External ATP triggers Ca^{2+} signals suited for synchronization of pancreatic β -cells

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Abstract

External ATP is supposed to trigger short-lived increases (transients) of cytoplasmic Ca^{2+} important for entraining insulin-secreting β -cells into a common rhythm. To get insight into this process, rises of the cytoplasmic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) induced by external ATP were compared with those obtained with acetylcholine, another neurotransmitter with stimulatory effects on the inositol trisphosphate (IP_3) production. A ratiometric fura-2 technique was used for measuring $[\text{Ca}^{2+}]_i$ in individual β -cells and small aggregates isolated from *ob/ob* mouse islets and superfused with a medium containing methoxyverapamil. ATP and acetylcholine induced temporary rises of $[\text{Ca}^{2+}]_i$ from a basal level manifested as solitary transients (<20 s) and bumps (≥ 20 s) superimposed or not with transients. Addition of ATP (1–100 μM) usually triggered transients whereas acetylcholine induced bumps lacking superim-

posed transients. After the initial rise there was a steady-state elevation of $[\text{Ca}^{2+}]_i$ in β -cells exposed to acetylcholine but not to ATP. Similar differences were seen comparing the responses of rat β -cells to 100 μM ATP and acetylcholine. Inhibition of the sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) pump (with 50 μM cyclopiazonic acid) prevented both the ATP-induced rise of $[\text{Ca}^{2+}]_i$ and the spontaneous firing of transients. Similar effects were seen after activation of protein kinase C (10 nM phorbol-12-myristate-13-acetate), whereas an inhibitor of this enzyme (2 μM bisindolylmaleimide) promoted the generation of transients. The results indicate that ATP fulfils the demands for a coordinator of the secretory activity of β -cells by generating distinct $[\text{Ca}^{2+}]_i$ transients without sustained elevation of basal $[\text{Ca}^{2+}]_i$.

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Introduction

The cytoplasmic concentration of Ca^{2+} ($[\text{Ca}^{2+}]_i$) is a key regulator of cellular events, including the release of insulin from pancreatic β -cells. Like other secretory processes, exocytotic release of insulin is subject to periodic variations (Stagner & Samols 1985, Lefèbvre *et al.* 1987, Hellman *et al.* 1992, Bergsten & Hellman 1993, Pørksen *et al.* 1995). It was shown in 1988 that cyclic variations of $[\text{Ca}^{2+}]_i$ is an intrinsic property of the β -cells, occurring also in the absence of neural pacemaker activity (Grapengiesser *et al.* 1988). Indeed, each β -cell is a biological oscillator, responding to glucose stimulation with slow oscillations of $[\text{Ca}^{2+}]_i$ resulting from rhythmic depolarization with subsequent entry of Ca^{2+} through voltage-activated channels (Hellman *et al.* 1992). Individual β -cells differ considerably with regard to duration and frequency of the $[\text{Ca}^{2+}]_i$ oscillations.

Within the islets both gap junctions (Meda 2003) and diffusible messengers (Grapengiesser *et al.* 2004) entrain the $[\text{Ca}^{2+}]_i$ oscillations into a common rhythm. Recent studies suggest that the coordinating action of diffusible messengers is linked to inositol trisphosphate (IP_3)–

induced generation of short-lived increases (transients) of $[\text{Ca}^{2+}]_i$ temporarily interrupting the Ca^{2+} entry into the β -cells by activating a repolarizing K^+ current (Grapengiesser *et al.* 1999, 2001, 2003, Lundquist *et al.* 2003, Hellman *et al.* 2004). Another effect of the $[\text{Ca}^{2+}]_i$ transients is to activate exocytosis, a process coupled to intermittent release of ATP (Hellman *et al.* 2004). The efficiency of the mechanisms for coordinating β -cells within an islet is illustrated by the observation that individual islets release insulin in pulses with a frequency unaffected by the islet size (Bergsten & Hellman 1993). Periodic variations of circulating insulin require that the β -cell oscillations of $[\text{Ca}^{2+}]_i$ appear in the same phase in the numerous islets of the pancreas. The data obtained so far make it attractive to postulate that neural activity with intermittent discharge of ATP accounts for the entrainment of the differently phased islets into a common rhythm (Grapengiesser *et al.* 2004).

