SUR1 Regulates PKA-independent cAMP-induced Granule Priming in Mouse Pancreatic B-cells

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ABSTRACT Measurements of membrane capacitance were applied to dissect the cellular mechanisms underlying PKA-dependent and -independent stimulation of insulin secretion by cyclic AMP. Whereas the PKA-independent (Rp-cAMPS–insensitive) component correlated with a rapid increase in membrane capacitance of ~80 fF that plateaued within ~290 ms, the PKA-dependent component became prominent during depolarizations >450 ms. The PKA-dependent and -independent components of cAMP-stimulated exocytosis differed with regard to cAMP concentration dependence; the $K_v$ values were 6 and 29 μM for the PKA-dependent and -independent mechanisms, respectively. The ability of cAMP to elicit exocytosis independently of PKA activation was mimicked by the selective cAMP-GEFII agonist 8CPT-2Me-cAMP. Moreover, treatment of B-cells with antisense oligodeoxynucleotides against cAMP-GEFII resulted in partial (50%) suppression of PKA-independent exocytosis. Surprisingly, B-cells in islets isolated from SUR1-deficient mice (SUR1−/− mice) lacked the PKA-independent component of exocytosis. Measurements of insulin release in response to GLP-1 stimulation in isolated islets from SUR1−/− mice confirmed the complete loss of the PKA-independent component. This was not attributable to a reduced capacity of GLP-1 to elevate intracellular cAMP but instead associated with the inability of cAMP to stimulate influx of Cl− into the granules, a step important for granule priming. We conclude that the role of SUR1 in the B cell extends beyond being a subunit of the plasma membrane $K_{ATP}$ channel and that it also plays an unexpected but important role in the cAMP-dependent regulation of Ca2+-induced exocytosis.

KEY WORDS: insulin • Ca2+ • cAMP • cAMP-GEFII • SUR1

INTRODUCTION Insulin secretion from the pancreatic B-cells is controlled by metabolic fuels, neurotransmitters released from intraslit nerve endings, paracrine mechanisms, and circulating hormones (Ashcroft and Rorsman, 1989; Satin and Kinard, 1998). Several modulators of insulin secretion act by activation of protein kinases and phosphatases (Nesher et al., 2002). For example, the capacity of glucagon, GLP-1, and GIP to enhance insulin secretion involves stimulation of adenylyl cyclase with resultant increase in the intracellular cAMP concentration (Gromada et al., 1998). Cyclic AMP initiates several processes that culminate in enhancement of insulin secretion. These include stimulation of electrical activity by a reduction of $K_{ATP}$ channel activity (Holz et al., 1993), increase of the L-type Ca2+-current (Ämmälä et al., 1993; Kanno et al., 1998), activation of depolarizing cation-permeable ion channels (Leech and Habener, 1997), mobilization of Ca2+ from intracellular stores (Gromada et al., 1995; Kang et al., 2001), and an effect on the release process itself (Jones et al., 1988). Many of these effects are likely to be mediated by activation of PKA. However, there is increasing evidence that PKA-independent mechanisms are also significant. For example, the ability of cAMP to mobilize Ca2+ from intracellular stores is mediated by the cAMP receptor protein cAMP-GEFII (Kang et al., 2001) and we and others have demonstrated previously that the effects of cAMP on secretion involves both PKA-dependent and -independent mechanisms (Renström et al., 1997; Ozaki et al., 2000). The latter pathway appears to be mediated by cAMP-GEFII and this protein has been suggested to play a prominent role in incretin-induced insulin secretion (Nakazaki et al., 2002). Exactly how cAMP, via binding to cAMP-GEFII, promotes exocytosis remains unestablished but the effect may involve Rim2 (Ozaki et al., 2000). RIM proteins have recently been shown to promote priming of granules for release in neurons (Wang et al., 1997; Schoch et al., 2002) and it is tempting to speculate that they fulfill a similar function in the B cell.

Here we have studied the PKA-dependent and -independent effects of cAMP on insulin secretion in B-cells.
taken from wild-type and SUR1 knockout mice. We demonstrate that the PKA-independent pathway of cAMP accounts for a rapid component of release that appears particularly significant for incretin-stimulated insulin secretion. We finally propose a working model for the regulation of exocytosis in the B cell that incorporates Rim2, cAMP-GEFII, and SUR1 in granule priming and release.

MATERIALS AND METHODS

Animals, Isolation of Islets, and Preparation and Culture of B-cells

Most experiments were performed on B-cells isolated from NMRI-mice (Bomholtgaard). The mice lacking the SUR1 receptor (SUR1+/− mice; 6–12 mo of age) used in this study were obtained from Dr. M. Magnuson (Shiota et al., 2002) and bred at the Karolinska Institute in Stockholm. The mice were killed by cervical dislocation, the pancreas was quickly removed, and islets were isolated by collagenase digestion. The procedures for sacrificing the animals were approved by the ethical committee at Lund University and by local authorities in Hamburg.

For electrophysiology, the islets were dissociated into single cells using a Ca2+-free solution. The resulting cell suspension was plated on Corning petri dishes or glass coverslips (for confocal microscopy) and maintained in tissue culture for 6–30 h in RPMI 1640 medium containing 10% (vol/vol) fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 i.u./ml penicillin.

The efficiency of the cAMP-binding protein cAMP-GEFII, which mediates cAMP-dependent but PKA-independent exocytosis (Ozaki et al., 2000), was tested by culturing intact islets from NMRI-mice in the presence of 4 μM of antisense phosphorothioate–substituted oligodeoxynucleotides (ODNs)* against mouse cAMP-GEFII (5′-CAACGGCCTTTATCC-3′) or control ODNs (5′-ACCTAGGTGACTACGT-3′) (provided by S. Seino, Chiba University, Japan) for 96 h. The control ODNs were made such that chemical properties were similar to that of antisense ODN (Ozaki et al., 2000). These islets were then dissociated into single cells and cultured as described above. Most cells prepared from islets cultured for 4 d had small exocytotic responses. The analysis of the effects of treatment with antisense cAMP-GEFII or the control ODN was therefore confined to the five cells in each group displaying the largest capacitance increases (the total number of cells analyzed was 114). Because of the difficulty maintaining secretion in primary B-cells, some experiments (Fig. 4, G–E) were instead performed using mouse insulinoma MIN6 cells (Miyazaki et al., 1990). These were either treated with the control and antisense ODNs specified above for 96 h or were subjected to transient transfection using mutant cAMP-GEFII expression vector pRSRO-cAMP-GEFII (G114E/G422D; provided by S. Seino) reconstituted in effector transfection reagent (QIAGEN). The cells were cotransfected with eGFP (CLONTECH Laboratories, Inc.) To facilitate the identification of the transfected cells. Electrophysiological experiments were performed ≥36 h after transfection.

