CELL STRUCTURE AND FUNCTION 25: 279–289 (2000)

P2Y-Purinoceptor Mediated Inhibition of L-type Ca^{2+} Channels in Rat Pancreatic β-Cells

Qi Gong, Masafumi Kakei*, Nobuyuki Koriyama, Mitsuhiro Nakazaki, Shingo Morimitsu, Kazuro Yaekura, and Chuwa Tei

First Department of Internal Medicine, Faculty of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan

ABSTRACT. **We used the patch-clamp technique to study the effects of extracellular ATP on the activity of ion channels recorded in rat pancreatic β-cells. In cell-attached membrane patches, action currents induced by 8.3 mM glucose were inhibited by 0.1 mM ATP, 0.1 mM ADP or 15 μM ADPβS but not by 0.1 mM AMP or 0.1 mM adenosine. In perforated membrane patches, action potentials were measured in current clamp, induced by 8.3 mM glucose, and were also inhibited by 0.1 mM ATP with a modest hyperpolarization to –43 mV. In whole-cell clamp experiments, ATP dose-dependently decreased the amplitudes of L-type Ca^{2+} channel currents (ICa) to 56.7±4.0% ($p<0.001$) of the control, but did not influence ATP-sensitive K^+ channel currents observed in the presence of 0.1 mM ATP and 0.1mM ADP in the pipette. Agonists of P2Y purinoceptors, 2-methylthio ATP (0.1 mM) or ADPβS (15 μM) mimicked the inhibitory effect of ATP on ICa, but PPADS (0.1 mM) and suramin (0.2 mM), antagonists of P2 purinoceptors, counteracted this effect. When we used 0.1 mM GTPγS in the pipette solution, ATP irreversibly reduced ICa to 58.4±6.6% of the control ($p<0.001$). In contrast, no inhibitory effect of ATP was observed when 0.2 mM GDPβS was used in the pipette solution. The use of either 20 mM BAPTA instead of 10 mM EGTA, or 0.1 mM compound 48/80, a blocker of phospholipase C (PLC), in the pipette solution abolished the inhibitory effect of ATP on ICa, but 1 μM staurosporine, a blocker of protein kinase C (PKC), did not. When the β-cells were pretreated with 0.4 μM thapsigargin, an inhibitor of the endoplasmic reticulum (ER) Ca^{2+} pump, ATP lost the inhibitory effect on ICa. These results suggest that extracellular ATP inhibits action potentials by Ca^{2+}-induced ICa inhibition in which an increase in cytosolic Ca^{2+} released from thapsigargin-sensitive store sites was brought about by a P2Y purinoceptor-coupled G-protein, PI-PLC and IP3 pathway.**

Key words: action potentials/Ca^{2+} channels/cytosolic Ca^{2+}/extracellular ATP/pancreatic β-cells/P2Y purinoceptor/G-proteins

In pancreatic islet β-cells, intracellular adenosine-5′-triphosphate (ATP) plays a central role in controlling insulin secretion (Petersen and Findlay, 1987). When the concentration of external glucose is increased, an elevation of intracellular levels of ATP or ATP/ADP ratio resulting from glucose metabolism causes the closure of ATP-sensitive K^+ (K_{ATP}) channels and depolarization of the plasma membrane potential (Ashcroft *et al*., 1984; Cook and Hales, 1984; Rorsman and Trube, 1985; Kakei *et al*., 1986; Yokoshiki *et al*., 1998). This leads to Ca^{2+} influx through activated voltage-dependent Ca^{2+} channels, a rise in the free cytosolic Ca^{2+} levels and eventually initiation of exocytosis of insulin (Boyd, 1992).

It has also been known that ATP co-released to the extracellular milieu with insulin or neurotransmitters upon exocytosis in β-cells or nerve endings may be of physiological importance in modulating islet functions (Dowdall *et al*., 1974; Morel and Meunier, 1981; Hutton *et al*., 1983; Gordon, 1986). Intravenous injection of ATP increased the plasma insulin levels in rats (Candela and Garcia-Fernandez, 1963a) and the stimulatory effect of ATP on insulin secretion was noted *in vitro* (Candela and Garcia-Fernan-

* To whom correspondence should be addressed: First Department of Internal Medicine, Faculty of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan.
Tel: +81–99–275–5318, Fax: +81–99–265–8447
E-mail: mkakei@med4.kufm.kagoshima-u.ac.jp

Abbreviations: BAPTA,1,2-bis(*0*-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid; ICa, Ca^{2+} channel current; KRBB, Krebs-Ringer bicarbonate buffer; PI-PLC, phosphatidyl inositol phospholipase C; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid; TEA, tetraethylammonium chloride; TTX, tetrodotoxin.

dez, 1963b). Thus, the current principle regarding extracellular ATP action is potentiation of insulin secretion by binding to cell surface receptors. Cellular responses to ATP have been thought to be mediated by P2 purinoceptors (Chapal and Loubatières-Mariani, 1981a; Petit *et al*., 1987; Hillaire-Buys *et al*., 1994; Tang *et al*., 1996), which are classified into three subclasses, P2X, P2Y and P2Z (Abbracchio and Burnstock, 1994). The signals of P2Y purinoceptors have been reported to be coupled to the G-proteins (Lustig *et al*., 1993; Simon *et al*., 1995), whereas P2X-type receptors are reportedly ligand-gated ion channels nonselective for cations and activated by ATP^{4-} (Valera *et al*., 1994). In pancreatic β-cells, the presence of both P2Y and P2X purinoceptors has been documented (Bertrand *et al*., 1987). The signaling mechanism from stimulation of P2Y purinoceptors to insulin secretion has been proposed to involve mobilization of intracellular Ca^{2+} through the formation of inositol-1,4,5-trisphosphate (IP3) via stimulating phosphatidyl inositol phospholipase C (PI-PLC) (Blachier and Malaisse, 1988; Li *et al*., 1991). On the contrary, extracellular ATP has been demonstrated to inhibit insulin secretion through decreasing electrical activity (Petit *et al*., 1989) and by interfacing with a G-protein-dependent activation of the serine/threonine protein phosphatase calcineurin (Poulsen *et al*., 1999) in mouse β-cells. In rat pancreatic β-cells, effects of external ATP on activity of ionic channels, which regulate insulin secretion, remain to be elucidated. In the present paper, extracellular ATP was demonstrated to inhibit action potentials by Ca^{2+}-induced ICa inhibition in which an increase in cytosolic Ca^{2+} released from thapsigargin-sensitive store sites was brought about by a P2Y purinoceptor-coupled G-protein, PI-PLC and IP3 pathway.

Materials and Methods

Preparations

Experiments were performed on male Wistar rats weighing 300–360 g using a previously described surgical procedure for isolating islets of Langerhans (Sutton *et al*., 1986). In brief, after the rat was anesthetized by an intraperitoneal injection of pentobarbital sodium (40 mg kg^{-1}), the abdomen was opened and 3 mg ml^{-1} collagenase (type 4, Funakoshi, Tokyo, Japan) dissolved in Krebs-Ringer bicarbonate buffer (KRBB) solution containing 5 mM Ca^{2+} was injected into the end of the common bile duct near the duodenum after the ligation of the duct at a site proximal to the liver to fill the pancreas with the injected solution. The animals were sacrificed by cutting the carotid artery for exsanguination. The pancreas was dissected out and incubated in KRBB solution for 17 min at 37°C, and digested into single islets of Langerhans. The collected islets were then dispersed into single islet cells after treatment of the islets with Ca^{2+}-free KRBB solution containing 1 mM ethylene glycol bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA). The cells were plated on coverslips (24 x 24 mm, 0.12 mm in thickness) in 35-mm tissue culture dishes and cultured for 1–4 days in Eagle's MEM medium containing 10% fetal calf serum, 100 μg ml^{-1} streptomycin, 100 IU ml^{-1} penicillin in an atmosphere of 95% O_2 and 5% CO_2 at 37°C. The coverslips was cut into small pieces, and the piece was placed in the recording chamber (1.25 ml) and superfused with the KRBB solution containing 2.8 mM glucose at least 20 min before experiments. The β-cells were identified by their responsiveness to high concentrations of glucose (>8.3 mM) with a closure of ATP-sensitive K^+ channels and subsequent appearance of action currents in cell-attached membrane patches.

