cAMP increases the sensitivity of exocytosis to Ca\textsuperscript{2+} primarily through protein kinase A in mouse pancreatic beta cells

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A B S T R A C T

Cyclic AMP regulates the late step of Ca\textsuperscript{2+}-dependent exocytosis in many secretory cells through two major mechanisms: a protein kinase A-dependent and a cAMP-GEF/Epac-dependent pathway. We designed a protocol to characterize the role of these two cAMP-dependent pathways on the Ca\textsuperscript{2+} sensitivity and kinetics of regulated exocytosis in mouse pancreatic beta cells, using a whole-cell patch-clamp based capacitance measurements. A train of depolarizing pulses or slow photo-release of caged Ca\textsuperscript{2+} were stimuli for the exocytotic activity. In controls, due to exocytosis after slow photo-release, the Ca\textsubscript{m} change had typically two phases. We observed that the Ca\textsuperscript{2+}-dependency of the rate of the first Ca\textsubscript{m} change followed saturation kinetics with high cooperativity and half-maximal rate at 2.9 ± 0.2 μM. The intracellular depletion of cAMP did not change amp1, while rate1 and amp2 were strongly reduced. This manipulation pushed the Ca\textsuperscript{2+}-dependency of the exocytotic burst to significantly lower [Ca\textsubscript{2+}]i. To address the question of which of the cAMP-dependent mechanisms regulates the observed shifts in Ca\textsuperscript{2+} dependency we included regulators of PKA and Epac2 activity in the pipette solution. PKA activation with 100 μM 6-Phe-cAMP or inhibition with 500 μM Rp-cAMPS in beta cells significantly shifted the EC\textsubscript{50} in the opposite directions. Specific activation of Epac2 did not change Ca\textsuperscript{2+} sensitivity. Our findings suggest that cAMP modulates Ca\textsuperscript{2+}-dependent exocytosis in mouse beta cells mainly through a PKA-dependent mechanism by sensitizing the insulin releasing machinery to [Ca\textsuperscript{2+}]; Epac2 may contribute to enhance the rates of secretory vesicle fusion.

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1. Introduction

Glucose is the main physiological stimulus for pancreatic beta cells to release insulin. It is currently believed that an increase in extracellular glucose concentration and increased uptake of glucose through GLUT2 increases the metabolism of glucose to form ATP. ATP closes ATP-dependent potassium channels (K\textsubscript{ATP} channels) and membranes of the beta cell syncytium depolarize, which increases the opening of voltage-activated Ca\textsuperscript{2+} channels [1]. Glucose increases cytosolic Ca\textsuperscript{2+} activity, which is a major trigger to promote the release of secretory granules. As in the synapse, exocytosis is managed by SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) fusion machinery [2,3]. The insulin release process was found to be modulated by second messengers like cAMP or phospholipase C (PLC) depending on which signalling pathway has been activated by physiological ligands [4,5].

Cyclic AMP is one of the central signalling molecules that has been identified to regulate release of various neurotransmitters and hormones, including insulin from pancreatic beta cells [4,6,7]. Two cAMP-dependent pathways have so far been reported to directly control Ca\textsuperscript{2+}-dependent exocytosis, either through activation of PKA or a cAMP–guanidine nucleotide exchange factor 2 (GEFII)/Epac2-dependent pathway [6,8,9]. The action of cAMP through PKA is believed to be a dominant pathway acting in synapses [10], chromaffin cells [11,12], pituitary cells [13,14] and was also suggested to be a major pathway in pancreatic beta cells [4,15,16]. However, some other studies, including our own, provide evidence that CAMP is also acting through an Epac-dependent pathway [17–21].

Hormones like incretins, including glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP), are known to potentiate glucose-induced insulin secretion by generating cAMP in pancreatic beta cells [8]. The significantly increased insulin

Abbreviations: 6-Phe-cAMP, N\textsuperscript{6}-phenyladenosine-3′,5′-cyclic monophosphate; 8-CPT-2′-O-Me-cAMP, 8-(4-chlorophenylthio)-2′-O-methyl-adenosine 3′,5′-cyclic monophosphate; Ca\textsubscript{m}, membrane capacitance; CSP, cysteine string protein; GEFII, guanidine nucleotide exchange factor 2; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide-1; LV, large vesicles; NSF, N-ethylmaleimide-sensitive factor; PKA, protein kinase A; PLC, phospholipase C.

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release due to incretins has a major contribution in the feedback mechanism by which insulin keeps plasma glucose levels within physiological limits. GLP-1 and GIP were described to exert cAMP actions through activation of PKA in pancreatic beta cells [22]. Furthermore, actions of GLP-1 mediated by PKA and Epac2 were described to include the recruitment and priming of vesicles, thereby increasing the number of vesicles available for release. Besides the direct effect on secretory vesicles, GLP-1 also promotes Ca²⁺ influx and mobilizes an intracellular source of Ca²⁺ [6,23]. Moreover, it has been reported that cAMP enhances translocation of granules and increases the size of the ready releasable pool and the rate of replenishment [24].

