$ and $P2Y_6$ receptors. RT-PCR analysis also indicated that $P2Y_1$ and $P2Y_6$ receptors were present in the islets. Our results are consistent with the view that the $P2Y_1$ receptor can regulate $[Ca^{2+}]_i$ in pancreatic β-cells [14, 31, 39] and insulinoma [37]. In addition to the $P2Y_1$ receptor, we found that UDP, a selective activator of the $P2Y_6$ receptor, elicited a transient $[Ca^{2+}]_i$ increase and inhibition of insulin release. These results suggested the possible involvement of the $P2Y_6$ receptor in the regulation of insulin secretion in the mouse islet. However, since UDP produced its effect at the relatively high concentration of 200 μM, we could not rule out the possibility that the effect of UDP may be mediated via other receptors. In our assay system, UDP-induced $[Ca^{2+}]_i$ rises were blocked by 1 μM MRS2179 in mouse islets (data not shown). There are several possibilities for the blockade. The first possibility is that various indirect effects could be produced when the nucleotides were incubated for a long time with the cells, since $[Ca^{2+}]_i$ measurements were performed under the static non-perfusion assay conditions. The second one is that the $P2Y_1$ receptor couples with the $P2Y_6$ receptor to form a heterodimer and therefore the UDP effect is influenced by the antagonist, since some functional recombinant heteromeric P2X receptors have been reported [20].

An earlier electrophysiological study also presented evidence indicating that suppression of Ca^{2+}-dependent exocytosis of insulin-containing granules was mediated via the $P2Y_1$ receptor in mouse primary islet β-cells [33]. It is also noteworthy that insulin secretion was significantly elevated in islets isolated from $P2Y_1$-deficient mice ($P2Y_1$ −/−) in comparison to those from wild-type animals [24], suggesting the involvement of the $P2Y_1$ receptor in inhibiting insulin secretion in the mouse islets. Since insulin secretory granules contain ATP [18] and ATP is co-released with insulin by pancreatic β-cells, it is possible that ATP exerts a negative control over insulin secretion in a feedback manner [33].

P2Y receptor activation enhanced moderate or high glucose-induced insulin release in rat islets, whereas such an enhancement was not observed in the presence of a low glucose concentration [4, 7, 11, 13, 31, 32]. It was also shown that pulsatile insulin secretion induced by high glucose in rat islets was enhanced in the presence of MRS2179 without affecting $[Ca^{2+}]_i$ oscillation [17, 36]. These results indicated that the $P2Y_1$ receptor plays a role in the regulation of insulin secretion in rat islets as well as in

mouse islets. In the present study, we found that stimulation of the $P2Y_1$ or $P2Y_6$ receptor resulted in a significant inhibition of high glucose (16.7 mM)-induced insulin secretion, while they showed a slight potentiation (not significant) at a normal glucose level (5.5 mM). The decreased insulin secretion was consistent with those amounts observed in the presence of stimulatory concentrations of glucose (10 or 15 mM) [30, 33]. Recently, we found that both ADP and UDP exhibited inhibitory effects on a sustained elevation and oscillations of $[Ca^{2+}]_i$ induced by high glucose (16.7 mM) in the islet (preliminary data). Taken together with the present results, it is suggested that the transient increases in $[Ca^{2+}]_i$ are associated with the slight stimulation, whereas the inhibitory effect on glucose-induced $[Ca^{2+}]_i$ rises is related to the significant inhibition of insulin release in mouse islets.

Very recently, Parandeh et al. reported that UDPβS, a selective agonist for the $P2Y_6$ receptor, stimulated insulin release in mouse islets in vitro [29]. Its stimulatory effect was observed at concentrations as low as 10 nM and reached the maximum at concentrations ranging from 100 nM to 1 μM that differed depending on the glucose concentration. On the other hand, in the present study we employed a high concentration of UDP, 200 μM, that increased $[Ca^{2+}]_i$ in mouse islets and found that it inhibited insulin secretion. It is clear that the opposite effect of UDP on insulin secretion is related to its concentration used in the two studies. Since we do not know the extracellular concentration of UDP during insulin secretion in mouse islets, it is not possible to evaluate the physiological significance of UDP effects observed at the two different concentrations. The possibility exists that UDP may produce its dual effects on insulin secretion in a concentration-dependent manner, i.e., a stimulatory effect at low concentrations and an inhibitory effect at high concentrations. However, we cannot rule out the possibility that the inhibitory effect of UDP at a high concentration is pharmacological and is mediated via other receptors.

Both $P2Y_1$ and $P2Y_6$ receptors are coupled to the Gq protein [23, 40]. We found that the effect of 2-MeSADP and UDP on $[Ca^{2+}]_i$ was inhibited by inhibitors of PLC and the IP_3 receptor and still persisted in the absence of extracellular Ca^{2+} in the medium. These results suggest that stimulation of the $P2Y_1$ or $P2Y_6$ receptor induces the $[Ca^{2+}]_i$ elevation by mobilizing from intracellular stores through the activation of a PLC/IP_3 pathway in the mouse islets. In a previous report, Zhao et al. showed that Ca^{2+} mobilization induced by ATP differed in duration between rat and mouse islet β-cells and suggested that the observed opposite pattern of insulin release, i.e., stimulation and inhibition between these two species, is due to the difference in the type of a store-operated calcium ion channel being involved [41].

It has been shown that protein kinases such as PKC and PKA or protein phosphatases play a crucial role in the P2Y receptor-mediated regulation of exocytosis in the islet β-cells [1, 10, 33]. Previous studies showed that activation of a cAMP/PKA pathway by the P2Y receptor enhanced insulin release in the rat [7], whereas activation of serine/threonine protein phosphatase calcineurin inhibited exocytosis of insulin-containing granules in the mouse system [33]. These findings suggested that activation of protein kinases and phosphatases can play a key role in controlling exocytosis of insulin-containing granules in the islet β-cells. In the present study, however, we found that the PKC inhibitor staurosporine had no effect on 2-MeSADP- or UDP-induced $[Ca^{2+}]_i$ rises. Thus, PKC did not appear to contribute to the generation of the nucleotide-induced $[Ca^{2+}]_i$ rises in the mouse islets.

The islet contains α-, β-, and δ-cells, and only β-cells produce and secrete insulin [14]. In order to examine whether the effect of 2-MeSADP and UDP on $[Ca^{2+}]_i$ was related to β-cells in the islet, we employed beta-TC6 insulinoma cells as another experimental β-cell model. We found that beta-TC6 cells, like the pancreatic islets, responded to the purinergic nucleotides in terms of $[Ca^{2+}]_i$ and insulin secretion. The response occurred in the presence of 5.5 mM glucose. Our previous study showed that these cells had a lower threshold for glucose so that 5.5 mM glucose elicited the maximal insulin secretion in these insulinoma cells [28]. We also found by RT-PCR analysis that the $P2Y_1$ and $P2Y_6$ receptors also were expressed in beta-TC6 cells. There is no clear evidence that a functional P2 receptor is present in other types of cells such as α-cells in the islet. These results suggested that the effect of nucleotides on $[Ca^{2+}]_i$ and insulin secretion in the mouse islet can be attributable to β-cells.

In summary, we present evidence indicating that the mouse islets and beta-TC6 cells possess a $P2Y_1$ or $P2Y_6$ receptor and respond to the purinergic agonists by increasing $[Ca^{2+}]_i$ via intracellular Ca^{2+} mobilization and modulation of insulin secretion. These purinergic receptors, especially the $P2Y_6$ receptor, may play a role as autocrine regulators of insulin release and may serve as a target for treatment of diabetes mellitus.

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