The concept that coordination of the β -cell rhythmicity is linked to mobilization of intracellular Ca^{2+} raises the question whether ATP is more suited as a trigger of a synchronizing Ca^{2+} signal than other external activators of phospholipase C (PLC). We now demonstrate that

Figure 1 Effects of micromolar concentrations of ATP and acetylcholine (ACh) on $[\text{Ca}^{2+}]_i$ in mouse β -cells superfused with a glucagon-free medium containing 20 mM glucose and 50 μM methoxyverapamil. Typical responses to ATP are a bump with a superimposed transient (A) or a solitary transient (B). Responses to acetylcholine were often manifested as a bump lacking transients followed by a sustained elevation of $[\text{Ca}^{2+}]_i$ (C, D).

micromolar concentrations of ATP differ from acetylcholine in essentially generating transients without subsequent elevation of basal $[\text{Ca}^{2+}]_i$.

Material and Methods

Chemicals

Reagents of analytical grade and deionized water were used. ATP (ultragate), acetylcholine, glucagon and methoxyverapamil were obtained from Sigma Chemical Co. (St Louis, MO, USA). Collagenase, BSA and Hepes were provided by Roche Diagnostics GmbH (Mannheim, Germany). Calbiochem (La Jolla, CA, USA) supplied cyclopiazonic acid (CPA), bisindolylmaleimide (BIM) and phorbol-12-myristate-13-acetate (PMA). The acetoxymethyl ester of fura-2 was bought from Molecular Probes (Eugene, OR, USA).

Preparation of β -cells

Adult *ob/ob* mice were taken from a non-inbred local colony (Hellman 1965) and killed by decapitation. Islets

were isolated with the aid of collagenase from the splenic part of the pancreas. These islets contain >90% β -cells, known to have a normal secretory response to glucose (Hahn *et al.* 1974). Single cells and small aggregates were prepared by shaking the islets in a Ca^{2+} -deficient medium. In some experiments the effects of ATP and acetylcholine were tested with cells prepared from islets of adult Sprague–Dawley rats. After suspension in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 30 $\mu\text{g}/\text{ml}$ gentamicin, the cells were allowed to attach to the central part of circular coverslips during 2–5 days of 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 nucleus/cytoplasm volume ratio compared with the islet cells secreting glucagon and somatostatin (Berts *et al.* 1995).

Measurements of cytoplasmic Ca^{2+}

The experiments were performed with a basal medium containing 0.5 mg/ml BSA and 125 mM NaCl, 4.8 mM KCl, 1.2 mM MgCl_2 , 2.6 mM CaCl_2 and 25 mM Hepes

Table 1 Proportions of mouse β -cells responding promptly with different types of $[\text{Ca}^{2+}]_i$ rise. Superfusion for 15 min with a medium containing 20 mM glucose and 50 μM methoxyverapamil was followed by exposure to ATP or acetylcholine. Data refer to observations made during the first 60 s of exposure to the additives. Percentages of cells with response are presented as means $\pm$ S.E.M. for five or six separate experiments with the total number of cells indicated. In the presence of glucagon spontaneous transients from the basal level were seen in $27 \pm 2\%$ of the cells. Note that solitary transients and bumps sometimes coexist in the cells.

Additive	Glucagon	Number of cells	Percentage of responding cells		
			Solitary transients	Bumps with transients	Bumps without transients
ATP (0.01 μM)	—	54	0 ± 0	0 ± 0	0 ± 0
ATP (1.0 μM)	—	46	7 ± 5	60 ± 9	15 ± 4
ATP (100 μM)	—	54	44 ± 11	40 ± 10	16 ± 7
Acetylcholine (0.01 μM)	—	50	0 ± 0	0 ± 0	0 ± 0
Acetylcholine (1.0 μM)	—	60	0 ± 0	13 ± 5	70 ± 4
Acetylcholine (100 μM)	—	50	9 ± 5	9 ± 6	81 ± 8
ATP (0.01 μM)	+	41	30 ± 6	0 ± 0	0 ± 0
ATP (0.1 μM)	+	33	41 ± 15	29 ± 12	29 ± 9
ATP (1.0 μM)	+	44	92 ± 17	24 ± 6	20 ± 9
ATP (100 μM)	+	39	86 ± 19	13 ± 10	0 ± 0
Acetylcholine (0.01 μM)	+	26	55 ± 12	0 ± 0	0 ± 0
Acetylcholine (0.1 μM)	+	35	78 ± 11	7 ± 7	16 ± 12
Acetylcholine (1.0 μM)	+	36	33 ± 10	33 ± 10	68 ± 10
Acetylcholine (100 μM)	+	39	8 ± 5	24 ± 7	73 ± 8