The precise mechanism of PKA action in beta cells is not fully understood. A plethora of proteins involved in the secretory machinery have been found to be phosphorylated by PKA in vitro and in vivo in different cell types. PKA phosphorylation has been described for several proteins including cysteine string protein (CSP), snapin, Rim1, SNAP-25, syntaphilin and synapsin [9]. The phosphorylation of CSPs by PKA has been reported to modify exocytotic kinetics [25]; phosphorylation of snapin by PKA caused a larger initial exocytic burst in chromaffin cells [26]. In addition to a larger number of available secretory vesicles and increase in the rate of their release, PKA was found to promote insulin secretion by increasing the number of vesicles that are highly sensitive to Ca²⁺ and thereby sensitizing the secretory machinery to Ca²⁺ [16]. More classic effects of PKA activation include larger L-type voltage-activated Ca²⁺ currents that upon activation result in higher cytosolic Ca²⁺ and therefore potentiate exocytosis from pancreatic beta cells [4,9] as well from pituitary cells [17]. As well as the effect on Ca²⁺ channels, PKA phosphorylation has been shown to partially increase membrane permeability to Na⁺ [9].

cAMP also potentiates Ca²⁺-dependent exocytosis by a PKA-independent mechanism. This mechanism involving the cAMP-binding protein Epac2 has been shown to increase the number of ready releasable vesicles [6,20]. Another hypothesis was recently presented where activation of Rap1 by cAMP through Epac2 mediates insulin secretion by increasing the size of the non-docked granule pool and by facilitating the recruitment of the granules to the plasma membrane [27]. Some actions of Epac2 are not mediated by Rap1 because it was reported that Epac2 interacts directly with insulin granule-associated proteins, Rim2 and Piccolo [28,29]. Epac2 was also identified as a molecule interacting with the sulfonyleurea receptor SUR1 [21] and thereby directly promoting insulin secretion. An Epac2-dependent pathway has also been described to be involved in increase of membrane permeability to Na⁺ [9].

In the present paper we assessed the role of cAMP during Ca²⁺-dependent exocytosis using electrophysiological approach. The addition of cAMP into cytosol of beta cells triggered exocytosis of the secretory vesicles at lowerintracellular Ca²⁺ concentration ([Ca²⁺]i) and the half-maximal rate of the release was achieved at lower [Ca²⁺]i. This suggests that cAMP sensitizes the secretory machinery to Ca²⁺. A direct activation of PKA, but not Epac2, also significantly shifted Ca²⁺ sensitivity. The present work suggests that the mechanism of action underlying such a change in the sensitiity to Ca²⁺ should be destabilization of the fusion pore.

2. Methods

2.1. Isolation of primary beta cells

Adult male NMRI mice were killed by cervical dislocation. Liberase (0.3 mg ml⁻¹; Roche, USA) dissolved in Hank’s buffered salt solution (Invitrogen, USA) was injected into the pancreas through the bile duct. The pancreas was removed and digested for 10–15 min at 37 °C. After digestion, 10 ml of ice cold Hanks’ balanced salt solution supplemented with 10 mM HEPES, 50 IU ml⁻¹ penicillin G and 0.05 mg ml⁻¹ streptomycin was added to the suspension to stop the reaction. Islets were collected by centrifugation, trypsinized into single cells and cultured on coverslips in RPMI 1640 medium (Invitrogen, USA) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 100 IU ml⁻¹ penicillin G and 0.1 mg ml⁻¹ streptomycin in a humidified atmosphere with 5% CO₂ at 37 °C. Cells were cultured 24–48 h before the electrophysiological experiments.

2.2. Preparation of mouse pancreatic tissue slices

Mouse pancreatic tissue slices were prepared as previously described [30]. Low-gelling agarose (Seaplaque GTG agarose, Lonza, USA; 0.475 g in 25 ml extracellular solution) was melted and kept on 37 °C. Adult male NMRI mice were killed by cervical dislocation. After opening the abdominal cavity, agarose was injected into the pancreas through bile duct. After injection, the pancreas was cooled with an ice-cold extracellular solution. The injected and hardened pancreas was then extracted and placed in ice-cold extracellular solution. Fat and connective tissue was removed from pancreas. Small pieces (3 mm × 3 mm) were cut from the pancreas and embedded into the agarose. Agarose-embedded pancreatic tissue slices were then immersed into the sample plate of the vibrotome (VT 1000 S, Leica, Nussloch, Germany). The pancreatic tissue was cut at 0.05 mm/sec at 70 Hz into 140 μm-thick slices. During slicing the tissue was kept in an ice-cold extracellular solution bubbled continuously with carbogen (95% O₂, 5% CO₂). After slicing the tissue slices were kept in carbogen-bubbled extracellular solution and used for electrophysiological experiments within the next 8 h.

