Cell. Mol. Life Sci. (2010) 67:445–453
DOI 10.1007/s00018-009-0191-3

Cellular and Molecular Life Sciences

RESEARCH ARTICLE

ADP receptor $P2Y_{13}$ induce apoptosis in pancreatic β-cells

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Received: 18 August 2009 / Revised: 22 October 2009 / Accepted: 22 October 2009 / Published online: 14 November 2009

Abstract Pancreatic β-cell loss represents a key factor in the pathogenesis of diabetes. Since the influence of purinergic signaling in β-cell apoptosis has not been much investigated, we examined the role of the ADP receptor $P2Y_{13}$ using the pancreatic insulinoma-cell line MIN6c4 as a model system. Real time-PCR revealed high expression of the ADP receptors $P2Y_1$ and $P2Y_{13}$. Adding the ADP analogue, 2MeSADP, to MIN6c4 cells induced calcium influx/mobilization and inhibition of cAMP production by activation of $P2Y_1$ and $P2Y_{13}$, respectively. 2MeSADP reduced cell proliferation and increased Caspase-3 activity; both these effects could be fully reversed by the $P2Y_{13}$ receptor antagonist MRS2211. We further discovered that blocking the $P2Y_{13}$ receptor results in enhanced ERK1/2, Akt/PKB and CREB phosphorylation mechanisms involved in β-cell survival. These results indicate that $P2Y_{13}$ is a proapoptotic receptor in β-cells as the $P2Y_{13}$ receptor antagonist MRS2211 is able to protect the cells from ADP induced apoptosis.

Keywords ADP · β-cells · Apoptosis · $P2Y_{13}$ · MIN6 cells

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Introduction

Purines and pyrimidines are important signaling molecules that affect a diverse array of cellular processes such as proliferation, differentiation, and cell death by interacting with purinergic (P2Y) receptors [1]. High glucose levels stimulate the release of ATP to the extracellular spatium from several tissues and cell types such as endothelial cells, blood vessels, mesangial cells, macrophages, and β-cells [2–7]. The net effects of extracellular ATP is difficult to predict since ATP by itself can stimulate $P2Y_2$, $P2Y_4$ (in rodents), $P2Y_{11}$, and $P2X_1$-$P2X_7$ receptors present on the cell surface [8]. Furthermore, ATP is rapidly degraded by ectonucleotidases to ADP [9], which can act on $P2Y_1$, $P2Y_{12}$, and PY_{13} receptors. ADP is then further degraded by ecto-5′-nucleotidase to adenosine which in turn can activate four different adenosine receptors [10].

A large number of studies indicate the importance of external ATP and ADP in modulation of insulin secretion [11–15]. The effect of ADP is pluripotent since it has been shown capable both of increasing [11–13] as well as decreasing [14, 15] insulin release in human β-cells. ADP activation of membrane bound P2 receptors stimulates calcium mobilization in beta cells which, in turn, may propagate a spread of the signal via intermittent release of ATP [16]. The reason for these discrepancies is likely due to involvement of several different purinoreceptors [15–18].

Maintenance of β-cell mass is critical for secretion of adequate amounts of insulin. Type 1 diabetes [19] results in excessive loss of β-cells, while progressive β-cell dysfunction and a reduction in beta-cell mass are features of type 2 diabetes [20]. A decreased β-cell mass is observed from the early stages of diabetes and is caused by accelerated apoptosis of the β-cells [21]. Thus, efforts to prevent

β-cell apoptosis during the pathogenesis of diabetes are necessary to ameliorate the course of both type 1 and type 2 diabetes.

The influence of extracellular ADP/ATP on apoptosis has not been much investigated in β-cells. In a recent publication using the rat insulinoma cell line INS-1e, Santini et al. [22] reported that ATP itself has little or no effect on apoptosis, as measured by translocation of phosphatidylserine to the outer layer of the plasma membrane and by DNA content. However, in glia cells, the ADP receptor agonist ADPβS enhanced expression of the early apoptosis marker Caspase-3 that eventually results in DNA fragmentation and cell death [23].

We have recently found that ADP via $P2Y_{13}$ receptors inhibits insulin release both in vitro and in vivo. We therefore wanted to examine if the $P2Y_{13}$ receptor could regulate cell survival for β-cells. To do so, we first quantified the transcriptional mRNA levels of the ADP specific purinergic receptors $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$, and based on these findings, we did pharmacological studies on cAMP, MAPkinase, Caspase-3, and Akt activity and on cell proliferation. We used the mouse insulinoma cell line MIN6c4 as our primary model system. The results indicate an important role for ADP acting on $P2Y_{13}$ receptors as a proapoptotic factor.

Materials and methods

Materials

All chemicals, unless otherwise stated, were obtained from Sigma-Aldrich and were either of analytic or laboratory grade. 2MeSADP and MRS2211 were purchased from Tocris (UK), The cyclic AMP kit was obtained from Cayman Chemical (USA). MAPKs kit, anti-phospho-SAPK/JNK, anti-phospho-p38, anti-phospho-ERK1/2, anti-GAPDH, anti-phospho-Akt, anti-Akt, anti-phospho-CREB, and anti-CREB antibodies were all obtained from Cell Signaling Technology (TX, USA). The EnzChek® Caspase-3 Assay Kit #2 was purchased from Molecular Probes (USA).

