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ATP-induced $[Ca^{2+}]_i$ changes and depolarization in GH3 cells

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1 Extracellular ATP is a neurotransmitter and mediates a variety of responses. In the endocrine system, there are data suggesting a physiological role for ATP in Ca^{2+} signalling and hormone secretion. However, the ATP receptor subtype involved has not been clearly elucidated in GH3 cells, a rat anterior pituitary cell line.

2 BzATP- and ATP-induced $[Ca^{2+}]_i$ responses had EC_{50} values of 18 and 651 μM, respectively. The maximal response to ATP was only 59±8% of that for BzATP. The BzATP-induced $[Ca^{2+}]_i$ increase was dependent upon the extracellular Ca^{2+} concentration. Preincubation with oxidized ATP (oATP) nearly abolished the ATP- and BzATP-induced $[Ca^{2+}]_i$ increases.

3 Both BzATP and ATP induced depolarization in GH3 cells, with EC_{50} values of 31 μM and 1 mM, respectively. The maximal depolarization to BzATP and ATP were 152±21 and 146±16% of that elicited by 30 mM KCl.

4 The rank order of agonist potency for $[Ca^{2+}]_i$ and depolarization responses was BzATP > > ATP > 2-MeSATP and purine derivatives such as ADP, AMP, adenosine were ineffective. Neither UTP nor α, β-methylene ATP showed any effect.

5 In low-divalent conditions BzATP evoked non-desensitizing inward currents, which were reversed at ~0 mV. This nonselective cationic conductance was increased by repeated applications of BzATP and the cells became very permeable to NMDG. Longer applications (30 min) of BzATP stimulated ethidium bromide influx in low divalent conditions, suggesting increased permeability to larger molecules. We also identified the existence of *P2X*$_7$ mRNA on GH3 cells by using reverse transcriptase (RT)-polymerase chain reaction (PCR).

6 These results suggest that the GH3 cells have an endogenous $P2X_7$ receptor and purinergic stimulation may play a potential role in neuroendocrine modulation on these cells.
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Keywords: $P2X_7$ receptor; GH3 cell; intracellular calcium; depolarization; nonselective cation current; membrane permeability

Abbreviations: BzATP, 2′- and 3′-o-(4-benzoyl-benzoyl) adenosine 5′-triphosphate; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; 2-MeSATP, 2-methylthioadenosine triphosphate; oATP, adenosine 5′-triphosphate 2′, 3′-dialdehyde

Introduction

The role of ATP in metabolism is well established, but its potential importance as an extracellular chemical messenger has been recognized only recently. Extracellular ATP mediates a variety of responses in a number of biological systems (Burnstock, 1990). It has been proposed that ATP plays important roles in both short-term physiologic events such as neurotransmission, exocrine and endocrine secretion and regulation of immune cell function, and long-term physiologic events such as cell growth, differentiation and proliferation in development (Abbracchio & Burnstock, 1998). These effects of extracellular ATP are mediated by purine-and pyrimidine-based nucleotide receptors, designated P2-receptors, expressed in many tissues throughout the body (Burnstock, 1990; Ralevic & Burnstock, 1998). P2-receptors have been classified into two main families: an ionotropic P2X receptor family consisting of ligand-gated ion channels, and a metabotropic P2Y receptor family consisting of G protein-coupled receptors (Buell *et al*., 1996; Burnstock, 1997; Ralevic & Burnstock, 1998). It has been proposed that many functional effects of extracellular ATP be related with the increase in $[Ca^{2+}]_i$ (Gargett *et al*., 1997). Stimulation of P2-receptors can elevate the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), either by influx from the extracellular medium *via* cation channels or by release of Ca^{2+} from the internal stores (Dubyak & El-Moatassim, 1993). Subclasses ($P2X_1$-$P2X_7$ and $P2Y_1$-$P2Y_6$, $P2X_{11}$) of these two main receptor families have been identified on the basis of recent molecular biology studies and the development of new selective agonists and antagonists (Abbracchio & Burnstock, 1994; Ralevic & Burnstock, 1998).

The $P2X_7$ receptor is known as an uniquely bifunctional molecule through which ATP can lead not only to an inward ionic currents, but also to an increase in cell membrane permeability (Surprenant *et al*., 1996; Rassendren *et al*., 1997; Virginio *et al*., 1997). The increased permeability caused by activation of the $P2X_7$ receptor results in large ion fluxes and leakage of small metabolites (Relevic & Burnstock, 1998). Activation of $P2X_7$ receptor also activates phospholipase D in murine and human macrophages (El-Moatassim & Dubyak, 1993; Humphreys & Dubyak, 1996) and human leukemic lymphocytes (Gargett *et al*., 1996; Fernando *et al*., 1999), although the mechanism is not fully understood. Surprenant *et al*. (1996) suggested that the $P2X_7$ receptor cloned from rat macrophage and brain is the cytolytic 'P2Z receptor' previously described in mast cells, macrophages, fibroblasts, lymphocytes, erythrocytes and erythroleukemia cells (Relevic & Burnstock, 1998).