with pH adjusted to 7.40 using NaOH. After rinsing, the cells were loaded with 0.5–1.0 μM fura-2 acetoxymethyl ester during 30–40 min incubation in the presence of 3 mM glucose. The coverslips with the attached cells were then washed and used as exchangeable bottoms of an open chamber connected to a two-channel peristaltic pump. The cells were superfused at a rate of 0.8 ml/min with a medium containing 20 mM glucose. The Ca^{2+} -channel blocker methoxyverapamil (50 μM) was included in the medium to allow recognition of Ca^{2+} transients without the background disturbance of slow oscillations. If not otherwise stated glucagon (20 nM) was present to counteract the depletion of cAMP known to occur in isolated β -cells (Schuit & Pipeleers 1985). The studies were performed with an inverted microscope (Nikon Diaphot) using a climate box maintained at 37 °C. The microscope was equipped for epifluorescence fluorometry with a 400 nm dichroic mirror and a 40 $\times$ Fluor oil immersion objective.

A xenon arc lamp was used for excitation of fura-2 at 340 and 380 nm with the aid of a monochromator (Cairn; Optoscan, Faversham, Kent, UK). Images were collected through a 30 nm half-bandwidth filter at 510 nm with an intensified CCD camera (Extended Isis-M; Photonic Science, Robertsbridge, East Sussex, UK). Pairs of 340 and 380 nm images, consisting of 10–16 accumulated video frames, were captured, followed by a delay resulting in measuring cycles of 2–5 s. The specimens were illum-

nated only during image capture and excitation light was kept at a minimum. Ratio frames were calculated after background subtraction, and $[\text{Ca}^{2+}]_i$ was estimated as described previously (Gryniewicz *et al.* 1985, Gylfe *et al.* 1991).

Evaluation of data

Each experiment refers to analyses of 4–12 cells or small aggregates (<10 cells) attached to coverslips. Temporary increases of $[\text{Ca}^{2+}]_i$ extending 50 nM were recognized as transients (<20 s) or bumps (≥ 20 s). Moreover, we distinguished between bumps superimposed or not with transients. Early effects of additions to the superfusion medium were evaluated by comparing the proportion of cells/aggregates generating an increase of $[\text{Ca}^{2+}]_i$ during a 60-s period preceding and following the exposure to the compounds. When the late effects were studied, the 60-s period following the exposure was excluded. Results are presented as means $\pm$ S.E.M. Statistical significances were evaluated by Student's *t*-test for paired and unpaired data.

Results

Initial response with temporary rise of Ca^{2+}

In the absence of glucagon, spontaneous transients of $[\text{Ca}^{2+}]_i$ were only seen occasionally. Addition of ATP or

Figure 2 Effects of ATP (A) and acetylcholine (ACh; B) on $[\text{Ca}^{2+}]_i$ in mouse β -cells superfused with a medium containing 20 mM glucose, 20 nM glucagon and 50 μM methoxyverapamil. Both agonists induced transients and/or bumps followed by suppression of the spontaneous transients. Solitary transients and bumps sometimes coexist as shown in (A). Prolonged exposure to acetylcholine resulted in steady-state elevation of $[\text{Ca}^{2+}]_i$. Representative of five experiments.

acetylcholine to medium lacking glucagon resulted in temporary rises of $[\text{Ca}^{2+}]_i$ (Fig. 1). The proportion of cells responding with solitary transients and/or bumps, superimposed or not with transients, is shown in Table 1. ATP was more effective than acetylcholine as a promoter of transients superimposed on bumps or starting from the basal level. Comparing the effects of different concentrations of ATP, it was found that 100 μM generated more solitary transients than 1 μM .

