Adrenaline inhibits depolarization-induced increases in capacitance in the presence of elevated $[Ca^{2+}]_i$ in insulin secreting cells

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Abstract Cell capacitance (C_m), cell conductance (G_m), access conductance (G_a) and membrane voltage (V_m) were measured simultaneously in insulin secreting cells using the dual frequency method. Depolarization and stimulation of the cells with secretagogues increased C_m. EGTA abolished the increase in $[Ca^{2+}]_i$ and prevented the rise of C_m. Adrenaline inhibited the augmentation of C_m without lowering $[Ca^{2+}]_i$. In pertussis toxin pretreated cells adrenaline had no effect. Thus, stimulation of insulin secretion is accompanied by an increase in C_m. Inhibition of exocytosis by adrenaline occurs even in the presence of elevated $[Ca^{2+}]_i$, i.e. at a more distal step of exocytosis.

Key words: Insulin secretion; Adrenaline; Capacitance measurement; Patch clamp method; Intracellular Ca^{2+} activity

1. Introduction

Membrane capacitance (C_m) directly correlates with the cell surface while it is largely independent of membrane conductance, composition and fluidity. Thus, exocytosis can be followed by measuring C_m since exocytosis comprises granule fusion with the plasma membrane resulting in an increase in the cell surface [1]. We established and evaluated a new method for measuring C_m using two sine waves of different frequencies as described by Rohlicek and Schmid [2]. This method makes it possible to simultaneously monitor C_m, membrane conductance (G_m), and access conductance (G_a).

Insulin secretion is stimulated by substances which increase $[Ca^{2+}]_i$ such as glucose and sulfonylureas [3–6]. In contrast, adrenaline, somatostatin and galanin inhibit secretion and lower $[Ca^{2+}]_i$ [7,8]. However, using a permeabilized cell preparation we have previously shown that inhibitors such as adrenaline, somatostatin and galanin lower secretion in a GTP-dependent but Ca^{2+}-independent way since Ca^{2+} was buffered with EGTA in these experiments [9–11]. The conclusion that inhibition of secretion occurs distal to the generation of second messengers, i.e. cAMP and Ca^{2+}, gave rise to the hypothesis that adrenaline, somatostatin and galanin exert direct effects on exocytosis [10]. The present study was undertaken to consider this hypothesis for the insulin secreting cell line INS-1 at the single cell level.

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2. Materials and methods

2.1. Beta-cell isolation and cell culture

Rat islets were isolated as described before [12]. Purified islets were further digested with trypsin (0.5 g/l) to obtain a single cell suspension. Thereafter cells were seeded on poly-L-ornithine (10 mg/l, Sigma, Munich, Germany) coated glass coverslips and cultured in RPMI 1640 supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin and 100 ml/l decomplemented fetal calf serum (Seromed, Berlin, Germany) for 2–6 days prior to the experiment. INS-1 cells were cultured in RPMI 1640 and prepared for patch clamp experiments as described before [13].

2.2. Capacitance measurements

Coverslips were mounted in a bath chamber on the stage of an inverted microscope (IM, Zeiss, Germany), kept at 37°C and perifused with a solution containing (in mmol/l) 140 NaCl, 5.6 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 0.5 glucose and 10 HEPES, pH 7.4. A flowing KCl electrode served as a reference and appropriate corrections for liquid junction voltages were made. The patch clamp pipettes (Clark-Medical, Reading, UK) with an input resistance of 4–6 MΩ were coated with beeswax to keep pipette capacitance (C_p) low and constant. They were filled with a solution containing (in mmol/l) 30 KCl, 95 K-gluconate, 1 $MgCl_2$, 1.2 NaH_2PO_4, 4.8 Na_2HPO_4, 5 Na_2ATP, 1 Na_3GTP, pH 7.2.

