

# Extracellular ATP Stimulates Exocytosis via Localized $\text{Ca}^{2+}$ Release from Acidic Stores in Rat Pancreatic $\beta$ Cells

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**Three different methods, membrane capacitance ( $C_m$ ) measurement, amperometry and FM dye labeling were used to investigate the role of extracellular ATP in insulin secretion from rat pancreatic  $\beta$  cells. We found that extracellular application of ATP mobilized intracellular  $\text{Ca}^{2+}$  stores and synchronously triggered vigorous exocytosis. No influence of ATP on the readily releasable pool of vesicles was observed, which argues against a direct modulation of the secretory machinery at a level downstream of  $\text{Ca}^{2+}$  elevation. The stimulatory effects of ATP were greatly reduced by intracellular perfusion of BAPTA but not EGTA, suggesting a close spatial association of fusion sites with intracellular  $\text{Ca}^{2+}$  releasing sites. ATP-induced  $\text{Ca}^{2+}$  transients and exocytosis were not blocked by thapsigargin (TG), by a ryanodine receptor antagonist or by dissipation of pH in acidic stores by monensin alone, but they were greatly attenuated by  $\text{IP}_3$  receptor inhibition as well as ionomycin plus monensin, suggesting involvement of  $\text{IP}_3$ -sensitive acidic  $\text{Ca}^{2+}$  stores. Taken together, our data suggest that extracellular ATP triggers exocytosis by mobilizing spatially limited acidic  $\text{Ca}^{2+}$  stores through  $\text{IP}_3$  receptors. This mechanism may explain how insulin secretion from the pancreas is coordinated through diffusible ATP that is co-released with insulin.**

**Key words:**  $\text{Ca}^{2+}$  stores, caged  $\text{Ca}^{2+}$ , exocytosis, extracellular ATP, insulin secretion

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Extracellular ATP acts as a signaling molecule that regulates a variety of cellular responses through binding to specific G protein coupling receptors –  $\text{P}_2$  purinoceptors. In pancreatic  $\beta$  cells, ATP is present in insulin-containing granules and is co-released with insulin to the extracellular environment. Released ATP in turn regulates insulin release (1,2), suggesting an autocrine or paracrine role of

ATP in insulin secretion. Extracellular ATP application is believed to act on plasma membrane  $\text{P}_2$  purinoceptors (3,4) and activate phospholipase C, mobilize intracellular  $\text{Ca}^{2+}$  release via  $\text{IP}_3$  generation and subsequently modulate insulin secretion (5,6).

The role of ATP on insulin secretion has been controversial. Extracellular ATP inhibits insulin secretion either by decreasing electrical activity (7) or by activating G protein-dependent serine/threonine protein phosphatase calcineurin in mouse pancreatic  $\beta$  cells (8). In rat pancreatic  $\beta$  cells, ATP increased  $\text{Ca}^{2+}$  release from thapsigargin (TG)-sensitive stores and inhibited action potential by  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  channel inactivation (9). However, as exocytosis was inhibited by ATP much more severely than  $\text{Ca}^{2+}$  influx was (8), it was postulated that the suppressive action of ATP could be due to a mechanism downstream of  $\text{Ca}^{2+}$  influx. Despite the abundance of reports on inhibitory effect of ATP, a stimulatory effect has also been reported throughout the literature. In rat pancreas, extracellular ATP application stimulated insulin secretion (10). ATP was also shown to evoke  $\text{Ca}^{2+}$ -dependent insulin secretion via facilitated  $\text{Ca}^{2+}$  influx and intracellular  $\text{Ca}^{2+}$  mobilization (11). Furthermore, ATP-induced  $\text{Ca}^{2+}$ -independent insulin secretion via direct triggering exocytosis in RINm5F cells (6). To resolve these discrepancies and better identify processes underlying ATP action on insulin secretion, we used a variety of electrophysiological methods to study the effects of ATP on insulin secretion in rat pancreatic  $\beta$  cells. We found that extracellular ATP application triggered transient  $\text{Ca}^{2+}$  elevation, which is not abolished by emptying endoplasmic reticulum (ER) stores alone but inhibited completely by the combined depletion of ER and acidic  $\text{Ca}^{2+}$  stores. ATP application also induced insulin secretion in these cells as confirmed by capacitance measurements, FM1-43 dye labeling and amperometry. This stimulatory effect is abolished by disruption of acidic  $\text{Ca}^{2+}$  pools by ionomycin and monensin. Therefore, our results suggest that ATP promptly triggers vigorous exocytosis in pancreatic  $\beta$  cells via localized  $\text{Ca}^{2+}$  release from acidic  $\text{Ca}^{2+}$  stores near exocytotic sites.

## Results

### **Extracellular ATP elevated $[\text{Ca}^{2+}]_i$**

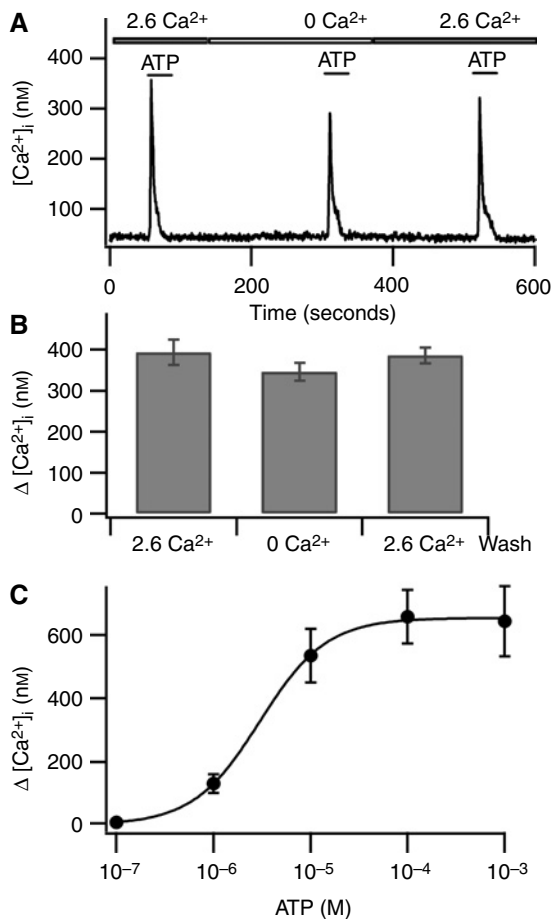
Extracellular application of ATP (0.1 mM) evoked a transient  $[\text{Ca}^{2+}]_i$  increase in normal bath solution containing 2.6 mM  $\text{Ca}^{2+}$  from intact pancreatic  $\beta$  cells as revealed by single cell microfluorometry (Figure 1). The average amplitude of  $[\text{Ca}^{2+}]_i$  elevation ( $\Delta[\text{Ca}^{2+}]_i$ ) was calculated to be  $393 \pm 31$  nM

( $n = 6$ ). As shown in Figure 1, removal of  $\text{Ca}^{2+}$  from the bath solution failed to affect ATP-evoked  $[\text{Ca}^{2+}]_i$  elevation significantly ( $386 \pm 19 \text{ nM}$ ), suggesting that the  $[\text{Ca}^{2+}]_i$  elevation was mainly due to  $\text{Ca}^{2+}$  release from intracellular stores.