Recording of ionic currents and analysis

Patch clamp experiments were performed as has been described (Hamill *et al*., 1981; James *et al*., 1991). The pipettes were pulled from borosilicate glass capillaries (Sutter Instrument Co., CA, USA) with a micropipette puller (Sutter Instrument Co.) coated with silicon resin to reduce stray capacitance, and fire-polished just before use. They had a resistance of 2–5 megohms when filled with the intracellular solution. An Axopatch 200B amplifier (AXON Instruments, CA, USA) was used to record patch membrane currents in the cell-attached mode, membrane potential in perforated-patch mode and K^+ or Ca^{2+} channel currents in standard whole-cell mode. For pulse generation and data collection or analysis, a DigiData 1200 (AXON Instruments) interface to a personal computer with patch clamp software (PCLAMP6) was used. The current data of cell-attached experiments and the membrane potential data of perforated patch experiments were stored on a PCM digital data recorder (TEAC, RD-125T, Tokyo, Japan) for the latter analysis. Replayed data were then processed by a low-pass filter (24dB/octave NF, E-3201A) at a cut-off frequency of 1 kHz, digitized at 5 kHz, and stored in the computer. The data of whole-cell clamp experiments were directly stored in the computer online. All experiments were performed at room temperature (22 to 25°C). All data were expressed as mean±S.E.M from n cells. The data were analyzed by a Scheffe test and Student's paired *t* test, with $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ taken as statistically significant.

Solutions

The KRBB solution contained (mM): NaCl 129; KCl 4.7; $CaCl_2$ 2.0; $MgCl_2$ 1.2; KH_2PO_4 1.2 and $NaHCO_3$ 5.0 (pH was adjusted to 7.4 with HEPES-NaOH). In cell-attached experiments for recording of action currents, the pipettes were filled with an extracellular solution composed of (mM): KCl 140; $CaCl_2$ 2; and HEPES 5.0 (pH was adjusted to 7.4 with KOH). In experiments on whole-cell configuration for recording of K^+ channel currents, the pipette solution contained (mM): KCl 50; K_2SO_4 35; $MgCl_2$ 3.0; EGTA 11; $CaCl_2$ 1; HEPES 11 (pH 7.2 adjusted with KOH) and 0.1 mM ATP plus 0.1 mM ADP were added to record the ATP-sensitive K^+ channel currents (Ashcroft *et al*., 1989). In experiments on the whole-cell configuration for recording of Ca^{2+} channel currents under the condition of inhibition of both K^+ and Na^+ currents, the bath solution was (mM): NaCl 125; KCl 4.8; $CaCl_2$ 10; $MgCl_2$ 1.2; tetraethylammonium chloride (TEA-Cl) 10; HEPES 5.0; tetrodotoxin (TTX) 0.3 μM (pH was adjusted to 7.4 with NaOH) and the pipette solution contained (mM): $MgCl_2$ 4; EGTA 10; ATP 3; GTP 0.1; HEPES 5 and CsCl 125 (pH was adjusted to 7.2 with CsOH). In perforated patch experiments, the pipette solution was (mM): L-

aspartic acid 100; KCl 50; HEPES 10 (pH was adjusted to 7.4 with KOH) and amphotericin B dissolved by DMSO (final concentration of 240 μg ml^{-1}) was added. ATP was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). 1,2-bis(*0*-aminophenoxy)ethane-N, N, N', N'-tetraacetic acid (BAPTA) was from Wako Life Science Reagents (Osaka, Japan) and was used to make the chelation of Ca^{2+} stronger and to suppress an increase in Ca^{2+} concentration at the interior of cells. Pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) and adenosine were from Research Biochemicals International (Natick, MA, USA). EGTA and thapsigargin were from Nacalai Tesque (Kyoto, Japan).

Results

Effects of extracellular ATP on the membrane potential

Fig. 1A–E shows the effects of extracellularly applied 0.1 mM ATP, ADP, AMP, adenosine and ADPβS (15 μM) on action currents evoked by an increase in the concentration of extracellular glucose from 2.8 mM to 8.3 mM in cell-attached membrane patches. When the β-cells were exposed to 2.8 mM glucose, single channel openings (downward deflections for inward current) with a unit amplitude of 3 pA (in the left part of each trace in Fig. 1A–E) were observed and these channel events were substantially inhibited by a subsequent increase in the glucose concentration to 8.3 mM. Noise levels of the baseline current were generally increased in association with the channel inhibition because of depolarization of the membrane. Labile fluctuation of the membrane potential may cause an increase in the noise of the baseline. These observations are consistent with the properties of ATP-sensitive K^+ (K_{ATP}) channel currents (Ashcroft *et al*., 1984; Rorsman and Trube, 1985; Petersen and Findlay, 1987). Action currents showing oscillatory deflecitons of the baseline current levels, which have been demonstrated in chromaffin cells (Fenwick *et al*., 1982), appeared within 2–5 min after exchanging the solution. External ATP reduced the amplitude of the action currents (n=17) and the effect was reversible on washout (Fig. 1A). These results were confirmed by recording membrane potentials using perforated current-clamp experiments (Fig. 1F). The membrane potential was −63 mV in the presence of 2.8 mM glucose. Elevation of glucose concentration to 8.3 mM depolarized the membrane and evoked the action

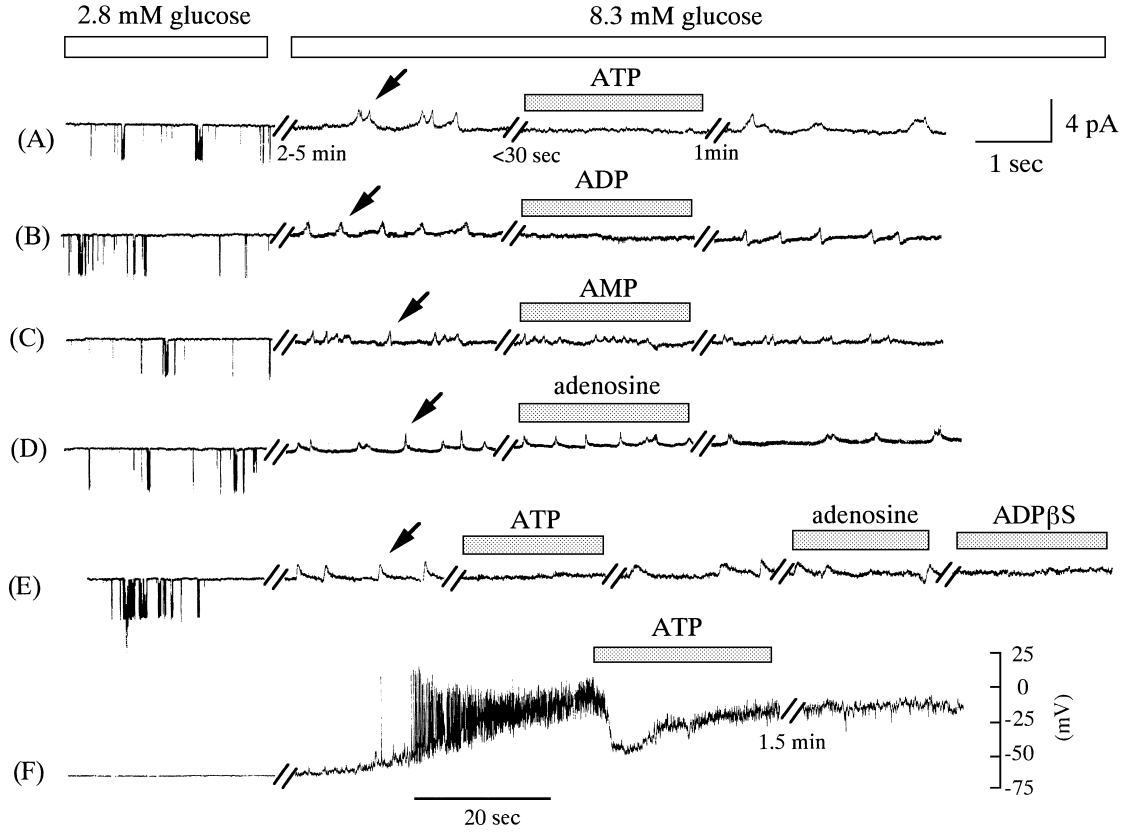

Fig. 1. Effects of nucleotides and adenosine on action currents or action potentials induced by 8.3 mM glucose. A–E: When the concentration of glucose was increased from 2.8 to 8.3 mM in the perfusion solution, the activity of the K_{ATP} channels was gradually decreased and the action current (indicated by arrows) was induced within 2–5 min. Data were recorded in cell-attached mode and filtered at 1 kHz. External application of 0.1 mM ATP (A, E), 0.1 mM ADP (B), 0.1 mM AMP (C), 0.1 mM adenosine (D and E) and 15 μM ADPβS (E) were indicated by columns above each trace. F: Recordings of membrane potential using perforated patch clamp technique showed the inhibition of glucose-induced action potentials by 0.1 mM ATP.