Cells and culture conditions

Mouse MIN6 pancreatic β-cell line subclone MIN6c4, a generous gift from Professor Jun-Ichi Miyazaki, Osaka, was grown in Dulbecco's modified Eagle's medium (DMEM) containing with Glutamax-1 and 25 mM glucose (Invitrogen, Paisley, UK) supplemented with 15% heat-inactivated FBS (Invitrogen), 60 μM β-mercaptothanol, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were maintained in a 37°C incubator with 5% CO_2.

Relative mRNA measurement by real time-PCR

Total RNA was extracted from MIN6c4 cells using TRIZOL reagent (Invitrogen, Grand Island, NY, USA). cDNA was synthesized from 3 μg RNA and random hexamer primers using the RT Kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions. Real-time quantitative PCR was performed in a LightCycler (Roche, Basel, Switzerland). A specific set of primers was chosen to obtain a product close to 200 bp. Primers were designed using VectorNTI software (Invitrogen/Informix, UK) with NCBI EntrezGene reference sequences as templates. The forward primers were 5′-GTTCAAGCAG AATGGAGACACG-3′ ($P2Y_1$), 5′-AGTATTCCCGGAGA CACTCATATC-3′ ($P2Y_{12}$), 5′-GCCTTTCAAAATCCTT TCCGA-3′ ($P2Y_{13}$), 5′-GGTCATCCCAGAGCTGAACG-3′ (GAPDH), and the reversed primers were 5′-GGAAAACC CTCACTCAGGTGG-3′ ($P2Y_1$), 5′-GAGAACCTGGGTG ATCTTGTAGTC-3′ ($P2Y_{12}$), 5′-TGTTTTTGCGAAAGC CGTCT-3′ ($P2Y_{13}$), 5′-TTGCTGTTGAAGTCGCAGGA-3′ (GAPDH).All quantitative real-time PCR (Q-RT-PCR) reactions were carried out in glass capillaries in 10-μl reactions containing 1 μl cDNA template, 3 mM $MgCl_2$, 0.5 μM of forward and reverse primers and 1× LightCycler DNA Master SYBR Green I mix (Roche, Basel, Switzerland). The PCR enzyme was activated by initial 600 s incubation at 95°C, followed by 45 PCR cycles (1 s denaturation at 95°C, 6 s annealing at primer specific temperatures, and 23 s elongation at 74°C). All measurements were normalized using gene GAPDH, a well-known housekeeping gene according to the ΔCT method [24].

Measurement of cAMP concentrations

MIN6c4 cells (4×10^5 cells/well) were cultured in a 24-well plate for 3 days. After washing with OptiMEM and addition of 25 μM Rolipram, the cells were incubated for 20 min in OptiMEM medium containing the test compounds. Extracting the monolayer with 200 μl 0.1 M hydrochloric acid terminated the incubations. The cell lysates were centrifuged at 100*g* for 10 min at 4°C. Cyclic AMP levels in cells extract were measured by ELISA using Cyclic AMP EIA kit (Cayman Chemical, USA) according to manufacturer's instructions.

Fluorescent measurements of $[Ca^{2+}]_i$ concentration

MIN6c4 cells were plated in 96-well plates at a density of 4×10^5 cells/well and allowed to grow for 2 days. The medium was removed and 20 μl FLUO-4 loading buffer, containing 4.5 μM FLUO-4 AM and 0.01% Pluronic in HBSS (Hank's BSS), was added per well. After incubating 1 h at 37°C, the loading buffer was removed and the cells

were washed once with 50 µl HBSS. Then, 50 µl HBSS +/−antagonists was added and the cells were incubated for 30 min at RT. Fluorescence (excitation 485 nm, emission 535 nm) was recorded, in a VICTOR microplate reader (PerkinElmer, Finland), after injection of 25 µl 10 µM 2MeSADP/well.

Western blot analysis

MIN6c4 cells, growing in 6-well plates were incubated for 30 min in the presence of different test agents. Cells were lysed in ice-cold SDS buffer containing a cocktail of protease inhibitors. Cellular lysate were prepared by sonication (15 s) and heating for 5 min at 99°C, and were then centrifuged. The protein content was determined by Dc protein assay (BIO-RAD) and lysate samples, representing 20 µg of total protein, were separated on a Precase 12% SDS–polyacrylamide gel (Lonza, USA) and transferred to Immobilon-P membranes (Millipore, Mass, USA). The intensities of bands were quantitated by scanning densitometry (Fluor-S Multi-Imager).

Measurement of Caspase-3 activity

Caspase-3 activity was determined using the EnzChek Caspase-3 Assay Kit #2 (Molecular Probes) according to the manufacturer's instructions. Briefly, after incubation with test substances for 36 h cells were washed with PBS and lysed and Caspase-3 activity in the extracts was measured by fluorometric assay. Fluorescent product of the substrate Z-DEVD-rhodamine 110 generated by Caspase-3 in the cell extract was detected by a microplate fluorometer (VICTOR; PerkinElmer, Finland) with excitation of 496 nm and emission of 520 nm. Background fluorescence was determined by including a specific Caspase-3 inhibitor (Ac-DEVD-CHO) in the reaction mixtures. The results were correlated to the protein concentration.

