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Biochemical Pharmacology 68 (2004) 667–674
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Pulses of external ATP aid to the synchronization of pancreatic β-cells by generating premature Ca^{2+} oscillations

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Received 10 March 2004; accepted 23 April 2004

Abstract

Pancreatic β-cells respond to glucose stimulation with increase of the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$), manifested as membrane-derived slow oscillations sometimes superimposed with transients of intracellular origin. The effect of external ATP on the oscillatory Ca^{2+} signal for pulsatile insulin release was studied by digital imaging of fura-2 loaded β-cells and small aggregates isolated from islets of ob/ob-mice. Addition of ATP (0.01–100 μM) to media containing 20 mM glucose temporarily synchronized the $[Ca^{2+}]_i$ rhythmicity in the absence of cell contact by eliciting premature oscillations. External ATP triggered premature $[Ca^{2+}]_i$ oscillations also when the sarcoendoplasmic reticulum Ca^{2+}-ATPase was inhibited with 50 μM cyclopiazonic acid and phospholipase C inhibited with 10 μM U-73122. The effect of ATP was mimicked by other activators of cytoplasmic phospholipase A_2 (10 nM acetylcholine, 0.1–1 μM of the C-terminal octapeptide of cholecystokinin and 2 μg/ml melittin) and suppressed by an inhibitor of the enzyme (50 μM *p*-amylcinnamoylanthranilic acid). Premature oscillations generated by pulses of ATP sometimes triggered subsequent oscillations. However, prolonged exposure to high concentrations of the nucleotide (10–100 μM) had a suppressive action on the β-cell rhythmicity. The early effects of ATP included generation of transients induced by inositol (1,4,5) trisphosphate and superimposed on the premature oscillation or on an ordinary oscillation induced by glucose. The results support the idea that purinergic activation of phospholipase A_2 has a co-ordinating effect on the β-cell rhythmicity by triggering premature $[Ca^{2+}]_i$ oscillations mediated by closure of ATP-sensitive K^+ channels.

Keywords: ATP; Ca^{2+} signaling; Oscillation; Pancreatic β-cells; Purinoceptor

1. Introduction

Like other secretory processes the release of insulin from the pancreatic β-cells is triggered by a rise of $[Ca^{2+}]_i$. Each β-cell is a biological oscillator, responding to a glucose stimulus with slow (0.1–0.5/min) oscillations of $[Ca^{2+}]_i$ resulting from rhythmic depolarization with subsequent entry of Ca^{2+} through voltage-activated channels [1]. Within an islet it is supposed that diffusible factors are complementary to gap junctions in co-ordinating the oscillatory activity of the β-cells [2–4]. Recent studies suggest that the synchronizing action of the diffusible factors is linked to generation of $[Ca^{2+}]_i$ transients, temporarily interrupting the entry of Ca^{2+} by activating a depolarizing K^+ current [5,6].

Cyclic variations of circulating insulin result from the pulsatile release of the hormone into the portal vein [7,8]. A prerequisite for this pulsatility is that the $[Ca^{2+}]_i$ oscillations are synchronized not only within but also among the numerous islets in the pancreas. Studies of insulin release from the perfused pancreas have resulted in the proposal that the islets communicate via nonadrenergic, noncholinergic (NANC) neurons [9]. In the search for a co-ordinator of the islet activity, attention was paid to ATP as a neural messenger [10] released from the β-cells themselves with mobilizing effects on the intracellular Ca^{2+} stores [11]. We now report that brief exposure to external ATP, like other ways for stimulating $cPLA_2$, has a co-ordinating

Abbreviations: ACA, *p*-amylcinnamoylanthranilic acid; $[Ca^{2+}]_i$, the cytoplasmic Ca^{2+} concentration; CCK-8, C-terminal octapeptide of cholecystokinin; $cPLA_2$, cytoplasmic phospholipase A_2; CPA, cyclopiazonic acid; IP_3, inositol (1,4,5) trisphosphate; K_{ATP} channel, ATP-sensitive K^+ channel; PLC, phospholipase C; SERCA, sarcoendoplasmic reticulum Ca^{2+}-ATPase

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doi:10.1016/j.bcp.2004.04.018

action on the β-cell rhythmicity by triggering premature oscillations of $[Ca^{2+}]_i$.