potentials. ATP inhibited the action potential with transient hyperpolarization to −43 mV in association with following reduction of action potential amplitudes. The same experimental protocol in cell-attached membrane patches was repeated with ADP, AMP, or adenosine instead of ATP (Fig. 1B, C and D). ADP at 0.1 mM also reduced the amplitudes of action currents (n=7). However, 0.1 mM AMP had no effect on the action current amplitude in 3 of 5 cells tested, and 0.1 mM adenosine in 8 of 9 cells. Fig. 1E shows that ATP and 15 μM ADPβS had similar inhibitory effects on the amplitude of action currents, but adenosine did not (n=4). As the effect of ATP was mimicked by ADP, we attempted to further test the effects of ATP and ADP at lower concentrations (1 μM). ATP decreased the amplitudes of the action currents in all cells tested (n=4), but ADP did so in only a part of the cells (1 cell out of 3 tested, data not shown). These results suggest that extracellular ATP potently inhibits the action potentials and the order of the inhibitory potency was ATP≥ADP>>AMP, adenosine.

Effects of extracellular ATP on Ca^{2+} channel currents

The most likely ionic current reflecting the inhibition of the action currents by ATP seemed to be L-type Ca^{2+} channels because the membrane potential was not hyperpolarized to the potassium equilibrium potential (Fig. 1F). We explored the effects of 0.1 mM ATP on Ca^{2+} channel currents using the standard whole-cell voltage clamp technique (Fig. 2). When the cell was depolarized to +10 mV from the holding potential of −90 mV at a frequency of 0.5 Hz, an inward current with a slowly inactivated current relaxation, which is characteristic of ICa, was evoked (Fig. 2A). The exposure of cells to 0.1 mM ATP reversibly reduced the peak amplitude of ICa. In Fig. 2B, the peak levels of ICa were plotted as a function of time. ATP inhibited ICa within 20–30 seconds to approximately 60% of the control level, followed by a gradual reduction of inhibitory efficacy. This apparent transient inhibition was also observed in recordings of action potentials (Fig. 1F). In some experiments, amplitudes of ICa declined with time (rundown of ICa; compare traces a and c in Fig. 2A). To evaluate inhibitory efficacy of ATP on ICa, the amplitude prior to exposure to ATP was taken as control and ratio of the minimal amplitude during exposure to ATP and the control was calculated. In Fig. 2C, the concentration-dependence of the inhibition of ICa by ATP was

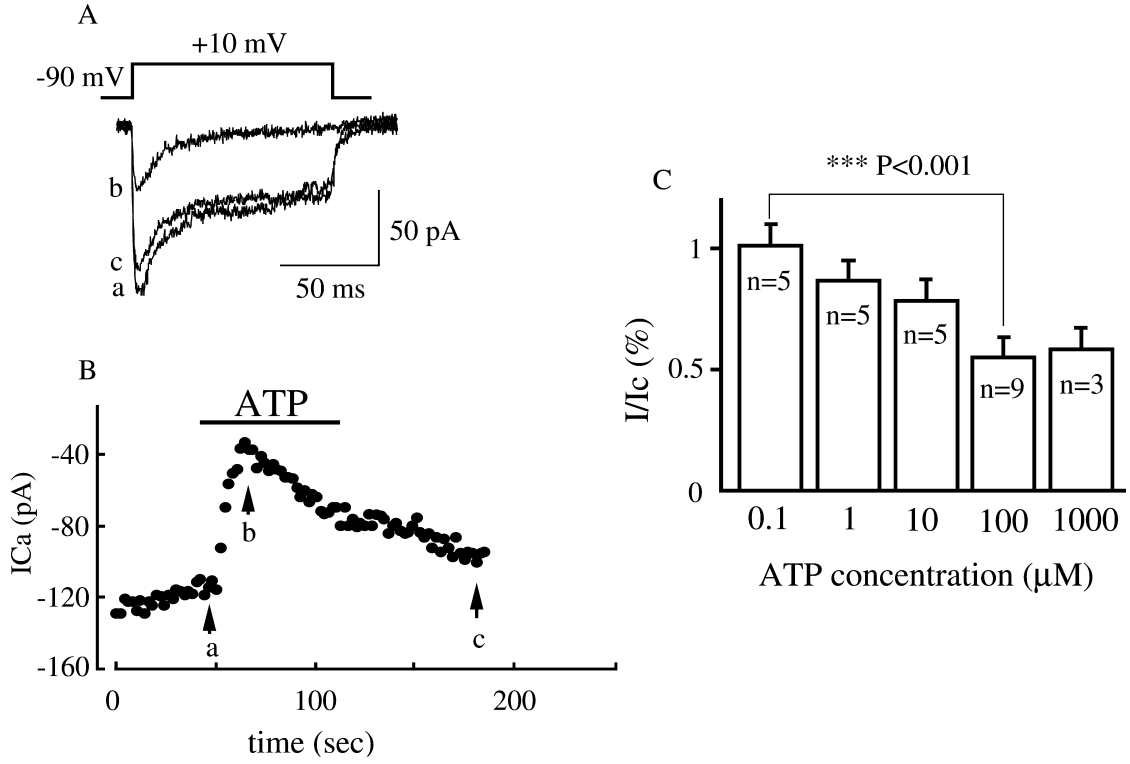

Fig. 2. Inhibition of Ca^{2+} channel currents by extracellular ATP in whole-cell mode. A: The cell was voltage-clamped at −90 mV and depolarized to +10 mV at 0.5 Hz. Current traces of ICa in the control (a), during application of 0.1 mM ATP (b) and after washout (c) were plotted. B: The peak inward current levels of ICa were plotted as a function of time. ATP was applied during the period indicated by the bar. a, b and c correspond to the current traces displayed in A. C: Concentration-dependent inhibition of ICa by ATP between 0.1 and 1000 μM. Numbers in columns are the number of cells tested. Peak amplitudes of ICa were measured at various ATP concentrations using the same voltage protocol in A. Data were normalized to the peak amplitude of ICa before application of ATP and the relative amplitude was plotted as mean±SEM. ***indicates P<0.001 by a Student's paired *t* test.

shown. ATP-induced ICa inhibition seemed to begin at 1 μM and become saturated at 100 μM. The inhibition was partial: ICa was reduced to 56.7±4.0% (n=9) at the minimum amplitude.

To test whether ATP reduces ICa via binding to a specific receptor on the membrane, 0.1 mM 2-methylthio ATP and 15 μM ADPβS, agonists of P2Y purinoceptors, were superfused during recording of the Ca^{2+} channel current evoked using the same protocol as in Fig. 2A and B. Both compounds decreased ICa to 50 and 55% of the control level, respectively (Fig. 3A and B). Addition of 0.1 mM UTP, an agonist of P2Y2 purinoceptors, was without effect (Fig. 3C), and 0.1 mM PPADS and 0.2 mM suramin, antagonists of P2 purinoceptors, prevented the inhibitory effect of ATP on ICa (Fig. 3D and E). The summarized data show the relative amplitudes of ICa during exposure to each analogue (Fig. 3F). ATP, 2-methylthio ATP, ADPβS and UTP reduced ICa to 56.7±4.0% (n=9, $p<0.001$), 52.7±6.2% (n=4, $p<0.001$), 71±5.2% (n=4, $p<0.01$) and 86.2±5.0% (n=4, $p>0.05$) of the control, respectively. The percent amplitudes of ICa during ATP exposure in the presence of suramin and PPADS were 96.4±1.9% (n=4, $p>0.05$) and 95.7±4.6% (n=4, $p>0.05$) of the control, respectively. These results suggested that inhibitory action of ATP on ICa was due to the binding of ATP to P2Y1 purinoceptors (Burnstock and King, 1996).