Solutions

The extracellular solution consisted of (in mM) 118 NaCl, 20 tetraethyammonium-chloride (TEA-Cl), 5.6 KCl, 1.2 MgCl2, 2.6 CaCl2, 5 D-glucose, and 5 HEPES (pH 7.4 with NaOH). In the perforated patch experiment, forskolin (2–10 μM; from Sigma-Aldrich) or GLP-1 (10 nM; Peninsula Laboratories) was added to increase intracellular cAMP levels and enhance exocytosis (Ammälä et al., 1993). PKA was inhibited by treating the cells with the membrane-permeant cAMP antagonists Rp-cAMPS or Rp-8-Br-cAMPS (Biologic) for ≥5 min. The pipette solution used for the perforated patch measurements contained (in mM) 76 CaSO4, 10 NaCl, 1 MgCl2, and 5 HEPES (pH 7.55 with CaOH2). The pipette solution in the standard whole-cell measurements in which the cells were stimulated with voltage-clamp depolarizations consisted of (in mM) 125 Cs-glutamate, 10 CsCl, 10 NaCl, 1 MgCl2, 0.05 EGTA, 3 Mg-ATP, 5 HEPES (pH 7.1 using CsOH; intracellular solution I). Cyclic AMP or the selective cAMP-GEFII-agonist 8-(4-chloro-phenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate (8CPT-2′Me-CAMP; Enserink et al., 2002) was added to the latter solution at the indicated concentrations. To block PKA,Rp-cAMPS was included at a concentration of 0.5 mM. When exocytosis was stimulated by infusion of a buffer with elevated [Ca2+]i (Figs. 4, C–E, 9, and 10), the pipette solution contained (in mM) 125 K-glutamate, 10 KCl, 10 NaCl, 1 MgCl2, 3 Mg-ATP, 5 HEPES (pH 7.15 with KOH), 5 or 9 CaCl2, and 10 EGTA (intracellular solution II). The free Ca2+ concentrations were estimated to be 0.17 and 1.5 μM using the binding constants of Marrell and Smith (1974).

Electrophysiology

Patch electrodes were made from borosilicate glass capillaries coated with Sylgard close to the tips and fire-polished. The pipette resistance ranged between 2 and 4 MΩ when the pipettes were filled with the intracellular solutions specified above. The zero-current potential of the pipette was adjusted in the bath before gigaseal formation. Either the perforated patch whole-cell technique (Figs. 1 and 4, A and B), in which the cells remain metabolically intact, or the standard whole-cell configuration (Figs. 2, 3, 4, C–E, 5, 7, 9, and 10), which allows intracellular application of reagents simply by including them in the pipette solution, was used. All measurements were conducted using EPC7 or EPC9 patch-clamp amplifiers and the Pulse software (version 8.30 or later; Heka Elektronik).

Exocytosis was detected as changes in cell capacitance, which was estimated by the Lindau-Neher technique (Gillis, 1995), implementing the “Sine + DC”-feature of the lock-in module (40 mV peak-to-peak and a frequency of 500–1,000 Hz). Secretion was elicited by single voltage-clamp depolarizations from −70 to 0 mV of variable duration or trains of 10 500-ms depolarizations (1-Hz stimulation frequency) to 0 mV. During the experiments, the cells were continuously superfused with the extracellular medium at a rate of <2 ml/min and the volume of the chamber reduced to 0.5 ml by a plastic insert. All electrophysiological measurements were performed at ≈33°C.

Immunocytochemistry

Primary B-cells and MIN6 cells were fixed in 3% paraformaldehyde in K-PIPERs (Sigma-Aldrich) and permeabilized with 0.1% Triton X-100. After blocking of nonspecific sites with 5% normal donkey serum, the cells were incubated for 2 h in the presence of goat-anti-rat-cAMP-GEFII anti-EPACII (C-19, 1:50, Santa Cruz Biotechnology, Inc.) and a guinea-pig polyclonal anti-insulin antibody (B 65–1, 1:500, Euro-diagnostica). Anti–goat-raised anti–cAMP-GEFII/anti-EPACII (C-19, 1:50, Santa Cruz Biotechnology, Inc.) and a guinea-pig polyclonal anti-insulin antibody (B 65–1, 1:500, Euro-diagnostica). Anti–goat-raised anti–cAMP-GEFII/anti-EPACII (C-19, 1:50, Santa Cruz Biotechnology, Inc.) and a guinea-pig polyclonal anti-insulin antibody (B 65–1, 1:500, Euro-diagnostica). Anti–goat-raised anti–cAMP-GEFII/anti-EPACII (C-19, 1:50, Santa Cruz Biotechnology, Inc.) and a guinea-pig polyclonal anti-insulin antibody (B 65–1, 1:500, Euro-diagnostica). Anti–goat-raised anti–cAMP-GEFII/anti-EPACII (C-19, 1:50, Santa Cruz Biotechnology, Inc.) and a guinea-pig polyclonal anti-insulin antibody (B 65–1, 1:500, Euro-diagnostica).
615 nm; Cy3) or a >650-nm filter (Cy5). The samples were scanned sequentially with the appropriate settings to minimize crosstalk.

Insulin Measurements

Islets were preincubated in Earle’s balanced Salt Solution (EBSS; Invitrogen) containing 0.1% BSA and 2.8 mM glucose for 30 min at 37°C. Groups of 10 islets were preincubated at 37°C for 35 cycles of 45 s at 94°C, 45 s at 58°C, and 45 s at 72°C with primers: Rim2 sense: 5’GCTCAAAACAGGTATGTTGCTG3’; Rim2 antisense: 5’CTCTACTGCTGCTGACATGCC3’; cAMPGEFII sense: 5’GGAGAGAGAAAGGTGCTGCT3’; and cAMPGEFII antisense: 5’CTCTAAAACTCCGCATAGAC3’.