2. Materials and methods

2.1. Chemicals

Reagents of analytical grade and deionized water were used. Collagenase, bovine serum albumin and HEPES were provided by Roche Diagnostics, GmbH and the SERCA inhibitor CPA was purchased from Calbiochem. ATP (ultragrade), arachidonic acid, tolbutamide and methoxyverapamil were obtained from Sigma and ACA was supplied by Biomol. Tocris Cookson Ltd was the source of mellitin, U-73122 and CCK-8. The acetoxymethyl ester of fura-2 was provided by Molecular Probes. A stock solution of 50 mM arachidonic acid was prepared in 100 mM Na_2CO_3/25% ethanol and the compound tested at concentrations up to 100 μM (molar ratio arachidonic acid/albumin 13.8).

2.2. Preparation of β-cells

Islets of Langerhans were collagenase-isolated from the splenic part of the pancreas of adult ob/ob-mice taken from a noninbred colony [12]. These islets contain more than 90% β-cells, which have a normal secretory response to glucose [13]. Single cells and small aggregates were prepared by shaking in a Ca^{2+}-deficient medium. After suspension in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 30 μg/ml gentamicin, the cells were allowed to attach to the central part of circular coverslips during 2–5 days culture at 37 °C in an atmosphere of 5% CO_2 in humidified air. The identification of the β-cells was based on their large size and low nucleo-cytoplasmic volume ratio compared with the islet cells secreting glucagon and somatostatin [14].

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

The experiments were performed with a basal medium containing 0.5 mg/ml bovine serum albumin and (in mM): NaCl 125, KCl 4, $MgCl_2$ 1.2, $CaCl_2$ 2.6 and HEPES 25, with pH adjusted to 7.40 with NaOH. After rinsing, the cells were loaded with 0.4–0.8 μM fura-2 acetoxymethyl ester during 30–40 min incubation at 37 °C. The coverslips with the attached cells were then washed and used as exchangeable bottoms of open chambers connected to a two-channel peristaltic pump. The wall of the original chamber was an 1 mm thick silicone rubber ring (19 mm inner diameter) pressed onto the coverslip by the threaded chamber mount and a stainless steel ring. In the pulse experiments the volume of the chamber was reduced using a silicone rubber wall with an elliptic opening (4 mm × 7 mm). This modification allowed more rapid wash out of substances around the cells, but increased the risk for cell detachment during the superfusion. It was verified that switching between media of similar composition did not affect the measurements. The chamber was placed on the stage of an inverted microscope (Nikon Diaphot) within a climate box, maintained at 37 °C by an air stream incubator. The microscope was equipped for epifluorescence fluorometry with a 400 nm dichroic mirror and a 40× fluor oil immersion objective.

A xenon arc lamp (150 W), attached to a monochromator (Cairn Optoscan), was used for excitation of fura-2 at 340 nm (half-bandwidth 1.94 nm) and 380 nm (half-bandwidth 1.81 nm). Images were collected through a 30 nm half-bandwidth filter at 510 nm with an intensified CCD camera (Extended ISIS-M, Photonic Science). Pairs of 340 and 380 nm images, consisting of 10 accumulated video frames, were captured, followed by a short delay resulting in measuring cycles of 1 or 2 s. The light exposure of the cells was restricted to the capture time. Ratio frames were calculated after background subtraction, and $[Ca^{2+}]_i$ was estimated as previously described [15,16] using the MetaFluor program (Universal Imaging Corp.)

2.4. Evaluation of data

Each experiment refers to analyses of three to nine cells or small aggregates (<10 cells) attached to a coverslip. Generation of premature oscillations was evaluated by comparing the incidence of $[Ca^{2+}]_i$ rises >50 nM during a period of 60 s before and during exposure to ATP and other compounds. It was tested whether the premature oscillations had a co-ordinating action on the β-cell rhythmicity by recording the incidence of glucose-induced oscillatory peaks appearing with a time difference <30 s. Statistical analyses were performed with the Student *t*-test using paired data.