In the presence of 20 nM glucagon, the proportion of cells generating spontaneous transients during a 60-s

period was estimated to $27 \pm 2\%$. Examples of the results obtained when ATP and acetylcholine were added to a glucagon-containing medium are shown in Fig. 2 and the effects of different concentrations of the agonists are summarized in Table 1. Whereas micromolar concentrations of ATP essentially triggered transients, the main effect of acetylcholine was to promote bumps without superimposed transients. Contrary to what was seen with ATP, there was a reduced firing of solitary transients when the concentration of acetylcholine was raised above 0.1 μM . Actually, the proportion of cells responding with

Figure 3 Effects of 50 μ M CPA on $[Ca^{2+}]_i$ in mouse β -cells exposed or not to ATP or acetylcholine (ACh) during superfusion with a medium containing 20 mM glucose, 20 nM glucagon and 50 μ M methoxyverapamil. Addition of CPA generated a bump with a superimposed transient, followed by disappearance of the spontaneous transients and steady-state elevation of $[Ca^{2+}]_i$ (A). Both 1 μ M ATP (B) and 1 μ M acetylcholine (C) lacked effects in the presence of CPA. Addition of 50 μ M CPA in the presence of 1 μ M ATP generated a bump lacking superimposed transients but followed by steady-state elevation of $[Ca^{2+}]_i$ (D). Addition of 50 μ M CPA in the presence of 1 μ M acetylcholine generated a small bump without superimposed transients or subsequent elevation of $[Ca^{2+}]_i$ (E). All observations are representative of five experiments.

solitary transients to 100 μ M acetylcholine ($8 \pm 5\%$) was lower than that recorded during the preceding 60-s period.

Testing various approaches for inducing prompt rise of $[Ca^{2+}]_i$ similar to that seen with ATP, it was found that CPA inhibition of the sarcoplasmic/endoplasmic

Table 2 Effects of the SERCA-pump inhibitor CPA on $[\text{Ca}^{2+}]_i$ in mouse β -cells exposed to ATP or acetylcholine. Effects of 50 μM CPA were tested in the absence and presence of 1 or 100 μM ATP and acetylcholine added to superfusion medium containing 20 mM glucose, 20 nM glucagon and 50 μM methoxyverapamil. The additives were present 10–15 min before CPA. Effects of CPA are presented as means $\pm$ S.E.M. for $[\text{Ca}^{2+}]_i$ increase with bumps and sustained elevation in five experiments

Additive	Effect of CPA	
	Bump (nM)	Steady-state elevation (nM)
None (control)	139 $\pm$ 12	25 $\pm$ 4
ATP (1 μM)	91 $\pm$ 19*	20 $\pm$ 5
ATP (100 μM)	92 $\pm$ 19*	31 $\pm$ 4
Acetylcholine (1 μM)	35 $\pm$ 8**	2 $\pm$ 1**
Acetylcholine (100 μM)	36 $\pm$ 4**	1 $\pm$ 1**

Statistical significances compared with control: * $P \approx 0.05$; ** $P < 0.001$.

reticulum Ca^{2+} -ATPase (SERCA) pump induced bumps, often superimposed with transients (Fig. 3A). Prolonged exposure to 50 μM CPA prevented the temporary increase of $[\text{Ca}^{2+}]_i$ otherwise seen in response to 1 μM ATP (Fig. 3B) and acetylcholine (Fig. 3C). After pre-exposure to micromolar concentrations of the agonists there was still a CPA effect resulting in bumps, but these became lower and lacked superimposed transients (Figs 3D and E; Table 2). The CPA-induced bumps of $[\text{Ca}^{2+}]_i$ were particularly affected by pre-treatment with acetylcholine.