The $P2X_7$ receptor has been classified by the following agonist selectivity: BzATP > > ATP > 2-MeSATP > ATPγS (Dubyak & El-Moatassim, 1993; Surprenant *et al*.,

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1996). This receptor shows selectivity for the ATP^{4-} species, but stimulation of this receptor requires a higher concentration of ATP than any other subtypes of P2X receptor (Kaiho *et al*., 1996). It has been reported that 2′, 3′-dialdehyde ATP (oATP) is an irreversible antagonist of the P2Z/ $P2X_7$ receptor (Murgia *et al*., 1993; Wiley *et al*., 1994; Surprenant *et al*., 1996). $P2X_7$ receptors are generally expressed in cells of hemopoietic origin, such as macrophages and thymocytes, but their function is not clear yet (Surprenant *et al*., 1996).

There are data suggesting a possible regulatory role for ATP in endocrine tissues, since ATP has been shown to increase $[Ca^{2-}]_i$ in hypothalamic neurons (Chen *et al*., 1994). Tomic *et al*. (1996) suggested that ATP represents a paracrine or autocrine factor in the regulation of $[Ca^{2+}]_i$ signaling and secretion of pituitary hormone in gonadotroph cells and this action may be mediated by P2X receptor. It is acknowledged that the rat pituitary growth hormone- and prolactin-secreting GH3 cell line is a useful and well studied model system for the study of pituitary cell signalling (Charles *et al*., 1999). However, it has not yet been elucidated which subtypes of P2 receptor are expressed in GH3 cells and which mechanisms are involved in purinergic stimulation. In the present study, we confirmed the presence of the endogenous $P2X_7$ receptor and characterized the receptor by combining of fluorimetric techniques, electrophysiological experiments and RT–PCR analysis.

Methods

Cell culture

GH3 cells obtained from the American Type Culture Collection (CCL-82.1; ATCC, MD, U.S.A.) were maintained in Ham's F10 (Sigma Chemical Company, St. Louis, MO, U.S.A.) supplemented with 15% (v v^{-1}) heat-inactivated horse serum, 2.5% (v v^{-1}) foetal calf serum and penicillin 100 (U ml^{-1}), streptomycin (100 μg ml^{-1}), fungizone (25 μg ml^{-1}; Gibco BRL, Grand Island, NY, U.S.A.). Cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was changed every 2 days and cells were subcultured about once every 2 weeks.

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

The intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured with fura-2, loaded into the cells as the acetoxymethyl ester (Suh & Kim, 1994). Cells were detached from culture dishes with 0.25% trypsin and washed twice by centrifugation ($1000 \times g$, 5 min) with Krebs-Ringer Henseleit (KRH) buffer, containing (in mM) NaCl 125, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 6, HEPES 25, $CaCl_2$ 2 to pH 7.4 and supplemented with 0.2% BSA. Cell suspensions (1×10^6 ml^{-1}) were incubated in KRH buffer with 4 μM fura-2/AM at 37°C for 40 min with continuous stirring. Cells were then washed with BSA-free KRH buffer (standard buffer). Sulfinpyrazone (250 μM) was added to standard buffer to prevent dye leakage. $[Ca^{2+}]_i$ measurements were carried out in a thermostatically regulated and magnetically stirred fluorometer cuvette (F2000 fluorescence spectrophotometer, Hitachi Ltd., Tokyo, Japan) at a concentration of 1×10^6 ml^{-1}. Changes in the fluorescence ratio were measured at dual excitation (ex) wavelengths of 340 and 380 nm and the emission (em) wavelength of 510 nm; ex and em wavelengths were monitored ten times a second at a pass band of 20 nm. The calibration of the fluorescence ratio in terms of $[Ca^{2+}]_i$ was performed according to Grynkiewicz *et al*. (1985).

Fluorometric measurement of plasma membrane potential

Changes in plasma membrane potential were measured with the fluorescent potential sensitive anionic dye bis [1, 3-diethylthiobarbiturate] trimethine oxonol (bisoxonol: Molecular Probes, Inc., Eugene, OR; Rink *et al*., 1980). Cultured cells were harvested and suspended in KRH buffer at a density of 1×10^7 ml^{-1} and stored on ice. Cells (1×10^6 ml^{-1}) were incubated with 200 nM bisoxonol for 5 min at 37°C prior to the addition of compounds or drugs. Fluorescence was monitored at the wavelength pair 540 (ex)–580 (em) nm. Changes of depolarization were represented either directly the intensity of fluorescence or relative fluorescence intensity evoked by 30 mM KCl.

Electrophysiological measurements

Cells were transferred to a recording chamber and superfused at 2 ml min^{-1} with normal bath solution. Ionic currents were recorded in whole-cell voltage clamp condition by using a patch clamp amplifier (Axopatch 1-D, Axon Instruments, Foster city, CA, U.S.A.). Patch pipettes were fabricated on a model P-97 Flaming/Brown micropipette puller (Sutter Instrument Co., CA, U.S.A.) from glass capillary (G-1.5, Narishinge, Tokyo, Japan). Pipettes were fire-polished prior to use and had a tip resistance of $3 \sim 5$ MΩ when dipped into normal bath solution. The dialyzed patch clamp technique was used to record whole cell currents at room temperature (20–22°C). The sampling rate to be digitized on AD converter (Digidata 1200, Axon Instruments) was 1 kHz, and an 8-pole Bessel filter was used to low-pass filter membrane currents at 1 kHz. To record non-selective cation currents, the pipette solution contained (in mM) NaCl 165, HEPES 10 and EGTA 11. Normal bath solution was (mM): NaCl 154, KCl 2, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 10, glucose 12 and 'low divalent' solution with no magnesium and 0.3 mM $CaCl_2$. Sodium was replaced by equimolar NMDG, when necessary. Internal and external solutions were maintained at pH 7.3 and 300 mOsm l^{-1}. Agonists were delivered by a fast-exchange flow system. Membrane potential was held at −60 mV, and continuously recorded with using MacLab and Chart v3.6/s (AD Instruments, Castle Hill, Australia).