Cellular viability

The effects of a sustained application of P2-receptor agonist or antagonist on cell viability of MIN6c4 cell line were studied using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) colorimetric assay. The MIN6c4 cells were seeded in 96-well plates at a concentration of 1×10^4 cells per well in a volume of 200 µl of cell culture medium per well. After 24 h, test substances were added and the plates were kept in the CO_2 incubator for 3 days, then 20 µl of the MTT reagent (5 mg/ml in PBS buffer) was added to each well, and the plates were incubated in a CO_2 incubator at 37°C for an additional 4 h. The medium was aspirated and replaced with 200 µl of DMSO (dimethyl sulfoxide solubilization solution) to dissolve the insoluble purple formazan product. The absorbance was quantified by measuring at 490 nm in a microplate reader (VICTOR; PerkinElmer).

Cellular proliferation

MIN6c4 cells were seeded in 24-well plates at a concentration of 1×10^4 cells per well in cell culture medium. The cells were counted every 2 days using a Bürkert chamber.

Statistical analysis

Results were expressed as mean and standard error of the mean (SEM) unless otherwise stated. The level of significance for the difference between sets of data was assessed by Student's unpaired *t* test using Graph-Pad InStat, Version 4.0 (GraghPad Software, SanDiego, CA, USA). Significant differences were considered at $p < 0.05$, $p < 0.01$, or $p < 0.001$ (two-tailed test).

Results

Expression of P2Y ADP receptors in MIN6c4 cells

The first thing we did was to determine the set-up and relative expression levels of P2Y ADP receptors in MIN6c4 cells. This was done by quantitative real-time PCR and normalization of the results against GAPDH. Three ADP receptors $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ are expressed on MIN6c4 cells. The rank order for the expression levels of P2Y ADP mRNA was $P2Y_{13} > P2Y_1 > P2Y_{12}$ (Fig. 1). Levels of

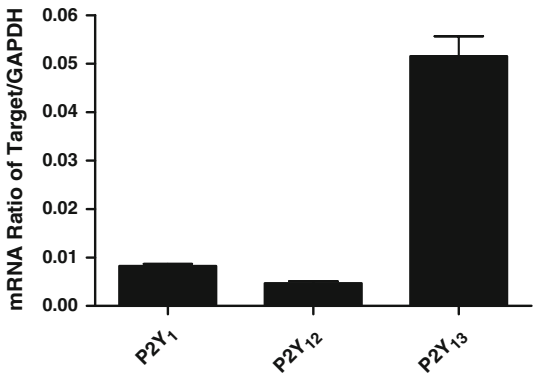

Fig. 1 mRNA quantification of P2Y ADP receptors in MIN6c4 cells. Real time PCR analysis of ADP receptor was performed using P2Y subtype-specific primer pairs in conjunction with SYBR Green dye I as described under "Material and methods". mRNA abundance was normalized to GAPDH and expressed as mean ± SE for three experiments as shown

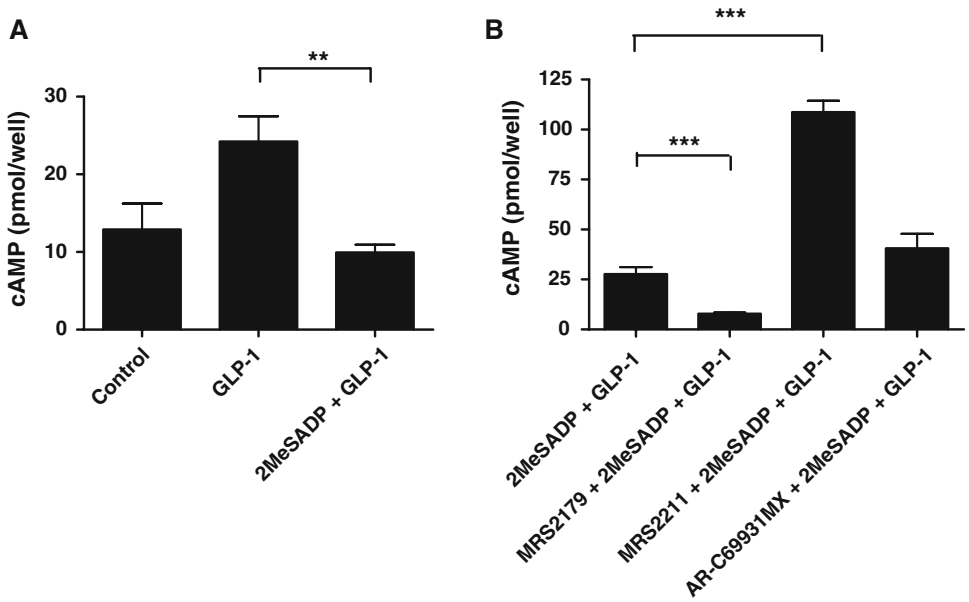

Fig. 2 Effects of ADP receptor ligands on GLP-1 stimulated cAMP production in MIN6c4 cells. MIN6c4 cells were treated with: **a** GLP-1 (0.1 μM) with or without 2MeSADP (10 μM), **b** GLP-1 (0.1 μM) and 2MeSADP (10 μM) in the presence or absence of MRS2179 (10 μM), MRS2211 (10 μM) or AR-C69931MX (10 μM). Cells growing in 6-well dishes were treated with the compounds for 30 min, in the presence of 25 μM Rolipram, and were then extracted with 200 μl of 0.1 M hydrochloric acid and analyzed for cAMP. Data are given as the mean ± SE ($n = 5$). Asterisks (*) denote probability level of random difference, by student's t test: $^{**}p < 0.01$ compared with GLP-1 treatment; $^{***}p < 0.001$ compared with GLP-1 + 2MeSADP treatment