Data Analysis

The kinetic model used for fitting experimental data (in Figs. 1–3 and 7) considers two pools of granules; the pool of immediately releasable granules (P) and the pool of fused granules (Pf).

Release can then be described as

$$P = k_{off}P_f,$$

where $k$ is

$$k = (1 - e^{-\frac{t}{\tau}})a_0.$$
GLP-1 potentiates exocytosis in B-cells. Increases in cell capacitance (ΔCm, bottom trace) elicited by progressively longer (5, 10, 15, 30, 50, 100, 150, 250, 350, 450, and 850 ms) depolarizations from −70 to 0 mV (V, top trace) under (A) control conditions and (B) 4 min after the inclusion of 10 nM GLP-1 in the extracellular medium. The interval between two successive depolarizations was 15 s for pulses ≤50 ms and 30 s for longer pulses. For clarity, only responses to the 30-, 100-, 250-, and 450-ms depolarizations are shown. The recording was performed using the perforated patch configuration. (C) Relationship between pulse duration (t) and increase in cell capacitance (ΔCm) under control conditions and in the presence of 10 nM GLP-1 as indicated. The curves were derived by fitting Eq. 3 to the data points for depolarizations ≤350 ms. The dotted lines represent linear fits to the values measured in response to the two longest depolarizations. Data are mean values ± SEM of seven paired experiments. ∗P < 0.05 and ∗∗P < 0.01.

The relationships between pulse length and the magnitude of the exocytotic responses under control conditions, with an intracellular cAMP concentration of 0.1 mM and in the simultaneous presence of cAMP and Rp-cAMPS are summarized in Fig. 2 D. Solutions to Eq. 3 were approximated to the data points and the average size of IRP determined under the different experimental conditions is presented in Table I (lines 4, 8, and 10). Cyclic AMP produced a 15-fold increase of IRP that was only partially reversed by Rp-cAMPS. The capacitance increase plateaued during depolarizations lasting ≥200 ms with a secondary stimulation being observed during longer depolarizations under both control conditions and in the presence of cAMP (50 fF/s under control conditions and 300 fF/s in the presence of cAMP). The latter component of capacitance increase was highly sensitive to inclusion of Rp-cAMPS and the ΔC/Δt-value in fact assumed a negative value in the presence of the PKA-inhibitor (−30 fF/s; i.e., less than what was observed under control conditions in the absence of cAMP), possibly indicative of endocytosis. These experiments confirm our earlier observation that cAMP stimulates exocytosis by both PKA-dependent and -independent mechanisms and provide the novel observation that the PKA-independent action is particularly prominent during brief depolarizations. Control experiments using 0.5 mM Rp-cAMPS alone re-
vealed that the compound did not exert any stimulatory effect on its own (Fig. 2 D).

**Differential cAMP Dependence of PKA-dependent and -independent Stimulation of Exocytosis**

We subsequently investigated the cAMP dependence of the PKA-dependent and -independent actions on exocytosis. Cyclic AMP was applied intracellularly at concentrations of 0, 1, 10, 50, 100, and 500 μM. The data are summarized in Fig. 3 A. Solutions of Eq. 3 were approximated to the data points and the values of Cₘₐₓ and ΔC/Δt₄₅₀–₈₅₀ are presented in Table I. Fig. 3 B shows the normalized increase in IRP (S) against the cAMP concentration (the increase in IRP at 500 μM of cAMP taken as unity). The Hill equation

\[ S = S_{\text{max}} \frac{[\text{cAMP}]^n}{K_d + [\text{cAMP}]^n} \]  

(4)

was fit to the data points to derive the Hill coefficient (n) and the Kᵣ value, the concentration of cAMP at which stimulation is half-maximal (Kᵣ). In Eq. 4, Sₘₐₓ represent the calculated maximal cAMP-induced stimulation. We thus obtained a Kᵣ value of 29 μM. For comparison, the normalized response seen in the presence of 0.1 mM cAMP and 0.5 mM Rp-cAMPS is shown (open circle). It is clear that the early cAMP-dependent component of exocytosis is little affected by PKA-inhibition.

The effects of varying the intracellular concentration of cAMP on exocytosis elicited by trains of 10 500-ms depolarizations from −70 to 0 mV (Fig. 3 C) were analyzed similarly. The total increase in cell capacitance elicited by the train in the absence of cAMP is quite variable but in this series of experiments averaged 31 ± 4 pF (n = 6). A low concentration of cAMP (1 μM) only marginally stimulated exocytosis, but much larger responses were obtained after inclusion of ≥10 μM cAMP in the pipette solution. Fig. 3 D summarizes the amplitude of the capacitance increases elicited by the last nine pulses of the train at the different concentrations of cAMP (1–500 μM). The Kᵣ value for this late component was estimated to 6 μM by approximating the data to Eq. 4. Unlike what was observed for the rapid component (Fig. 3 B), the late cAMP-dependent effect was highly sensitive to Rp-cAMPS and in the presence of this antagonist, the increase in cell capacitance elicited by the last nine pulses was in fact less than that observed in the complete absence of cAMP (Fig. 3 D, open circle).

**cAMP Mediates its PKA-independent Component via cAMP-GEFII**

The cAMP-binding protein cAMP-GEFII binds cAMP at concentrations ≈10 μM and has been reported to mediate PKA-independent effects of the nucleotide on insulin secretion (Ozaki et al., 2000). We next confirmed that this effect also contributes to the cAMP-induced enhancement of exocytosis monitored as increases in membrane capacitance using the perforated patch whole-cell configuration (Fig. 4, A and B). The PKA-dependent effects were prevented by pretreatment of the cells with 500 μM of the membrane-permeant PKA inhibitor 8-Br-Rp-cAMPS. After pretreatment with control oligonucleotide, exocytosis elicited by a 500-ms depolarization from −70 to 0 mV applied in the presence

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**Table 1**

Summary of the Effects of cAMP and PKA Inhibition in B-cells from Wild-type and SUR1⁻/⁻ Mice