Changes in plasma membrane permeability

ATP-dependent increases in plasma membrane permeability were measured with ethidium bromide, an extracellular fluorescent tracer (Falzoni *et al*., 1995). For ethidium bromide uptake, cell suspensions were incubated for 30 min at 37°C in low divalent solution containing 250 μM sulfinpyrazone and 20 μM ethidium bromide, and stimulated with 300 μM BzATP. Control cells were incubated in the same solution without BzATP. After several washings to remove the extracellular dye, cells were analysed with an inverted fluorescence microscope (Olympus IMT2-RFL; Olympus Optical Co., Ltd., Tokyo, Japan) equipped with a 10 × objective and a fluorescein filter.

Reverse transcriptase (RT)-polymerase chain reaction (PCR)

Total RNAs from GH3 cells and RAW 264.7 cells, murine macrophages obtained from ATCC, were prepared using a modified guanidium thiocyanate-phenol-chloroform extraction method. Random hexamer-primed complementary DNA (cDNA) synthesis was performed in a final volume of 25 μl, containing 20 units of RNase inhibitor (Promega, WI, U.S.A.)

and 200 units of murine leukemia virus reverse transcriptase (Promega) at 37°C for 60 min. Then, PCR amplification of $P2X_7$ gene was performed in a volume of 50 μl, containing 0.25 units of Taq polymerase (Perkin-Elmer, CT, U.S.A.) and 10 pmol of oligonucleotide primer pairs, which was sequence specific for rat and mouse $P2X_7$ gene; upstream 5′-AAACAAAGTCACCCGGATCC-3′ and downstream 5′-AAGTTTTCGGCGCTCCTCAA-3′ (519 bp of product size). Amplification of β-*actin* gene fragment (540 bp) was performed using commercial primers (Clontech, CA, U.S.A.) to enable the semi-quantitative normalization between GH3 and RAW 264.7 cells. The PCR conditions for $P2X_7$ included an initial denaturing for 5 min, and then 40 cycles as follows: denaturing for 30 s at 94°C, annealing for 30 s at 56°C and extending for 1 min at 72°C using GeneAmp (Perkin-Elmer). For β-*actin* gene, optimal product yield was obtained at 24 cycles at the same annealing temperature. Aliquots of PCR reactions were loaded onto a 1.1% agarose gel containing ethidium bromide, electrophoresed, visualized and exposed to Polaroid film. Quantification was performed with densitometry (Vilber Lourmat, France).

Drugs

Horse serum, foetal calf serum and antimycotic-antibiotic were purchased from GIBCO (Grand Island, NY, U.S.A.), and fura-2/AM, pluronic and bisoxonol were obtained from Molecular probes (Eugene, OR, U.S.A.). ATP, ADP, AMP, adenosine, UTP, Benzoyl-Benzoyl ATP (BzATP), oxidized ATP (oATP), sulfinpyrazone, ethidium bromide, EGTA, N-methyl-D-glucamine (NMDG), nimodipine, caffeine, BSA, 0.25% trypsin, and Ham's F10 were purchased Sigma Chemical Co. (St. Louis, MO, U.S.A.). 2-methylthio ATP (2-MeSATP) and α, β-methylene ATP were obtained from Research Biochemicals Inc (Natick, MA, U.S.A.).

Data analysis

Data were expressed as mean ± s.e.mean. Statistical differences between groups were evaluated using Student's *t*-test. Differences were considered significant at $P<0.05$.

Results

Effects of extracellular nucleotides on $[Ca^{2+}]_i$

The effects of extracellular ATP and BzATP on $[Ca^{2+}]_i$ levels in GH3 cells were measured using the Ca^{2+}-sensitive fluorescent dye, fura-2. Figure 1 shows that ATP (A) and BzATP (B) increase the intracellular calcium levels in GH3 cells. ATP and BzATP triggered an initial rapid increase in $[Ca^{2+}]_i$ followed by a long lasting plateau. The amplitude of the $[Ca^{2+}]_i$ increase was greater for BzATP compared to ATP. The $[Ca^{2+}]_i$ increases induced by BzATP (3–300 μM) and ATP (0.3–2 mM) were concentration dependent with EC_{50} values of 18.4 ± 3.6 μM ($n=6$) and 651 ± 52.8 μM ($n=8$), respectively (Figure 2). The maximal response for ATP was only 59.1 ± 7.9% ($n=6$) of that for BzATP.