The stimulatory effect of ATP is dose dependent. In Figure 1C, we plotted the average amplitudes of  $[\text{Ca}^{2+}]_i$  elevation against the concentration of ATP and fitted the curve with the Hill equation. The fit reveals an  $\text{EC}_{50}$  of ATP concentration at  $301 \text{ nM}$  and a Hill coefficient of 1.27. The maximal ATP effect is reached at concentrations =  $100 \mu\text{M}$ .

#### ATP-induced exocytosis from pancreatic $\beta$ cells

$[\text{Ca}^{2+}]_i$  elevation is directly coupled to insulin secretion. To examine whether the ATP-triggered  $\text{Ca}^{2+}$  mobilization



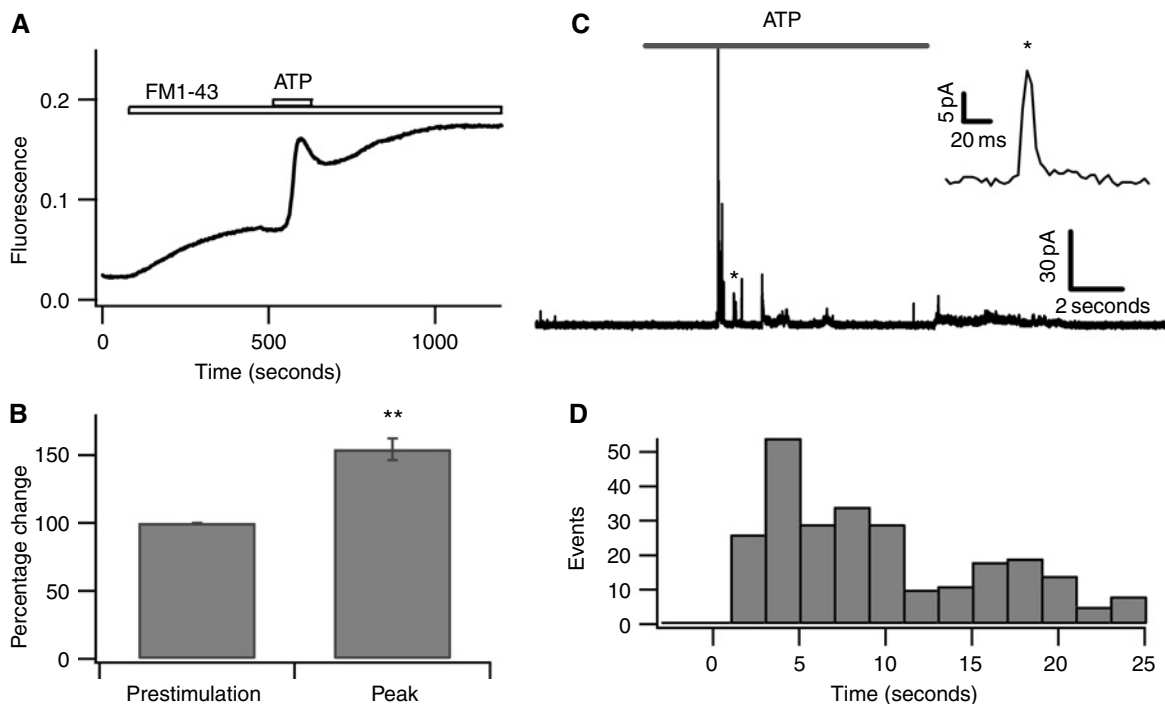
**Figure 1: ATP-induced  $[\text{Ca}^{2+}]_i$  elevation in pancreatic  $\beta$  cells.**

(A) Perfusion of ATP ( $0.1 \text{ mM}$ ) triggered transient  $[\text{Ca}^{2+}]_i$  elevation in the bath solution containing  $2.6 \text{ mM}$   $\text{Ca}^{2+}$  and  $0 \text{ Ca}^{2+}$ . (B) Summary of  $\Delta[\text{Ca}^{2+}]_i$  evoked by ATP application in  $2.6 \text{ mM}$   $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$ -free bath solution, respectively. Data are presented as mean values  $\pm$ SEM from six cells. There is no significant difference between the  $[\text{Ca}^{2+}]_i$  increase evoked by ATP in bath solution containing  $2.6 \text{ mM}$   $\text{Ca}^{2+}$  or  $0 \text{ Ca}^{2+}$ . (C) Dose-response curve of  $\Delta[\text{Ca}^{2+}]_i$  induced by different concentration of ATP. Each data point represents average of 10–15 samples. We estimated the  $\text{EC}_{50}$  to be  $301 \pm 37 \text{ nM}$  and the Hill coefficient to be  $1.27 \pm 0.12$ .

affected exocytosis, we simultaneously recorded the  $C_m$  and  $[\text{Ca}^{2+}]_i$  traces by combining whole cell capacitance recordings with intracellular  $\text{Ca}^{2+}$  measurements using fura-2. When  $0.1 \text{ mM}$  ATP was applied locally, a significant increase in  $C_m$ , synchronized with the elevation of  $[\text{Ca}^{2+}]_i$  in single rat  $\beta$  cells was observed (data not shown). The ATP-induced  $C_m$  increment ( $\Delta C_m$ ) was not significantly different in either normal or  $\text{Ca}^{2+}$ -free bath solution (see Figure 4), with an average amplitude of  $350 \pm 38 \text{ fF}$  ( $n = 8$ ) and  $338 \pm 16 \text{ fF}$  ( $n = 12$ ), respectively. This result suggests that extracellular application of ATP stimulates exocytosis mainly by releasing  $\text{Ca}^{2+}$  from intracellular  $\text{Ca}^{2+}$  stores.

Whole cell capacitance recording measures the net change in cell membrane surface area. Therefore, it cannot separate exocytosis from endocytosis when they occur simultaneously. We further verified our hypothesis by using two other different methods, FM1-43 and amperometry, to measure ATP evoked exocytosis in these cells. FM dye cannot diffuse through membranes and selectively stains the external leaflet of the lipid bilayer when applied via bath solution. Upon insertion into the lipid bilayer, the quantum yield of FM1-43 increases  $\sim 350$  times. Thus FM1-43 staining has been successfully implemented in monitoring granular exocytosis because the fluorescence of FM 1–43 is proportional to the total amount of bath-accessible cell membrane (12,13). An example of FM1-43 fluorescence measurement from a pancreatic  $\beta$  cell in  $\text{Ca}^{2+}$ -free bath solution is shown in Figure 2A. Upon perfusing FM1-43, we observed a first phase of increase in FM1-43 fluorescence, which is indicative of the insertion of the extracellular dye into the plasma membrane. After the fluorescence reached a plateau, application of  $0.1 \text{ mM}$  ATP elicited a further increase, which should be proportional to the increase of cell membrane area due to vesicle fusion. Figure 2B summarizes the effect of ATP on the fluorescence change. The fluorescence intensity was normalized to the base value immediately prior to the ATP application. ATP stimulation induces a  $54 \pm 8\%$  increase ( $n = 13$ ). The amount of exocytosis measured with FM1-43 fluorescence was larger when compared with that of  $C_m$ , probably reflecting that the concurrently occurred endocytosis may underestimate the amount of exocytosis assayed in  $C_m$  measurement (14,15).