$P2Y_{13}$ receptor mRNA was five times higher than $P2Y_1$ and ten times higher than $P2Y_{12}$ receptor in the MIN6c4 cell (Fig. 1). $P2Y_2$ and $P2Y_4$ mRNA levels were too low to be detected with real-time PCR.

Effect of $P2Y_{12}$ and $P2Y_{13}$ on MIN6c4 cell cyclic AMP level

We next investigated the functionality of the Gαi linked receptors $P2Y_{12}$ and $P2Y_{13}$. First, we examined the effect of the stable agonist, 2-methylthioadenosine diphosphate (2MeSADP), on GLP-1 stimulated cAMP production. We found that 10 μM 2MeSADP reduced cAMP accumulation by 59% ($p < 0.01$; Fig. 2a). Addition of the $P2Y_1$ antagonist, MRS2179, reduced cAMP levels by another 71% ($p < 0.001$; Fig. 2b) while the $P2Y_{13}$ antagonist, MRS2211, increased the levels by four times ($p < 0.001$). However, the $P2Y_{12}$ receptor antagonist, AR-C69931MX, had no significant effect on cAMP level.

Effect of $P2Y_1$ on MIN6c4 cell calcium level

Activation of $P2Y_1$ is linked to PLC activation and calcium mobilization. To prove that there are functional $P2Y_1$ receptors we loaded MIN6c4 cells with Fluo-4 and stimulated them with 2MeSADP, in the presence and in the absence of the $P2Y_1$ specific antagonist MRS2179.

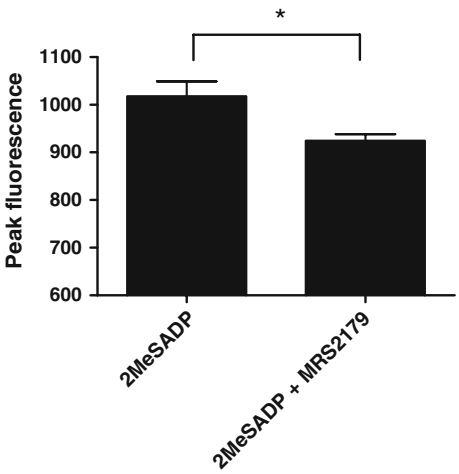

Fig. 3 Effects of ADP receptor ligands on the intracellular calcium concentration of MIN6c4 cells. Cells growing in white 96-well dishes were loaded with 5 μM FLUO-4 loading buffer for 1 h at 37°C. After washing once with HBSS, cells were treated with: 2MeSADP (10 μM) with or without $P2Y_1$ receptor antagonist MRS2179 (10 μM) in HBSS for 30 min. Emission was recorded, at 535 nm for 30 s, in a Victor 3 multilabel counter. Results are expressed as peak fluorescence. $^{*}p < 0.05$ ($n = 6$) compared with 2MeSADP treatment

We found that blocking the $P2Y_1$ receptor resulted in a decrease of intracellular Ca^{2+} level in MIN6c4 cells (Fig. 3).