2.3. Electrophysiology

The coverslip with cells or an agarose-embedded slice was transferred from the incubator to the perfusion chamber and fixed with a U-shaped platinum frame. When using dispersed cells 1.5 ml of extracellular solution consisting of 150 mM NaCl, 10 mM HEPES, 3 mM glucose, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, pH 7.2 and osmolarity 300 ± 10 mOsm was added to the chamber. For pancreatic slices, extracellular solution containing 125 mM NaCl, 2.5 mM KCl, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 2 mM sodium pyruvate, 0.25 mM ascorbic acid, 3 mM myo-inositol, 6 mM lactic acid, 1 mM MgCl₂, 2 mM CaCl₂ and 3 mM glucose, adjusted to pH 7.3 by gassing with carbogen for at least 30 min before the experiment, was continuously supplied to the perfusion chamber. When using depolarization protocol for triggering Cₘ change, 5 mM CaCl₂ instead of 2 mM CaCl₂ was included in the extracellular solution. The pipette solution used for Ca²⁺–induced capacitance and current measurements was composed of 5 mM NP-EGTA (Invitrogen, USA), 4 mM CaCl₂, 0.1 mM Fura 6F (Invitrogen, USA) together with 125 mM CsCl, 40 mM HEPES, 2 mM MgCl₂, 20 mM TEA–Cl, 2 mM Na₂ATP at pH 7.2 and osmolality 300 ± 10 mOsm. The current measurements in isolated beta cells turned out to be a useful tool to differentiate among the different Cₘ phases, particularly helpful to exclude the possibility of the contamination of the Cₘ changes by the endocytotic processes. The latter were almost completely absent in the beta cells still inside the whole pancreas slice. However, the current characteristics remained the same as in beta cell in dispersed cell cultures. The competitive antagonist of cAMP binding to PKA, Rp-adenosine 3′,5′-cyclic-monophosphorothioate triethylammonium salt (Rp-cAMPS; Biolog Life Sci., Germany) and PKA agonist, 6-Phe-cAMP (Biolog Life Sci., Germany), were included in the pipette solution at concentrations of 0.5 mM and 0.1 mM, respectively. A selective cAMP-GEF/Epac agonist, 8-(4-chlorophenylthio)-2-
O-methyl-adenosine 3',5'-cyclic monophosphate (8-CPT-2'-O-Me-cAMP; Biolog Life Sci., Germany), was added to the pipette solution to a final concentration of 0.1 mM. All chemicals were purchased from Sigma-Aldrich (Germany) unless otherwise indicated.

Cells were visualized with an upright Nikon Eclipse E600 FN microscope (Nikon, Tokyo, Japan) and a mounted CCD camera (Cohu, San Diego, CA, USA). Patch pipettes were pulled from borosilicate glass capillaries (GC150F-15, Harvard Apparatus, USA) using a horizontal pipette puller (P-97, Sutter Instruments, USA). The pipette resistance was 2–3 MΩ in Cs-based solution. Fast pipette capacitance (Cfast) was compensated before and slow membrane capacitance (Cslow) as well as series conductance (Gs) were compensated after whole-cell breakthrough. Only experiments with Gs > 50 nS were processed. Recordings were performed in the standard whole-cell mode via a patch-clamp lock-in amplifier (SWAM llc, Celica, Slovenia), low-pass filtered, transferred to a PC via an A/D converter (12 bit, Nidaq PCI-6035E, National Instruments) and recorded on the hard disk using WinWCP V3.9.6 software (John Dampster, University of Strathclyde, UK). The same software was used to apply the voltage protocol for identifying beta cells by their Na+ current inactivation pattern. A continuous sine voltage (1600 Hz, 11 mV RMS amplitude) was applied to measure Cm, a parameter that is proportional to membrane surface area [31]. Resting membrane potential in voltage-clamp mode was −80 mV.

In beta cells within the intact pancreatic tissue slices all currents were analyzed and presented after leak subtraction. Secretory activity was triggered by trains of depolarization. To determine the evoked ΔCm, Cm was first averaged over the 30 ms preceding the depolarization to obtain a baseline value that was subtracted from the value estimated after the depolarization averaged over a 40-ms pulse. The first 30 ms after the depolarization were excluded from the Cm measurement to avoid contamination by nonsecretory capacitive transients related to gating charge movement [32]. Only experiments not displaying crosstalk between Cm and membrane conductance monitored in parallel were used for analysis. To calculate Ca2+ charge entry, the first 2 ms of the inward current integral were omitted to reduce contamination with Na+ current.

All electrophysiological experiments were performed at room temperature or at 32 °C when using isolated cells or tissue slices, respectively. Control experiments (no cAMP in the patch pipette) were performed on adult male beta cells. Signal processing and curve fitting was done using Matview (Wise Technologies, Ljubljana, Slovenia) and Matlab (Mathworks, USA). Estimations of the number of released vesicles are based on a value of 3.6 fF per vesicle for adult beta cells [33,34].

2.4. Ca2+-measurements

Fura 6F (0.1 mM in pipette solution; Molecular Probes, USA) was used to measure the intracellular Ca2+ concentration ([Ca2+]i) simultaneously with the patch-clamp recordings. Fura 6F was excited at 380 nm with a monochromator (Polychrome IV; TILL Photonics, Germany). A long-pass dichroic mirror reflected the monochromatic light nm to the perfusion chamber and transmitted the emitted fluorescence above 400, which was further filtered through a 420-nm barrier filter. The fluorescence intensity was measured with a photodiode (TILL Photonics, Germany). [Ca2+]i was calculated as described previously [35].

2.5. Statistical analysis

Statistical analysis was performed using Sigmaplot 11.0 (SPSS, USA). Data and the results are reported as means ± S.E.M. for the indicated number of experiments. The unpaired Student’s t-test
was used to evaluate the statistical significance, unless otherwise indicated. For multiple comparisons, one-way ANOVA and Holm–Sidak post hoc test were used. A p value <0.05 was considered statistically significant.