Moreover, using amperometry we directly measured co-released serotonin from insulin-containing granule in pancreatic  $\beta$  cells artificially preloaded with serotonin. It has been demonstrated that pancreatic  $\beta$  cells from a variety of species, take up serotonin selectively as compared to other islet cells. They sequester serotonin into granules, and then secrete serotonin along with insulin when exposed to insulin secretagogues like glucose (16–18). A comparison study on the dynamics of insulin secretion detected with chemically modified electrodes versus those using normal carbon fiber electrode to detect serotonin secretion demonstrates that these two



**Figure 2: ATP-triggered exocytosis in pancreatic  $\beta$  cells.** (A) The cell was exposed to  $2 \mu\text{M}$  FM1-43 in  $\text{Ca}^{2+}$ -free bath solution. The initial increase in fluorescence intensity after application of the dye reflected staining of surface-accessible plasma membrane. Addition of  $0.1 \text{ mM}$  ATP-induced significant increase in fluorescence intensity, suggesting vesicle fusion to the plasma membrane. (B) The fluorescence intensity at 1 second before the application of ATP was set to 1. The fluorescence intensity after ATP application was normalized accordingly. ATP administration induced 50% increase in the FM1-43 fluorescence in rat pancreatic  $\beta$  cells. (C) Following ATP ( $0.1 \text{ mM}$ ) application to the  $\beta$  cell in  $\text{Ca}^{2+}$ -free bath solution, spikes of 5-HT oxidation current were detected using a carbon fiber electrode with voltage held at  $+650 \text{ mV}$ . The insert showed an enlarged single spike. (D) Time histogram of ATP-evoked exocytosis events. Exocytosis peaked at 4–5 seconds after perfusion of ATP.

amperometry methods agree well with each other (19). As the representative trace in Figure 2C shows, numerous amperometric spikes were detected upon perfusion of ATP ( $0.1 \text{ mM}$ ) in  $0\text{-Ca}^{2+}$  bath solution. We plotted the latency histogram of release events in Figure 2D, revealing that the rate of insulin secretion peaked at 4–5 seconds after ATP perfusion. Based on these experiments, we confirmed that extracellular application of ATP did induce insulin secretion in rat pancreatic  $\beta$  cells.

#### **ATP exerts no effect on the kinetics of secretion and the size of RRP**

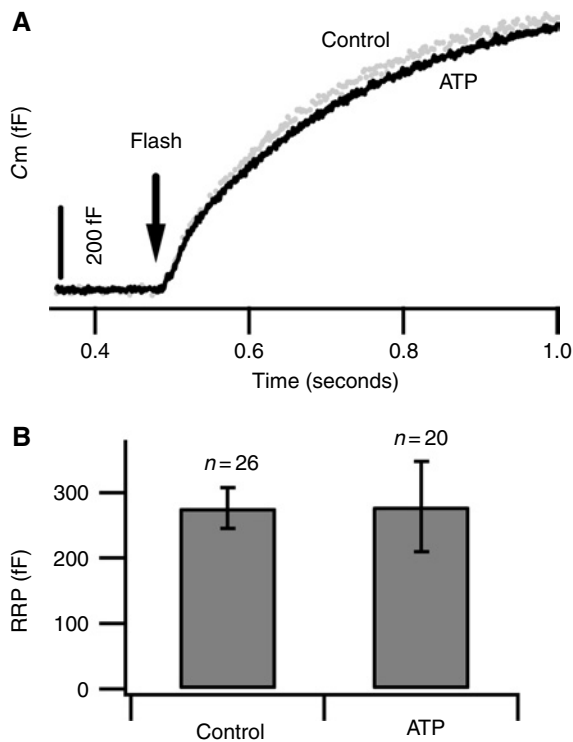
Sometimes we observed robust exocytosis without significant  $[\text{Ca}^{2+}]_i$  elevation in our whole cell experiments (data not shown). Therefore, it remains to be determined whether there exists ATP-triggered  $\text{Ca}^{2+}$ -independent exocytosis as previously suggested (6), or if the local  $[\text{Ca}^{2+}]_i$  microdomain induced by ATP is too small to be detected in the averaged whole cell  $\text{Ca}^{2+}$  signals.

To explore the possibility of direct action of extracellular ATP on the secretory machinery, we took advantage of homogeneous elevation of  $[\text{Ca}^{2+}]_i$  by photorelease of caged  $\text{Ca}^{2+}$  and did high time-resolution capacitance measurements. In response to the step-like  $[\text{Ca}^{2+}]_i$  elevations generated by

flash photolysis, we observed two distinct phases of exocytosis: an initial, rapid phase, often termed 'exocytotic burst', which happens within a fraction of a second, and a slower, sustained phase on a time scale of several to 10 seconds. It is generally held that the exocytotic burst represents the release of a pool of vesicles that are 'readily releasable' (the readily releasable pool, RRP). The sustained phase of secretion has been thought to represent the release from a depot pool of vesicles that are in an early maturation stage at the onset of stimulation (20). We compared the averaged  $C_m$  traces from control ( $n = 26$ ) and ATP-treated cells ( $n = 20$ ) responding to similar step-like  $[\text{Ca}^{2+}]_i$  elevations (Figure 3A). The similarity of the kinetics of the exocytotic burst implies that the secretory machinery of exocytosis is not modulated by ATP treatment. Furthermore, the size of the RRP was estimated to be  $278 \pm 68 \text{ fF}$  after ATP treatment, which is not significantly different from  $276 \pm 31 \text{ fF}$  for control cells (Figure 3B). These results indicate that neither the fusion machinery nor the size of RRP is the site of extracellular ATP action.

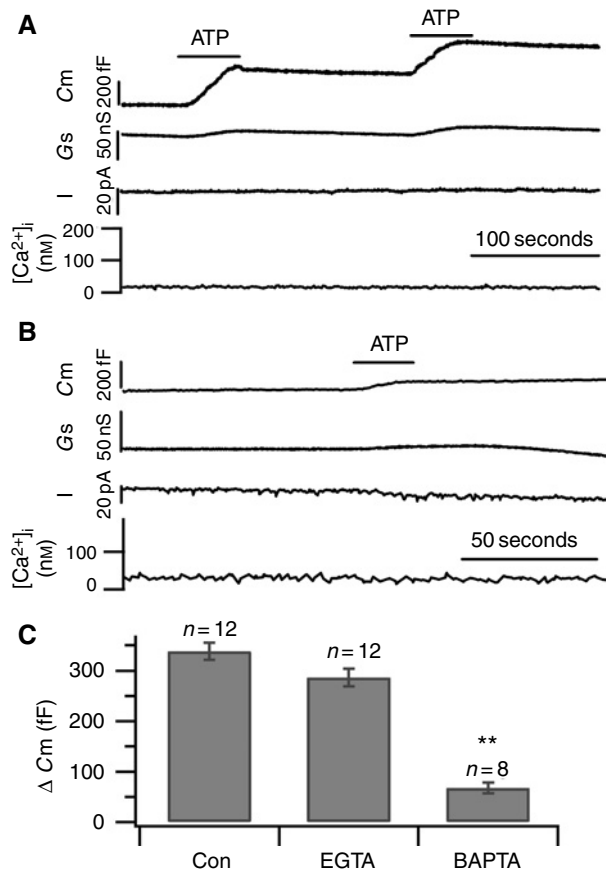
#### **ATP-induced exocytosis through localized $\text{Ca}^{2+}$ elevation**

To further address the possibility of ATP-evoked localized  $\text{Ca}^{2+}$  elevation, we perfused the cell interior via whole



**Figure 3: No alternation of exocytotic kinetics or the size of readily releasable pool by ATP.** (A) Averaged  $C_m$  traces from control (grey line) and ATP treated (black line) cells. Arrows indicates the time point of UV flashes. ATP was perfused for 2–3 min before the onset of the flashes. (B) Comparison of the size of RRP between the control ( $n = 26$ ) and ATP treated ( $n = 20$ ) cells. No significant effect of ATP was observed.