Two dual lockin amplifiers (U. Fröbe and R. Busche, this institute) generated sine wave voltages of 250 and 800 Hz both of an amplitude of ±12.5 mV. Phase shifts due to the setup were first compensated with the reference phases of both lockin amplifiers with the open pipette in immediate proximity of the cell. After a GΩ seal was obtained, C_p was compensated with C_{fast}. C_p (4.3 ± 0.04 pF, $n = 27$) varied maximally by 0.7 pF from one pipette to the next (mean 0.35 ± 0.05 pF, $n = 27$). Measurements were performed with $G_a > 30$ nS (51 ± 4 nS, $n = 24$ for INS-1 cells and 50 ± 3 nS, $n = 61$ for β-cells) in the voltage clamp mode. Values for G_a, G_m and C_m were continuously calculated by the computer and displayed on a pen recorder (Rikadenki, Freiburg, Germany). During the experiment membrane voltage (V_m) was directly measured by switching to the current clamp mode of the patch clamp amplifier (U. Fröbe and R. Busche, this institute) and was also displayed on the pen recorder. Basal C_m was 4.7 ± 0.6 pF ($n = 23$) for β-cells and 5.6 ± 0.28 pF ($n = 24$) for INS-1 cells. Membrane potential of β-cells and of INS-1 cells depolarized upon stimulation from −67 ± 2 mV ($n = 62$) to −40 ± 1.7 mV ($n = 45$) and from −63 ± 2.5 mV ($n = 43$) to −46 ± 2.6 mV ($n = 22$), respectively.

2.3. $[Ca^{2+}]_i$ measurements

For $[Ca^{2+}]_i$ measurements the pipette solution was supplemented with 500 μmol/l fura-2 acid. After direct access between the pipette and the cell had been established, diffusion of fura-2 into the cell occurred within 1–2 min. The fluorescence of the patched cell was monitored with a photomultiplier system [14] at excitation wavelengths of 340, 360 and 380 nm with a filter rotation rate of 10 per second. As a measure of $[Ca^{2+}]_i$ the fluorescence emission ratio at 340/380 nm excitation was calculated after subtraction of the autofluorescence as described previously [14].

2.4. Ca^{2+} influx measurements

Measurements of Ca^{2+} influx were performed simultaneously with C_m measurements as inward currents in the presence of 20 mmol/l TEA-HCl (tetraethylammoniumchloride) and a pipette solution con-

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taining (in mmol/l) 110 CsCl, 10 TEA-HCl, 1 $MgCl_2$, 5 HEPES, 10 NaCl, pH 7.2 (CsOH).

2.5. Statistical analysis

Data are presented as mean ± S.E.M. Student's paired *t*-test ($P < 0.05$) was used for statistical analysis.

3. Results

3.1. Stimulation of β-cells

We first examined whether C_m of isolated rat β-cells is increased by substances which stimulate insulin secretion. When C_m was monitored at a voltage clamp condition of −70 mV, C_m was stable or slowly declining. Often G_a slowly declined in parallel. To prevent changes of G_m tolbutamide was present in most experiments and the membrane voltage was clamped to −70 mV. Under these conditions G_m was very low and stable (near zero). Addition of 100 μmol/l tolbutamide itself did not change C_m ($n = 11$, not shown).

Stimulation of voltage-dependent Ca^{2+} influx by application of four successive depolarizing voltage pulses to 0 mV induced an increase in C_m of 100.4 ± 18 fF, $n = 24$. After stimulation of the cells with 16.7 mmol/l glucose, 50 μmol/l tolbutamide, 5 μmol/l forskolin and 100 μmol/l IBMX the voltage pulse induced increase in C_m was significantly higher (199.6 ± 25 fF, $n = 24$, cf. also Fig. 1A), reflecting the synergistic effect of forskolin, glucose and tolbutamide on secretion as has been demonstrated previously [11].

3.2. Inhibition of β-cells

To examine whether adrenaline inhibits increases in C_m we added 1 μmol/l adrenaline in the presence of 16.7 mmol/l glucose, 50 μmol/l tolbutamide, 5 μmol/l forskolin and 100 μmol/l IBMX. As shown in Fig. 1, adrenaline lowered the increase in C_m due to depolarizing voltage pulses by 50% (from 275 ± 27 fF to 133 ± 30 fF, $n = 6$). Pretreatment of the cells with 100 μg/l pertussis toxin prior to the experiment abolished the inhibitory effect of adrenaline, suggesting that a pertussis toxin sensitive G-protein mediates the effect of adrenaline on C_m (Fig. 2).