Steady-state elevation of Ca^{2+}

Prolonged exposure to acetylcholine, but not to ATP, induced a steady-state elevation of $[\text{Ca}^{2+}]_i$ both in the absence (Fig. 1; Table 3) and presence (Fig. 2; Table 3) of glucagon. A similar elevation was seen when blocking the

Table 3 Steady-state elevation of $[\text{Ca}^{2+}]_i$ in mouse β -cells exposed to ATP, acetylcholine or CPA. Superfusion for 15 min in the absence and presence of 20 nM glucagon with a medium containing 20 mM glucose and 50 μM methoxyverapamil was followed by exposure to ATP, acetylcholine and CPA. Data refer to the steady-state elevation in 26–60 cells reached after the initial rise of $[\text{Ca}^{2+}]_i$. Means $\pm$ S.E.M. for five experiments are shown

Additive	Steady-state elevation (nM)	
	Without glucagon	With glucagon
ATP (0.01 μM)	0 $\pm$ 0	0 $\pm$ 0
ATP (1.0 μM)	0 $\pm$ 0	0 $\pm$ 0
ATP (100 μM)	0 $\pm$ 0	0 $\pm$ 0
Acetylcholine (0.01 μM)	0 $\pm$ 0	1 $\pm$ 1
Acetylcholine (1.0 μM)	18 $\pm$ 2	17 $\pm$ 7
Acetylcholine (100 μM)	26 $\pm$ 6	27 $\pm$ 2
CPA (50 μM)	–	25 $\pm$ 4

SERCA pump with CPA (Fig. 3A; Table 3). Addition of CPA resulted in steady-state elevation of $[\text{Ca}^{2+}]_i$ also in the presence of 1 μM ATP (Fig. 3D; Table 2). However, there was no additional increase of steady-state $[\text{Ca}^{2+}]_i$ when CPA (Fig. 3E; Table 2) or ATP (Fig. 4A) was added to cells exposed to 1 μM acetylcholine.

Suppression of spontaneous Ca^{2+} transients

Both ATP and acetylcholine interfered with the generation of spontaneous $[\text{Ca}^{2+}]_i$ transients (Fig. 2). The suppression obtained after addition of the agonists to a glucagon-containing medium is shown in Table 4. Whereas prolonged exposure to 0.1 μM acetylcholine lacked significant effect, the same concentration of ATP removed 81% of the transients. Studying the combined effects of the agonists it was found that 100 μM ATP induced a temporary rise of $[\text{Ca}^{2+}]_i$ in 23 $\pm$ 6% ($n=5$) of the cells lacking spontaneous transients due to the presence of 1 μM acetylcholine (Fig. 4A). However, when the transients were removed with 1 μM ATP almost all cells (96 $\pm$ 2%; $n=5$) responded to 100 μM acetylcholine with an initial rise of $[\text{Ca}^{2+}]_i$ followed by sustained elevation (Fig. 4B).

As stimulators of PLC, both ATP and acetylcholine promote the generation of diacylglycerol, a well-known activator of protein kinase C (PKC). PMA, another activator of PKC, effectively removed spontaneous $[\text{Ca}^{2+}]_i$ transients (Fig. 4C). This effect was coupled to a loss of the initial $[\text{Ca}^{2+}]_i$ rises seen in the presence of 10 μM ATP or acetylcholine (not shown). Added at a concentration of 100 μM , acetylcholine but not ATP sometimes induced a temporary rise of $[\text{Ca}^{2+}]_i$ in the presence of PMA (Fig. 4D). Consistent with the suppressive action of PKC an inhibitor of this enzyme, BIM, promoted the generation of spontaneous $[\text{Ca}^{2+}]_i$ transients (Fig. 5A). After 5 min of pre-exposure to 2 μM BIM, continued presence of this PKC inhibitor increased the frequency of the transients by 96 $\pm$ 22% ($P < 0.01$; $n=6$). Prolonged exposure to 1 μM ATP (Fig. 5B) or acetylcholine (Fig. 5C) resulted in the disappearance of spontaneous transients also in the presence of BIM. In the case of acetylcholine, an initial rise of $[\text{Ca}^{2+}]_i$ was followed by sustained elevation.