Previous work indicates that 2-MeSATP and ATPγS are agonists for the $P2X_7$ receptor, since both induce $[Ca^{2+}]_i$ increases and inward currents (Gargett *et al*., 1997; Surprenant *et al*., 1996). In GH3 cells, they also increased $[Ca^{2+}]_i$. However, the amplitude of the $[Ca^{2+}]_i$ increase by 2-MeSATP (2 mM) was only 3.14 ± 0.96% of the maximal response to BzATP (Figure 2). The EC_{50} value for 2-MeSATP was not determined, as the response to the highest concentration of 2-MeSATP tested was not maximal. Table 1 shows the maximal $[Ca^{2+}]_i$ responses to different nucleotide agonists, together with their EC_{50} values. The order of agonist potencies was BzATP > > ATP > 2-MeSATP (Figure 2, Table 1). However, α, β-methylene ATP, ADP, AMP, UTP and adenosine were ineffective within tested concentration ranges (300–1000 μM). The amplitude of BzATP-induced $[Ca^{2+}]_i$ increase was proportional to the concentration of extracellular Ca^{2+} (10 μM–3 mM; Figure 3A,B). Most of intracellular calcium response by BzATP and ATP were largely attenuated in prior treatment of nimodipine (Figure 3C) and under calcium-free conditions. Also, prior treatment of caffeine (1 mM) did not significantly affect the calcium response by BzATP and ATP compared to control (data not shown). Therefore, these data imply that calcium influx was the major route for intracellular calcium increase in response to the purinergic stimulation.

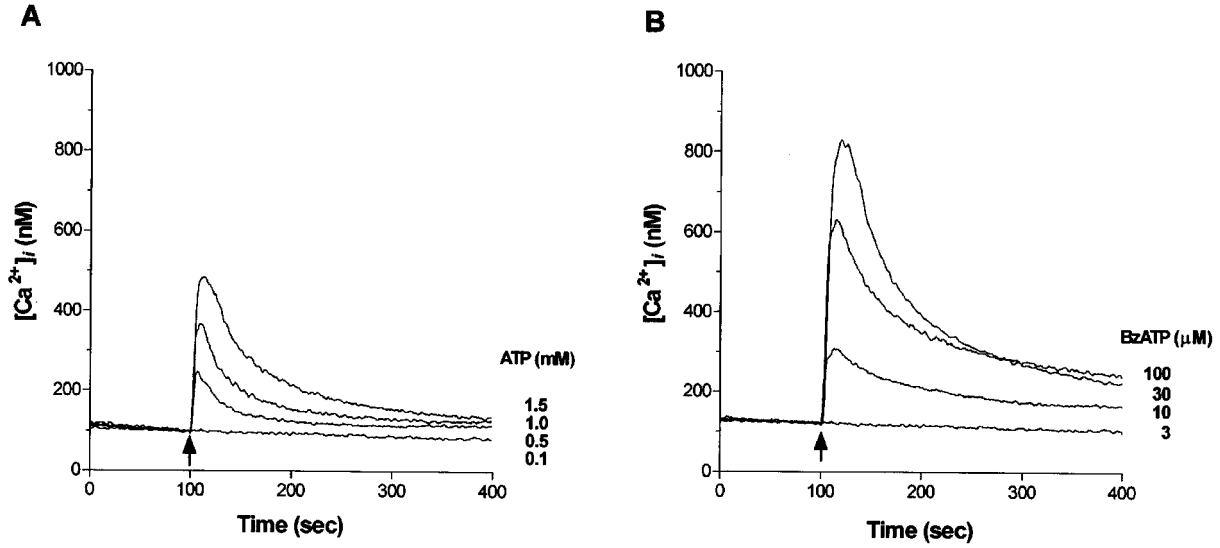

Figure 1 ATP and BzATP increase $[Ca^{2+}]_i$ in a concentration-dependent manner. Shown here are representative traces illustrating $[Ca^{2+}]_i$ changes evoked by the addition (arrows) of extracellular nucleotides. Fura-2 loaded GH3 cells were suspended at 1×10^6 ml^{-1} in KRH buffer. Cells were preincubated at 37°C for 5 min in a stirred fluorometer cuvette before stimulation with ATP (A) and BzATP (B).

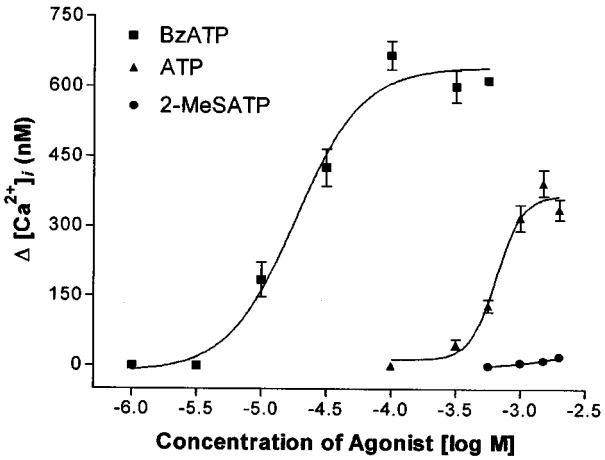

Figure 2 Concentration-response curves for $[Ca^{2+}]_i$ increases in response to ATP and two analogues. The curves for ATP, BzATP and 2-methylthio ATP were drawn using non-linear regression analysis. Data are the mean ± s.e.mean from at least six experiments.

Table 1 Effect of various ATP analogues in $[Ca^{2+}]_i$ increase

Compounds	*EC_{50}*	*Maximal response*
BzATP	18.4 μM	100 ± 4.5 (6)
ATP	651 μM	59.1 ± 7.9 (6)
2-MeSATP	—	3.14 ± 0.9 (4)
α-β-MeATP	—	—
ADP	—	—
AMP	—	—
UTP	—	—
Adenosine	—	—
oATP (300 μM)	—	—

Maximal responses for $[Ca^{2+}]_i$ increase are expressed as a percentage relative to maximal responses to BzATP. Data are the mean ± s.e.mean from (*n*) observations. Maximal rate for $[Ca^{2+}]_i$ increase were calculated by non-linear regression analysis of concentration-response plots using Prism software (GraphPad).