<table>
<thead>
<tr>
<th>Line</th>
<th>Condition</th>
<th>ΔCₘₐₓ,∞ (=IRP)</th>
<th>ΔCₘₐₓ/Δt₄₅₀–₈₅₀</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type B-cells</td>
<td>Control</td>
<td>45 ± 7</td>
<td>6 ± 8</td>
<td>7</td>
</tr>
<tr>
<td>Perforated patch</td>
<td>GLP-1 (10 nM)</td>
<td>104 ± 15</td>
<td>56 ± 17</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>Forskolin (2 mM)</td>
<td>123 ± 30</td>
<td>152 ± 58</td>
<td>11</td>
</tr>
<tr>
<td>Wild-type B-cells</td>
<td>cAMP (1 μM)</td>
<td>11 ± 2</td>
<td>25 ± 12</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>cAMP (10 μM)</td>
<td>18 ± 6</td>
<td>16 ± 4</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>cAMP (50 μM)</td>
<td>57 ± 10</td>
<td>150 ± 53</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>cAMP (100 μM)</td>
<td>83 ± 20</td>
<td>94 ± 18</td>
<td>5</td>
</tr>
<tr>
<td>9</td>
<td>cAMP (500 μM)</td>
<td>157 ± 27</td>
<td>317 ± 119</td>
<td>7</td>
</tr>
<tr>
<td>10</td>
<td>cAMP (100 μM) and Rp-cAMPS (0.5 μM)</td>
<td>176 ± 38</td>
<td>306 ± 135</td>
<td>6</td>
</tr>
<tr>
<td>SUR1⁻/⁻ B-cells</td>
<td>Control</td>
<td>16 ± 6</td>
<td>43 ± 19</td>
<td>5</td>
</tr>
<tr>
<td>Standard whole-cell</td>
<td>cAMP (0.1 μM)</td>
<td>68 ± 18</td>
<td>77 ± 40</td>
<td>8</td>
</tr>
<tr>
<td>13</td>
<td>cAMP (0.1 μM) and Rp-cAMPS (0.5 μM)</td>
<td>18 ± 3</td>
<td>3 ± 10</td>
<td>6</td>
</tr>
</tbody>
</table>

The size of IRP was estimated from the approximation of the solution of Eq. 3 to the data points in Figs. 1 C, 2 D, and 7, D and E. ΔCₘₐₓ/Δt₄₅₀–₈₅₀ is the rate of capacitance increase occurring between 450- and 850-ms data points. Data are mean values ± SEM of indicated number of cells tested. Statistical significances refer to comparisons within the same column.

*P < 0.01 versus line 1; *P < 0.01 versus line 4; **P < 0.05 versus line 4; ***P < 0.02 versus line 8; **P < 0.05 versus line 11; **P < 0.05 versus line 8; **P < 0.05 versus line 12.
The cAMP effector protein cAMP-GEFII was originally identified by two yeast hybrid screening of a MIN6-cell library using the sulfonylurea receptor SUR1 as the bait (Ozaki et al., 2000). We have reported previously that sulfonylureas, such as tolbutamide and glibenclamide, stimulate exocytosis by a late effect on the exocytotic machinery that does not involve closure of plasma membrane KATP channels and that culminates in accelerated priming of the secretory granules by a PKA-dependent mechanism (Eliasson et al., 1996; Barg et al., 1999, 2001a). We analyzed the significance of cAMP-GEFII for sulfonylurea-stimulated exocytosis using clonal MIN-6 cells.

In control cells (pretreated with control oligonucleotides or untransfected cells, no difference being observed between the two sets of cells), the capacitance increased steadily after establishment of the whole-cell configuration at a rate (ΔC/Δt) of 9 ± 1 fF/s (n = 17; Fig. 4 C). Inclusion of 0.1 mM cAMP in the pipette solution almost doubled the rate of exocytosis and the ΔC/Δt-value rose to 17 ± 2 fF/s (n = 18, P < 0.001 vs. control). Addition of tolbutamide (0.1 mM) produced a further 30% stimulation of exocytosis and ΔC/Δt amounted to 22 ± 1 fF/s (n = 13; P < 0.05 vs. rate measured with cAMP alone). When the same type of experiment was repeated in cells pretreated with antisense cAMP-GEFII oligonucleotides (Fig. 4 D), ΔC/Δt averaged 12 ± 3 fF/s (n = 12) under control conditions, 16 ± 3 fF/s (n = 14) in the presence of cAMP alone and 14 ± 3 fF/s in the simultaneous presence of cAMP and tolbutamide (n = 14; P < 0.05 vs. rate observed in cells exposed to cAMP and tolbutamide but incubated with the control oligonucleotide). Thus, reduced expression of cAMP-GEFII not only abolished the effect of cAMP on exocytosis, it also interfered with the ability of tolbutamide to stimulate exocytosis. This conclusion is reinforced by experiments performed in MIN-6 cells, which had been transfected with a dominant-negative mutant of cAMP-GEFII (G114/G422D; Ozaki et al., 2000; Fig. 4 E). In this series of experiments, ΔC/Δt averaged 8 ± 2 fF/s (n = 16) under control conditions, 8 ± 1 fF/s (n = 15) in the presence of cAMP alone and 8 ± 2 fF/s in the simultaneous presence of cAMP and tolbutamide (n = 13). The effects of treatment with the antisense oligonucleotide on exocytosis echo those on cAMP-GEFII immunoreactivity (Fig. 4 F). Whereas both MIN6-cells and primary mouse B-cells treated with the control oligonucleotide ex-

of forskolin amounted to 91 ± 8 fF (n = 5). This value is about twice that measured in cells pretreated with antisense cAMP-GEFII oligonucleotide, which averaged 48 ± 5 fF (n = 5; P < 0.01). Exocytosis in the presence of Rp-cAMPS alone amounted to 5 ± 5 fF (n = 5) and 10 ± 4 (n = 5) after pretreatment with control and antisense ODNs, respectively. We conclude that cAMP-GEFII mediates >55% of the PKA-independent component of exocytosis (i.e., 100% * [1 - (48 fF - 10 fF)/ (91 fF - 5 fF)]).

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hibited clear cAMP-GEFII/EPACII immunoreactivity, that only weakly colocalized with insulin, cells treated with the antisense oligonucleotide showed little cAMP-GEFII immunoreactivity. Notably, the antisense treatment did not affect insulin immunoreactivity.