Ca^{2+} responses in rat β -cells

Differences in the effects of ATP and acetylcholine on rat β -cells were found both for the initial responses and the presence of a sustained elevation of $[\text{Ca}^{2+}]_i$ (Fig. 6). The proportion of rat β -cells generating transients and/or bumps in the presence of 100 μM of the agonists is shown in Table 5. Whereas most cells (82%) rapidly responded to ATP with transients superimposed or not on bumps, the addition of acetylcholine usually resulted in bumps

Figure 4 Effects of ATP, acetylcholine (ACh) and PMA on $[Ca^{2+}]_i$ in mouse β -cells superfused with a medium containing 20 mM glucose, 20 nM glucagon and 50 μ M methoxyverapamil. After removal of spontaneous transients by 15 min exposure to 1 μ M acetylcholine, addition of 100 μ M ATP induced a temporary rise of $[Ca^{2+}]_i$ (106 ± 16 nM) in $23 \pm 6\%$ of the cells ($n=5$; A). After removal of spontaneous transients by 15 min exposure to 1 μ M ATP, addition of 100 μ M acetylcholine induced a temporary rise of $[Ca^{2+}]_i$ (175 ± 22 nM) in $96 \pm 2\%$ of the cells ($n=5$; B). Spontaneous transients disappeared in all of six experiments in the presence of 10 nM PMA (C). Pulse additions (60 s) of 100 μ M acetylcholine but not of ATP induced a temporary rise of $[Ca^{2+}]_i$ in all of six experiments when spontaneous transients were removed by PMA (D).

lacking superimposed transients (83%). Accordingly, the ATP-induced rise of $[Ca^{2+}]_i$ above the basal level often exceeded that found with acetylcholine. The proportion of

cells responding with a temporary rise of $[Ca^{2+}]_i$ above 1000 nM was $27 \pm 7\%$ ($n=6$) for ATP and $4 \pm 3\%$ ($n=8$) for acetylcholine.

Table 4 Effects of ATP and acetylcholine on the generation of spontaneous $[\text{Ca}^{2+}]_i$ transients in mouse β -cells. Superfusion for 10–12 min with a medium containing 20 mM glucose, 20 nM glucagon and 50 μM methoxyverapamil (control period) was followed by exposure to ATP or acetylcholine (test period). The tests were performed during a 10 min period starting 60 s after addition of the substances. Effects are presented as means $\pm$ S.E.M. for five or six experiments

Additive	Transients in 10 min		
	Control period	Test period	Effect
ATP (0.01 μM)	2.87 $\pm$ 0.60	2.36 $\pm$ 0.59	−0.51 $\pm$ 0.25
ATP (0.1 μM)	2.63 $\pm$ 0.58	0.50 $\pm$ 0.24	−2.13 $\pm$ 0.46**
ATP (1.0 μM)	2.49 $\pm$ 0.40	0.07 $\pm$ 0.03	−2.42 $\pm$ 0.38***
ATP (100 μM)	2.97 $\pm$ 0.44	0.03 $\pm$ 0.03	−2.94 $\pm$ 0.43***
Acetylcholine (0.01 μM)	2.55 $\pm$ 0.26	2.01 $\pm$ 0.32	−0.53 $\pm$ 0.43
Acetylcholine (0.1 μM)	2.21 $\pm$ 0.47	1.63 $\pm$ 0.53	−0.58 $\pm$ 0.22
Acetylcholine (1.0 μM)	2.50 $\pm$ 0.63	0 $\pm$ 0	−2.50 $\pm$ 0.35*
Acetylcholine (100 μM)	2.38 $\pm$ 0.38	0.03 $\pm$ 0.03	−2.35 $\pm$ 0.35***

* $P \approx 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Discussion

Effects of ATP on $[\text{Ca}^{2+}]_i$ were studied without the background disturbance of slow oscillations by including the Ca^{2+} -channel blocker methoxyverapamil in the superfusion medium. This approach has been instrumental for exploring whether $[\text{Ca}^{2+}]_i$ transients, triggered by neurotransmitters, propagate to adjacent β -cells via release of ATP associated with exocytosis of secretory granules (Hellman *et al.* 2004). The presence of methoxyverapamil neither prevents the generation of $[\text{Ca}^{2+}]_i$ transients nor the synchronization of the transients seen in β -cells lacking physical contact (Grapengiesser *et al.* 1999). After isolation the β -cells will be depleted of cAMP, due to separation from the glucagon-producing α -cells (Schuit & Pipeleers 1985). Most experiments were therefore performed with media containing glucagon, a protocol facilitating the analyses by promoting the generation of spontaneous $[\text{Ca}^{2+}]_i$ transients (Grapengiesser *et al.* 1999, 2001).