Effect of extracellular nucleotides on membrane potential of GH3 cells

Extracellular ATP has been shown to either depolarize or hyperpolarize the membrane potential depending on the cell type (De Souza *et al*., 1995; Ferrari *et al*., 1996). In order to assess the effects of ATP on GH3 cells, changes in membrane potential were measured using the lipophilic anionic fluorescent dye, bisoxonol. Negatively charged oxonol dye molecules partition across the cell membrane according to the membrane potential (Rink *et al*., 1980). Thus, depolarization increases the uptake of dye and the intensity of emitted fluorescence (Rink *et al*., 1980). Figure 4 shows the fluorescence profiles of GH3 cells in suspension equilibrated with 200 nM bisoxonol and stimulated with increasing concentration of KCl (15–90 mM) to depolarize the cells. There was an increase in the fluorescence intensity with the addition of KCl. The change of fluorescence intensity was well fitted to the logarithm concentration of KCl, which follows the membrane potential calculated by the Nernst equation.

Figure 5A and B show that the addition of ATP and BzATP evoked a rapid and sustained increase in bisoxonol fluorescence intensity in the same manner as KCl. BzATP was more potent that ATP in evoking depolarization. Analysis of the concentration-response curves (Figure 5C) for BzATP and ATP gave EC_{50} values of 31.9 ± 6.3 μM ($n = 6$) and 1.05 ± 0.05 mM ($n = 6$), respectively. The maximal responses to ATP and BzATP were 146.1 ± 15.9% and 152.3 ± 21.7% of 30 mM KCl-induced depolarization, respectively. However, 2-MeSATP, α, β-methylene ATP, ADP, AMP, UTP and adenosine did not elicit depolarization within tested concentrations (up to 1 mM).

Effects of oxidized ATP on ATP and BzATP-induced changes in $[Ca^{2+}]_i$

Although ionotropic P2 receptors have been recently identified in inflammatory cells, their physiologic roles are not yet clear. To clarify the subtype of ionotropic P2 receptors, it would be most desirable to develop specific pharmacologic antagonists. We used oxidized ATP (oATP) as an irreversible antagonist of the P2Z/ $P2X_7$ receptor, which was known to inactivate the $P2X_7$ receptor (Murgia *et al*., 1993). In agreement with previous reports on human and mouse macrophages (Murgia *et al*., 1993), oATP obliterated the early spike and the sustained $[Ca^{2+}]_i$ rise. Preincubation with 100 μM oATP for 2 h, followed by washing out oATP, inhibited the BzATP and ATP-induced $[Ca^{2+}]_i$ increases more than 95% (Figure 6A,B). Concentration-response curves for oATP-induced inhibition showed that a maximal inhibitory dose of oATP was nearly 300 μM, which closely matched that reported for mouse microglial cells (Ferrari *et al*., 1996). Oxidized-ATP potently antagonized BzATP-induced $[Ca^{2+}]_i$ increase with an IC_{50} of 37.65 ± 1.1 μM ($n = 6$) (Figure 7A,B). Both ATP and BzATP-induced $[Ca^{2+}]_i$ increases were completely inhibited by 300 μM oATP.

Electrophysiological experiments

In a low divalent cation containing solution, BzATP induced whole-cell inward currents at a holding potential of −60 mV (Figure 8). The currents were linearly dependent on membrane potential (−110 to 10 mV); reversal potential was nearly 0 mV (Figure 8C,D). Maximal inward currents obtained from repetitive application of BzATP in the same cell were significantly larger than those observed for the first exposure; the large cation N-methyl-D-glucamine (NMDG) could permeate the ion channels activated by BzATP. (Figure 8A: first application and B: 5th application).

Ethidium bromide uptake

In low divalent solutions, application of 300 μM BzATP for 30 min caused marked cellular uptake of ethidium bromide. In the absence of BzATP, less than 5% of cells displayed the fluorescence by ethidium bromide (Figure 9A), while approximately 90% of all cells displayed a strong fluorescence in the presence of BzATP (Figure 9B).

Detection of mRNA of $P2X_7$ receptor by RT–PCR

We investigated whether the mRNA of $P2X_7$ receptor was present in GH3 cells by RT–PCR. We also compared the steady-state level of $P2X_7$ mRNA in GH3 cells with those in RAW 264.7 cells. As shown in Figure 10, single bands, predicted as 519 bp, were observed from RT–PCR products from GH3 cells and RAW 264.7 cells. The steady-state mRNA level of $P2X_7$ receptor, normalized to that of β-actin, was not different between GH3 cells and RAW 264.7 cells (0.45 ± 0.12 vs 0.47 ± 0.04). These results indicate that $P2X_7$ receptor expression in GH3 cells is similar to that in macrophages, which has been shown to