**A Selective cAMP-GEFII Agonist Promotes Rapid Exocytosis**

The data of Fig. 4 suggest that cAMP-GEFII is responsible for the PKA-independent component of cAMP-stimulated exocytosis. We next tested the effects of the selective cAMP-GEFII agonist 8CPT-2Me-cAMP on B cell exocytosis. Secretion was elicited by a train of 10 depolarizations from −70 to 0 mV under control conditions, in the presence of 0.1 mM 8CPT-2Me-cAMP alone and in the simultaneous presence of both the agonist and 0.5 mM Rp-cAMPS (Fig. 5 A). The total increase in capacitance elicited by the train averaged 99 ± 14 fF (n = 6) under control conditions, 274 ± 51 fF (n = 6; P < 0.01 vs. control) in the presence of 0.1 mM 8CPT-2Me-cAMP and 178 ± 30 fF (n = 5) in the simultaneous presence of 0.1 mM 8CPT-2Me-cAMP and 0.5 mM Rp-cAMPS. The latter value is statistically different from the control value (P < 0.05) but not from that observed in the presence of 8CPT-2Me-cAMP alone. Fig. 5 B illustrates the increase in cell capacitance elicited by the individual pulses during the train. It is clear that the action of 8CPT-2Me-cAMP is particularly pronounced during the first part of the train and that this effect was unaffected by PKA-inhibition (pulses 1 and 2). Surprisingly, an exocytotic component sensitive to Rp-cAMPS was observed during the middle
part of the train (pulses 3–5). We speculate that Ca\(^{2+}\)/H11001-entry associates with some Ca\(^{2+}\)/H11001-dependent activation of adenylate cyclase leading to sufficient generation of cAMP to promote granule mobilization by the PKA-dependent mechanism (Fig. 3 D). This scenario would also explain the slow increase in capacitance that occurs in the complete absence of cyclic AMP (Figs. 3 C and 5 A) and that can likewise be suppressed by Rp-cAMPS (Figs. 2 C and 3 D; Renström et al., 1997).

Impaired Insulinotropic Action of Glucose and the Incretin GLP-1 in vitro

It has been reported recently that insulin secretion from SUR1 knockout mice (SUR1\(^{-/-}\)) exhibits a much lower sensitivity to intracellular cAMP and appears unaffected by stimulation with incretins (Shiota et al., 2002; Nakazaki et al., 2002). We have repeated these experiments focusing on the ability of GLP-1 and forskolin to stimulate glucose-induced insulin secretion and the effects of inhibiting PKA using Rp-cAMPS in wild-type and SUR1\(^{-/-}\) islets (Table II). Wild-type islets had low basal insulin secretion, which increased five- to ninefold in response to stimulation with 1 \(\mu\)M glibenclamide or 20 mM glucose. GLP-1 and forskolin potentiated the stimulatory action of glucose 2.8- to 3.6-fold. The latter effects were partially (65–70%) counteracted by Rp-8-Br-cAMPS. In islets from SUR1\(^{-/-}\) mice, basal secretion was increased >1.4-fold relative to
that seen in wild-type islets. As expected, glibenclamide had no effect but glucose retained a 4.5-fold stimulatory action. GLP-1 and forskolin potentiated glucose-induced secretion from SUR1\(^{-/-}\) islets 2.2- to 2.8-fold; i.e., not much less than the relative stimulation in wild-type islets. However, unlike the situation in the wild-type islets, the effects of forskolin and GLP-1 were fully (\(\approx 90\%\)) antagonized by Rp-cAMPS.

The smaller secretory responses in SUR1\(^{-/-}\) B-cells are not attributable to degranulation and islet insulin content averaged 280 ± 10 ng insulin/islet (\(n = 6\)) and 253 ± 10 ng/islet (\(n = 6\)) in wild-type and SUR1\(^{-/-}\) mice, respectively. GLP-1 increased cAMP content more than sevenfold in SUR1\(^{-/-}\) islet; similar to the ninefold elevation seen in the wild-type islets (Fig. 6). The effects of GLP-1 were dose-dependent and bell-shaped in both wild-type and SUR1\(^{-/-}\) islets. The maximum cAMP levels were observed at 10 nM and at this concentration the content approached the values observed in response to 10 \(\mu\)M forskolin.

**Lack of an Early PKA-independent Component in B-cells from SUR \(^{-/-}\) Mice**

Fig. 7, A–C, shows the effects of intracellular cAMP and PKA inhibition on exocytosis measured as a capacitance increase in B-cells from SUR1\(^{-/-}\) mice using the same protocol as in Fig. 2. Under basal conditions (no cAMP, Fig. 7 A), the exocytotic responses were small and \(\approx 20 \text{ fF}\) for depolarizations lasting 450 ms. As was the case in wild-type B-cells, inclusion of 0.1 mM cAMP in the intracellular medium resulted in much larger exocytotic responses (Fig. 7 B). However, unlike the situation in wild-type B-cells, the effects of cAMP were fully antagonized by 0.5 mM Rp-cAMPS (compare Figs. 2 C and 7 C). The relationships between pulse length and exocytosis under control conditions, in the presence of cAMP and in the simultaneous presence of both cAMP and Rp-cAMPS are summarized in Fig. 7 D. The size of IRP under the different experimental conditions was determined by fitting Eq. 3 to the observed data points (Table I, lines 11–13). It is apparent that cAMP produced a fourfold increase in pool size but that it is \(\approx 90\%\) smaller than in wild-type animal (compare lines 8 and 12 of Table I). The effect of cAMP in B-cells from SUR1\(^{-/-}\) mice, unlike what was observed in their wild-type counterparts, was completely inhibited by Rp-cAMPS. Moreover, the fast component of exocytosis (detectable during the first 100 ms) was more prominent in wild-type than in SUR1\(^{-/-}\) islets (Fig. 7 E). Thus, the value of \(\tau\) (see Eq. 2) was 3.5-fold higher in B-cells from SUR1\(^{-/-}\) mice than in their wild-type counterparts (45 ± 13 ms vs. 12 ± 4 ms; \(P < 0.05\)). We can discard the possibility that the differences between wild-type and SUR1\(^{-/-}\) B-cells were due to a reduction of voltage-gated Ca\(^{2+}\)-entry. The Ca\(^{2+}\)-current amplitude during depolarizations to 0 mV averaged \(-57 ± 5\) pA.
(n = 12) and −64 ± 8 pA (n = 8) in SUR1−/− and wild-type B-cells, respectively.

cAMP-GEFII and Rim2 are Transcribed in SUR1−/− B-cells

We tested whether the failure of cAMP to exert a PKA-independent stimulatory action on exocytosis in SUR1−/− B-cells results from loss of transcription of the putative effector proteins. However, applying RT-PCR to islets isolated from SUR1−/− mice indicate that both cAMP-GEFII and Rim2 remain expressed in islets from the knockout animals (Fig. 8).