3. Results

3.1. ATP generation of premature Ca^{2+} oscillations

Isolated β-cells/aggregates usually responded to glucose stimulation with slow (0.1–0.5/min) oscillations of $[Ca^{2+}]_i$. Addition of ATP to media containing 20 mM glucose resulted in a premature rise of $[Ca^{2+}]_i$, resembling an ordinary oscillation. The premature oscillations were triggered by pulses with as little as 10 nM ATP (Fig. 1; Table 1), a concentration increasing the incidence of $[Ca^{2+}]_i$ rises just as much as 1 μM of the nucleotide. A similar effect was noted with 1 μM ADP but not with UTP (Table 1). It was possible to induce premature oscillations by suddenly raising the concentration of ATP (Fig. 2). The incidence of premature $[Ca^{2+}]_i$ rises

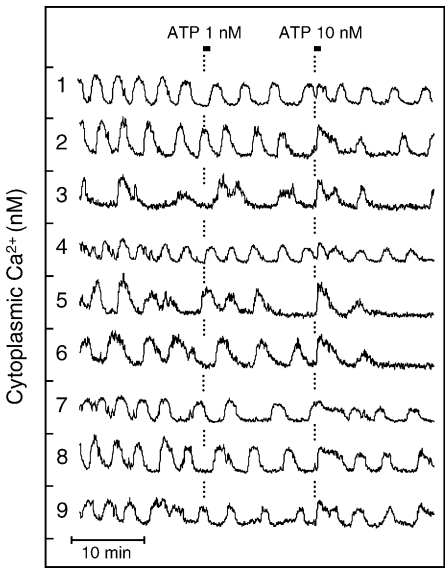

Fig. 1. Effects of 60 s pulses of ATP added to a superfusion medium containing 20 mM glucose. Premature oscillations of $[Ca^{2+}]_i$ were induced with 10 nM but not with 1 nM ATP. Each mark on the ordinate indicates 0 nM $[Ca^{2+}]_i$ for the trace above and/or the 400 nM level for the trace below. Representative for four experiments.

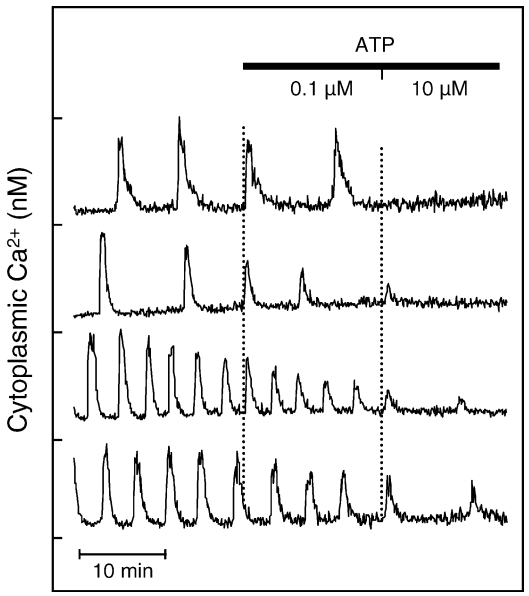

Fig. 2. Premature oscillations induced by addition of 0.1 μM ATP and the subsequent increase of the concentration to 10 μM during superfusion with a medium containing 20 mM glucose. Prolonged exposure to 10 μM ATP resulted in disappearance of the $[Ca^{2+}]_i$ oscillations in most cells. Each mark on the ordinate indicates 0 nM $[Ca^{2+}]_i$ for the trace above and/or the 400 nM level for the trace below. Representative for five experiments.

increased with 30 ± 3% when ATP was elevated from 0.1 to 10 μM ($P < 0.001$; $n = 6$). Prolonged exposure to high concentrations of ATP often resulted in disappearance of the glucose-induced $[Ca^{2+}]_i$ oscillations (Fig. 2). The number of $[Ca^{2+}]_i$ oscillations during 10 min exposure to 10 μM ATP was half of that seen at 0.1 μM ATP ($P < 0.001$; $n = 5$).

Using a protocol with recurrent pulses of ATP it was possible to replace all glucose-induced oscillations with premature ones. When repeating the ATP pulse with an interval allowing generation of ordinary $[Ca^{2+}]_i$ oscillations, these tended to be synchronized (Fig. 3). Comparing the concordance of the ordinary oscillations seen in the presence of 20 mM glucose before and after two consecutive pulses of 100 μM ATP, the percentage of peaks coinciding within 30 s had increased from 38 ± 5 to 53 ± 6 ($P < 0.001$; $n = 5$).