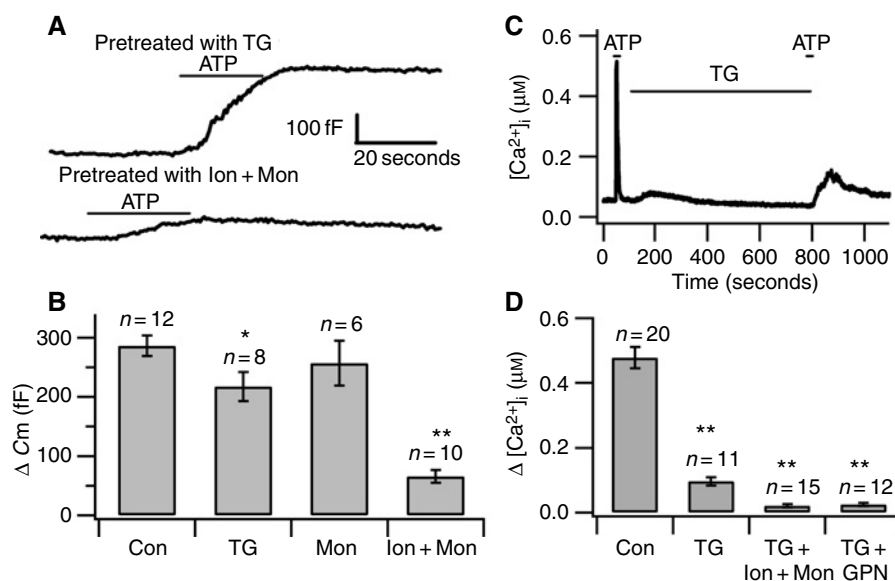
cell patch pipette with an EGTA or a BAPTA containing solution to chelate intracellular  $Ca^{2+}$  to a low level. When 10 mM EGTA was included in the pipette solution, ATP still induced significant  $C_m$  increase (Figure 4A). The ATP-evoked average increase in capacitance was  $286 \pm 17$  fF ( $n = 12$ ), which was slightly smaller than the value recorded from experiments with normal pipette solution containing 0.1 mM EGTA (Con, Figure 4C). This increase in  $C_m$  was not accompanied by concurrent changes in series conductance ( $G_s$ ) or membrane conductance (inferred from membrane current,  $I$ ) during the recording, suggesting that it indeed represented fusion of vesicles with the plasma membrane. Due to the slow on-rate of  $Ca^{2+}$  binding to EGTA, it is possible that EGTA is not fast enough to buffer  $Ca^{2+}$  in microdomains generated around  $Ca^{2+}$  release sites. Therefore, we used the much faster  $Ca^{2+}$  chelator, BAPTA. As shown in Figure 4B,C, ATP-induced  $C_m$  increase was attenuated significantly to a mean value of  $67 \pm 11$  fF ( $n = 8$ ) when 10 mM BAPTA was included in the pipette solution. Based on these results, we conclude that the majority of ATP-induced exocytosis is due to localized  $Ca^{2+}$  release near the vesicle fusion sites in rat pancreatic  $\beta$  cells.



**Figure 4: ATP-induced near granular  $Ca^{2+}$  triggered membrane capacitance increase.** ATP-induced increase in membrane capacitance in the absence of extracellular  $Ca^{2+}$ . Either 10 mM EGTA (A) or BAPTA (B) were included in the pipette solution. The individual traces are membrane capacitance ( $C_m$ ), series conductance of the equivalent circuit ( $G_s$ ), membrane current ( $I$ ) and  $[Ca^{2+}]_i$ , respectively. ATP applications were indicated by the black bars. ATP (0.1 mM) was applied 3 min after establishment of the whole-cell configuration. (C) Summary of the ATP-induced  $\Delta C_m$  in  $Ca^{2+}$ -free bath solution with pipette solution containing either 0.1 mM EGTA (Con), 10 mM EGTA (EGTA) or 10 mM BAPTA (BAPTA). Data were collected from 8 to 12 cells.

#### Acidic $Ca^{2+}$ pools participated in ATP-induced exocytosis

Next, we wanted to address the cellular location of this ATP-evoked localized  $Ca^{2+}$  release in  $\beta$  cells. Single  $\beta$  cells were preincubated for 15 min with TG (2  $\mu$ M), a selective non-reversible ER  $Ca^{2+}$ -ATPase (SERCA) inhibitor, to test whether the ER  $Ca^{2+}$  store is the target of ATP action. SERCA inhibition decreased but did not abolish the ATP-induced capacitance increase in  $Ca^{2+}$ -free bath solution. The averaged  $\Delta C_m$  in the presence of TG was  $217 \pm 24$  fF ( $n = 8$ ), only slightly smaller than that of control cells ( $286 \pm 17$  fF,  $n = 12$ ) (Figure 5A,B). On the other hand, ATP-induced  $[Ca^{2+}]_i$  transients were much more pronouncedly inhibited by the same TG treatment. As shown in Figure 5C,D, ATP-triggered  $\Delta[Ca^{2+}]_i$  from intact



**Figure 5: ATP-induced exocytosis and thapsigargin (TG)-insensitive  $\text{Ca}^{2+}$  elevation was blocked by depleting intracellular acidic compartment.** (A) ATP-induced increase in  $C_m$  persisted after TG treatment ( $2 \mu\text{M}$ ) in  $\text{Ca}^{2+}$ -free bath solution but inhibited by treatment of ionomycin ( $1 \mu\text{M}$ ) plus monensin ( $2 \mu\text{M}$ ). (B) Summary of ATP-induced increase in  $C_m$  in control  $\beta$  cells (Con), TG treated cells (TG), monensin ( $2 \mu\text{M}$ ) treated cells (Mon) and ionomycin ( $1 \mu\text{M}$ ) plus monensin ( $2 \mu\text{M}$ ) treated cells (Ion + Mon). (C) The effect of ATP on  $[\text{Ca}^{2+}]_i$  before and after treatment of TG ( $2 \mu\text{M}$ ) in  $\text{Ca}^{2+}$ -free bath solution. Treatment of TG for more than 10 min failed to completely block the ATP-induced  $[\text{Ca}^{2+}]_i$  elevation. (D) The ATP-induced, TG-insensitive  $[\text{Ca}^{2+}]_i$  increase was completely abolished by the treatment of ionomycin ( $1 \mu\text{M}$ ) plus monensin ( $2 \mu\text{M}$ ), as well as GPN ( $50 \mu\text{M}$ ).