Sulfonylureas Stimulate Exocytosis in B-cells from SUR1−/− Mice

It has been suggested that sulfonylureas stimulate exocytosis in B-cells by a direct effect on the exocytotic machinery (Eliasson et al., 1996). We investigated whether this effect is retained in SUR1−/− B-cells using a Ca2+ infusion protocol (intracellular solution II supplemented with 0.1 mM cAMP). In SUR1−/− B-cells, tolbutamide (0.1 mM) increased the rate of capacitance increase ≥2.5-fold at both 0.17 and 1.5 μM intracellular free Ca2+ (Fig. 9, A and B); from 5 ± 1 fF/s (n = 8) to 14 ± 3 fF/s (n = 11; P < 0.05) at low Ca2+ and from 16 ± 3 fF/s (n = 6) to 37 ± 9 fF/s (n = 6; P < 0.05) at the higher intracellular Ca2+-concentration (Fig. 9, A and B). These rates and the extent of stimulation are comparable to effects reported previously (Barg et al., 1999, 2001a). The failure of glibenclamide to stimulate insulin secretion in B-cells from SUR1−/− mice (Table II) we attribute to the glucose concentration in this series of experiments. The direct action of sulfonylureas on exocytosis is particularly pronounced at high glucose, whereas it is without effect at low concentrations of the sugar (unpublished data).

Acidification of the granule interior has been identified as an important reaction in the preparation of the granules for release (Barg et al., 2001a; Renström et al., 2002a,b). As shown in Fig. 9 C, extracellular applica-

### TABLE II

<table>
<thead>
<tr>
<th>Line</th>
<th>Conditions</th>
<th>Wild-type (SUR1+/+)</th>
<th>Knockout (SUR1−/−)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.8 mM glucose</td>
<td>0.27 ± 0.04 (n = 5)</td>
<td>0.39 ± 0.06 (n = 5)</td>
</tr>
<tr>
<td>2</td>
<td>2.8 mM glucose +1 μM glibenclamide</td>
<td>1.34 ± 0.10 (n = 5)</td>
<td>0.41 ± 0.09 (n = 5)</td>
</tr>
<tr>
<td>3</td>
<td>20 mM glucose 10 nM GLP-1</td>
<td>2.53 ± 0.23 (n = 5)</td>
<td>1.76 ± 0.27 (n = 5)</td>
</tr>
<tr>
<td>4</td>
<td>20 mM glucose 10 nM GLP-1 +100 μM Rp-8-Br-cAMPS</td>
<td>7.01 ± 0.51 (n = 5)</td>
<td>3.87 ± 0.37 (n = 5)</td>
</tr>
<tr>
<td>5</td>
<td>20 mM glucose 10 nM GLP-1 +100 μM Rp-8-Br-cAMPS</td>
<td>4.11 ± 0.33 (n = 5)</td>
<td>1.91 ± 0.31 (n = 5)</td>
</tr>
<tr>
<td>6</td>
<td>20 mM glucose +10 μM forskolin</td>
<td>8.97 ± 0.62 (n = 5)</td>
<td>4.88 ± 0.49 (n = 5)</td>
</tr>
<tr>
<td>7</td>
<td>20 mM glucose +10 μM forskolin</td>
<td>4.54 ± 0.46 (n = 5)</td>
<td>2.07 ± 0.43 (n = 5)</td>
</tr>
</tbody>
</table>

Impaired PKA-independent incretin effect in islet from SUR1−/− mice. Secretion of insulin from isolated islets taken from wild-type (SUR1+/+) and SUR1 knockout (SUR1−/−) in the absence and presence of different insulin secretagogues and inhibitors. Incubation was performed with five islets in each tube at either 2.8 or 20 mM glucose. Data are presented as mean values ± SEM of indicated number of experiments (n).

*P < 0.01 versus line 1; †P < 0.001 versus line 1; ‡P < 0.02 versus line 2; ††P < 0.001 versus line 3; ‡‡P < 0.01 versus line 4; †††P < 0.001 versus line 6; ‡‡‡P < 0.01 versus line 3; †P < 0.01 versus line 6; ‡P < 0.001; †P < 0.01 versus corresponding value in wild-type islets; ‡P < 0.05 versus corresponding value in wild-type islets.

![Figure 6. GLP-1–induced cAMP production in wild-type and SUR1−/− B-cells.](image-url)

The Journal of General Physiology
tion of tolbutamide resulted in a pronounced acidification of the granules (detected as an increase in granular LSG fluorescence), an effect that was maintained in B-cells from SUR1/−/− mice (Fig. 9 D), although the average effect was reduced by ~30% (Fig. 9 E). Apparently, the ability of tolbutamide to promote granule priming is largely unperturbed by ablation of SUR1.

Cyclic AMP Stimulates Influx of Cl− by a PKA-independent Mechanism in Wild-type but Not in SUR1/−/− B-cells