Table 1
Generation of $[Ca^{2+}]_i$ rise by tolbutamide and putative stimulators of $cPLA_2$

Compound tested	Percentage incidence of $[Ca^{2+}]_i$ rises		
	Before exposure	During exposure	Effect
Tolbutamide (10 μM)	8 ± 3	85 ± 4	77 ± 5 (4)*
ATP (10 nM)	20 ± 4	75 ± 5	55 ± 4 (4)*
ATP (1 μM)	22 ± 4	83 ± 3	61 ± 4 (8)*
ADP (1 μM)	10 ± 2	81 ± 4	71 ± 3 (5)*
UTP (1 μM)	11 ± 8	8 ± 4	−3 ± 4 (5)
Acetylcholine (10 nM)	13 ± 2	79 ± 4	66 ± 4 (4)*
CCK-8 (1 μM)	12 ± 4	83 ± 6	71 ± 5 (4)*
Melittin (2 μg/ml)	9 ± 3	81 ± 3	72 ± 4 (6)*

The incidence of $[Ca^{2+}]_i$ rises observed 60 s before and during exposure to 60 s pulses of various compounds are indicated. In the case of melittin the registration was postponed 60 s to compensate for delay of its action. Data are presented as mean ± S.E.M. for the number of experiments shown within parenthesis.

* $P < 0.001$.

3.2. Effects on premature Ca^{2+} oscillations by inhibitors of SERCA and PLC

ATP acted as a trigger of premature oscillations when SERCA was inhibited with CPA (Fig. 4). After 15 min

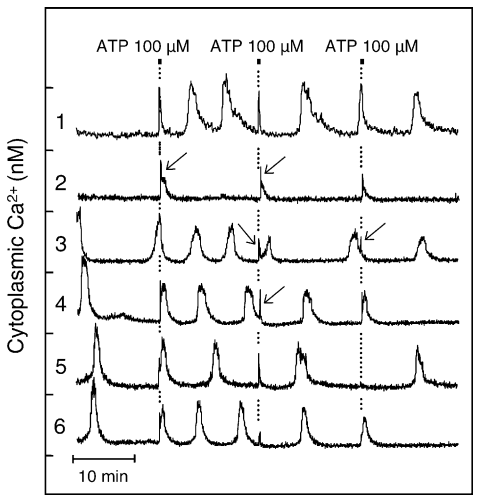

Fig. 3. Effects of repeated 30 s pulses of 100 μM ATP added to a superfusion medium containing 20 mM glucose. Arrows indicate transients of $[Ca^{2+}]_i$ superimposed on premature or ordinary oscillations. Each mark on the ordinate indicates 0 nM $[Ca^{2+}]_i$ for the trace above and/or the 400 nM level for the trace below. Representative for five experiments.

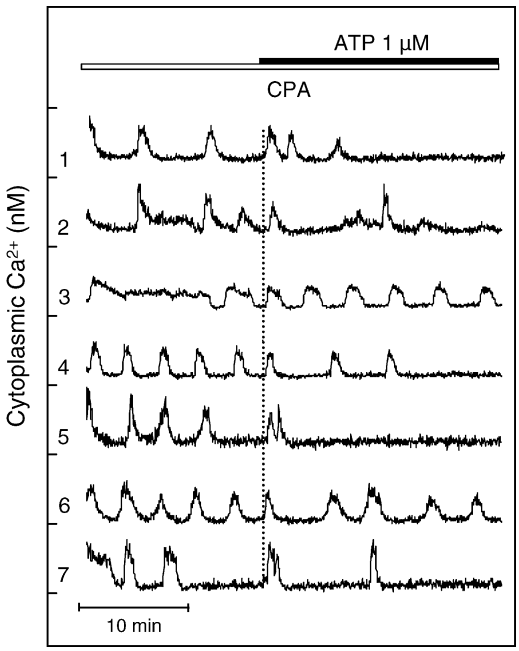

Fig. 4. Premature oscillations induced by 1 μM ATP added to a superfusion medium containing 50 μM CPA and 20 mM glucose. Each mark on the ordinate indicates 0 nM $[Ca^{2+}]_i$ for the trace above and/or the 500 nM level for the trace below. Representative for four experiments.