3.3. Stimulation of INS-1 cells

For comparison, measurements were also performed using insulin secreting INS-1 cells [15]. Under control conditions, application of depolarizing voltage pulses to 0 mV increased C_m by 73.9 ± 10.1 fF, $n = 21$. Like in rat β-cells, stimulation of the same cells by depolarizing voltage pulses in the presence of 16.7 mmol/l glucose, 50 μmol/l tolbutamide, 5 μmol/l forskolin and 100 μmol/l IBMX induced a significantly larger increase in C_m than under control conditions (125.7 ± 16.9 fF, $n = 21$).

3.4. Inhibition of INS-1 cells

Adrenaline, 1 μmol/l, in the presence of 16.7 mmol/l glucose, 50 μmol/l tolbutamide, 5 μmol/l forskolin and 100 μmol/l IBMX inhibited voltage pulse induced C_m increases by 50% in INS-1 cells (from 93 ± 22 fF to 41 ± 10 fF, $n = 7$, Fig. 3). Measurements of $[Ca^{2+}]_i$ in voltage clamped cells revealed that $[Ca^{2+}]_i$ was not significantly changed by substances added to the perifusion solution (Fig. 4A,B). Moreover, the integrated Ca^{2+} influx induced by one depolarizing voltage

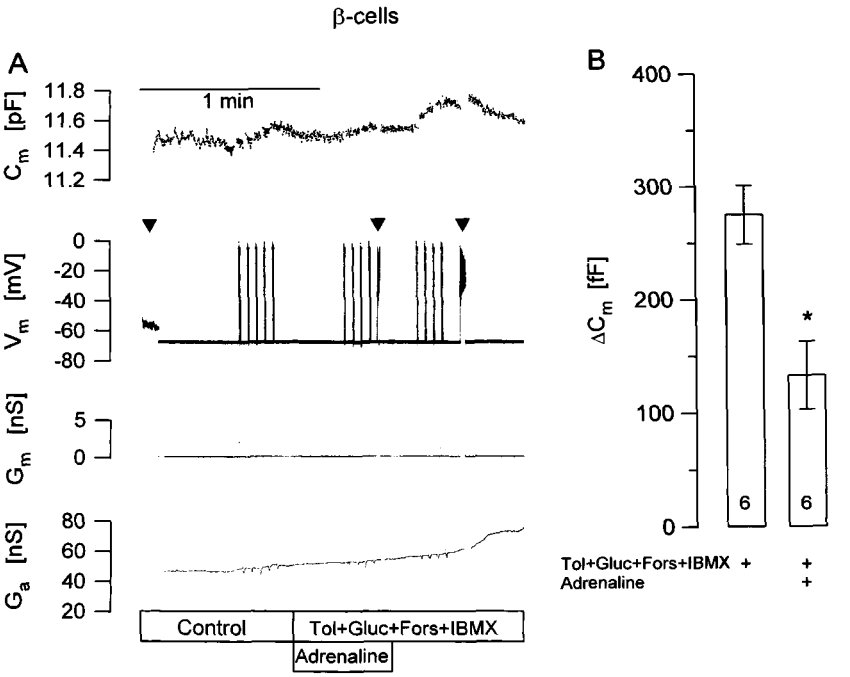

Fig. 1. Measurements of C_m, V_m, G_m and G_a in isolated rat β-cells. A: From top to bottom registrations of C_m, V_m, G_m and G_a are shown. In the beginning C_m of the cell was 11.45 pF. V_m was −60 mV (first arrowhead indicates V_m measurements at current clamp condition) and voltage was held at −70 mV at voltage clamp condition. Five or four successive depolarizing voltage pulses were applied firstly under control conditions (0.5 mmol/l glucose), secondly in the presence of 16.7 mmol/l glucose, 50 μmol/l tolbutamide, 5 μmol/l forskolin, 100 μmol/l IBMX and 1 μmol/l adrenaline and finally after withdrawal of adrenaline. G_m was stable and near zero nS, G_a was slowly increasing. Note that adrenaline attenuated the depolarization induced increase in C_m. In current clamp condition action potentials could be observed in the presence of stimuli (second and third arrowheads). B: Mean ± S.E.M. of the increase in C_m due to four successive voltage pulses from six experiments. Statistical significance is indicated by the asterisk.