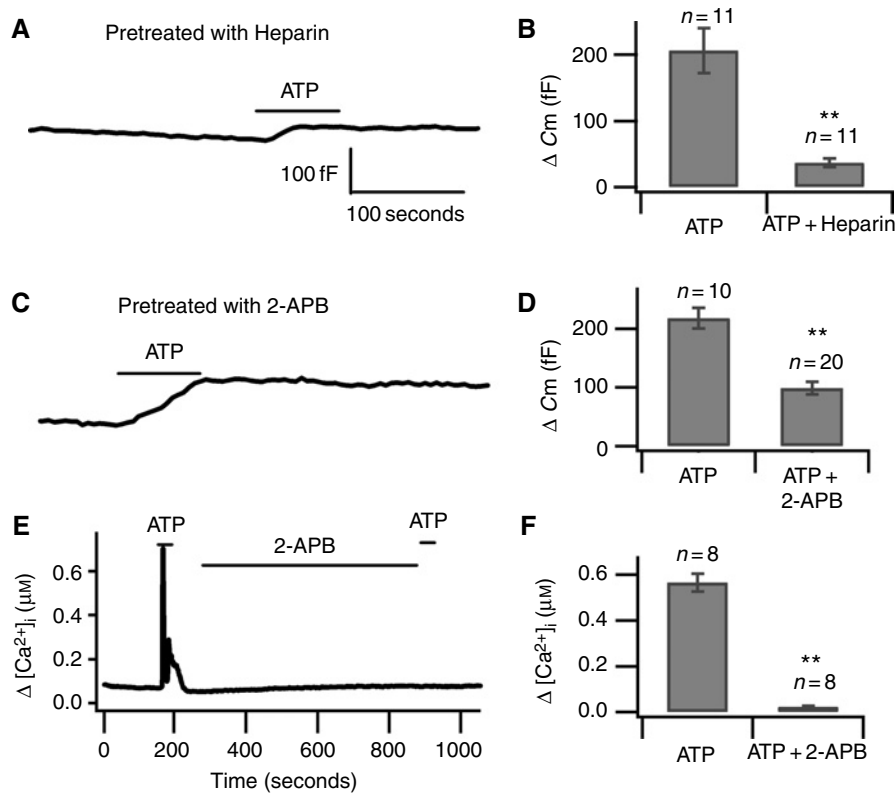
single  $\beta$  cell decreased from an averaged control value of  $477 \pm 32$  to  $96 \pm 13$  nM after pretreatment with TG for about 10 min, suggesting that 80% of the ATP-evoked  $[\text{Ca}^{2+}]_i$  elevation comes from the TG-sensitive ER store and the rest of the 20% comes from TG-insensitive one.

Because vesicles have been proposed to serve as a  $\text{Ca}^{2+}$  reservoir in a number of cell types (21–28), our next goal was to test whether atypical acidic  $\text{Ca}^{2+}$  stores such as secretory granules contribute to this TG-insensitive  $[\text{Ca}^{2+}]_i$  elevation and exocytosis induced by ATP. Monensin is a protonophore that neutralizes the pH of intracellular acidic organelles. However, application of monensin ( $2 \mu\text{M}$ ) alone failed to abolish ATP-induced exocytosis ( $257 \pm 38$  fF,  $n = 6$ ) in  $\beta$  cells (Figure 5B). When monensin ( $2 \mu\text{M}$ ) was perfused with ionomycin ( $1 \mu\text{M}$ ) for 10 min in the bath solution, ATP-triggered exocytosis was significantly inhibited to a mean value of  $66 \pm 11$  fF (Figure 5B;  $n = 10$ ). As ionomycin and monensin treatment has been shown to dissipate  $\text{Ca}^{2+}$  gradients from all organelles (29) including acidic organelles, we next tested whether they inhibited the ATP evoked TG-insensitive  $[\text{Ca}^{2+}]_i$  transient as well. Application of ionomycin and monensin completely abolished ATP evoked TG-insensitive  $\text{Ca}^{2+}$  transient, as summarized in Figure 5D. As ionomycin- and monensin-treated cells still respond to  $\text{Ca}^{2+}$  elevation with an increase in capacitance (data not shown), we believed those were live cells retaining intact fusion machinery after treatment. TG-insensitive  $[\text{Ca}^{2+}]_i$  increase was similarly inhibited when cells were perfused for 10 min with

TG and glycyphenylalanine 2-naphthylamide (GPN,  $50 \mu\text{M}$ ), another agent that selectively disrupts acidic organelles. In a separate study, we have shown that GPN pretreatment diminished NPY-EGFP labeled fluorescence puncta (representative of insulin-containing granule) in INS-1 cells and UV-flash evoked exocytosis in mouse pancreatic  $\beta$  cells (Duman et al. manuscript in review). Therefore, we propose this ATP-induced exocytosis may be mainly stimulated by localized  $\text{Ca}^{2+}$  release from TG-insensitive granule stores in these rat pancreatic  $\beta$  cells.

#### Participation of $\text{P}_2\text{Y}$ and $\text{IP}_3$ receptor in ATP-triggered exocytosis

Extracellular application of ATP activates membrane  $\text{P}_2\text{Y}$  receptor and generates  $\text{IP}_3$  to release  $\text{Ca}^{2+}$  from intracellular stores (5,30). In order to identify the signaling pathway underlying ATP effects, we preincubated cells with a  $\text{P}_2\text{Y}$  receptor blocker, suramin ( $100 \mu\text{M}$ ) for 30 min. This treatment decreased ATP-induced  $\Delta C_m$  to  $47 \pm 13$  fF ( $n = 11$ , data not shown), suggesting that the ATP stimulates exocytosis by activation of  $\text{P}_2\text{Y}$  receptors. Secretory granules have been identified as an  $\text{IP}_3$ -sensitive intracellular  $\text{Ca}^{2+}$  store in different types of cells (23,31,32). To test whether  $\text{IP}_3$  participates in the ATP-induced exocytosis and  $[\text{Ca}^{2+}]_i$  elevation in rat pancreatic  $\beta$  cells, we used two  $\text{IP}_3$  receptor blockers. First, with intracellular perfusion of heparin ( $1 \text{ mg/mL}$ ) via patch pipette, ATP-induced capacitance increase was almost completely abolished ( $\Delta C_m = 37 \pm 6$  fF,  $n = 11$ ), as shown in Figure 6A,B. Furthermore, when we preincubated cells with another



**Figure 6: IP<sub>3</sub> receptor blockers inhibited ATP evoked exocytosis and [Ca<sup>2+</sup>]<sub>i</sub> elevation in single rat pancreatic β cell.** (A) ATP-induced increase in C<sub>m</sub> was inhibited by intracellular perfusion with pipette solutions containing an IP<sub>3</sub> receptor blocker, heparin. (B) Summary of ATP-induced ΔC<sub>m</sub> in the absence and presence of heparin. (C) An ATP-induced increase in C<sub>m</sub> was also attenuated in cells pretreated with another cell permeable IP<sub>3</sub> inhibitor 2-APB (100 μM) for 15 min. (D) Summary of ATP-induced ΔC<sub>m</sub> in the absence and presence of 2-APB. (E) Perfusion of 2-APB abolished ATP-evoked [Ca<sup>2+</sup>]<sub>i</sub> elevation in a normal bath solution. (F) Summary of the ATP-induced increase in [Ca<sup>2+</sup>]<sub>i</sub> in control cells and cells pretreated with 2-APB.

cell membrane permeable IP<sub>3</sub> receptor blocker, 2-APB (100 μM) for 15 min, the ATP evoked exocytosis was again decreased to 99 ± 11.0 fF (n = 20) (Figure 6C,D). Single-cell [Ca<sup>2+</sup>]<sub>i</sub> measurement from intact cells also demonstrated that 2-APB treatment abolished [Ca<sup>2+</sup>]<sub>i</sub> elevation induced by ATP (Figure 6E,F). Therefore, we propose that the IP<sub>3</sub> receptors couple extracellular ATP stimulation to exocytosis in rat pancreatic β cells.