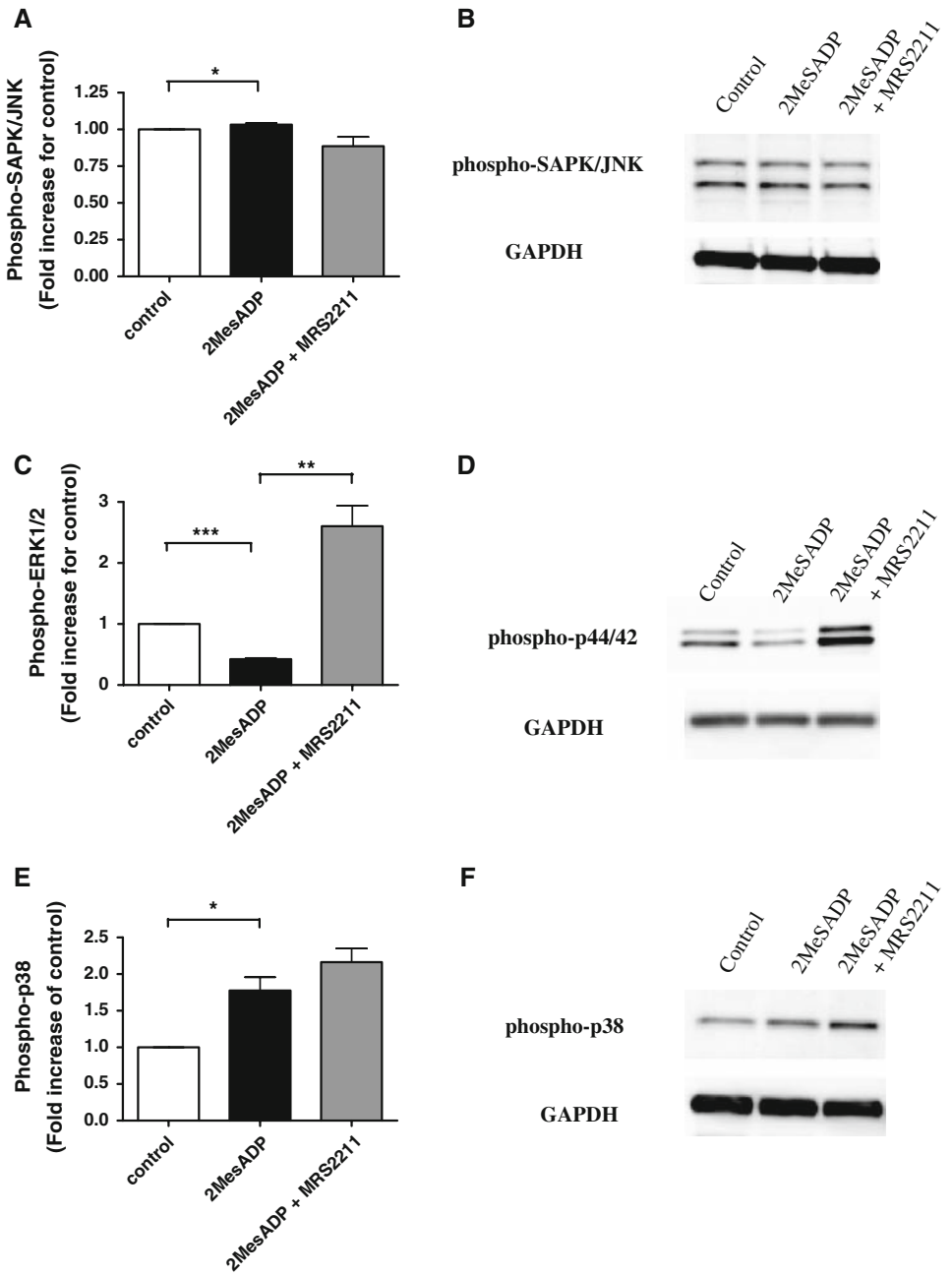

Fig. 4 Effects of ADP receptor ligands on the MAP kinases phosphorylation status of MIN6c4 cells. MIN6c4 cells were incubated for 30 min in medium containing PBS control, 2MeSADP (10 μM) with or without MRS2211 (10 μM). Cell lysates were prepared and then subjected to western blot analysis (20 μg protein/lane), using antibodies against phosphorylated MAPkinases. **a**,**b** SAPK/JNK. **c**,**d** p44/42. **e**,**f** p38. Membranes were reprobed with the anti-GAPDH antibody. Each *bar* represents the fold increase of phospho-SAPK/JNK, phospho-ERK1/2, or phospho-p38 relative to control after normalizing against total total GAPDH. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ ($n = 3$). Data for western blots (**b**,**d**,**f**) are representative for three independent experiments in each group

Effect of $P2Y_{13}$ on MIN6c4 MAPK activities

MAPkinases are important regulators of proliferation and survival, which is why our next step was to determine if $P2Y_{13}$ activation/inhibition results in phosphorylation of these pathways. MIN6c4 cell were stimulated with 2MeSADP in the absence or presence of MRS2211 for 30 min and then analyzed for changes in phosphorylation of SAPK/JNK, p38, and p42/44 kinase (referred to as ERK1/2), using western blots. Addition of 10 μM 2MeSADP to the incubation media resulted in a very minor increase in activation of SAPK/JNK (Fig. 4a) and p38 (Fig. 4e) in the MIN6c4 cells. When cells were examined after 30 min of 2MeSADP treatment, very little ERK1/2 phosphorylation was observed. However, MIN6c4 cell exposed to 2MeSADP, in the presence of MRS2211, showed a sustained and significant ERK1/2 phosphorylation (Fig. 4c, d), while 10 μM MRS2179 did not have any effect on p38, and caused a minor increase in ERK1/2 in the presence of 2MeSADP (data not shown).

Effects of 2MeSADP with or without MRS2211 on Caspase-3 activity in MIN6c4 cells

We next investigated whether 2MeSADP could influence Caspase-3 level in MIN6c4 insulinoma cells cultured for 36 h and to what extent this effect could be modulated by