Two nonhydrolyzable analogues of guanine nucleotides, GTPγS and GDPβS, were used to test whether G-protein has a functional role in ATP-induced inhibition of ICa. When 0.1 mM GTPγS was included in the pipette solution, the application of ATP reduced the ICa. The magnitude of the ICa inhibition was close to that seen in the presence of GTP (Fig. 4A and C), but the inhibition was irreversible (Fig. 4A). In contrast, the presence of 0.2 mM GDPβS in the pipette solution abolished the inhibitory effect of ATP (Fig. 4B). Pertussis toxin (PTX), which ADP-ribosylates the α-subunit of G-proteins and thereby makes them inactive, was used to determine which subtype of G-protein was involved in the ATP-induced ICa inhibition. In five β-cells pretreated with 400 nM PTX for 18 h, the inhibitory effect of ATP on ICa was similarly observed in all cells tested, as compared to the results when GTP or GTPγS was present in the pipette solution (Fig. 4C). These results indicate that PTX-insensitive G-proteins may be involved in the transduction pathway from P2Y purinoceptors to ICa inhibition. Fig. 4C shows a summary of the data about the effects of ATP on ICa under the various conditions described above. The amplitude of ICa during exposure to 0.1 mM ATP in the presence of GTP, GTPγS, GDPβS and GTP with the PTX pretreatment was 56.7±4.0% (n=9, $p<0.001$), 58.4±6.6% (n=4, $p<0.001$), 96.9±1.1% (n=3, $p>0.05$) and 63.5±4.9% (n=4, $p<0.001$), respectively.

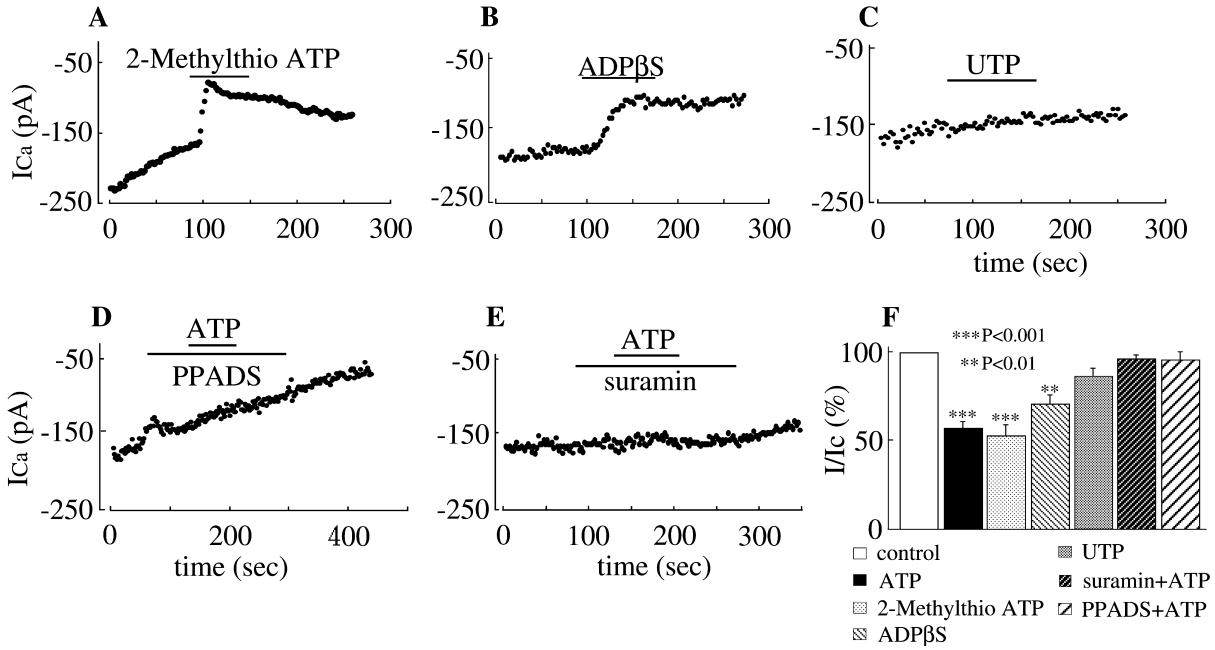

Fig. 3. Effects of agonists or antagonists of P2Y receptors on ICa. Amplitudes of ICa were recorded by depolarizing the membrane potential from –90 mV to +10 mV at 0.5 Hz and plotted against time. The test solution was superfused during the period indicated by the bar above the data points. Effects of 0.1 mM 2-methylthio ATP (A), 15 μM ADPβS (B) and 0.1 mM UTP (C) on ICa are shown. Effects of ATP on ICa in the presence of 0.1 mM PPADS (D) and 0.2 mM suramin (E), which are antagonist of P2 receptors, are shown. ATP did not reduce the ICa during exposure to P2 antagonists. F: Summarized data of relative amplitudes of ICa in the presence of P2 receptor agonists or antagonists. Statistical evaluations of each compound vs control by a Scheffe test are indicated.

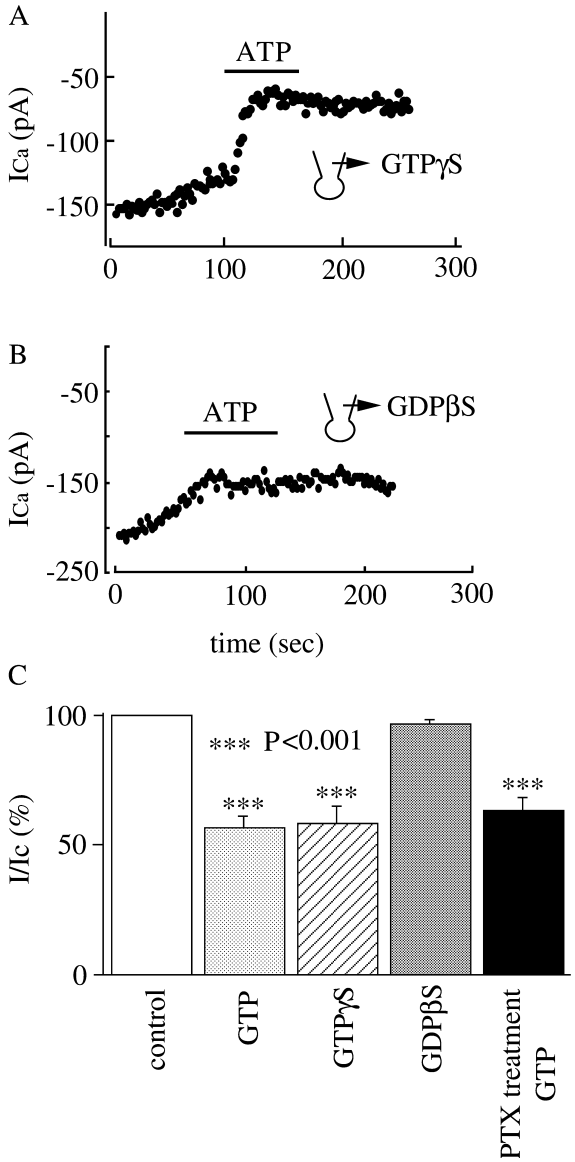

Fig. 4. Involvement of G-proteins in ATP-induced ICa inhibition. The voltage protocol was the same as in Fig. 2A and B. The bars above the data points indicate the period during exposure to ATP. A: The effect of extracellular ATP (0.1 mM) on ICa was irreversible when GTP was replaced by 0.1 mM GTPγS in the pipette solution. B: 0.2 mM GDPβS in the pipette solution abolished the inhibitory effect of 0.1 mM ATP. C: Summary of data on the effect of ATP on ICa under various conditions.

To further explore the signaling pathway distal to G-proteins of the P2Y-mediated ICa inhibition, we used 20 mM BAPTA (a greater chelator of Ca^{2+}) in the pipette solution instead of 10 mM EGTA. Under these conditions, ATP failed to produce any inhibitory effect on ICa (Fig. 5A, n=4). When we used compound 48/80 (0.1 mM), which is a blocker of PLC (Bronner *et al*., 1987), ATP was also without effect (Fig. 5B, n=4). Staurosporine at 1 μM (Yang *et al*., 1997) was then used to determine if ATP reduces ICa by influencing the activity of PKC. ATP inhibited ICa to a similar extent in the presence and absence of staurosporine (Fig. 5C and E, n=4). In β-cells treated with thapsigargin of 0.4 μM for 4 min (Aizawa *et al*., 1995), ATP did not reduce amplitudes of ICa (Fig. 5D). Percent amplitudes of ICa during exposure to 0.1 mM ATP in the presence of EGTA, BAPTA, compound 48/80, thapsigargin pretreatment and staurosporine were 56.7±4.0% (n=9, $p<0.001$), 85.7±4.1% (n=4, $p>0.05$), 84.2±4.8% (n=4, $p>0.05$), 88.3±4.1% (n=4, $p<0.001$) and 62.0±3.1% (n=4, $p<0.001$), respectively (Fig. 5E). These results suggested that ICa inhibition by ATP was dependent on the increase in the concentration of cytosolic Ca^{2+} which may have been released from thapsigargin-sensitive Ca^{2+}stores, but not on PKC.