ClC3 channels have been shown previously to play an important role in granule priming by providing the shunt conductance required for the granules to acidify (Barg et al., 2001a; Renström et al., 2002a). Given that the stimulatory effects of both the PKA-independent mechanism of cAMP and those of the sulfonylureas on exocytosis appear to involve cAMP-GEFII (Fig. 4, C–E), it is tempting to speculate that their actions converge at the level of granular ClC3-channels. In wild-type B-cells, inclusion of cAMP accelerated granule deprotonation (corresponding to stimulation of Cl− influx) fourfold over that seen under control conditions, an effect resistant to PKA inhibition (Fig. 10, A and C). Interestingly, the ability of cAMP to accelerate Cl− uptake into the granules was almost abolished in B-cells from SUR1/−/− mice (Fig. 10, B and C) and the rate of fluorescence decrease was not significantly different from that observed in the absence of cAMP. We are not implying that granules in B-cells from SUR1/−/− mice are unable undergo priming. Indeed the ability of Ca2+ alone to acidify the granules was the same in wild-type and SUR1/−/− B-cells (Fig. 10 C). It is evident from both the capacitance measurements and insulin release experiments that SUR1/−/− B-cells contain a large pool of release-competent granules. We postulate, however, that the ability of cAMP to accelerate priming is much reduced in the SUR1/−/− B-cells and that this accounts for the poor incretin effects in these cells.
The ability of cAMP-increasing agents to stimulate insulin secretion is well established (Wollheim and Sharp, 1981), but the precise cellular processes involved are not fully understood. Here we have combined measurements of insulin release with high-resolution capacitance measurements, antisense oligonucleotide technology, and the use of transgenic animals to dissect the mechanisms by which cAMP accelerates exocytosis. We confirm that cAMP enhances insulin secretion by both PKA-dependent and -independent mechanisms by which cAMP accelerates exocytosis. We propose that the PKA-independent component of exocytosis can be used to estimate the cAMP concentration at the release sites. Under basal conditions, the size of IRP amounted to 45 fF, which rose to ≈100 fF and ≈120 fF after stimulation with 10 nM GLP-1 or 2 μM forskolin (Table I). Using the dose–response curve in Fig. 3 B, these values correspond to a cAMP concentrations of 8, 42, and 70 μM under basal conditions in the presence of GLP-1 and after stimulation with forskolin. Thus, the cAMP concentration sensed by the exocytotic machinery may vary by almost a factor of 10 under different experimental conditions. It is interesting to compare the above concentrations with the reported cAMP levels in the absence and presence of forskolin. In one study performed in the absence of any phosphodiesterase inhibitor, the cAMP content of isolated mouse islets was found to increase from a basal 4 fmol/islet to ≈65 fmol in the presence of 10 μM forskolin (Eddlestone et al., 1985). The above values can be converted to global intracellular cAMP concentrations taking the intracellular water volume to be ≈2 nl in mouse islets (Ashcroft et al., 1980). We thereby estimate that cAMP increases from a basal concentration of ≈2 to ≈30 μM in the presence of forskolin. Since
the latter value is only ≈40% of that suggested by the capacitance measurements, it appears that fairly steep intracellular gradients of cAMP may exist within the B cell. This is perhaps not so unexpected given that adenylate cyclase is situated in the plasma membrane, i.e., close to the release site. It is also worthy of note that already the basal concentration (2 μM) is sufficient to allow some limited PKA-dependent stimulation of granule mobilization (Fig. 3 D).

**Evidence for PKA-dependent and -independent Components of Insulin Release in vitro in Wild-type and SUR1−/− mice**

Although SUR1−/− B-cells lack functional K_ATP-channels, these islets retain some glucose dependence of insulin secretion (3.5-fold stimulation; Table II). Contrary to what was reported recently by others (Nakazaki et al., 2002), islets from SUR1−/− mice also respond well to GLP-1 and forskolin although the magnitude of the responses is only 50% of those seen in wild-type islets. However, an important difference exists with respect to the effects of PKA inhibition. Whereas 30–35% of insulin secretion elicited by GLP-1 or forskolin is resistant to Rp-cAMPS in wild-type islets, the PKA-independent component is absent in the SUR1−/− islets. Consistent with data reported by others (Nakazaki et al., 2002), this difference cannot be explained by a reduced ability of GLP-1 and forskolin to increase intracellular cAMP, which was only marginally reduced in the knockout islets. Indeed, the lack of a rapid PKA-independent component of insulin secretion was confirmed using capacitance measurements in which B-cells from SUR1−/− mice were dialyzed with 0.1 mM cAMP (Fig. 7 E). These findings therefore raise the interesting alternative possibility that SUR1 somehow is involved in the exocytotic process. It is important to note that both cAMP-GEFII and Rim2 are transcribed in the SUR1−/− B-cells (Fig. 8). Although PCR data cannot be equated to protein levels, these results nevertheless argue that the loss of PKA-independent cAMP-induced exocytosis cannot simply be attributed to the absence of the effector proteins. It may seem surprising that whereas our data indicate that GLP-1 retains a good stimulatory action in isolated islets, in vivo experiments on the same SUR1−/− mouse strain suggest the complete loss of GLP-1–stimulated secretion (Shiota et al., 2002 and unpublished data). Although we are unable to provide a simple explanation to this discrepancy, this observation might indicate that the PKA-independent component of cAMP-induced exocytosis plays a particularly important role for incretin-stimulated insulin secretion in vivo. It should be noted that the PKA-independent action of cAMP is operational at higher intracellular cAMP concentrations than the PKA-dependent mechanism (Fig. 3 D) and one possible explanation is therefore that stimulation with GLP-1 resulted in a larger increase in intracellular cAMP levels in the in vitro experiments than that attained in vivo. This concept would in fact be in accordance with the

**Figure 9.** Sulfonylureas retain ability to stimulate exocytosis in SUR1−/− B-cells. (A-B) Changes in cell capacitance (∆Cm) under control conditions (ctrl) and in the presence of 0.1 mM tolbutamide (tolb) in SUR1−/− B-cells dialyzed with intracellular solution II with free Ca2+-concentrations of 0.17 μM (A) or 1.5 μM (B), respectively. Cyclic AMP was included at a concentration of 0.1 mM to promote granule mobilization (Renström et al., 1997). Traces shown are representative of 6–11 experiments. (C and D) Tolbutamide-induced granular acidification in wild-type (C) and SUR1−/− B-cells (D). Acidification is estimated as the relative increase (in %) in the initial LSG-signal (∆F/F0) after addition of tolbutamide. (E) Relative change in LSG fluorescence in wild-type (wt) and SUR1−/− B-cells 60 s after addition of tolbutamide. Data are mean values ±SEM of 7–8 experiments.
observations of Nakazaki et al. (2002), who found that whereas GLP-1 failed to stimulate insulin secretion, forskolin retained the ability to potentiate insulin secretion and especially so when applied in the presence of IBMX.