3. Results

3.1. Trains of depolarization triggered Ca\(^{2+}\)-dependent exocytosis at lower Ca\(^{2+}\) current in cAMP treated cells compared to the control beta cells

In beta cells within the pancreatic tissue slices we used a voltage pulse protocol with 50 40-ms long depolarization pulses (from −80 mV to +10 mV, Fig. 1A, upper panel) to mimic the glucose induced-spiking activity and thereby elicit inward Ca\(^{2+}\) current (Fig. 1A, lower panel, black line). Increase in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) triggered Ca\(^{2+}\)-dependent exocytosis which we detected as a change in C\(_{m}\) (Fig. 1A, middle panel, black circles). As previously described [30] at least one third of the beta cells did not show voltage dependent inward current during the voltage protocol. Next we included 200 μM cAMP in the pipette solution. Faster exocytotic responses were observed in cAMP treated cells (Fig. 1A, middle panel, gray circles) compared to the controls (Fig. 1A, middle panel, black circles) while there were no differences in the amplitude of the inward current (Fig. 1A, lower panel, black and gray lines for control and cells with elevated cAMP concentration, respectively). Next we plotted change in C\(_{m}\) as a function of cumulative charge (ΣQ) triggered with depolarization pulses [36]. In cells treated with 200 μM cAMP (Fig. 1B, gray circles) C\(_{m}\) was triggered at lower Ca\(^{2+}\) charge entry compared to the controls (Fig. 1B, black circles).

3.2. Kinetic phases of exocytosis at increased [Ca\(^{2+}\)]\(_{i}\) in pancreatic beta cells

Since at least one third of beta cells did not react to the depolarization protocol and because of the high variability in Ca\(^{2+}\) current density during voltage protocol we decided to elevate [Ca\(^{2+}\)]\(_{i}\) uniformly through the cell using slow photo-release of caged Ca\(^{2+}\). Under control conditions, slow photo-release of Ca\(^{2+}\) caged to NP-EGTA buffer released Ca\(^{2+}\) ions in the [Ca\(^{2+}\)]\(_{i}\) range of several μM (Fig. 2A) with a rate of change comparable with global [Ca\(^{2+}\)]\(_{i}\) changes during the train of depolarizing pulses [30]. Increased [Ca\(^{2+}\)]\(_{i}\) stimulated exocytosis change (Fig. 2B). We perceived exocytosis as a change in C\(_{m}\) using high-resolution whole-cell patch-clamp recordings. In this representative experiment, [Ca\(^{2+}\)]\(_{i}\) reached a threshold level at Ca\(_{tr}\), followed by a biphasic membrane capacitance with maximal amplitudes amp1 (311 ± 37 fF, n = 28) and amp2 (1589 ± 225 fF, n = 26) for the first and the second phase, respectively. The first phase reached its maximal amplitude within the first second after the start of C\(_{m}\) change. The second phase saturated within the next 5 s after the onset of C\(_{m}\) change (Fig. 2B). The time derivatives for the C\(_{m}\) traces showed two separate kinetic exocytotic components, fast and slow phase, with the maximal rates of C\(_{m}\) change within each phase (rate1 and rate2; Fig. 2C). The maximal rate of the first phase was compa-
Fig. 3. Types of $C_m$ change observed during slow photo-release of caged Ca$^{2+}$. (A) Increased [Ca$^{2+}$]$_i$ triggered $C_m$ change in a Ca$^{2+}$-dependent manner. If [Ca$^{2+}$]$_i$ did not reach the threshold level, no $C_m$ was observed (lower trace). When [Ca$^{2+}$]$_i$ reached the $C_{at}$ level but did not exceed $C_{a2}$, only the first phase of $C_m$ was observed. On the other hand, [Ca$^{2+}$]$_i$ higher than $C_{a2}$ triggered the first and the second phase of $C_m$ change with amp2 proportional to the [Ca$^{2+}$]$_i$ change (upper two traces). (B) In addition to Ca$^{2+}$-dependent exocytosis, Ca$^{2+}$-dependent endocytosis was observed with amp2 proportional to the [Ca$^{2+}$]$_i$ change.

Fig. 4. Increased [Ca$^{2+}$]$_i$ triggered a biphasic increase in $C_m$ and biphasic current change. (A) Increase in [Ca$^{2+}$]$_i$, after slow photo-release. $C_{a0}$ and $C_{a2}$ indicate the [Ca$^{2+}$]$_i$ where the first and the second phase of current were triggered, respectively. (B) A biphasic increase in $C_m$ with the first and the second phase if we extend the $C_m$ trace to the baseline to mark the onset of the second phase (dark gray, dashed line). (C) A biphasic current change triggered after increase in [Ca$^{2+}$]$_i$. In the control, the first phase of the current change was triggered at [Ca$^{2+}$]$_i$, which is 2 pmol lower than [Ca$^{2+}$]$_i$ at which the first phase of $C_m$ was triggered; the second phase of current change was triggered simultaneously with the second phase of $C_m$ change.
the second exocytotic phase, which is otherwise mostly hidden in the initial exocytotic burst. To mark the onset of the second phase of \( C_m \) change we extrapolated the \( C_m \) trace to the base line (Fig. 4B, dark gray dash line).