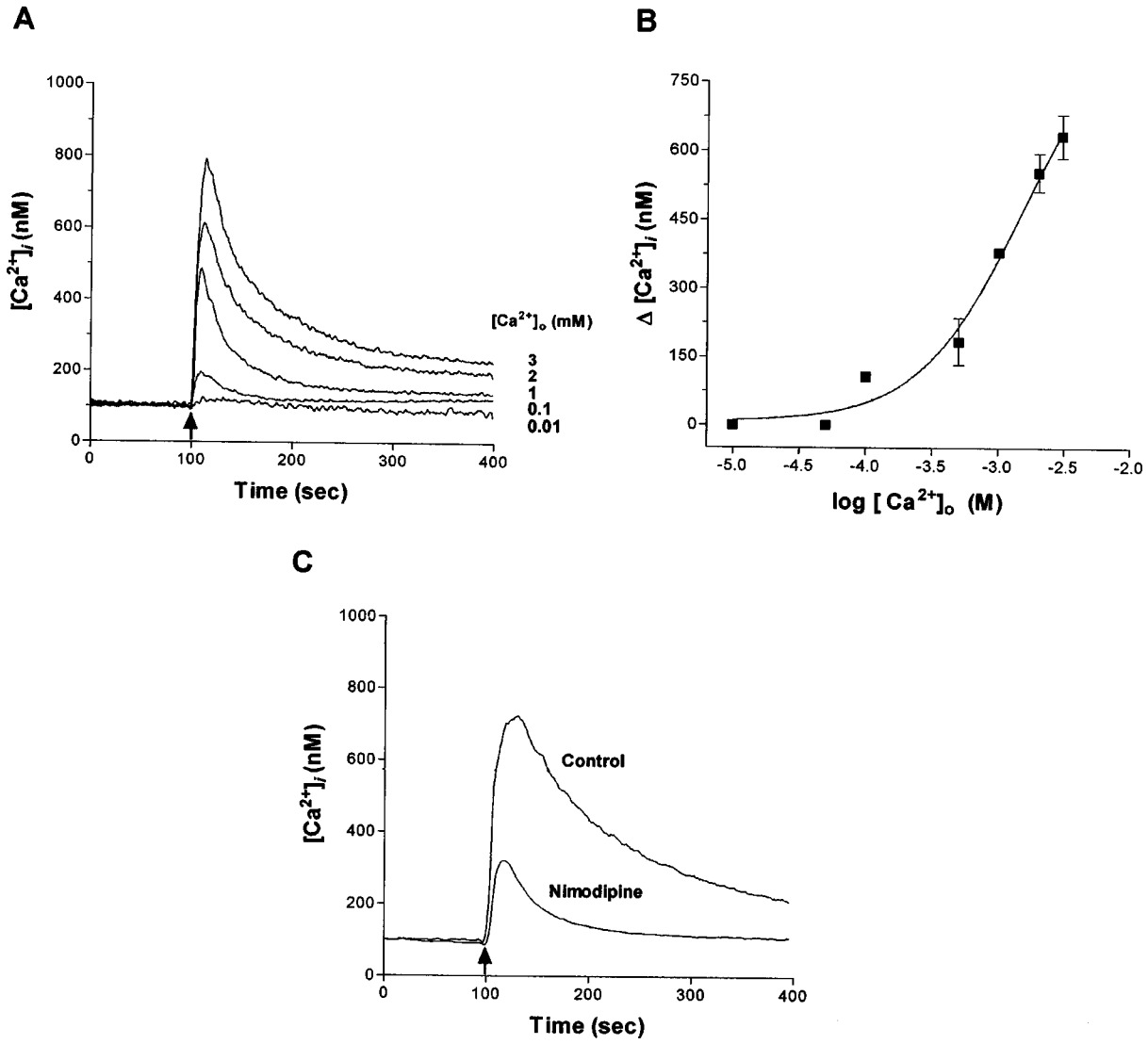

Figure 3 Effect of the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) and the Ca^{2+} channel blocker nimodipine on BzATP-induced $[Ca^{2+}]_i$ responses. Representative fluorescence traces show that the magnitude of BzATP induced $[Ca^{2+}]_i$ responses depends upon $[Ca^{2+}]_o$ (A) (added at arrows). Group data show the relationship between the extracellular Ca^{2+} concentration and BzATP-induced $[Ca^{2+}]_i$ increases (B). Nimodipine (10 μM) was added 2 min before stimulation with 100 μM BzATP (C). Data are the mean $\pm$ s.e.mean from at least six experiments.

have $P2X_7$ receptors (Suprenant *et al*., 1996; Chessell *et al*., 1998).

Discussion

The expression of purinergic receptors and the effects of ATP on calcium signaling and hormone secretion in rat anterior pituitary cells have been reported previously (Tomic *et al*., 1996). Villalobos *et al*. (1997) reported that functional ionotropic P2 receptors, which are typical of neural tissue, are also in gonadotroph cells from the pituitary gland and could contribute to regulation of the hormone secretion. The $P2X_7$ (P2Z) receptor is known as an uniquely bifunctional molecule through which ATP can open a small cationic channel typical of ionotropic receptors and also induces membrane permeability to large molecules (Virginio *et al*., 1997). In the present study, we characterized the pharmacological properties of purinoceptors and determined their subtype of endogenous purinoceptors in GH3 cells, a model system for the study of pituitary cell signalling (Charles *et al*., 1999).