pretreatment with 50 μM CPA, pulse addition of 10 μM ATP increased the incidence of $[Ca^{2+}]_i$ rises from 23 ± 6% to 51± 6% ($P < 0.01$; $n = 5$). High concentrations of the PLC inhibitor U-73122 did not prevent ATP induction of premature oscillations (Fig. 5, panel A). After 15 min pretreatment with 10 μM U-73122, a pulse addition of 10 μM ATP increased the incidence of $[Ca^{2+}]_i$ rises from 15 ± 5% to 80 ± 4% ($P < 0.001$; $n = 4$).

3.3. Generation of premature Ca^{2+} oscillations by tolbutamide and stimulators of $cPLA_2$

Tolbutamide, a blocker of the K_{ATP} channels, induced premature oscillations when added to a medium containing 20 mM glucose (Fig. 6, panel A). The presence of 10 μM tolbutamide increased the incidence of $[Ca^{2+}]_i$ rises as effective as 0.01–1 μM ATP (Table 1). Premature oscillations (Fig. 6, panels B and C) with higher incidence of $[Ca^{2+}]_i$ rises (Table 1) were generated by brief exposure to acetylcholine and CCK-8. Acetylcholine was equally potent as ATP, inducing premature oscillations already at 10 nM. The minimal effective concentration of CCK-8 was 0.1–1 μM. Pulse addition of the bee venom melittin, another activator of $cPLA_2$, induced temporary rise of $[Ca^{2+}]_i$ after a delay of about 60 s (Fig. 6, panel D; Table 1). However, there was no effect when arachidonic acid was added at concentrations up to 100 μM ($n = 5$; not shown). Inhibition of $cPLA_2$ with 50 μM ACA was accompanied by disappearance of the premature oscillations induced by ATP as well of the ordinary ones seen in glucose-stimulated β-cells (Fig. 5, panel B).

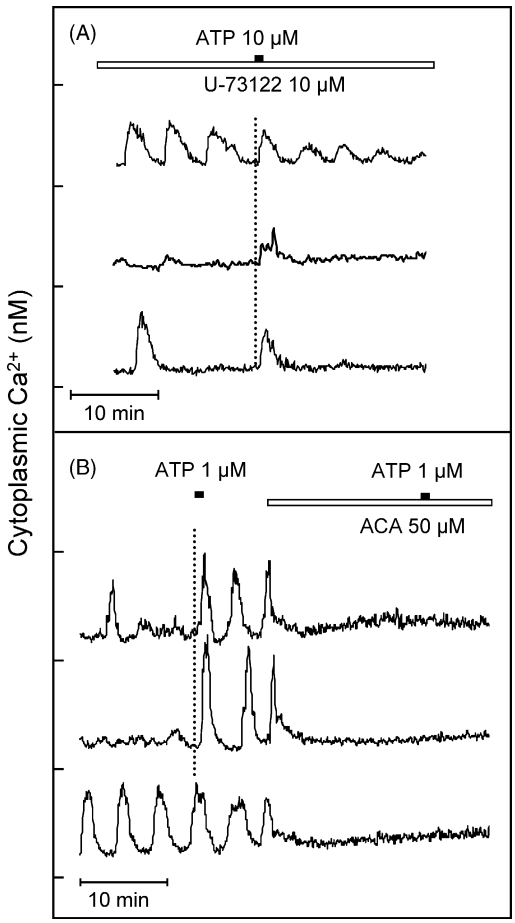

Fig. 5. Effects of 60 s pulses of ATP on β-cells exposed to 10 μM U-23172 (panel A) or 50 μM ACA (panel B) during superfusion with a medium containing 20 mM glucose. Each mark on the ordinate indicates 0 nM $[Ca^{2+}]_i$ for the trace above and/or the 400 nM level for the trace below. Representative for four experiments with each substance.

3.4. ATP generation of transients superimposed on premature Ca^{2+} oscillations

The observation of ATP induction of premature oscillations made it important to analyze whether there was a concomitant generation of IP_3-mediated $[Ca^{2+}]_i$ transients. This was found to be the case when the β-cells were exposed to high (>1 μM) concentrations of ATP. Distinct transients of $[Ca^{2+}]_i$ were superimposed either on the premature oscillation or on the ordinary oscillations coinciding with the addition of the nucleotide (Fig. 3).