In rat β-cells, L- and T-type Ca^{2+} channels have been identified (Ashcroft *et al*., 1990). To isolate the L-type ICa from T-type ICa, the membrane potential was held at −40 mV and depolarized to +10 mV at 0.2 Hz (Narahashi *et al*., 1987). Under these conditions, an inward current with a slowly inactivated process, which is the characteristic of L-type ICa, was recorded in the control (●) and during the application of ATP (■) (Fig. 6A). In current-voltage relationship of L-type Ca^{2+} channels (Fig. 6C), ATP reduced the peak levels of L-type ICa from −20±1.3 to −7.9±1.8 pA/pF at +10 mV (n=6). When the membrane potential was held at −90 mV and depolarized to −30 mV at 0.5 Hz, an inward current with more rapid inactivation, which is the characteristic of T-type ICa, was observed (Fig. 6B). ATP had no inhibitory effect on this type of Ca^{2+} channel current (n=4). These results suggested that extracellular ATP inhibited only the L-type Ca^{2+} channels.

Effect of extracellular ATP on K^+ channel currents

Likewise, we tested the effects of ATP (0.1 mM) on ATP-sensitive K^+ (K_{ATP}) channels using the conventional whole-cell voltage clamp technique. In Fig. 7A, the cell was depolarized from −70 to −60 mV to evoke the K_{ATP} channel current using the pipette solution containing 0.1 mM ATP and 0.1mM ADP. Because 0.1 mM tolbutamide totally inhibited the current, this current is due to opening of the K_{ATP} channels. Exposure to extracellular ATP did not influence the amplitude of the K_{ATP} channel current. Regarding the current-voltage relationship, ATP did not change the K_{ATP} channel current, but slightly reduced the voltage-dependent K^+ channel current at membrane potentials positive to −10 mV (Fig. 7B).

Discussion

In the present study, we demonstrated that: 1) extracellular ATP inhibited action potentials which were evoked by high concentrations of glucose in pancreatic β-cells; 2) the inhibitory effect of ATP on the action potential was mediated by an inhibition of L-type Ca^{2+} channels via ATP binding to

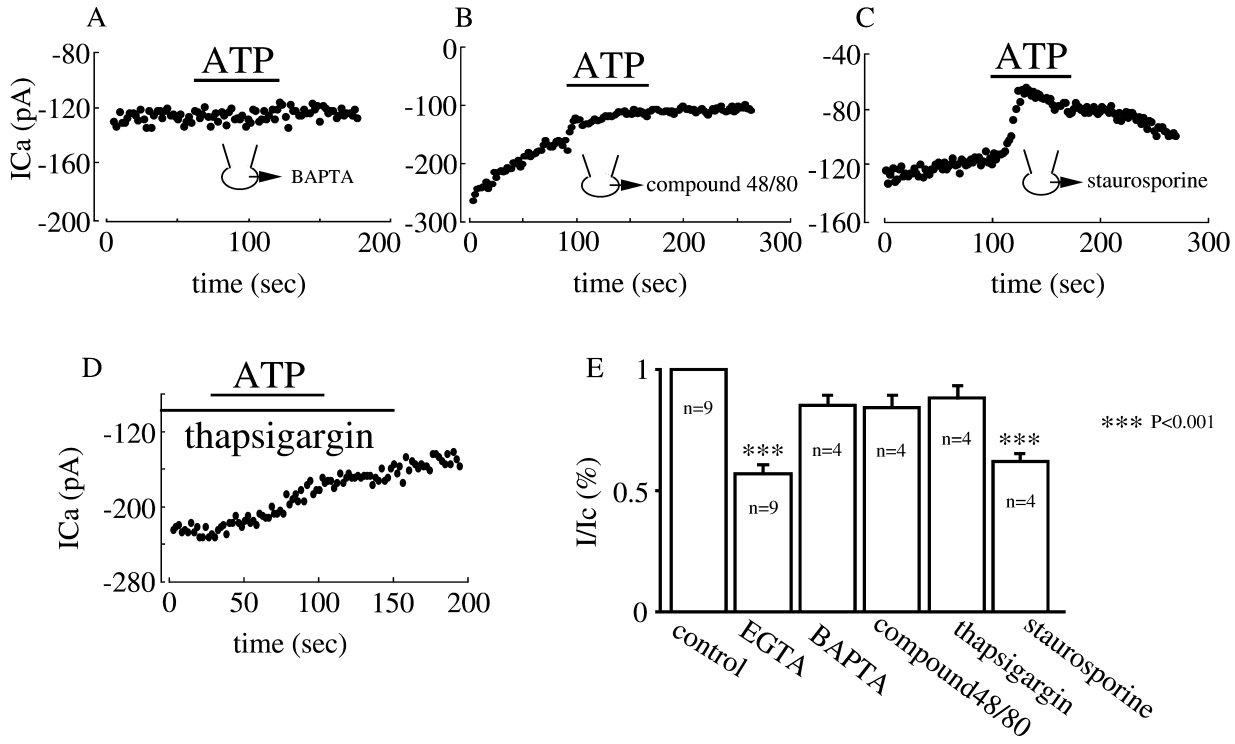

Fig. 5. Effects of ATP on ICa amplitude in the presence of Ca^{2+} chelator, PI-PLC blocker, PKC blocker and a Ca^{2+} pump inhibitor in the cytosol. A: The inhibitory effect of extracellular ATP (0.1 mM) on ICa was prevented when 10 mM EGTA was replaced by 20 mM BAPTA in the pipette solution. B: The presence of 0.1 mM compound 48/80 abolished the inhibitory effect of ATP. C: Staurosporine at the concentration of 1 μM in the pipette solution did not affect the inhibition of ICa by ATP. D: Thapsigargin at the concentration of 0.4 μM prevented the ATP-induced ICa inhibition. E: Summary of data on the effects of ATP on ICa under various conditions.

P2Y purinoceptors, but not due to either activation of K_{ATP} channels or inhibition of T-type Ca^{2+} channels. Activation of voltage-dependent K^+ channel current did not seem to be involved because we did not observe an increase in the current-voltage relationship at membrane potentials positive to −40 mV (Fig. 7B); 3) PTX-insensitive G-proteins may be involved in the ATP-induced Ca^{2+} channel inhibition. It has been established that extracellular ATP stimulates insulin secretion by binding to its receptors, of which signaling results in Ca^{2+} movement from its store sites via activation of PI-PLC coupled to G-proteins and the consequent production of IP3 (Blachier and Malaisse, 1988; Li *et al*., 1991). A rise in cytosolic Ca^{2+} as a consequence of activation of PI-PLC and presumably following IP3 pathway may be a main mechanism underlying the ATP-induced ICa inhibition because the presence of BAPTA, compound 48/80 or thapsigargin prevented ATP-induced ICa inhibition. Elevation of cytosolic Ca^{2+} concentrations may reduce the driving force for L-type Ca^{2+} channels, followed by an inhibition of the channels. Ca^{2+}-sensitive inactivation and facilitation on L-type Ca^{2+} channels have been reported (McCarron *et al*., 1992; Zühlke et al., 2000). Thus, the ATP-induced inhibition of L-type Ca^{2+} channels reported here is a novel action of extracellular ATP.

In cell-attached membrane patches, biphasic current deflections of baseline levels were usually elicited in the presence of 8.3 mM glucose (Fig. 1). A similar current fluctuation has previously been observed in pancreatic β-cells (Ashcroft *et al*., 1984; Rorsman and Trube, 1985) and chromaffin cells (Fenwick *et al*., 1982), and was interpreted as changes in patch membrane current accompanied by action potentials. When we simply tested the effects of extracellular ATP on action currents, extracellular ATP at 0.1 mM effectively inhibited the action currents (Fig. 1A). The inhibition of action currents was not associated with the opening of K_{ATP} channels (Figs. 1 and 7). Since the amplitudes of action currents should be determined by the resistance of membrane patches, the apparent disappearance of action currents during exposure to nucleotides may be caused by either inhibition of membrane potential accompanied by changes in ionic currents or simply by an increase in the patch membrane resistance at the pipette tip. Assuming that the patch membrane resistance was constant throughout these particular experiments, the inhibitory effects of 0.1 mM ATP or 15 μM ADPβS were greater than that of 0.1 mM adenosine (Fig. 1E). Thus, we concluded that the inhibitory potency of various nucleotides and adenosine on the action current was ATP≥ADP>>AMP, adenosine.