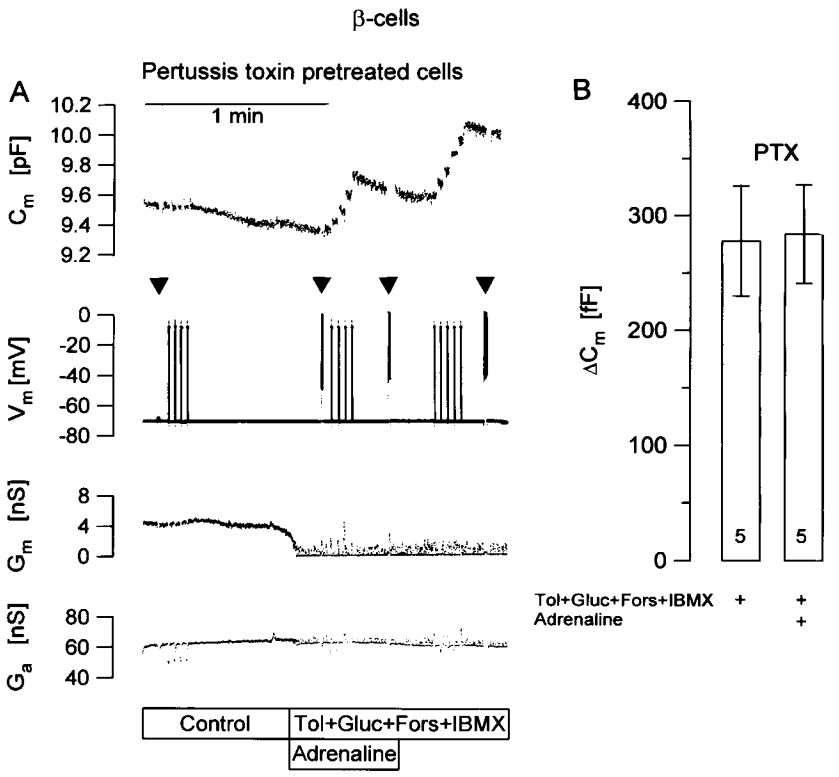

Fig. 2. Measurements of C_m, V_m, G_m and G_a in isolated rat β-cells pretreated with pertussis toxin. Pertussis toxin pretreatment (100 μg/l, 3–4 h) was performed prior to the experiment. A: From top to bottom registrations of C_m, V_m, G_m and G_a are shown. Initially C_m of the cell was 9.57 pF. V_m was −70 mV (first arrowhead indicates V_m measurements at current clamp condition). Voltage was held at −70 mV at voltage clamp condition. G_m was 4 nS under control condition and declined to 0 nS after addition of stimuli. G_a was stable. Voltage pulses and substances were applied as indicated in Fig. 1. Note that adrenaline had no effect on depolarization induced increases in C_m. Under stimulation action potantials were observed in current clamp condition (arrowheads). B: Mean ± S.E.M. of the increase in C_m due to four successive voltage pulses from five experiments.

pulse in the presence of stimuli was not significantly altered by the addition of adrenaline (108 ± 32% vs. 100 ± 29%, $n = 7$, in the presence and absence of adrenaline, respectively).

Thus, the adrenaline effect on C_m (Fig. 3) was not accompanied by changes of Ca^{2+} influx or of $[Ca^{2+}]_i$ (Fig. 4) indicating an action of adrenaline distal to the rise of $[Ca^{2+}]_i$.