It is likely that IP_3 -induced mobilization of Ca^{2+} from endoplasmic reticulum (ER) is important for coordinating the $[\text{Ca}^{2+}]_i$ rhythmicity in pancreatic β -cells. Evidence has been provided that spontaneous transients of $[\text{Ca}^{2+}]_i$ appear in synchrony in β -cells lacking contact (Grapengiesser *et al.* 1999), and that these transients entrain the oscillatory activity into a common rhythm (Grapengiesser *et al.* 2003). In the present study initial rises of $[\text{Ca}^{2+}]_i > 50$ nM were classified as transients and bumps. The observation that the bumps were often superimposed with one or several transients justifies their recognition as a distinct type of $[\text{Ca}^{2+}]_i$ increase. As indicated from the CPA experiments, inhibition of the SERCA pump was sufficient to induce bumps with superimposed transients. Similar bumps can be evoked by gaseous NO, a messenger supposed to be complementary to ATP as a coordinator of the β -cell rhythmicity (Grapengiesser *et al.* 2001).

Discussing the physiological role of the observed $[\text{Ca}^{2+}]_i$ rises, it should be kept in mind that the presence of methoxyverapamil prevents voltage-activated Ca^{2+} entry mediated by closure of K_{ATP} channels. In media lacking methoxyverapamil both ATP and acetylcholine induce premature oscillations of $[\text{Ca}^{2+}]_i$, an effect also obtained with other activators of cytoplasmic phospholipase A_2 (Grapengiesser *et al.* 2004). The bumps now observed sometimes mimic premature oscillations. Nevertheless, pre-treatment with CPA, which is known to leave most of the premature oscillations unaffected (Grapengiesser *et al.* 2004), resulted in a complete removal of the bumps. Transients are mediated by steeper increases to higher levels of $[\text{Ca}^{2+}]_i$ than seen with bumps. When superimposed on slow $[\text{Ca}^{2+}]_i$ oscillations the transients can generate a repolarizing K^+ current, temporarily interrupting the electrical activity (Ämmälä *et al.* 1993, Dryselius *et al.* 1999). Accumulating data support the idea that this effect provides a coupling force for synchronization of pancreatic β -cells (Grapengiesser *et al.* 2003), in analogy to what has been shown for other pulse-coupled oscillators (Gilbey 2001, Strogatz 2001).

Consistent with a previous report (Hellman *et al.* 2004) continuous exposure to ATP (0.1 μM or above) evoked initial rise of $[\text{Ca}^{2+}]_i$ followed by disappearance of spontaneously occurring transients. The presence of a suppressive component in the ATP action emphasizes the importance of an intermittent release of the nucleotide from the β -cells. We now report that prolonged exposure to acetylcholine of 1 μM or more results in a similar loss of transients. In the case of ATP the disappearance of transients cannot be explained by depletion of Ca^{2+} stored in the ER, since the SERCA-pump inhibitor CPA evoked a pronounced increase of $[\text{Ca}^{2+}]_i$ also in the presence of the nucleotide. The observation that ATP has a suppressive action on the

Figure 5 Effects of BIM, ATP and acetylcholine (ACh) on $[\text{Ca}^{2+}]_i$ in mouse β -cells superfused with a medium containing 20 mM glucose, 20 nM glucagon and 50 μM methoxyverapamil. The number of spontaneous $[\text{Ca}^{2+}]_i$ transients increased in the presence of 2 μM BIM (A). The presence of 1 μM ATP (B) or 1 μM acetylcholine (C) resulted in disappearance of spontaneous $[\text{Ca}^{2+}]_i$ transients in a medium containing 2 μM BIM. Representative of four–six experiments.