The receptors for different nucleotides and adenosine have been classified into two subtypes. P1 purinoceptors are

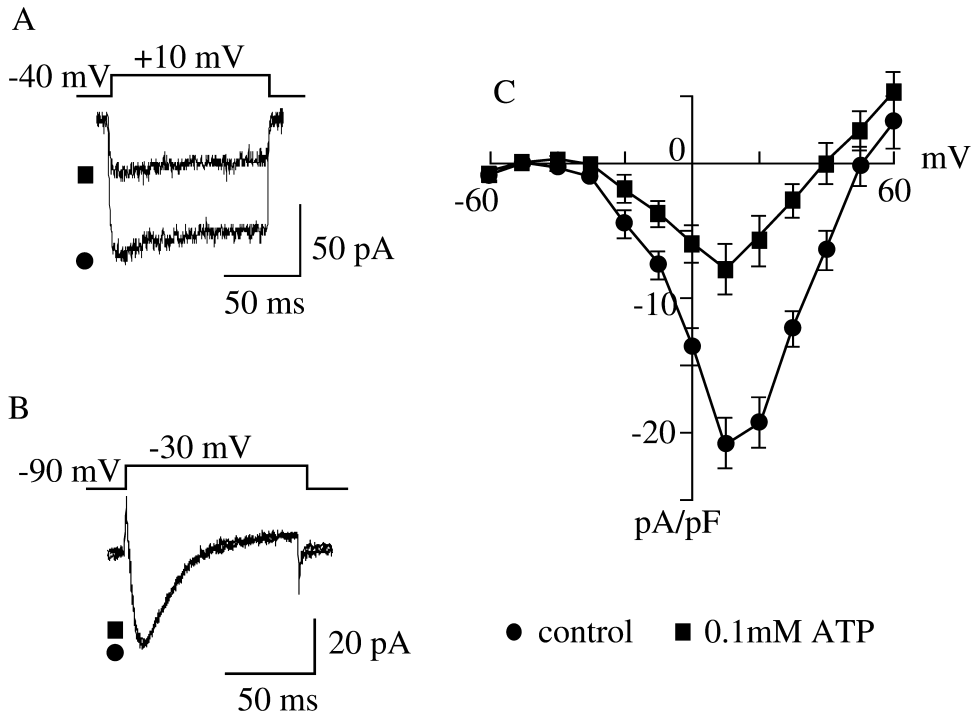

Fig. 6. Inhibition of L-type Ca^{2+} channels by ATP. A: L-type ICa evoked by depolarizing pulses from –40 mV to +10 mV at 0.2 Hz in the control (●) and during application of 0.1 mM ATP (■) was illustrated. B: T-type ICa evoked by depolarizing pulses from –90 mV to –30 mV at 0.5 Hz was not influenced by 0.1 mM ATP. C: Current-voltage relationships (I-V curve) of L-type Ca^{2+} channel current recorded from 8 cells were plotted. The data were expressed as mean±S.E.M of current density. ATP decreased the L-type Ca^{2+} channel current without changing a voltage dependency of the I-V curve.

the most sensitive to adenosine, whereas P2 purinoceptors are sensitive to ATP and ADP. It has been suggested that these subtypes are present in pancreatic β-cells (Hillaire-Buys *et al*., 1994) and insulin secretion is differentially modulated by these types of purinoceptors (Chapal and Loubatières-Mariani, 1981a; Petit *et al*., 1987; Hillaire-Buys *et al*., 1994; Tang *et al*., 1996). Our results indicated that the order of potency of inhibiting action currents was ATP>adenosine, suggesting that these responses were mediated by P2 rather than P1 purinoceptors. The observation that P2 purinoceptor antagonists, PPADS and suramin, attenuated the inhibitory effect of ATP on Ca^{2+} channel currents further supported this idea (Fig. 3). Recently P2 purinoceptors were divided into P2X, P2Y and P2Z. P2X has four subclasses (P2X1-4) with ligand-gated ion channels, P2Y seven subclasses (P2Y1-7) with coupling to G-proteins, and P2Z non-selective pore channels activated by ATP^{4-} (Abbracchio and Burnstock, 1994). When we used selective agonists of P2 purinoceptors to further characterize the subtypes, the order of potency of the effects on inhibition of ICa was found to be 2-methylthio ATP=ATP >ADPβS>>UTP. Although 2-methylthio ATP is also the agonist of P2X, our observations that the ICa inhibition by ATP was mediated via G-proteins suggest that ATP-induced ICa inhibition was mediated by P2Y1 purinoceptors (Burnstock and King, 1996) but not P2X purinoceptors. This is supported by the results that extracellular ATP did not influence the holding current levels, which should be changed if P2X purinoceptors were stimulated (Fig. 7).

P2Y purinoceptors are coupled to a class of G-proteins. It has been reported that the P2Y purinoceptors activate PI-PLC by PTX-insensitive mechanisms (Nanoff *et al*., 1990; Dubyak and el-Moatassim, 1993). Taylor *et al*. (1991) demonstrated that the PI-PLCβ1 isoform can be activated by α-subunits of the Gq class of heterotrimeric G-proteins in liver membranes. As shown in Fig. 4, our results indicated that ICa inhibition mediated by P2Y purinoceptor stimulation was coupled to pertussis toxin-insensitive G-proteins. Two possible hypotheses may be proposed for signals from G-proteins to L-type Ca^{2+} channel inhibition: 1) Involvement of second messengers for the L-type Ca^{2+} channel inhibition through the stimulation of PI-PLC, and Ca^{2+} release from the stores sensitive to IP3 produced from phosphatidylinositol-4,5-bisphosphate (PIP_2). The cytosolic Ca^{2+} elevation may therefore be involved in ATP-induced Ca^{2+} channel inhibition. Production of IP3 by ATP stimulation has been reported in RINm5F cells and isolated rat pancreatic islet cells (Blachier and Malaisse, 1988; Li *et al*., 1991). 2) The second possibility is involvement of directly coupled inhibition of Ca^{2+} channel via G-proteins. Swandulla *et al*. (1991) suggested that the inhibition of Ca^{2+} channels induced by either neurotransmitters or intracellular GTP was due to a

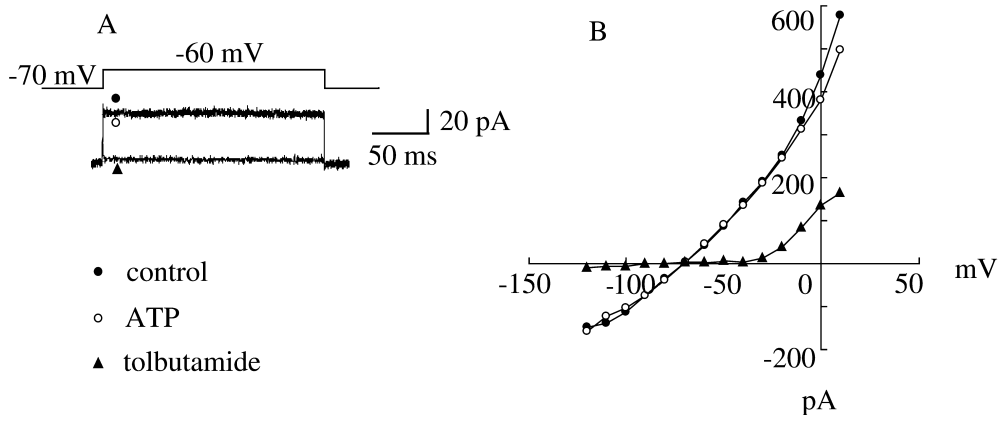

Fig. 7. ATP did not inhibit K_{ATP} channel currents. The K_{ATP} channel current was evoked by decreasing the ATP concentration in the pipette solution to 0.1 mM and adding 0.1 mM ADP. After reaching a steady state level of background current as determined by monitoring the current level depolarized from −70 mV to −60 mV, 0.1 mM ATP or 0.1 mM tolbutamide was superfused. A: The K_{ATP} channel current indicated by closed circles (●) was inhibited by tolbutamide (▲). Exposure to extracellular ATP did not change the amplitude of K_{ATP} channels (○). B: The I-V curve of the K_{ATP} channel current was not influenced by ATP.

direct coupling of G-protein to the channels. Our results supported the former idea. When we used 20 mM BAPTA instead of 10 mM EGTA in the pipette solution, 0.1 mM ATP failed to inhibit the ICa. Furthermore, when the upstream portion of the signaling pathway was blocked by 0.1 mM compound 48/80, which is a specific inhibitor of PI-PLC, ICa inhibition by ATP was also attenuated. Equal inhibition of ICa by ATP in the presence of staurosporine and ineffectiveness of ATP on amplitudes of ICa in the cells pretreated with thapsigargin strongly suggests involvement of Ca^{2+} release from the Ca^{2+} store sites sensitive to thapsigargin but not of PKC. Similar ICa inhibition was observed when 250 μM acetylcholine (ACh) was tested instead of ATP (data not shown).