Previous studies have reported that murine macrophage cell express P2Y, P2U and P2Z receptors. BzATP activated mainly the P2Z receptor and potently stimulated influx of Ca^{2+} from the medium in this cell (El-Moatassim & Dubyak, 1992). This study showed that the effects of ATP and BzATP were likely mediated by $P2X_7$ receptor, the activation of which was more potent by BzATP in GH3 cells. Several lines of evidences support this conclusion. Firstly, maximal responses to BzATP were greater than those to ATP for two different $P2X_7$-stimulated responses; $[Ca^{2+}]_i$ increase and depolarization of membrane potential. Secondly, an irreversible nonselective P2 antagonist, oATP completely inhibited the ATP and BzATP-induced $[Ca^{2+}]_i$ increase. Thirdly, in low-divalent conditions BzATP evoked non-desensitizing inward currents which were reversed at $\sim$0 mV. This nonselective cationic conductance was increased by repeated applications of BzATP and the cells became very permeable to NMDG. BzATP also stimulated the uptake of ethidium bromide. Fourthly, we also identified the expression of endogenous $P2X_7$ receptor in GH3 cells by using RT–PCR.

Previous studies have indicated that the activation of $P2X_7$ ionotropic receptors with BzATP directly opens non-selective

cation channels, leading to an increase in $[Ca^{2+}]_i$ and membrane depolarization (Inoue & Brading, 1990) in other cell types, including macrophages (Picello *et al*., 1990), thymocytes (Pizzo *et al*., 1991), oocytes (Nuttle & Dubyak, 1994), and neuroblastoma × NG108-15 cells (Brater *et al*., 1999). Membrane depolarization activates voltage-sensitive

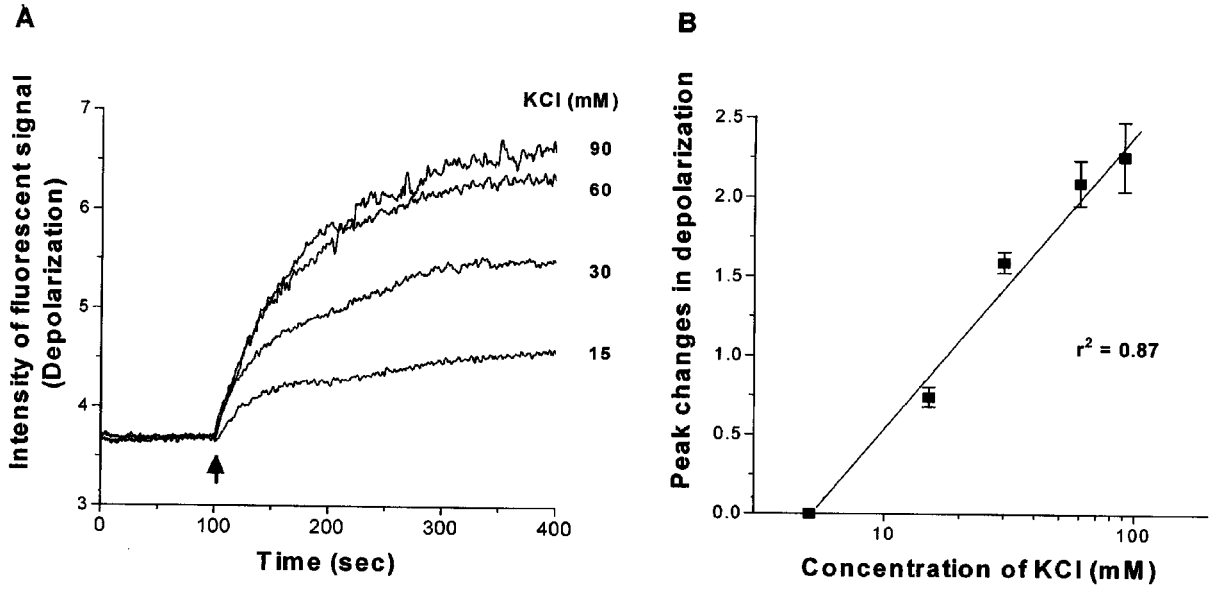

Figure 4 Adding extracellular KCl depolarizes GH3 cells, as measured by bisoxonol fluorescence. The addition (arrow) of increasing concentrations of KCl (15–90 mM) to GH3 cells (1.0×10^6 ml^{-1}), in suspension containing 200 nM bisoxonol, progressively increases fluorescence intensity (A). A Schild plot shows the relationship between the concentration of KCl and the depolarization (B).