### 3.3. The effect of cAMP on Ca\(^{2+}\)-dependent exocytosis

To assess how cAMP affects the Ca\(^{2+}\)-dependent exocytosis in pancreatic \( \beta \)-cells we first included 200 \( \mu \)M cAMP in the pipette solution to provide saturating conditions [17]. The maximal capacitance rate in cells dialysed with cAMP did not differ from the control (Fig. 5B, left panel); the half-maximal rate was achieved at 1.6 \( \pm \) 0.2 \( \mu \)M of free Ca\(^{2+}\) (\( n = 23, p < 0.001 \), Fig. 5B, right panel) which is significantly lower compared with the control. The Ca\(^{2+}\)-concentration triggering Ca\(^{2+}\)-dependent exocytosis (\( C_{\text{tr}} \)) was significantly lower (1.5 \( \pm \) 0.2 \( \mu \)M, \( n = 28, p < 0.001 \), Fig. 5C), and amp1 was indistinguishable from the control (Fig. 6A). We did not observe any significant differences in amp2 (Fig. 6B) or rate2 (Fig. 6D).

Next we performed the wash out of ATP, which should result in reduced cAMP production. Slow photo-release of caged Ca\(^{2+}\) shows the same pattern including two separate exocytotic components. This manipulation did not differ in amp1 (Fig. 5A) or in rate1 (Fig. 5B, left panel) compared with the control. The [Ca\(^{2+}\)], required for triggering \( C_m \) change (\( C_{\text{tr}} \)) was also comparable with the control (Fig. 5C). Cells dialysed with 0 mM ATP exhibit a shift in Ca\(^{2+}\)-dependency towards higher Ca\(^{2+}\) concentrations (3.2 \( \pm \) 0.2 \( \mu \)M, \( n = 10, p < 0.001 \); Fig. 5B, right panel) compared with the cells with increased cAMP concentration, which could suggest an important role of cAMP in sensitizing the secretory machinery to Ca\(^{2+}\).

Although ATP depletion did not attenuate the amplitude of the first phase of \( C_m \) change, we expected the second phase to be depleted as previously reported [37]. We observed a strongly reduced amp2 (664 \( \pm \) 156 \( \mu \)F, \( n = 14, p < 0.05 \); Fig. 6B) and rate2 (98 \( \pm \) 25 \( \mu \)F/s, \( n = 12, p < 0.05 \); Fig. 6D) in contrast to control cells and cells treated with cAMP; the \( C_{\text{tr}} \) at which the second phase of \( C_m \) was triggered was comparable with the control (Fig. 6C).

### 3.4. PKA-dependent and PKA-independent pathway of insulin release

To assess whether cAMP acts through a PKA- or EPAC2-sensitive mechanism we first included 100 \( \mu \)M N\(^{6}\)-phenyladenosine-3',5'-cyclic monophosphate (6-Phe-cAMP) in the pipette solution. 6-Phe-cAMP is a potent site-selective activator of PKA showing a strong preference for site A of both isozymes (type I and type II) and at the same time does not activate Epac2 [38]. Glucose-stimulated insulin secretion in pancreatic beta cells has been reported to be both PKA- and Epac2-dependent [18]. The cumulative \( C_m \) change under this condition did not significantly differ from the control. The amp1 (Table 1) and amp2 (Table 1) were comparable with the control as well as rate1 (Fig. 7D). On the other hand, activation of PKA by 6-Phe-cAMP triggered an additional high Ca\(^{2+}\)-sensitive phase (Fig. 7A, gray line) at [Ca\(^{2+}\)], comparable with those in cells treated with cAMP (Fig. 7C). The half-maximal rate of \( C_m \) change was also achieved at significantly lower EC\(_{50}\) (Fig. 7B, gray solid line).
line) compared with the control conditions, suggesting that cAMP acts through a PKA-dependent mechanism.

We next decided to remove the PKA activity by adding 0.5 mM Rp-cAMPS, a competitive antagonist of cAMP binding to PKA, to the pipette solution. The C<sub>m</sub> change was triggered at C<sub>tr</sub> (Fig. 7C) which is indistinguishable from the control. No changes were observed in amp1 (Table 1), rate1 (Fig. 7D), amp2 (Table 1) and rate2 (Fig. 7F). Despite unchanged amplitudes of C<sub>m</sub> change, we observed that Rp-cAMPS pushed the EC<sub>50</sub> towards a higher [Ca<sup>2+</sup>]<sub>i</sub> (p < 0.001; Fig. 7B, gray dash line) compared with the cells treated with cAMP and 6-Phe-cAMP, which indicates that activation of PKA is important to determine the Ca<sup>2+</sup> sensitivity of the fusion machinery.