The inhibitory effect of ATP on Ca^{2+} channel currents was transient (Fig. 2B). This may be explained by the observation that the ATP-induced cytosolic Ca^{2+} concentration increase is reportedly transient (Theler *et al*., 1992; Nakazaki *et al*., 1998). Time constant for the falling phase of the transient Ca^{2+} increase evoked by ATP was calculated to be 0.25±0.04 min (Nakazaki *et al*., 1998), and it was comparable with our observations (Fig. 2B). Another possible mechanism involved in the transient inhibition of ICa by ATP is a desensitization of ATP stimulation. However, since a non-hydrolyzable analogue of ADP, ADPβS, did not show transient changes in ICa inhibition as compared to that caused by ATP (Fig. 3B), the hydrolysis of ligands near P2Y purinoceptors or on the plasma membrane of β cells might cause the transient inhibition of L-type Ca^{2+} channels.

Isolated insulin secretory granules have been reported to contain ATP and other adenine nucleotides as well as adenosine (Hutton *et al*., 1983). These intragranular constituents should be co-released with insulin. Likewise, ATP may be released simultaneously with acetylcholine from the stimulated cholinergic synaptosomes surrounding β cells (Dowdall *et al*., 1974). It has been estimated that the concentration of ATP is 3.5±0.6 mM in isolated insulin-secretory granules (Hutton *et al*., 1983). Recently, it was reported that the mean local concentration of ATP at the surface of β cells stimulated by glucose exceeds 25 μM, as determined by a biosensor technique using PC12 cells expressing P2X2 purinoceptors (Hazama *et al*., 1998). We found that the effective concentration of ATP was between 0.1 and 1000 μM and the half-maximum concentration of ATP was approximately 6.8 μM for the inhibition of ICa (Fig. 2C). The inhibitory efficacy was saturated at 0.1 mM ATP. Thus, these concentrations found in the present study correspond well to the concentrations reported for the stimulation of P2Y purinoceptors, which increase insulin secretion (Loubatières-Mariani *et al*., 1979; Chapal and Loubatières-Mariani, 1981b; Arkhammar *et al*., 1990; Li *et al*., 1991; Squires *et al*., 1994), and the concentration in the extracellular space (Gandia et al., 1993). Similar extracellular ATP-induced inhibitory effects on voltage-gated Ca^{2+} channels have been reported with the same effective concentration ranges (Diverse-Pierluissi *et al*., 1991; Qu *et al*., 1993; Mühlen *et al*., 1997).

Extracellular ATP may inhibit insulin secretion by reducing electrical activity in mouse β-cells (Petit *et al*., 1989), although there have been a great deal of reports revealing stimulation of insulin secretion by extracellular ATP via P2Y-purinoceptors (Chapal and Loubatières-Mariani,1981a; Blachier and Malaisse, 1988; Li *et al*., 1991). However, Poulsen *et al*. (1999) indicated that extracellular ATP inhibited exocytosis without modulation of Ca^{2+} channels activity in mouse β-cells. The difference between our results and those by Poulsen *et al*. might be due to the difference of signaling pathway from P2Y purinoceptors or experimental conditions for temperature performed (33–37°C) and electrophysiological protocol (2 min intervals of depolarizing

pulses vs. 2 s for us). Extracellular ATP may play an important physiological role in regulating insulin secretion and β-cell function. When the glucose concentration is increased to a level above normal, extracellular ATP co-released with insulin by exocytosis may further increase insulin secretion in a positive feedback mechanism by increasing cytosolic Ca^{2+} levels. In contrast, co-released ATP may transiently inhibit L-type Ca^{2+} channels in a negative feedback mechanism demonstrated in the present paper. The latter mechanism may contribute to prevention of an excessive increase in Ca^{2+} concentration in the cytosol.

Acknowledgments. This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (to M.K.).

References

Abbracchio, M-P. and Burnstock, G. 1994. Purinoceptors: are there families of P2X and P2Y purinoceptors? *Pharmacol. Ther.*, **64**: 445–475.

Aizawa, T., Yada, T., Asanuma, N., Sato, Y., Ishihara, F., Hamakawa, N., Yaekura, K., and Hashizume, K. 1995. Effects of thapsigargin, an intracellular Ca^{2+} pump inhibitor, on insulin release by rat pancreatic B-cell. *Life Sci.*, **57** (14): 1375–1381.

Arkhammar, P., Hallberg, A., Kindmark, H., Nilsson, T., Rorsman, P., and Berggren, P.O. 1990. Extracellular ATP increases cytoplasmic free Ca^{2+} concentration in clonal insulin-producing RINm5F cells. *Biochem. J.*, **265**: 203–211.

Ashcroft, F.M., Harrison, D.E., and Ashcroft, S.J.H. 1984. Glucose induces closure of single potassium channels in isolated rat pancreatic β-cells. *Nature*, **312**: 446–448.

Ashcroft, F.M., Kakei, M., Gibson, J.S., Gray, D.W., and Sutton, R. 1989. The ATP- and tolbutamide-sensitivity of the ATP-sensitive K-channel from human pancreatic B cell. *Diabetologia*, **32**: 591–598.

Ashcroft, F.M., Kelly, R.P., and Smith, P.A. 1990. Two types of Ca^{2+} channels in rat pancreatic β-cells. *Pflügers Arch.*, **415**: 504–506.

Bertrand, G., Chapal, J., Loubatières-Mariani, M.M., and Roye, M. 1987. Evidence for two different P_2-purinoceptors on beta cell and pancreatic vascular bed. *Br. J. Pharmacol.*, **91**: 783–787.

Blachier, F. and Malaisse, W.J. 1988. Effect of exogenous ATP upon inositol phosphate production, cationic fluxes and insulin release in pancreatic islet cells. *Biochim. Biophys. Acta*, **970**: 222–229.

Boyd, A.E. 1992. The role of ion channels in insulin secretion. *J. Cell Biochem.*, **48**: 235–241.

Bronner, C., Wiggins, C., Monté, D., Märki, F., Capron, A., Landry, Y., and Franson, R.C. 1987. Compound 48/80 is a potent inhibitor of phospholipase C and a dual modulator of phospholipase A_2 from human platelet. *Biochim. Biophys. Acta*, **920**: 301–305.

Burnstock, G. and King, B.K. 1996. Numbering of cloned P2 purinoceptors. *Drug Dev. Res.*, **38**: 67–71.

Candela, J.L. and Garcia-Fernandez, M.C. 1963a. Stimulation of secretion of insulin by adenosine triphosphate. *Nature*, **197**: 1210.

Candela, J.L. and Garcia-Fernandez, M.C. 1963b. Stimulation of insulin secretion in vitro by adenosine triphosphate. *Nature*, **197**: 1304.

Chapal, J. and Loubatières-Mariani, M.M. 1981a. Attempt to antagonize the stimulatory effect of ATP on insulin secretion. *Eur. J. Pharmacol.*, **74**: 127–134.

Chapal, J. and Loubatières-Mariani, M.M. 1981b. Effects of phosphate-modified adenine nucleotide analogues on insulin secretion from perifused rat pancreas. *Br. J. Pharmacol.*, **73**: 105–110.

Cook, D.L. and Hales, C.N. 1984. Intracellular ATP directly blocks K^+ channels in pancreatic B-cells. *Nature*, **311**: 271–273.

Diverse-Pierluissi, M., Dunlap, K., and Westhead, E.W. 1991. Multiple actions of extracellular ATP on calcium currents in cultured bovine chromaffin cells. *Neurobiology*, **88**: 1261–1265.

Dowdall, M.J., Boyne, A.F., and Whittaker, V.P. 1974. Adenosine triphosphate a constituent of cholinergic synaptic vesicles. *Biochem. J.*, **140**: 1–12.