4. Discussion

A number of studies with insulin-secreting cell lines [17–19] and primary β-cells [11,20–22] indicate that ATP binding to purinoceptors results in mobilization of intracellular Ca^{2+} stores. Moreover, there is convincing evidence that increased entry of Ca^{2+} contributes to the ATP-induced increase of $[Ca^{2+}]_i$ [17,19,21]. The complex effects of high concentrations of ATP are illustrated

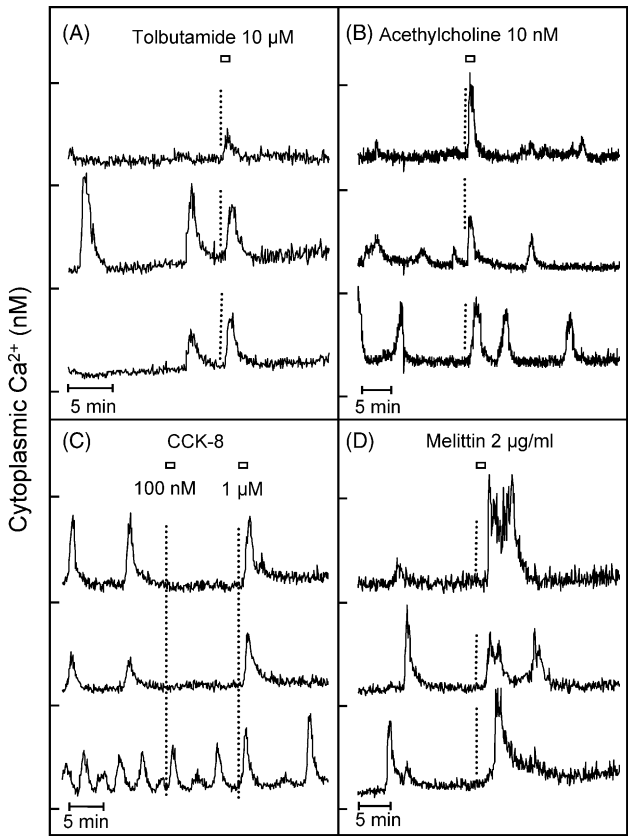

Fig. 6. Effects of 60 s pulses of 100 μM tolbutamide (panel A), 10 nM acetylcholine (panel B), 0.1 and 1 μM CCK-8 (panel C) and 2 μg/ml melittin (panel D) added to a superfusion medium containing 20 mM glucose. All agents evoke temporary rises of $[Ca^{2+}]_i$, resembling premature oscillations. In the case of melittin the response was delayed about 60 s. Each mark on the ordinate indicates 0 nM $[Ca^{2+}]_i$ for the trace above and/or the 400 nM level for the trace below. Representative for four experiments with each substance.

from the early observation that the initial increase of $[Ca^{2+}]_i$ is followed by lowering below the starting level when the entry of Ca^{2+} is maximally stimulated with glucose [11].

Pancreatic β-cells have an intrinsic ability to generate cyclic variations of $[Ca^{2+}]_i$ resulting in pulsatile release of insulin [1,23]. We have now demonstrated that a major effect of purinoceptor activation is to trigger a premature oscillation, followed by ordinary oscillations induced by glucose. The ATP induction of premature $[Ca^{2+}]_i$ oscillations was mimicked by ADP but not by UTP, suggesting involvement of the same purinoceptors as recently found to promptly generate $[Ca^{2+}]_i$ transients in mouse β-cells [24]. Premature oscillations were seen in the presence of inhibitors of PLC (U-73122) and SERCA (CPA), indicating that they are mediated by enhanced entry of Ca^{2+} rather than by IP_3-induced mobilization of intracellular stores. External ATP has been reported to rapidly depolarize insulin-releasing RINm5F cells [18,19] as well as primary mouse β-cells [22]. It is therefore likely that Ca^{2+} entry via voltage-activated channels is essential for the ATP induction of premature $[Ca^{2+}]_i$ oscillations.