### Table 1

<table>
<thead>
<tr>
<th>Condition</th>
<th>Amp1 (fF)</th>
<th>Rate1 (fF/s)</th>
<th>EC50 (μM)</th>
<th>Amp2 (fF)</th>
<th>Rate2 (fF/s)</th>
<th>C&lt;sub&gt;A&lt;/sub&gt; (μM)</th>
<th>C&lt;sub&gt;A2&lt;/sub&gt; (μM)</th>
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<tbody>
<tr>
<td>Control</td>
<td>311 ± 37</td>
<td>329 ± 42</td>
<td>2.9 ± 0.2</td>
<td>1588 ± 225</td>
<td>209 ± 30</td>
<td>2.6 ± 0.1</td>
<td>4.2 ± 0.2</td>
</tr>
<tr>
<td>cAMP</td>
<td>263 ± 44</td>
<td>302 ± 48</td>
<td>1.6 ± 0.2</td>
<td>1332 ± 184</td>
<td>225 ± 35</td>
<td>1.5 ± 0.2</td>
<td>4.1 ± 0.3</td>
</tr>
<tr>
<td>Without ATP</td>
<td>221 ± 44</td>
<td>333 ± 62</td>
<td>3.2 ± 0.2</td>
<td>671 ± 155</td>
<td>98 ± 25</td>
<td>2.8 ± 0.2</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>6-Phe-cAMP</td>
<td>320 ± 41</td>
<td>402 ± 105</td>
<td>1.8 ± 0.4</td>
<td>1464 ± 158</td>
<td>334 ± 69</td>
<td>1.7 ± 0.4</td>
<td>4.5 ± 0.5</td>
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<tr>
<td>Rp-cAMPS</td>
<td>316 ± 30</td>
<td>452 ± 60</td>
<td>3.3 ± 0.2</td>
<td>1445 ± 156</td>
<td>265 ± 48</td>
<td>2.8 ± 0.2</td>
<td>3.9 ± 0.3</td>
</tr>
<tr>
<td>8-pCPT-O-2′-Me-cAMP</td>
<td>286 ± 45</td>
<td>610 ± 153</td>
<td>2.7 ± 0.2</td>
<td>1536 ± 197</td>
<td>404 ± 43</td>
<td>2.2 ± 0.2</td>
<td>3.6 ± 0.1</td>
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Fig. 7. cAMP increases the sensitivity to Ca^{2+} of exocytosis primarily through PKA. (A) Time derivatives of C_mates of the first phase in control cells and 6-Phe-cAMP-treated cells show an additional high Ca^{2+}-sensitive phase in PKA-activated cells. (B) Normalized C_m rate versus [Ca^{2+}] in control cells (black, dotted line), cells with increased cAMP concentration (black, solid line), cells treated with PKA activator (light gray, solid line) and inhibitor (light gray, dashed line), and cells treated with specific Epac activator (dark gray, solid line). The half-effective Ca^{2+} concentration was achieved at significantly lower [Ca^{2+}] in cells with increased cAMP concentration and cells dialysed with PKA activator 6-Phe-cAMP compared with the control. (C) Bar chart represents the [Ca^{2+}] at which the first phase of C_m change was triggered (Catr). Catr was significantly lower in cells treated with cAMP and selective PKA activator 6-Phe-cAMP compared with the control and PKA inhibitor Rp-cAMPS. (D) Bar chart represents the rate of the first phase of C_m change (rate1). Rate1 was significantly increased in cells treated with Epac activator 8-pCPT-2′O-Me-cAMP compared with the controls and cells with increased cAMP concentration. Data was analyzed using one-way ANOVA and the results are presented as means ± S.E.M., *p < 0.05, **p < 0.01, ***p < 0.001. The number of cells tested is given on bars.

It has recently been shown [18,39] that PKA-dependent and PKA-independent pathways regulate exocytosis in endocrine cells. The presence of an alternative cAMP-dependent pathway through Epac2 in secretion was tested with 8-pCPT-2′-O-Me-cAMP, a potent, specific and membrane-permeant activator of the Epac cAMP receptor. When 8-pCPT-2′-O-Me-cAMP was used, no significant changes were observed in amp1 (Table 1) or amp2 (Table 1). On the other hand, rate1 (p < 0.001; Fig. 7D) and rate2 (p < 0.05; Fig. 7F) were significantly higher compared with the controls and cells treated with 200 μM cAMP. The Catr (Fig. 7C) and EC_{50}
gested that the first phase of 
lem of loss of Ca2+ and Na+ channels during cell isolation procedure. we preferred to use slow photo-release of caged Ca2+ to trigger at least one third of beta cells did not respond to this protocol [43]. We believe that Ca2+ microdomains are not necessary to explain fast release in beta cells [42]. All subsequent studies on the rate of secretory granule fusion was around 400 fF/s. This is indeed sig- nificantly slower compared to the original estimates on isolated beta cells [36,43]. Longer depolarization pulses produced significantly slower Cm changes in the range of 50 fF/s [43]. We believe that Ca2+ microdomains are not necessary to explain fast release in beta cells and that uniform increase of Ca2+ universally in the cell and thereby avoids the problem of loss of Ca2+ and Na+ channels during cell isolation procedure. This approach bypasses any modulatory effects that act on electrical excitability and Ca2+ influx [41] and enables the detailed study of Ca2+ sensitivity.

Our aim was to assess the effect of cAMP on the Ca2+-dependent exocytosis triggered by slow photo-release in mouse pancreatic beta cells. The secretory rates observed in isolated pancreatic beta cells were comparable with those observed by trains of depolarizations in pancreatic tissue slices where the maximal rate of secretory granule fusion was around 400 fF/s. This is indeed significantly slower compared to the original estimates on isolated beta cells [42]. All subsequent studies on the rate of Cm changes triggered by short (40–50 ms) depolarization pulses yielded comparable rates (see fig. 3B in [36,43]). Longer depolarization pulses produced significantly slower Cm changes in the range of 50 fF/s [43]. We believe that Ca2+ microdomains are not necessary to explain fast release in beta cells and that uniform increase of Ca2+ is a good measure of a Ca2+-dependent change during the physiological stimula- tion. In slow photo-release when the Hill function was fitted through Ca2+-dependent data, the half-maximal rate of the Cm change was achieved at EC50 which is comparable with the data obtained from pancreatic slices for beta cells (Fig. 2D, inset) and α-cells (data not shown) but not adrenal chromaffin cells or pituitary cells (data not shown). Furthermore, if we estimate the number of vesicles released during the first phase of Cm change, we can see that approximately 86 large vesicles (LV) with average diameter of 3.6 fF [34] were fused, although the first phase of Cm change may also partially represent a group of synaptic vesicles [18]. This is the reason why we chose to express the exocytotic process as dCm/dt instead of a per vesicle rate. In control cells the first phase amplitude was comparable with previous reports where a first phase ampli- tude was comparable with previous reports where a first phase ampli- tude was comparable with previous reports where a first phase ampli- tude was comparable with previous reports where a first phase ampli- tude was comparable with previous reports where a first phase ampli- tude was comparable with previous reports where a first phase ampli- tude was comparable with previous reports where a first phase ampli- 5. PKA and [Ca2+]i dependent Cm changes in beta cells