#### **No involvement of ryanodine receptor in ATP-induced exocytosis**

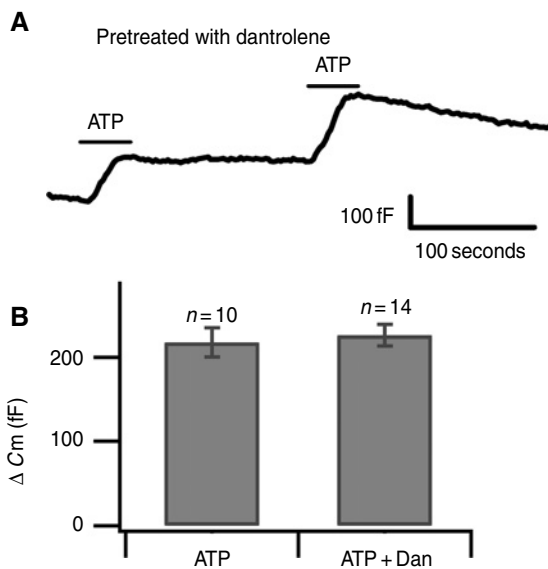
A previous report demonstrated that ryanodine receptor type I (RyR I) is present on insulin-containing secretory vesicles in MIN6 cells (26). However, when rat β cells were pretreated for 20 min with dantrolene (10 μM), an inhibitor of RyR I and III channels (33), ATP-induced exocytosis was not affected (Figure 7A). As summarized in Figure 7B, C<sub>m</sub> increments were similar in both control cells (218 ± 17 fF, n = 10) and cells treated with dantrolene (226 ± 13 fF, n = 14).

Caffeine, an agonist of ryanodine receptors, also triggered substantial exocytosis (394 ± 46 fF, n = 10) from rat pancreatic β cells at a concentration of 10 mM as well

(Figure 8A). However, we found that the caffeine-induced exocytosis was sensitive to ER depletion. Preincubation with TG largely abolished the stimulatory effect of caffeine on secretion, leaving a remaining ΔC<sub>m</sub> of 59 ± 9 fF (n = 8, Figure 8A,B), in contrast to the large TG-resistant component of exocytosis induced by ATP. [Ca<sup>2+</sup>]<sub>i</sub> transients induced by caffeine in Ca<sup>2+</sup>-free bath solution were also completely abolished by TG (Figure 8C,D). These results hint that ryanodine receptors are unlikely to be the target of ATP action in rat pancreatic β cell.

## **Discussion**

ATP co-localizes with insulin in pancreatic β cells and is co-released with insulin (1,2). An autocrine or paracrine role for ATP in regulating insulin secretion has been proposed. However, the exact role of extracellular ATP on insulin release is controversial in the literature. Extracellular ATP has been proposed to inhibit insulin release by decreasing electrical activity (7) or by inhibiting Ca<sup>2+</sup> influx through activation of specific purinoceptors (8). On the other hand, it was also reported that exogenous ATP stimulates insulin secretion by acting on specific P<sub>2</sub> receptors in rat (10) and

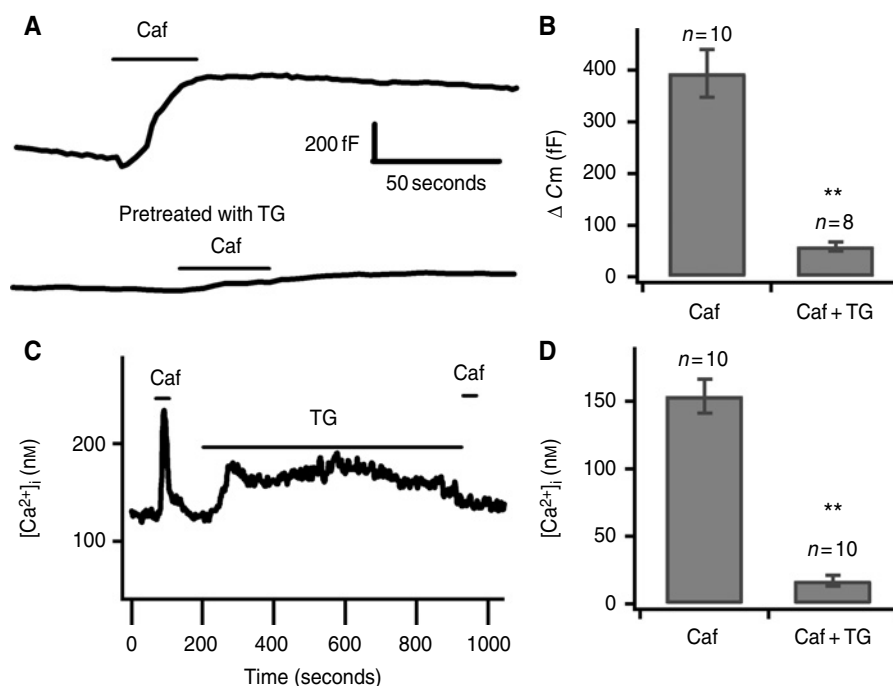


**Figure 7: Dantrolene did not block ATP-induced exocytosis in single rat pancreatic  $\beta$  cell.** (A) Pancreatic  $\beta$  cells were pretreated with ryanodine receptor blocker, dantrolene (10  $\mu$ M) for 20 min. Following application of ATP, a similar increase in membrane capacitance was observed as compared with that of control cells. (B) Summary of  $\Delta C_m$  in control (Con) and dantrolene-pretreated (Dan) cells. Data were presented as mean values  $\pm$ SEM and were collected from 10 and 14 cells, respectively.

human (34) pancreatic islets. Recently, ATP has also been proposed to act as a diffusible messenger to coordinate the rhythmic characteristics of insulin release by generating synchronized  $[Ca^{2+}]_i$  oscillations in  $\beta$  cells within and among the islets in the pancreas (35). Using rat  $\beta$  cells as

our cell model, we explored the role of extracellular ATP on insulin secretion using different methods. We confirmed that ATP slightly blocked the depolarization-induced  $Ca^{2+}$  current (data not shown) as previously described (9). However, ATP inhibits depolarization-induced exocytosis much more than its effects on the  $Ca^{2+}$  influx pathway, and it was postulated that the inhibitory effect of ATP could be due to an additional direct mechanism downstream of  $Ca^{2+}$  influx (9). On the other hand, a direct triggering of exocytosis by ATP independent of  $Ca^{2+}$  had also been proposed (6). We decided to approach this problem and solve the discrepancy by uniformly elevating  $[Ca^{2+}]_i$  via photolysis of caged- $Ca^{2+}$  and analyzing the dynamic of granules fusion by using membrane capacitance measurement. Our results suggested that neither the size of the RRP nor the kinetics of exocytosis is influenced by ATP treatment, arguing against a direct role of ATP on exocytosis at a level downstream of  $Ca^{2+}$  influx.