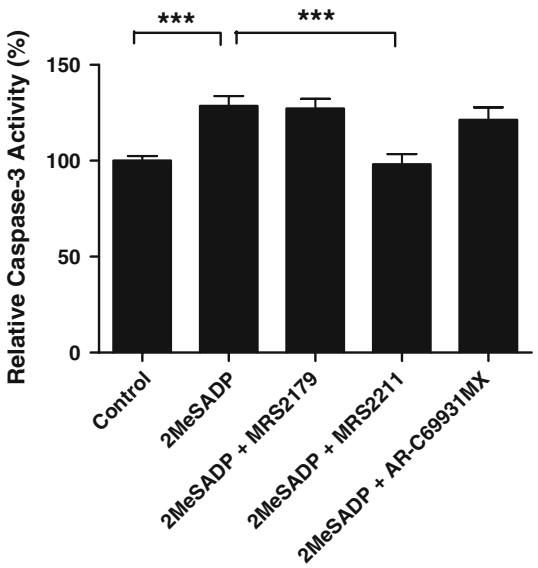

Fig. 5 Effects of ADP receptor ligands on Caspase-3 activity in MIN6c4 cells. MIN6c4 cells growing in 6-well dishes were treated for 36 h with: 2MeSADP (10 μM) in the absence or presence of MRS2179 (10 μM), MRS2211 (10 μM), or AR-C69931MX (10 μM). After completing the incubation, the cells were washed and lysed on ice and then centrifuged at 500*g* for 3 min. Supernatants were transferred to 96-well plates and analyzed for Caspase-3 activity using a commercial kit. Result of Caspase-3 was normalized to protein concentration and expressed as the percentual change in specific activity. $***p < 0.001$ ($n = 3$)

MRS2211. As shown in Fig. 5, 10 μM of 2MeSADP induced a significant increase in Caspase-3 activity in MIN6c4 cells. The effect of 2MeSADP was markedly suppressed by 10 μM of MRS2211. Moreover, the $P2Y_1$ antagonist MRS2179 and $P2Y_{12}$ antagonist AR-C69931MX did not have any significant effect on Caspase-3 level induced by 2MeSADP, thus indicating that the observed increase in Caspase-3 activity is solely mediated by $P2Y_{13}$.

Activation of Akt and CREB by blocking the $P2Y_{13}$ receptor

To determine the role of $P2Y_{13}$ receptor signal transduction on Akt and CREB activation we carried out western blot experiments using antibodies against Thr-308 phospho-Akt and Ser-133 phospho-CREB. Addition of 10 μM 2MeSADP to the incubation medium resulted in a suppression of Akt activation (Fig. 6a, b). This effect was completely blocked by the $P2Y_{13}$ receptor antagonist, MRS2211, resulting in a markedly enhanced Akt (Fig. 6a, b). These results were paralleled by the results of the CREB activation experiment, since $P2Y_{13}$ inhibition also produced a strong phosphorylation of CREB (Fig. 6c, d), while 10 μM of MRS2179 did not have any effect on Akt activation (data not shown).

Effect of activating or blocking $P2Y_{13}$ receptor on viability and proliferation of MIN6c4 cell

To investigate the functional consequences of $P2Y_{13}$ activation in pancreatic beta cell, the changes in the cell viability and proliferation were determined by means of MTT assay and by cell growth. MIN6c4 cells were seeded at a low cell density and cultured in cell culture medium, in the presence or absence of different stimulants. As measured by the MTT assay 3 days after treatment, the growth of MIN6c4 (Fig. 7a) cells was inhibited by treatment with 10 μM 2MeSADP. However, when cells were incubated with MRS2211, at a concentration of 10 μM, the effect was inverted and cells proliferated more efficiently (Fig. 7a). These results were confirmed by the cell growth also showing an increased proliferation in the presence of MRS2211 (Fig. 7b), while 10 μM of MRS2179 did not have any effect on the MIN6c4 cell MTT assay activation (data not shown). The results of the MIN6c4 cell MTT assay and the cell proliferation study show that blocking the $P2Y_{13}$ receptor promotes both the viability and proliferation.

Discussion

The purine nucleotides ATP and ADP are pluripotent mediators involved in a multitude of different processes. Purinergic nucleotides are released from several tissues and act extracellularly via specific P2 receptors as autocrine and paracrine regulators [25]. In the endocrine pancreas, the insulin-producing pancreatic β-cells are crucial for regulation of glucose homeostasis, and its impairment leads to diabetes mellitus, the most common metabolic disorder in man. Briefly, glucose is taken up by β-cells, via glucose transporters, where it is metabolized in glycolysis and Krebs cycle, resulting in an increase in the cytoplasmic ratio of ATP to ADP. This ratio determines the plasma membrane potential, which in turn regulates the release of insulin. Although being instrumental in insulin release, several reports have shown that ATP is also released from pancreatic islets and thus may be involved in autoregulative mechanisms. Purinergic receptors have been described in β-cells from humans as well as rodents, and purines have been reported to modulate insulin release in rat pancreas, islets, and in insulin-secreting cell lines [7, 11–15]. Although a role of adenine nucleotides in insulin secretion is slowly emerging, little is known about other potential functions. The present study aims at addressing the potential influence of purinergic receptors on β-cell mass and survival.