As mentioned earlier slow photo-release of caged Ca2+ produced changes in Cm and I
. Detailed analysis did not reveal any signif- icant differences in the [Ca2+]i (Ca0, data not shown) or [Ca2+]i (Ca2+; Fig. 7E) at which the first and the second phase of inward current was triggered, respectively. In control cells, the first phase of current change was not associated with a change in Cm (Fig. 8). However, in cells treated with cAMP, the first phase of Cm change started at the same time as the first phase of Cm change. Similarly, with direct activation of PKA when 6-Phe-cAMP was included in the pipette solution, the first phase of current change was again triggered simultaneously with the Cm change.

4. Discussion

In beta cells within the intact pancreatic tissue slice, change in Cm was triggered using voltage depolarization protocol. Since at least one third of beta cells did not respond to this protocol we preferred to use slow photo-release of caged Ca2+ to trigger Ca2+-dependent exocytosis. Slow photo-release of caged Ca2+ is an effective method for studying regulated exocytosis because the ramp-like increase in [Ca2+]i leads to robust secretion in pancreatic beta cells [40]. Furthermore, slow photo-release of caged Ca2+ increases [Ca2+]i uniformly in the cell and thereby avoids the problem of loss of Ca2+ and Na+ channels during cell isolation procedure. This approach bypasses any modulatory effects that act on electrical excitability and Ca2+ influx [41] and enables the detailed study of Ca2+ sensitivity.

Fig. 8. Increased [Ca2+]i evoked biphasic Cm change as well as biphasic current change. Increased [Ca2+]i (upper panel) evoked biphasic Cm change (middle panel) as well as biphasic current change (lower panel). The first phase of current change was triggered at the same [Ca2+]i in control cells, with increased cAMP concentration and in cells treated with PKA activator 6-Phe-cAMP. In cAMP- and 6-Phe-cAMP-treated cells the changes in Cm were also related to change in the first phase of current change: in controls, the first phase of current change was not associated with Cm change. In controls, the first phase of Cm change was triggered at almost 2 μM higher [Ca2+]i, compared with [Ca2+]i, at which the first phase of current was triggered.

If cAMP stimulates insulin granule mobilization, an increase in the size of the pool available for immediate release should be observed. According to our results, when the intracellular cAMP concentration was increased to 200 μM, the amplitude as well as the rate of the first phase of Cm change did not differ from the con-
trol and the cumulative \( C_m \) change was also indistinguishable from control cells. On the other hand, the \( C_m \) change was triggered at significantly lower \([\text{Ca}^{2+}]_i\) (Fig. SC). Furthermore, the \( \text{Ca}^{2+} \) dependency of the secretory rate obtained in \( C_m \) recordings from cells treated with cAMP was pushed to significantly lower EC\(_{50}\) (Fig. SB, right panel). It is not clear whether this first phase of \( C_m \) change represents the same pool of secretory granules as elicited in controls or the cAMP triggers the fusion of a high \( \text{Ca}^{2+} \)-sensitive pool of vesicles as suggested recently [16]. The low affinity release-ready vesicles fuse later. Our findings indicate another effect of cAMP on regulated exocytosis in pancreatic beta cells where cAMP does not influence the number of vesicles available for release but rather it sensitizes these granules to \( \text{Ca}^{2+} \) and thus triggers \( C_m \) increase at lower \([\text{Ca}^{2+}]_i\).

Our study also provides evidence that cAMP, through activation of PKA, regulates \( \text{Ca}^{2+} \)-dependent exocytosis in pancreatic beta cells [4,9,47]. PKA activation by 6-Phe-cAMP triggered a triphasic \( C_m \) change at \([\text{Ca}^{2+}]_i\), comparable with those in cells treated with cAMP with an additional high \( \text{Ca}^{2+} \)-sensitive phase similar to data obtained from cells with increased cAMP concentration (Fig. 8, middle panel). The half-maximal rate of \( C_m \) change was also achieved at significantly lower \([\text{Ca}^{2+}]_i\) concentrations in cells treated with 6-Phe-cAMP (Fig. 7B), which indicates that activation of PKA is essential for altering the \( \text{Ca}^{2+} \)-dependency of the rate of the exocytotic burst. That action of cAMP mediated mainly through PKA was confirmed with the addition of Rp-cAMPS, a competitive antagonist of cAMP binding to PKA, where the change in sensitivity was not observed because the results did not differ from the controls (Fig. 7B). Moreover, in cells treated with Rp-cAMPS, vesicles started to fuse at \( C_{tr} \), which was also comparable with the control.