Dubyak, G. R. and el-Moatassim, C. 1993. Signal transduction via P_2-purinergic receptors for extracellular ATP and other nucleotides. *Am. J. Physiol.*, **265**: C577–C606.

Fenwick, E.M., Marty, A., and Neher, E. 1982. A patch-clamp study of bovine chromaffin cells and of their sensitivity to acetylcholine. *J. Physiol.*, **331**: 577–597.

Gandia, L., Garcia, A.G., and Morad, M. 1993. ATP modulation of calcium channels in chromaffin cells. *J. Physiol.*, **470**: 55–72.

Gordon, J.L. 1986. Extracellular ATP: effects, sources and fate. *Biochem. J.*, **233**: 309–319.

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F.J. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.*, **391**: 85–100.

Hazama, A., Hayashi, S., and Okada, Y. 1998. Cell surface measurements of ATP release from single pancreatic β cells using a novel biosensor technique. *Pflügers Arch.*, **437**: 31–35.

Hillaire-Buys, D., Chapal, J., Bertrand, G., Petit, P., and Loubatières-Mariani, M.M. 1994. Purinergic receptors on insulin-secreting cells. *Fundam. Clin. Pharmacol.*, **8**: 117–127.

Hutton, J.C., Penn, E.J., and Peshavaria, M. 1983. Low-molecular-weight constituents of isolated insulin-secretory granules. *Biochem. J.*, **210**: 297–305.

James, R., Kim, C., Peter, G., and Mitchell, W. 1991. Low access resistance perforated patch recordings using amphotericin B. *J. Neurosci. Methods*, **37**: 15–26.

Kakei, M., Kelly, R.P., Ashcroft, S.J.H., and Ashcroft, F.M. 1986. The ATP-sensitivity of K^+ channels in rat pancreatic B-cells is modulated by ADP. *FEBS Lett.*, **208**: 63–66.

Li, G., Milani, D., Dunne, M.J., Pralong, W.F., Theler, J.M., Petersen, O.H., and Wollheim, C.B. 1991. Extracellular ATP causes Ca^{2+}-dependent and -independent insulin secretion in RINm5F cells. *J. Biol. Chem.*, **266**: 3449–3457.

Loubatières-Mariani, M.M., Chapal, J., Lignon, F., and Valette, G. 1979. Structural specificity of nucleotides for insulin secretory action from the isolated perifused rat pancreas. *Eur. J. Pharmacol.*, **59**: 277–286.

Lustig, K.D., Shiau, A.K., Brake, A.J., and Julius, D. 1993. Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Pharmacology*, **90**: 5113–5117.

McCarron, J.G., McGeown, J.G., Reardon, S., Ikebe, M., Fay, F.S., and Walsh, J.V. Jr. 1992. Calcium-dependent ehhancement of calcium current in smooth muscle by calmodulin-dependent protein kinase II. *Nature*, **357**: 74–77.

Morel, N. and Meunier, F.M. 1981. Simultaneous release of acetylcholine and ATP from stimulated cholinergic synaptosomes. *J. Neurochem.*, **36**: 1766–1773.

Mühlen, F.V.Z., Gonska, B.D., and Kreuzer, H. 1997. Inhibition of the voltage-dependent calcium current by extracellular ATP in hamster ventricular cardiomyocytes. *Am. J. Physiol.*, **273**: H250–H256.

Nakazaki, M., Ishihara, H., Kakei, M., Inukai, K., Asano, T., Miyazaki, J. I., Tanaka, H., Kikuchi, M., Yada, T., and Oka, Y. 1998. Repetitive mitochondria Ca^{2+} signals synchronize with cytosolic Ca^{2+} oscillations in the pancreatic beta-cell line, MIN6. *Diabetologia*, **41**: 279–286.

Nanoff, C., Freissmuth, M., Tuisl, E. and Schütz, W. 1990. P_2-, but not P_1-purinoceptors mediate formation of 1,4,5-inositol trisphosphate and its metabolites via a pertussis toxin-insensitive pathway in the rat renal cortex. *Br. J. Pharmacol.*, **100**: 63–68.

Narahashi, T., Tsunoo, A., and Yoshii, M. 1987. Characterization of two types of calcium channels in mouse neuroblastoma cells. *J. Physiol.*, **383**: 231–249.

Petersen, O.H. and Findlay, I. 1987. Electrophysiology of the pancreas. *Physiol. Rev.*, **67**: 1054–1116.

Petit, P., Manteghetti, M., Puech, R. and Loubatières-Mariani, M.M. 1987. ATP and phosphate- modified adenine nucleotide analogues effects on insulin secretion and calcium uptake. *Biochem. Pharmacol.*, **36**: 377–380.

Petit, P., Bertrand, G., Schmeer, W., and Henquin, J.C. 1989. Effects of extracellular adenine nucleotides on the electrical, ionic and secretory events in mouse pancreatic β-cells. *Br. J. Pharmacol.*, **98**: 875–882.

Poulsen, C.R., Bokvist, K., Olsen, H.L., Hoy, M., Capito, K., Gilon, P., and Gromada, J. 1999. Multiple sites of purinergic control of insulin secretion in mouse pancreatic β-cells. *Diabetes*, **48**: 2171–2181.

Qu, Y., Campbell, D.L., and Strauss, H.C. 1993. Modulation of L-type Ca^{2+} current by extracellular ATP in ferret isolated right ventricular myocytes. *J. Physiol.*, **471**: 295–317.

Rorsman, P. and Trube, G. 1985. Glucose dependent K^+-channels in pancreatic β-cells are regulated by intracellular ATP. *Pflügers Arch.*, **405**: 305–309.

Simon, J., Webb, T.E., King, B.F., Burnstock, G., and Barnard, E.A. 1995. Characterization of a recombinant P2Y purinoceptor. Eur. *J. Pharmacol.*, **291**: 281–289.

Squires, P.E., James, R.F.L., London, N.J.M., and Dunne, M.J. 1994. ATP-induced intracellular Ca^{2+} signals in isolated human insulin-secreting cells. *Pflügers Arch.*, **427**: 181–183.

Sutton, R., Peters, M., McShane, P., Gray, D.W.R., and Morris, P.J. 1986. Isolation of rat pancreatic islets by ductal injection of collagenase. *Transplantation*, **42**: 689–691.

Swandulla, D., Carbone, E., and Lux, H.D. 1991. Do calcium channel classifications account for neuronal calcium channel diversity? *Trends Neurosci.*, **14**: 46–51.

Tang, J., Pugh, W., Polonsky, K.S., and Zhang, H.E. 1996. Preservation of insulin secretory responses to P_2 purinoceptor agonists in Zucker diabetic fatty rats. *Am. J. Physiol.*, **270**: E504–E512.

Taylor, S.J., Chae, H.Z., Rhee, S.G., and Exton, J.H. 1991. Activation of the β1 isozyme of phospholipase C by α subunits of the G_q class of G proteins. *Nature*, **350**: 516–518.

Theler, J.M., Mollard, P., Guérineau, N., Vacher, P., Pralong, W.F., Schlegel, W., and Wollheim, C.B. 1992. Video imaging of cytosolic Ca^{2+} in pancreatic β-cells stimulated by glucose, carbachol, and ATP. *J. Biol. Chem.*, **267**: 18110–18117.

Valera, S., Hussy, N., Evans, R.J., Adami, N., North, R.A., Surprenant, A., and Buell, G. 1994. A new class of ligand-gated ion channel defined by P_{2x} receptor for extracellular ATP. *Nature*, **371**: 516–519.

Yang, R., Fu, W., Wang, S., Liu, T., and Lin-Shiau, S. 1997. Mechanism of the morphological changes induced by staurosporine in rat osteoblasts. *Calcif. Tissue Int.*, **61**: 68–73.

Yokoshiki, H., Sunagawa, M., Seki, T., and Sperelakis, N. 1998. ATP-sensitive K^+ channels in pancreatic, cardiac, and vascular smooth muscle cells. *Am. J. Physiol.*, **274**: C25–C37.

Zühlke, R.D., Pitt, G.S., Tsien, R.W., and Reuter, H. 2000. Ca^{2+}-sensitive inactivation and facilitation of L-type Ca^{2+} channels both depend on specific amino acid residues in a consensus calmodulin-binding motif in the a1c subunit. *J. Biol. Chem.*, **275**: 21121–21129.

(*Received for publication, August 30, 2000*
and in revised form, October 10, 2000)