4. Discussion

The present approach of combining zero current clamp measurement (V_m) and voltage clamp measurement (I_m) as well as fura-2 measurements enabled us to examine on the same cell simultaneously C_m, G_m, and V_m or V_m and $[Ca^{2+}]_i$ as well as the effects of stimulators and inhibitors of insulin secretion on these parameters. The standard whole cell configuration was used, since the access conductance obtained with amphotericin B permeabilized patches hardly exceeded 50 nS. A low access conductance, however, not only compromised stable C_m measurements but was also limiting for reliably clamping the membrane voltage of the cells, a prerequisite for standardized stimulation by depolarization from −70 mV to 0 mV. Increases in C_m varied substantially from cell to cell and from one stimulation to the next. In the standard whole cell mode 39% of β-cells and 44% of INS-1 cells did not respond to voltage pulses with any change in C_m (not shown). If responding cells were stimulated two or three times with four successive voltage pulses, half of the responding cells showed a declining responsiveness to depolarizing voltage pulses during the time course of the experiment. However, most of the responsive cells (85%) responded 2–4 times to stimulation. The increase in C_m was transient as it was slowly reversible. Stimulation of rat β-cells and INS-1 cells increased C_m by 275 ± 27 (Fig. 1) and 125.7 ± 16.9 fF, respectively, which may represent the readily fusable pool of secretory granules. The increase in C_m was larger in rat β-cells than in INS-1 cells. This difference may be explained by the 10 times fewer granules present in INS-1 cells compared to rat β-cells [13]. However, the commonly used insulin secreting cell line RINm5F has been shown to contain even 100 times fewer insulin secretory granules than normal rat β-cells [16]. Thus, the expected maximal increase in C_m due to fusion of the entire pool of insulin containing granules of RINm5F cells will most likely not exceed 100 fF. Although qualitatively similar results to those reported here have been shown in a study using RINm5F cells, the C_m changes in RINm5F cells exceeded 1 pF and can, thus, probably not account for exocytosis of insulin granules [17]. This makes the INS-1 cells more suitable for such studies. Our results are in the expected range and confirm studies on mouse β-cells [2].

There are several lines of evidence that changes in C_m reflect changes in insulin secretion. Firstly, during stimulation of insulin secretion membrane capacitance C_m increased in β-cells and in INS-1 cells. Thus, increases in $[Ca^{2+}]_i$ by action potentials or by depolarizing voltage pulses were accompanied by increases in C_m (cf. Figs. 1–3). Addition of EGTA completely abolished the depolarization induced augmentation of

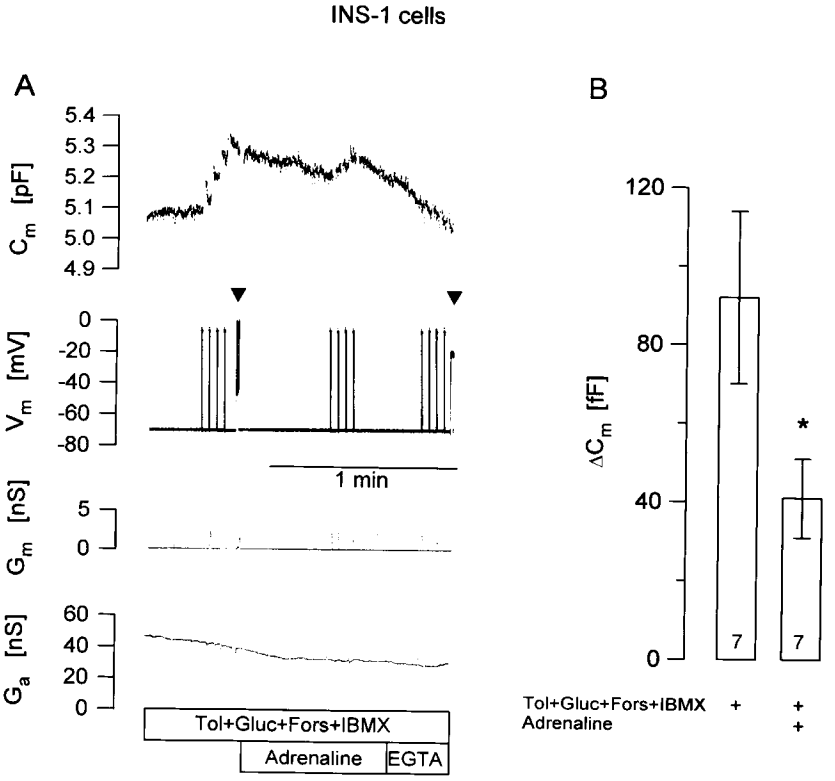

Fig. 3. Measurements of C_m, V_m, G_m and G_a in INS-1 cells. A: From top to bottom registrations of C_m, V_m, G_m and G_a are shown. In the beginning, C_m of the cell was 5.08 pF. Voltage was held at −70 mV. G_m was 0 nS. G_a decreased from 48 to 30 nS. Arrowheads indicate measurements of V_m (zero current clamp). Four successive depolarizing voltage pulses were given during perifusion of the cells with substances as indicated (see also Fig. 1). The concentration of EGTA was 5 mmol/l. Note that adrenaline attenuated and EGTA inhibited depolarization induced increases in C_m. B: Mean ± S.E.M. of the increase in C_m due to four successive voltage pulses from seven independent experiments. Asterisk indicates statistical significance.