Besides the PKA-dependent pathway, a PKA-independent pathway has also been described to be involved in cAMP-mediated insulin secretion [9,20,27]. Epac2 is known to be present in pancreatic beta cells [22]; therefore, we used 8-pCPT-2′-O-Me-cAMP, a potent, specific and membrane-permeant activator of the Epac receptor, and we did not observe any significant difference in amp1 and amp2 or in \( \text{Ca}^{2+} \) sensitivity compared with the control. On the other hand, the rate of fusion is increased when Epac2 is activated (Fig. 7DF). The underlying molecular mechanism is not yet known but we can speculate from our data that specific Epac activation increases the number of ready releasable vesicles as previously suggested [6,20] or by increasing the size of the non-docked granule pool and by facilitating the recruitment of the granules to the plasma membrane [27]. If this is the case, then the question arises why increased secretory rates are not observed in cells with increased cAMP concentration? The possible explanation is that PKA activation by cAMP is the dominant pathway through which cAMP is affecting insulin exocytosis and thereby Epac activation seems to vanish. Because, in the presence of cAMP, the insulin secretion is triggered at significantly lower \([\text{Ca}^{2+}]_i\) by activation of PKA, it can be assumed that at this \([\text{Ca}^{2+}]_i\) the effect of the Epac2-dependent pathway is not yet fully operational. From our data it is clear that the Epac-selective analogue, 8-pCPT-2′-Me-cAMP, does not mimic the effect of cAMP by changing the sensitivity of the secretory machinery to \([\text{Ca}^{2+}]_i\).

At present the nature of the observed \( I_m \) change is not clear (Figs. 4C and 8). It may be completely independent of vesicle fusion as it seems from the control conditions as published previously [44]. However it is well coordinated with the \( C_m \) change in cAMP-stimulated conditions. One possibility is that the current represents a current through the fusion pore that is still too tight to allow the measurement of the vesicle capacitance. As seems from the Fig. 4, the second phase vesicles fuse simultaneously as the \( I_m \) change, likely through the fusion pore expansion which is sufficient to allow also the measurement of the vesicle capacitance. One of the reasons why such isolated \( I_m \) change has not been noticed before is that flash photolysis experiments raised \([\text{Ca}^{2+}]_i\), to several tens of \( \mu \)M and at this concentration the second phase overtake the fastest component in slow photo-release. Why does cAMP coordinate the earliest \( I_m \) phase by the fastest \( C_m \) phase? A possible explanation for this phenomenon is the ability of cAMP to destabilize the tight form of fusion pore and thereby enabling secretory vesicles to fuse with plasma membrane at lower \([\text{Ca}^{2+}]_i\). In control conditions, a stable fusion pore complex could be formed but the physiological stimulus would not be sufficient to trigger the full fusion. As has been reported before, the \( C_m \) measurements are sometimes less sensitive as amperometry or current measurements [48]. In cells treated with 6-Phe-cAMP, the high \( \text{Ca}^{2+} \)-sensitive phase of \( C_m \) change was again well coordinated with the change in the first phase of \( I_m \), which could suggest that cAMP acts through a PKA-dependent mechanism.

When the second phase of \( C_m \) change was analyzed in cells treated with cAMP, no significant differences compared with the control were observed. The amplitude and the rate of the second phase were indistinguishable from the control and the second phase of \( C_m \) change was triggered at the same \([\text{Ca}^{2+}]_i\) (Fig. 6A–D). These findings suggest that cAMP does not change the sensitivity or the number of secretory granules available for release during the second phase. On the other hand, ATP depletion caused a significant reduction in the amplitude and the rate of the second phase of \( C_m \) change (Fig. 6A–D). This can be a consequence of reduced cAMP production or the effect of ATP depletion because it seems that ATP is required for a vesicle-recruitment step prior to docking as well as for ATP-dependent priming reactions that may occur after vesicle docking [49]. Although amp2 was reduced compared with the control, there were no differences in amp1, which represents the pool of granules that are capable of undergoing exocytosis in an ATP-independent manner [37].

We can conclude that cAMP stimulated exocytosis of insulin granules in a PKA-dependent and PKA-independent manner. Our data suggest that cAMP does not seem to increase the number of secretory granules available for release but rather it sensitizes the exocytotic machinery to \( \text{Ca}^{2+} \) and thereby promotes insulin exocytosis at lower \( \text{Ca}^{2+} \) concentration. The increased \( \text{Ca}^{2+} \) sensitivity by cAMP is consistent with previously published data from experiments using depolarization-evoked insulin exocytosis where cAMP enhanced the size of a pool of vesicles available for immediate release and in addition accelerated refilling of a releasable pool of vesicles [50]. Furthermore, cAMP acts mainly through activation of PKA and protein kinase-dependent phosphorylation is a major method of regulation of sensitivity to glucose in pancreatic beta cells. In contrast, Epac2 may contribute to enhance the rates of secretory vesicle fusion. As GLP-1 and GIP were described to exert cAMP actions through activation of PKA in pancreatic beta cells we suggest that they modify the \( \text{Ca}^{2+} \) sensitivity and enhance the beta cell reaction to weak stimulus like glucose.

**Conflict of interest**

No conflict of interest exists.

**Author contributions**

Both authors designed the study and wrote the paper. M.S. performed and analyzed the depolarization experiments and slow photo-release experiments at the Faculty of Medicine, University of Maribor, Slovenia.
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