On the other hand, we confirmed that extracellular ATP-induced elevation of  $[Ca^{2+}]_i$  via mobilization of intracellular  $Ca^{2+}$  in rat pancreatic  $\beta$  cells. These  $[Ca^{2+}]_i$  transients may then trigger pulses of insulin secretion. Sometimes we also observed an ATP-evoked large  $C_m$  increase in the absence of obvious global  $[Ca^{2+}]_i$  elevation, similar to the so-called  $Ca^{2+}$ -independent stimulation of insulin secretion described previously (6). As the direct modulation of exocytosis by ATP is ruled out, we asked whether there might be localized  $Ca^{2+}$  microdomains proximal to the release sites that are globally undetected. To address this possibility, we dialyzed cells intracellularly with different  $Ca^{2+}$  chelators, EGTA and BAPTA. We find that both



**Figure 8: Caffeine-induced exocytosis and  $[Ca^{2+}]_i$  elevation were abolished by thapsigargin (TG) treatment.** (A) Caffeine (Caf) induced increase in  $C_m$  was abolished by pretreatment of TG (2  $\mu$ M). (B) Summary of caffeine-induced  $\Delta C_m$  in the control and TG-pretreated pancreatic  $\beta$  cells. (C) Caffeine-induced increase in  $[Ca^{2+}]_i$  was completely blocked by pretreatment of TG. (D) Summary of caffeine-induced  $[Ca^{2+}]_i$  elevation in control and TG-pretreated pancreatic  $\beta$  cells.

EGTA and BAPTA we are effective in preventing global  $[Ca^{2+}]_i$  elevation induced by ATP, but only BAPTA-dialyzed cells exhibited a suppressed capacitance increase. As BAPTA binds to  $Ca^{2+}$  150 times faster than EGTA (21), it is much more potent in sequestering local  $Ca^{2+}$  elevations than EGTA. Therefore, our data suggest close spatial colocalization of ATP-triggered intracellular  $Ca^{2+}$  release sites with granule fusion sites.

Local  $Ca^{2+}$  microdomains have been suggested in a variety of cell types and are important in regulating the kinetics of exocytosis. Flux through voltage-gated  $Ca^{2+}$  channels set up  $[Ca^{2+}]_i$  gradients both in nerve terminals (36,37) and in chromaffin cells (38), such that  $[Ca^{2+}]_i$  near the vesicle exocytotic sites is higher than elsewhere in the cytosol. In an elegant experiment,  $Ca^{2+}$  microdomains were able to selectively trigger the release of vesicles that were docked within 300 nm of the plasma membrane and decrease the distance between the docked vesicles and  $Ca^{2+}$  entry sites (39).  $Ca^{2+}$  release from intracellular stores has also been suggested to couple with exocytosis. In pituitary corticotropes extracellular  $Ca^{2+}$  entry and intracellular  $Ca^{2+}$  release generated a local  $[Ca^{2+}]_i$  gradient near the secretory granules and has similar efficacy in triggering exocytosis (40). In pituitary gonadotropes, intracellular  $Ca^{2+}$  stores were shown to be tightly coupled with exocytotic sites (41).

It is generally assumed that ATP-induced  $Ca^{2+}$  signals are mainly generated through mobilization from TG-sensitive  $Ca^{2+}$  stores (8,42). To our surprise, we found that the depletion of ER stores with TG fails to block the exocytosis induced by ATP in rat pancreatic  $\beta$  cells. Consistent with this result, TG pretreatment largely suppresses but does not completely abolish the ATP-induced  $[Ca^{2+}]_i$  elevation, suggesting that the TG-insensitive  $Ca^{2+}$  stores participate in the localized triggering of exocytosis.

Besides traditional ER  $Ca^{2+}$  stores, a variety of intracellular compartments have recently been proposed to serve as the  $Ca^{2+}$  store in many kinds of cells, such as the lysosome in sea urchin eggs (22), bovine corneal endothelium (43) and macrophage (44), the Golgi apparatus in HeLa cell (45), the endosome in fibroblast (46), the nuclear envelope in pancreatic acinar cells (47) and  $\beta$  cells (27). Recent papers also propose secretory granules as an important  $Ca^{2+}$  store in chromaffin cells (25), mast cells (28), pancreatic acinar cells (23) and  $\beta$  cell lines (24,26). Indeed, it has been demonstrated that alkalization of the granule interior can cause release of granular  $Ca^{2+}$  in adrenal chromaffin cells and that this localized  $Ca^{2+}$  release is sufficient to initiate exocytosis (48). This kind of exocytosis has been shown to be independent of TG-treatment and external  $Ca^{2+}$ , but could be suppressed by BAPTA not EGTA (49), similar to the ATP-induced exocytosis described here. In our hands, the TG-insensitive exocytosis and  $[Ca^{2+}]_i$  elevation are also greatly reduced after application of ionomycin plus monensin, which dissipates the proton gradient and hence empties  $Ca^{2+}$  from acidic

cellular compartments. This result suggests a possible role for acidic  $Ca^{2+}$  stores in the ATP-evoked signaling pathway. Application of GPN also abolishes TG-insensitive residual  $[Ca^{2+}]_i$  elevation evoked by ATP (Figure 5D). Possible targets of this GPN action include ER, granules, lysosomes, endosomes and mitochondria. We have experiments which showed that GPN diminished the number of granules in insulin-secreting cells while leaving the ER  $Ca^{2+}$  handling ability intact (Duman et al. manuscript in review). Although endosomes also contain acid peptidases and proteases, they are not disrupted by GPN treatment probably due to a pH-dependence of this disruption process (50). Neither are mitochondria likely to be the major source of this TG-insensitive Ca transient because it has been demonstrated that mitochondria contribute very little to the  $Ca^{2+}$  signaling in mouse  $\beta$  cell (51). Although our data cannot exclude lysosomes as the putative  $Ca^{2+}$  stores, lysosomes comprise only 0.4% of the cell volume in  $\beta$  cells (52), whereas secretory granules occupy a much higher proportion of cell volume [11.5–15% (52,53)]. In addition, the highly co-localized nature of the  $Ca^{2+}$ -release and fusion sites prompted us to speculate that ATP may induce local  $Ca^{2+}$  release from insulin-containing granules themselves.

Another question concerning the stimulatory effects of ATP is to determine the intracellular messengers that mediate the release of  $Ca^{2+}$  store. External ATP stimulates generation of  $IP_3$  via activation of G-protein-coupled  $P_2Y$  receptors (5,30). Consistent with this idea, we showed that  $P_2Y$  receptor antagonist suramin effectively blocked the ATP-induced exocytosis, and we confirmed that the two  $IP_3$  receptor antagonist, heparin and 2-APB, inhibited the stimulatory effect of ATP. Mobilization of dense core granule  $Ca^{2+}$  by  $IP_3$  has been suggested in chromaffin cells (25,31), whereas the presence of  $IP_3$  receptors on vesicle membranes from other cells has been controversial.  $IP_3$  receptors were reported on granule membranes from insulin-secreting cells by immunolabeling (54) and from pancreatic acinar cells by cell fractionation (23). However, subsequent experiments either showed that the  $IP_3$  antibody used cross-reacted with insulin (55) or that ER contaminated the granule fraction used in the fractionation study (56), challenging the idea of  $IP_3$  receptors on the vesicle membrane. In contrast, using a granule-associated membrane protein aequorin to report vesicular  $[Ca^{2+}]_v$  ( $[Ca^{2+}]_v$ ), it was found that cytosolic  $IP_3$  accumulation in MIN6 cells induced no attenuation in  $[Ca^{2+}]_v$ , whereas ryanodine receptor activation with caffeine or 4-chloro-3-ethylphenol caused a dramatic fall in  $[Ca^{2+}]_v$  (26). Although RyR I and nicotinic acid adenine dinucleotide phosphate (NAADP) receptors have been demonstrated in the same MIN6 cells (24), the colocalization of ryanodine receptor and insulin in human  $\beta$  cells was challenged in a recent immunofluorescence study (57). In our hands, the ATP-induced exocytosis was not blocked by the ryanodine receptor antagonist dantrolene. Moreover, caffeine-induced exocytosis and  $[Ca^{2+}]_i$  signals



were sensitive to TG-pretreatment. Taken together, our data seems to suggest that IP<sub>3</sub> receptors but not ryanodine receptors participate in this localized Ca<sup>2+</sup> release from acidic Ca<sup>2+</sup> stores triggered by ATP in rat pancreatic  $\beta$  cells. However, our experiment did not rule out the possibility of other receptors like ryanodine or NAADP receptor locating on the granule membrane and participating in other physiological processes after stimulation.