We started by first mapping the P2Y receptor expression profile in MIN6c4 cells, our chosen model system. P2Y

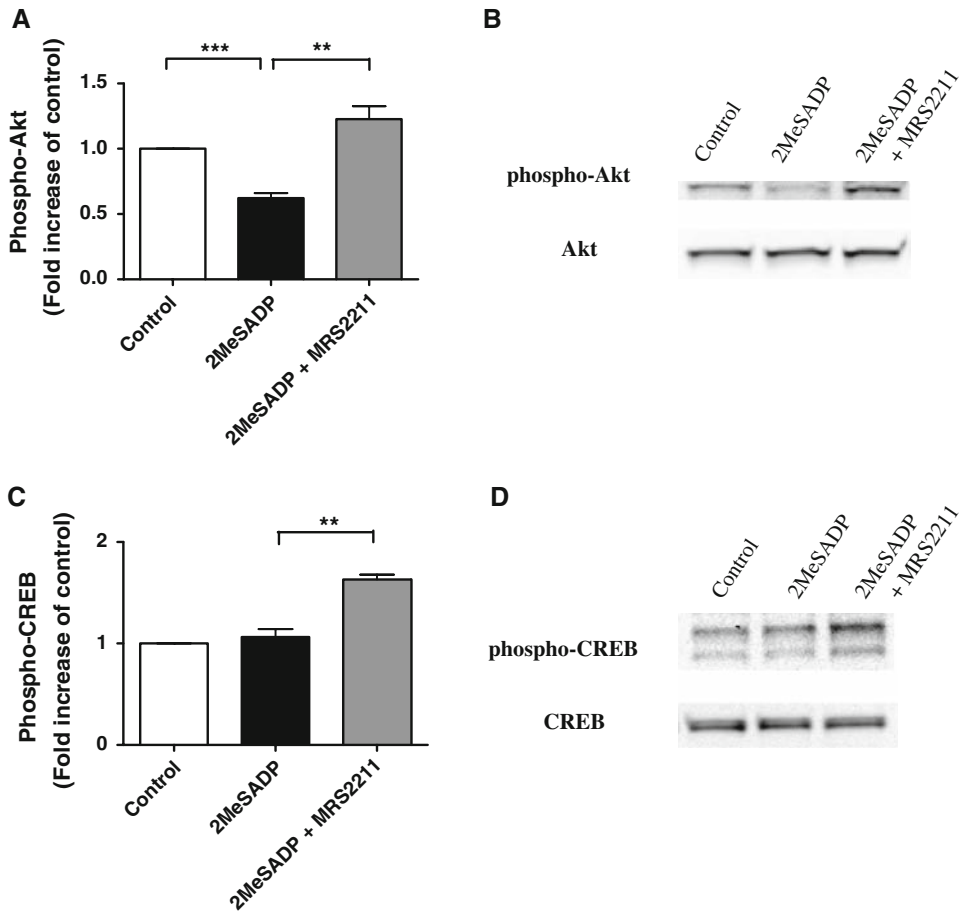

Fig. 6 Effects of ADP receptor ligands on the phosphorylation status of Akt/PKB and CREB in MIN6c4 cells. MIN6c4 cells were incubated for 30 min in medium containing: PBS control, 2MeSADP (10 μM) with or without MRS2211 (10 μM). Cell lysates were prepared and then subjected to western blot analysis (20 μg protein/lane), using antibodies against phosphorylated Akt/PKB and ser-133-CREB, respectively. **a**,**b** Akt/PKB and **c**,**d** ser-133-CREB. Membranes were reprobed with the anti-Akt and CREB antibody. Each *bar* represents the fold increase of phospho-Akt or phospho-CREB relative to control after normalizing against total non-phosphorylated Akt or CREB. $^{**}p < 0.01$, $^{***}p < 0.001$ ($n = 3$). Data for western blots (**b** and **d**) are representative for three independent experiments in each group

receptors have previously been reported in rat INS-1 [26] and in MIN6 cells [27]. However, the mapping of the MIN6 cell line was not complete as several P2Y receptors were yet to be discovered and the identities of some receptors were not conclusive. Our results showed high mRNA levels of the ADP receptor $P2Y_{13}$, low to moderate levels of $P2Y_1$ and $P2Y_{12}$, while the ATP receptors $P2Y_2$ and $P2Y_4$ were undetectable. Functional analysis revealed that both the $P2Y_{13}$ and the $P2Y_1$ are active in this cell type as 2MeSADP simultaneously inhibited GLP-1 stimulated cAMP production through $P2Y_{13}$, and triggered calcium mobilization through $P2Y_1$.

Activation of mitogen-activated protein kinases (MAPK) are well known to determine the fate of β-cells, it has been shown that, while ERKs are important for cell proliferation and survival, SAPK/JNKs and p38-MAPKs are stress responsive and thus involved in apoptosis [28]. We found that the stable ADP analog, 2MeSADP, simultaneously inhibited ERK1/2 while stimulating p38, thus giving rise to a combined pro-apoptotic signal. Pharmacological intervention with MRS2211 indicated $P2Y_{13}$ being responsible for the ERK1/2 inhibition.

In Fig. 4d, MRS2211 increases p44/42 more than control, despite the inhibitory effects of 2MeSADP. This could indicate an autocrine effect of ADP mediated via $P2Y_{13}$ receptors. Such endogenous presence of ADP/ATP have been reported previously and could be important for β-cell survival. We are currently investigating these possible autocrine effects.