**PKA-independent Effect of cAMP Involves Stimulation of Cl\(^{-}\) Influx and Granular Acidification**

We and others have demonstrated previously that sulfonylureas stimulate exocytosis in B-cells via an effect exerted on the secretory machinery itself and that is not mediated by closure of plasma membrane K\(_{ATP}\)-channels (Flatt et al., 1994; Eliasson et al., 1996; Tian et al., 1998; Barg et al., 1999). This effect remained observable in the presence of forskolin but disappeared when PKC was maximally activated by the phorbol ester PMA or inhibited by bisindolylmaleimide (Eliasson et al., 1996). The ability of sulfonylureas to stimulate exocytosis in SUR1\(^{-/-}\) B-cells therefore suggests that inactivation of this gene with resultant suppression of the PKA-independent action does not interfere with the ability of PKC activation to stimulate secretion. Indeed, it has been reported recently that the potency of PMA to stimulate insulin secretion is at least as strong in the SUR1\(^{-/-}\) islets as in their wild-type counterparts (Nakazaki et al., 2002).

The ability of sulfonylureas to stimulate exocytosis in B-cells may not only be of pharmacological significance. It can be speculated that it contributes to the metabolic regulation of insulin secretion (i.e., the “augmenting” or “K\(_{ATP}\) channel-independent” effects of glucose on insulin release; Henquin, 2000; Henquin et al., 2002). The importance of the metabolic state for exocytosis is witnessed by the prompt (within a few seconds) inhibition when the cytoplasmic ATP/ADP-ratio is lowered by flash photolysis of caged ADP even if ATP and Ca\(^{2+}\) are present at levels that would otherwise be stimulatory (Barg et al., 2002a). This effect of ADP can be reversed by tolbutamide and in the simultaneous presence of the sulfonylurea and ADP, exocytosis proceeds at the same rate as in the presence of ATP alone (Barg et al., 2001a).

A recent model postulates that the inhibitory and stimulatory effects of ADP and tolbutamide on exocytosis are secondary to changes in granular Cl\(^{-}\) influxes and pH; stimulation of exocytosis occurs upon intragranular acidification and activation of Cl\(^{-}\) influx (Barg et al., 2001a; Renström et al., 2002a). We now extend these observations and demonstrate that binding of cAMP to cAMP-GEFII stimulates exocytosis via the same mechanism. We also provide evidence that the poor incretin effect characterizing B-cells from SUR1\(^{-/-}\) mice associates with the failure of cAMP to activate these processes (Fig. 10).

**Model**

In Fig. 11 we outline a hypothetical model that appears to account for our observations. We propose that

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![Figure 10](image-url)
SUR1, in addition to its well-known role in the formation of functional $K_{ATP}$-channels (see i.e., Ashcroft and Gribble, 1998), also facilitates the interaction between cAMP-GEFII and downstream effector proteins including RIM2. The ability of SUR1 itself to associate with cAMP-GEFII is suggested by the original identification of cAMP-GEFII in insulin-secreting cells by yeast-two-hybrid screening of a mouse cDNA library using SUR1 as the bait (Ozaki et al., 2000). RIM proteins have been proposed to subserve scaffolding roles in neurons (compare Wang et al., 1997; Schoch et al., 2002) and it is easy to imagine how SUR1, Rim2, and cAMP-GEFII can give rise to a molecular net of protein–protein interactions in the B cell that culminates in the priming of the insulin secretory granules. The ability of sulfonylureas to stimulate exocytosis in B-cells from SUR1$^{-/-}$ mice reinforces previous data, indicating that the granular sulfonylurea-binding protein (gSUR) is distinct from SUR1 (Barg et al., 1999; Renström et al., 2002a). We have suggested that gSUR, via interaction with the ClC3 channels and regulation of granular Cl$^-$/H$^+$ fluxes, modulates the release competence of the insulin granules (Barg et al., 2001a). We now extend this concept and propose that cAMP-GEFII, by assembling with the gSUR–ClC3 complex, promotes granule priming. A tight association of cAMP-GEFII, gSUR, and ClC3 is indeed suggested by the finding that the stimulatory action of sulfonylureas is lost following down-regulation of cAMP-GEFII using antisense ODNs or a dominant negative construct (Fig. 4). It is unlikely that the sulfonylureas bind directly to cAMP-GEFII because the latter protein has a molecular weight of 110 kD and no sulfonylurea-binding proteins of this molecular weight have been identified. We propose that the binding of sulfonylureas to gSUR stabilizes the interaction between cAMP-GEFII/gSUR and ClC3 thus facilitating granule priming. We acknowledge that this scenario by necessity is speculative because the identity of gSUR remains enigmatic and the interaction with other proteins and effects of sulfonylureas have accordingly not been possible to test.

To account for the loss of PKA-independent stimulation of exocytosis in SUR1$^{-/-}$ B-cells, we propose that the cAMP-GEFII–gSUR1 complex fails to associate correctly with the granular ClC3-channel in the absence of SUR1. Accordingly, the ability of cAMP to exert its PKA-independent effect on granular Cl$^-$/H$^+$ fluxes (Fig. 10) and exocytosis (Fig. 7) is lost. This concept would account for the observations that the effects of both cAMP and sulfonylureas converge at the level of granular acidification. We finally emphasize that although SUR1, cAMP-GEFII, ClC3, and gSUR are important for granule priming, membrane fusion still depends on the SNARE proteins VAMP2, syntaxin-1, SNAP25, and the Ca$^{2+}$-sensor synaptotagmin (Jacobsson et al., 1994; Lin and Scheller, 2000; Satin, 2000) and exocytosis can therefore proceed in the absence of cAMP or in SUR1-deficient B-cells, albeit at a lower rate than in wild-type B-cells.

The interactions between cAMP and sulfonylureas and their respective receptors are clearly very complex. However, based on the present findings as well as those published by others (Nakazaki et al., 2002; Shiota et al., 2002) it now seems justifiable to conclude that the role of SUR1 in the B cell extends beyond being a subunit of the $K_{ATP}$-channel and that it also plays an unexpected but important role in the control of the insulin secretory machinery.

Lena Eliasson, Xiaosong Ma, and Erik Renström contributed
equally to this study and their names appear in alphabetical order. We thank Dr. Mark Magnusson for generating the SUR1−/− mice and Susumo Seino for kindly providing us with the antisense cAMP-GEF II and control ODNs, as well as the dominant negative mutant of cAMP-GEFII. We also express our gratitude to Kristina Borglid and Britt-Marie Nilsson for skilled technical assistance.

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