$[\text{Ca}^{2+}]_i$ transients may not only reflect desensitization of the purinoceptors. It has been proposed that the action of ATP on β -cells involves autocrine feedback inhibition

of exocytosis via G-protein-dependent activation of the serine/threonine phosphatase calcineurin (Poulsen *et al.* 1999).

Figure 6 Effects of ATP and acetylcholine (ACh) on $[\text{Ca}^{2+}]_i$ in rat β -cells superfused with a glucagon-free medium containing 20 mM glucose and 50 μM methoxyverapamil. Addition of 100 μM ATP usually resulted in transients superimposed on bumps (A). Most β -cells responded to 100 μM acetylcholine with a bump lacking transients but followed by sustained elevation of $[\text{Ca}^{2+}]_i$ (B). Representative of six–eight experiments.

Both ATP and acetylcholine activate PLC with resulting generation of diacylglycerol, the natural stimulator of PKC. It has been reported that PMA stimulation of PKC effectively removes the spontaneous $[\text{Ca}^{2+}]_i$ transients (Liu *et al.* 1996). We now observe that PMA is a suppressor also of the temporary $[\text{Ca}^{2+}]_i$ rises evoked by ATP and acetylcholine. The suppression was particularly pronounced in the case of ATP, as indicated by the absence of a $[\text{Ca}^{2+}]_i$ rise after addition of 100 μM of the nucleotide. BIM, an established inhibitor of PKC (Toullec *et al.* 1991), promoted the generation of $[\text{Ca}^{2+}]_i$ transients but did not prevent their disappearance during prolonged exposure to ATP and acetylcholine. The decisive role of cAMP for the generation of $[\text{Ca}^{2+}]_i$ transients (see above) raises the question of whether activation of PKC results in inhibition of adenylate cyclase or protein kinase A. Pancreatic islets express several subtypes of these enzymes, which are known to react differently to agents affecting PKC (Tian & Laychock 2001, Gao *et al.* 2002).

Like ATP, acetylcholine induced a temporary rise of $[\text{Ca}^{2+}]_i$ followed by removal of the spontaneous transients. Comparing the initial responses to the agonists in β -cells from both *ob/ob* mice and rats it was found that micromolar concentrations of ATP essentially triggered transients, whereas the main effect of acetylcholine was to generate bumps without superimposed transients. Moreover, prolonged exposure to acetylcholine, but not to ATP, resulted in a steady-state elevation of Ca^{2+} similar to what is seen when capacitative entry of Ca^{2+} is activated by depleting the ER stores of Ca^{2+} (Dyachok & Gylfe 2001). This observation should not be taken to indicate that capacitative entry of Ca^{2+} is the only mechanism by which acetylcholine induces sustained elevation of $[\text{Ca}^{2+}]_i$. It is likely that entry of Na^+ via non-specific cation channels is essential for the steady-state elevation of $[\text{Ca}^{2+}]_i$ observed after activation of the muscarinic receptors (Saha & Grapengiesser 1995, Gilon & Henquin 2001).

Table 5 Proportion of rat β -cells responding promptly to ATP and acetylcholine with different types of $[\text{Ca}^{2+}]_i$ rise. Superfusion for 15 min in the absence of glucagon with a medium containing 20 mM glucose and 50 μM methoxyverapamil was followed by exposure to ATP or acetylcholine. Data refer to observations made during the first 60 s of exposure to the additives. Percentages of responding cells are presented as means $\pm$ S.E.M. for six and eight experiments with the total number of cells indicated

Additive	Number of cells	Percentage of responding cells		
		Solitary transients	Bumps with transients	Bumps without transients
ATP (100 μM)	76	4 $\pm$ 3	78 $\pm$ 2	18 $\pm$ 3
Acetylcholine (100 μM)	94	0 $\pm$ 0	18 $\pm$ 6	83 $\pm$ 6

In summary, previous studies have shown improved coordination of the β -cell rhythmicity after increasing the number of synchronous [Ca²⁺]_i transients (Grapengiesser *et al.* 2003). We now demonstrate that external ATP is better suited than acetylcholine for coordinating the activity of the β -cells by generating distinct [Ca²⁺]_i transients without sustained elevation of [Ca²⁺]_i.

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