The pro-apoptotic effect was confirmed as 2MeSADP activated Caspase-3 in a clearly $P2Y_{13}$-specific way. It is likely that a major trigger of these effects is lowering of the cAMP concentration by activation of Gαi. These results are well in line with the report by Costes et al. [29] and Jhala et al. [30] showing that glucose stimulated cAMP production results in increased β-cell survival through ERK1/2-mediated phosphorylation of CREB. As these studies, and several other similar reports, point to CREB as a major survival factor in β-cells. We next investigated the effect of 2MeSADP on Ser-133-phosphorylation of CREB. We found that blocking $P2Y_{13}$ with MRS2211 resulted in a significant increase in ser-133-CREB phosphorylation. This increase was, in turn, paralleled by a simultaneous increase in phosphorylation of Akt/PKB [30]. Akt/PKB is major factor of β-cell survival, and it has previously been reported that activation of the cAMP/PKA/CREB pathway results in induction of IRS2 and thus amplification of insulin stimulated PI3kinase phosphorylation of Akt/PKB.

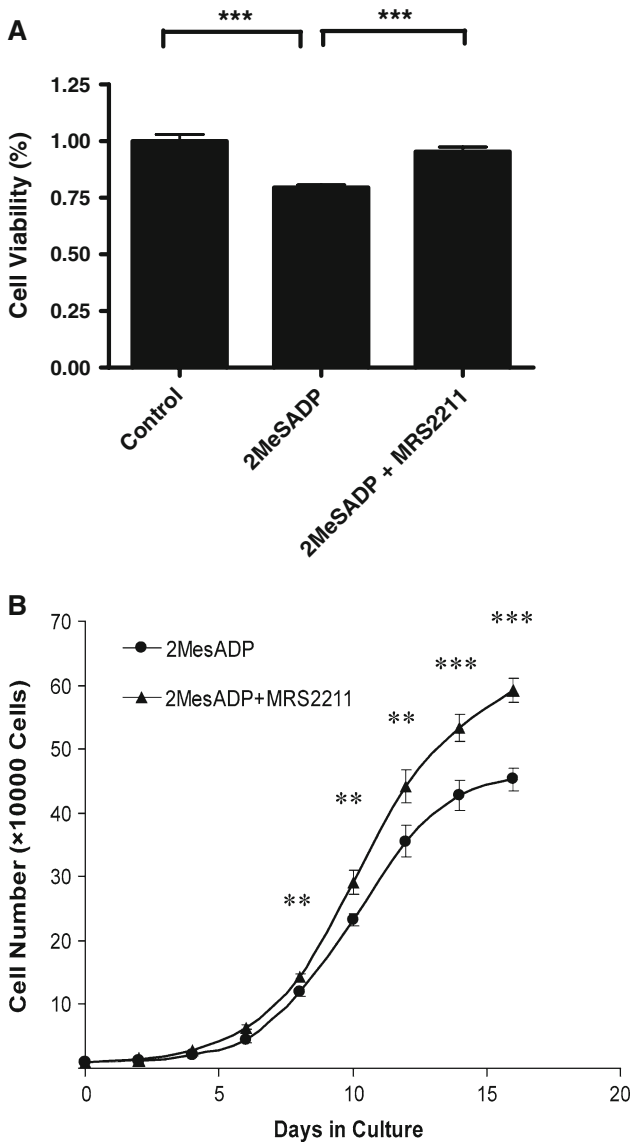

Fig. 7 Effects of ADP receptor ligands on the viability and proliferation of MIN6c4 cells. Cell viability was determined by MTT assay and cell proliferation was estimated by cell growth assay. **a** MIN6c4 cells (1×10^4 cells/well) growing in 96-well plate were incubated with 2MeSADP (10 μM) with or without MRS2211 (10 μM). After 3 days in culture, the cellular viability was determined using a colorimetric MTT assay. **b** MIN6c4 cells (1×10^4 cells/well) were seeded in 24-well plates were allowed to grow in the presence of 2MeSADP (10 μM) with or without MRS2211 (10 μM). The cell proliferation curves were obtained by counting the cells every 2 days. $^{**}p < 0.01$, $^{***}p < 0.001$ ($n = 4$)

Akt is a primary regulator of β-cell mass and overexpression leads to islet hyperplasia and resistance to apoptosis [31]. We believe the hyperplastic response that is received upon activation of Akt is reflected in the increased viability and proliferation rate we obtained for MIN6c4 cells grown in the presence 2MeSADP and the $P2Y_{13}$ antagonist MRS2211.

Finally, we examined the effect of $P2Y_{13}$ activation on cell viability and proliferation. The stable ADP analogue 2MeSADP clearly reduced cell viability in an MTT assay, an effect that was completely reversed by the $P2Y_{13}$ antagonist MRS2211. Furthermore, addition of MRS2211 to cells growing in the presence of 2MeSADP increased the number of MIN6 cells indicating a growth inhibitory effect mediated by ADP via activation of $P2Y_{13}$ receptors.

To conclude, we have shown that activation of the $P2Y_{13}$ receptor of mouse MIN6c4 cells exercises a pronounced proapoptotic effect and that this effect is mediated by inhibiting the cAMP/ERK/CREB/Akt pathway. Many factors have been reported to regulate β-cell mass by effecting survival and proliferation. We propose that autocrine/paracrine ADP signalling is one of the components of complex mechanism acting in synergy with factors like food intake and insulin release to regulate β-cell mass.

Acknowledgments This study was supported by the Swedish Heart and Lung Foundation, Swedish Scientific Research Council, The Vascular Wall Program, and Lund University. David Erlinge is a holder of The Lars Werkö distinguished research fellowship from the Swedish Heart and Lung Foundation.

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