C_m and the rise of $[Ca^{2+}]_i$ indicating that Ca^{2+} influx was the crucial event for stimulation of exocytosis (cf. Fig. 3). Secondly, the increase in C_m due to depolarizing voltage pulses was twice as big under stimulatory as under control conditions both for rat β-cells and for INS-1 cells. We have previously shown that forskolin potentiates secretion 3–4-fold in INS-1 cells [18]. Since forskolin depolarized INS-1 cells and increased the frequency of action potentials, at least some of its effect may be due to an elevation of $[Ca^{2+}]_i$. However, in voltage clamped INS-1 cells, the elevation of $[Ca^{2+}]_i$ due to voltage pulses is not augmented by forskolin (not shown) indicating a Ca^{2+}-independent action on secretion. Thirdly, adrenaline, an inhibitor of secretion, diminished the increase in C_m.

We previously observed that adrenaline almost abolished the insulin secretion induced by glucose, tolbutamide and forskolin, repolarized the cells, inhibited action potentials [19] and transiently lowered $[Ca^{2+}]_i$ (unpublished observation). In RINm5F cells a direct inhibitory effect of adrenaline on

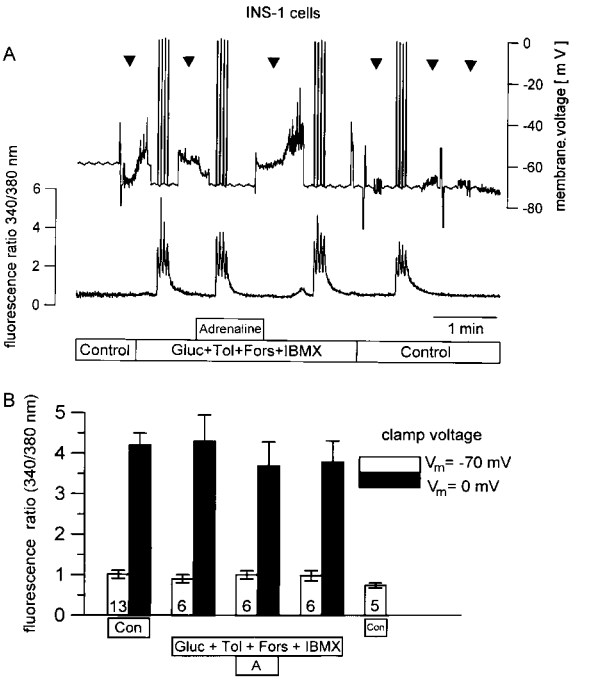

Fig. 4. Measurements of V_m and cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) as indicated by the fluorescence ratio in INS-1 cells. A. V_m (upper trace) was −70 mV under control condition (arrowheads indicate V_m measurements at current clamp condition). Addition of glucose, tolbutamide, forskolin and IBMX depolarized the cell to −55 mV (first and third arrowheads). $[Ca^{2+}]_i$ (lower trace) was stable at the clamp voltage of −70 mV. Depolarization by voltage pulses induced transient increases in $[Ca^{2+}]_i$. Substances were added as indicated (see also Fig. 1). Note that the depolarization induced $[Ca^{2+}]_i$ increases were unaltered by the addition both of stimulators and of the inhibitor adrenaline. B: Mean ± S.E.M. of maximal increase in $[Ca^{2+}]_i$ due to voltage pulses of six independent experiments.

dihydropyridine sensitive Ca^{2+} channels has been demonstrated [20]. In contrast, in the present report the large increases in $[Ca^{2+}]_i$ due to depolarizing voltage pulses were not lowered by adrenaline. In accordance to this adrenaline did not inhibit depolarization induced Ca^{2+} influx. Thus, the inhibitory effect of adrenaline on C_m occurred in the presence of elevated $[Ca^{2+}]_i$ indicating an action of adrenaline distal to the rise in $[Ca^{2+}]_i$.

The present experiments demonstrate that the simultaneous monitoring of the dynamic changes of G_m, G_a, C_m and V_m is a powerful approach to evaluate changes of C_m and hence exocytosis. It is shown that adrenaline attenuates exocytosis at a step distal to $[Ca^{2+}]_i$ also in INS-1 cells.

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