Exploring the alternatives of how external ATP can induce a depolarization resulting in premature $[Ca^{2+}]_i$ oscillations, attention should be paid to the type of purinoceptors involved. Results of immunohistochemical studies of mouse β-cells [25] are difficult to reconcile with an increased permeability for cations mediated by ionotropic P2X receptors. Premature oscillations of $[Ca^{2+}]_i$ were generated with 10 nM ATP, a concentration lower than that found to elicit transients of intracellular origin in the present as well as in a previous study [24] from our laboratory. Evidently, mechanisms other than capacitative entry of cations account for the purinoceptor depolarization of the β-cells, since premature oscillations were seen when CPA was used for depleting the endoplasmatic reticulum of Ca^{2+}. K_{ATP} channels have a key role in controlling the membrane potential of the β-cells [26]. As previously reported [27,28], it was found that tolbutamide, an established blocker of the K_{ATP} channels, triggers oscillations of $[Ca^{2+}]_i$ similar to those evoked by external ATP.

Stimulation of purinoceptors is known to activate $cPLA_2$ in isolated mouse islets [22]. The products of this enzyme have a suppressive action on K_{ATP} channels both in insulin-secreting HIT cells [29] and in mouse β-cells [22,30]. The importance of $cPLA_2$ for the β-cell function is far from established and there is recent information that underexpression of the enzyme does not attenuate the $[Ca^{2+}]_i$ response to glucose and other depolarizing stimuli in pseudoislets of clonal insulin-releasing MIN6 cells [31,32]. Evaluating the role of $cPLA_2$ for ATP induction of premature $[Ca^{2+}]_i$ oscillations we now observed that activators of the enzyme are stimulatory, whereas the inhibitor ACA had a suppressive action at half the concentration reported to leave PLC activity and glucose oxidation unaffected in rat islets [33]. Acetylcholine stimulation of muscarinic receptors, that is known to activate $cPLA_2$ [34,35] and inhibit the K_{ATP} channels [36] in pancreatic β-cells, mimicked the effect of external ATP by generating premature $[Ca^{2+}]_i$ oscillations at a concentration as low as 10 nM. Another G-protein-coupled stimulator CCK-8, that preferentially activates the Ca^{2+}-independent form of $cPLA_2$ [37], was found to induce premature oscillations at 100 nM or above. The honey bee venom melittin can penetrate the plasma membrane due to a marked hydrophobicity, enabling its use as potent stimulator of the $cPLA_2$ generation of arachidonic acid in mouse islet cells [35]. In support for the idea that premature oscillations result from activation of $cPLA_2$, pulse addition of melittin induced a temporary rise of $[Ca^{2+}]_i$. It was recently reported that the secretory form of PLA_2 ($sPLA_2$) is released together with insulin [38]. When applied to the outside of the β-cells arachidonic acid opens rather than closes the K_{ATP} channels [29]. In accordance with this report we now observed that addition of arachidonic acid to the superfusion medium does not result in premature oscillations of $[Ca^{2+}]_i$.

Prolonged exposure to high concentrations of ATP (10 μM or more) often resulted in a disappearance of the glucose-induced $[Ca^{2+}]_i$ oscillations. The inhibitory component in the ATP action may reflect an enhanced extrusion of Ca^{2+}, mediated by activation of protein kinase C, and/or closure of the voltage-dependent Ca^{2+} channels. Evidence has been provided that stimulation of protein kinase C sometimes results in a disappearance of the oscillations with a steady-state of $[Ca^{2+}]_i$ slightly above the basal level [39]. Moreover, it has been reported that external ATP acts as a G-protein-coupled inhibitor of L-type Ca^{2+} channels in rat β-cells [40] but not in mouse β-cells [22]. Uncertainties how external ATP affects the Ca^{2+} channels and whether it has stimulatory or inhibitory effects on insulin release may be due to species differences in the types of purinoceptors involved and/or conversion of ATP to the P1 receptor agonist adenosine [41].