Although localized intracellular Ca<sup>2+</sup> release has been proposed as an important route for stimulating exocytosis, the physiological relevance of this pathway remains largely unclear. In the current study, we have provided evidence that the physiological stimulation by ATP triggers Ca<sup>2+</sup> release from acidic Ca<sup>2+</sup> stores. This localized rise in [Ca<sup>2+</sup>]<sub>i</sub> is highly efficient in triggering the exocytosis from the docked readily releasable granules. Given the co-release of ATP with insulin, we propose that the ATP synchronizes  $\beta$  cells in pulsatile release of insulin from the pancreas via mobilizing Ca<sup>2+</sup> from insulin-containing granules.

## Materials and Methods

### Cell preparation and solutions

Primary cultures of pancreatic  $\beta$  cells were prepared as described (58). Briefly, pancreatic islets were isolated by collagenase digestion of the pancreas from male wistar rats. The islets were further digested by dispase II to dissociate single  $\beta$  cells. The dispersed  $\beta$  cells were plated on glass coverslips and kept in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal calf serum (Invitrogen), 100  $\mu$ g/mL penicillin and 100  $\mu$ g/mL streptomycin (Invitrogen).

The normal bath solution for experiments contained (in mM): 138 NaCl, 5.6 KCl, 1.2 MgCl<sub>2</sub>, 2.6 CaCl<sub>2</sub>, 5 D-glucose, 10 HEPES (pH 7.4 adjusted with NaOH). The Ca<sup>2+</sup> free external bath solution consisted of similar components, except that CaCl<sub>2</sub> was substituted by 2 mM EGTA. The cells were cultured on small square coverglass (roughly 5 × 5 mm) and were transferred to a recording chamber prior to each recording. Only one recording was performed on each coverglass, after which the coverglass was removed and the recording chamber was washed. Then we added fresh bath solution to the chamber and started a new recording.

The standard pipette solution contained (in mM): 125 Cs-Glutamate, 2 Mg-ATP, 0.3 Na<sub>2</sub>-GTP, 1 MgCl<sub>2</sub>, 0.1 EGTA and 0.1 fura-2 (pH 7.2 with CsOH). For flash experiments, the DM-nitrophen-containing pipette solution contained (in mM): 112 Cs-Glutamate, 5 DM-nitrophen, 4.7 CaCl<sub>2</sub>, 2 Mg-ATP, 0.3 Na<sub>2</sub>-GTP and 0.2 fura-6F. All experiments were performed at 32–33 °C on cells with diameter of 11–12  $\mu$ m. Conservatively, these cells have a >80–90% probability of being  $\beta$  cells (59). Frequent tests showed that these cells possessed K<sub>ATP</sub> channels and responded to high glucose with Ca<sup>2+</sup> elevation.

DM-nitrophen, fura-2 and fura-6F were purchased from Molecular Probes (Eugene, OR, USA). Unless otherwise stated, all reagents were purchased from Sigma (St Louis, MO, USA).

### Electrophysiology

Patch pipettes were produced with borosilicate glass capillaries, coated with Sylgard and heat-polished. The pipette resistance ranged between 3 and 4 MO when filled with intracellular solutions. An EPC-9 patch-clamp amplifier controlled by Pulse software (Heka Electronics, Lambrecht, Germany) was used. Capacitance measurements were performed using

the 'sine + DC' mode (60) of the software lock-in extension in the Pulse software. An 800 Hz, 20 mV peak to peak sinusoid voltage stimulus was applied to DC holding potential of –70 mV. The capacitance changes to flash photolysis were fitted with a double exponential function and the amplitude of the faster component was taken as the size of the RRP (20). Long-term capacitance measurements were recorded using the X-Chart plug-in module of the Pulse software.

### Amperometric recordings of catecholamine release

Amperometric recordings were performed with polypropylene insulated carbon fibers (Thornel T-300, diameter 7  $\mu$ m; Amoco Performance Products, Ridgefield, CT, USA); electrodes were held at +650 mV using an EPC-9 amplifier (HEKA, Lambrecht, Germany). Cells were preincubated in culture medium supplemented with oxidizable neurotransmitter serotonin (5-HT, 0.5 mM) and its precursor 5-OH tryptophan (1 mM) for 4–6 h (61). ATP-evoked co-release of serotonin from individual insulin-containing granules was detected by the electrode as spikes of oxidation current. Amperometric signals were filtered at 500 Hz and sampled at 1 kHz. Amperometric events were viewed and analyzed with Minianalyses (Synaptosft, Decatur, GA, USA).

### Photolysis of caged Ca<sup>2+</sup> and photometry

For photolysis of caged Ca<sup>2+</sup> and [Ca<sup>2+</sup>]<sub>i</sub> measurements, flashes of ultraviolet light and fluorescence-exciting light were generated as previously described (62). [Ca<sup>2+</sup>]<sub>i</sub> was measured by dual-wavelength excitation (340/380 nm) microfluorometry using either fura-2 or fura-6F as the Ca<sup>2+</sup> indicator. In some experiments,  $\beta$  cells were loaded by incubation with fura2-AM for 15-min at 37 °C in the culture medium. The recording was conducted on an inverted microscope (Olympus IX71, Olympus, Tokyo, Japan) equipped with polychromatic Xenon light source (TILL photonics, Gräfeling, Germany). For simultaneous C<sub>m</sub> and [Ca<sup>2+</sup>]<sub>i</sub> measurements, fura-2 potassium salt was directly perfused into the cytosol through the patch pipettes. In the flash experiments, Ca<sup>2+</sup> was introduced using the caged-Ca<sup>2+</sup> compound, DM-nitrophen and [Ca<sup>2+</sup>]<sub>i</sub> was measured using fura-6F. Because the [Ca<sup>2+</sup>]<sub>i</sub> would decay significantly during 10 seconds of C<sub>m</sub> measurement after flashes (62), we used the fluorescence excitation light to measure [Ca<sup>2+</sup>]<sub>i</sub> and simultaneously to photolyze Ca<sup>2+</sup> after the flashes in order to keep [Ca<sup>2+</sup>]<sub>i</sub> more or less constant. [Ca<sup>2+</sup>]<sub>i</sub> was calculated from the fluorescence ratio *R* according to equation (63).

FM1-43 was used to monitor total surface membrane and exocytosis (12). The fluorescence was excited at 488 nm employing the TILL polychrome IV. The emission was collected at 530 ± 30 nm with a photodiode controlled by the TILL photometry system (TILL photonics) and X-Chart extension of the Pulse software.

### Data analysis

Data analysis was conducted using IGOR Pro 4.06 (Wavemetrics, Portland, OR, USA). Averaged results were presented as mean ± SEM with the number of experiments indicated. Statistical significance was evaluated using Student's *t*-test. Asterisks \* and \*\* denote statistical significance as compared with control, with *p*-value less than 0.05 and 0.01, respectively.

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