In the physiological situation there is an intermittent rather than a continuous exposure of β-cells to ATP. As a transmitter or co-transmitter released from nerves ATP can be expected to be delivered in pulses. Another source of external ATP with intermittent effects on the β-cells is that released during the exocytosis of the secretory granules. Studies on isolated islets from different species, including ob/ob-mice, indicate that the glucose-induced cycles of insulin release can be resolved into distinct periods of enhanced activity [42]. Moreover, it has been reported that single β-cells have brief events of insulin release [43]. Another study [44] has shown fluctuations in the micromolar range of external ATP close to the β-cell surface. In the test system now employed, 10 nM ATP was sufficient to elicit premature oscillations of $[Ca^{2+}]_i$. Moreover, premature oscillations appeared in the continued presence of ATP after increase of the concentration. Acting in this way ATP fulfills the criteria for an external signal, standing out against the background noise of ATP released from the β-cells.

Individual β-cells differ considerably with regard to the duration and frequency of the glucose-induced $[Ca^{2+}]_i$ oscillations. When coupled, the β-cells interact with mutual entrainment of the $[Ca^{2+}]_i$ oscillations into a common rhythm mediated by gap junctions and diffusible messengers. Accordingly, it is possible to demonstrate release of insulin in pulses from isolated islets, the frequency of which is fairly constant irrespective of islet size [45]. The present studies indicate that the depolarization obtained by brief exposure to ATP aids to the co-ordination of the β-cell rhythmicity by replacing ordinary oscillations with premature ones. Moreover, there was a tendency to synchronization of ordinary oscillations when the ATP pulses were repeated. The latter observation suggests that repetitive depolarization of the β-cells provides a coupling force for synchronization of ordinary oscillations in analogy to what has been shown for other pulse-coupled oscillators [46–48]. Methodological problems, including the harmful effects of the UV-light used for excitation of fura-2 [1,49], have so far prevented analysis of the synchronization in long-term experiments.

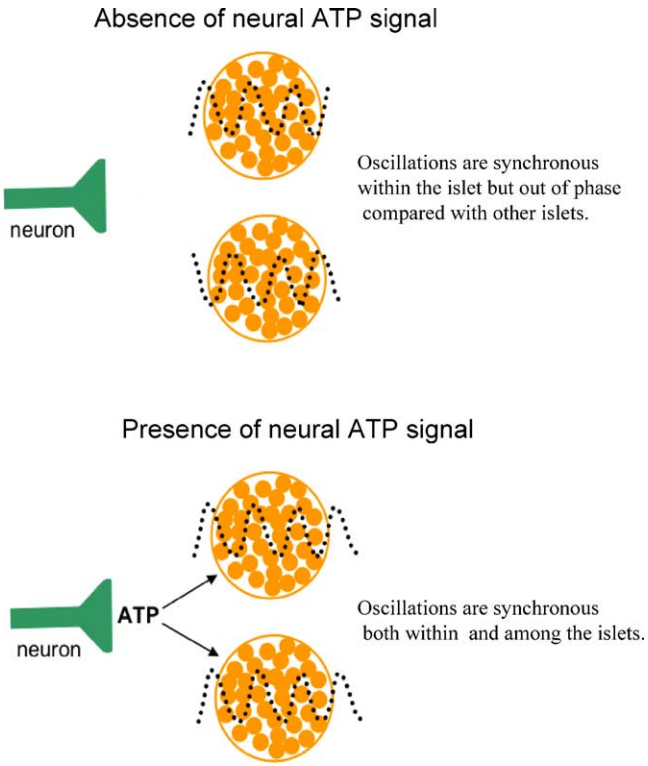

Fig. 7. Importance of neural ATP signals for entrainment of the β-cell rhythmicity in the different islets of the pancreas. The synchronization is supposed to occur both by replacement of ordinary oscillations with premature ones and by phase-shift of existing oscillations due to the coupling force provided by repetitive depolarization.

Periodic variations of circulating insulin require that the β-cell oscillations of $[Ca^{2+}]_i$ within an islet appear in the same phase as those in the other islets in the pancreas. Our observations make it attractive to propose that neural activity with intermittent discharge of ATP entrains differently phased islets into a common rhythm (Fig. 7). Such a synchronization is supposed to occur both by replacement of ordinary oscillations with premature ones and by phase-shift of existing oscillations due to the coupling force provided by repetitive depolarizations.

Acknowledgments

This study was supported by the Swedish Research Council (72X-562), the Swedish Diabetes Association and the Family Ernfors Fund.

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