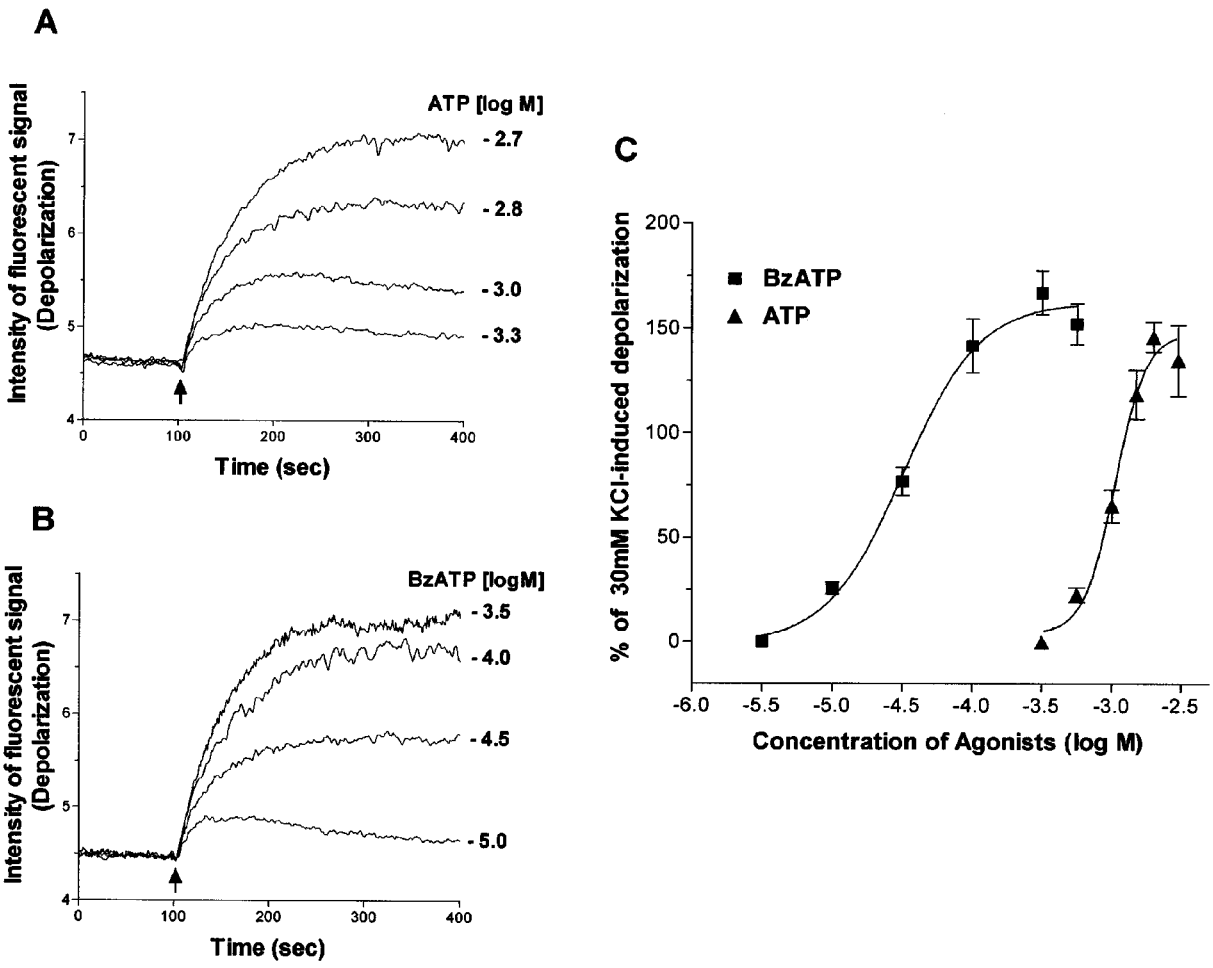

Figure 5 ATP and BzATP depolarize GH3 cells. ATP (A) or BzATP (B) were added to GH3 cells in suspension containing 200 nM bisoxonol. Depolarizations induced by ATP and BzATP were expressed as a percentage of the response to 30 mM KCl, which was defined as 100% (C). Data are the mean ± s.e.mean from at least six experiments.

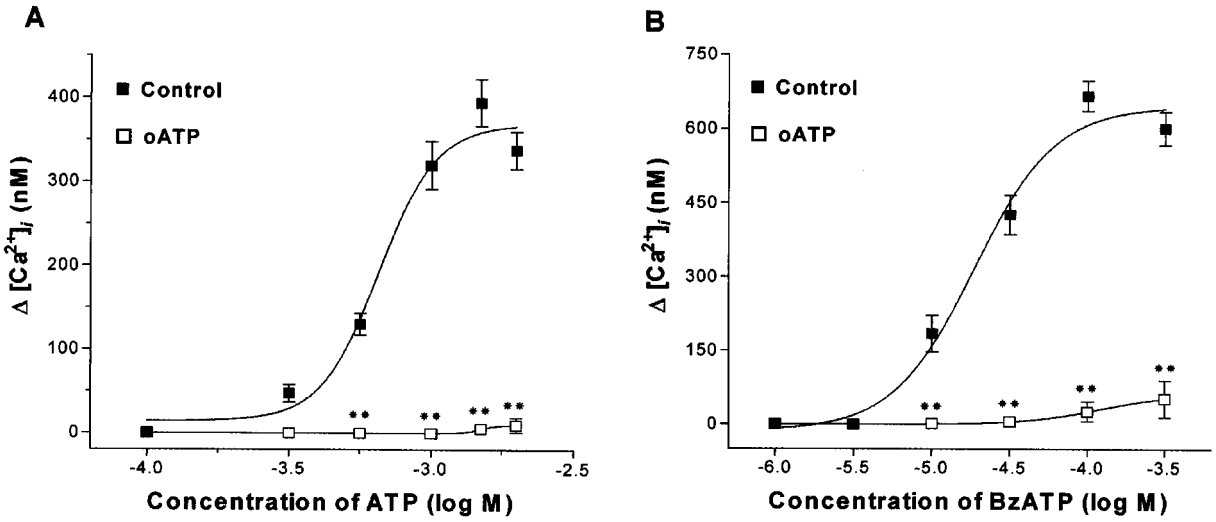

Figure 6 Oxidized ATP (oATP) inhibits ATP and BzATP-induced $[Ca^{2+}]_i$ increases. GH3 cells (1×10^6 ml^{-1}), preincubated at 37°C for 2 hrs with or without 100 μ M oATP, were suspended in a fluorometer cuvette and stimulated with either ATP (A) or BzATP (B). Data are the mean ± s.e.mean from at least six experiments. $^{**}P < 0.05$.

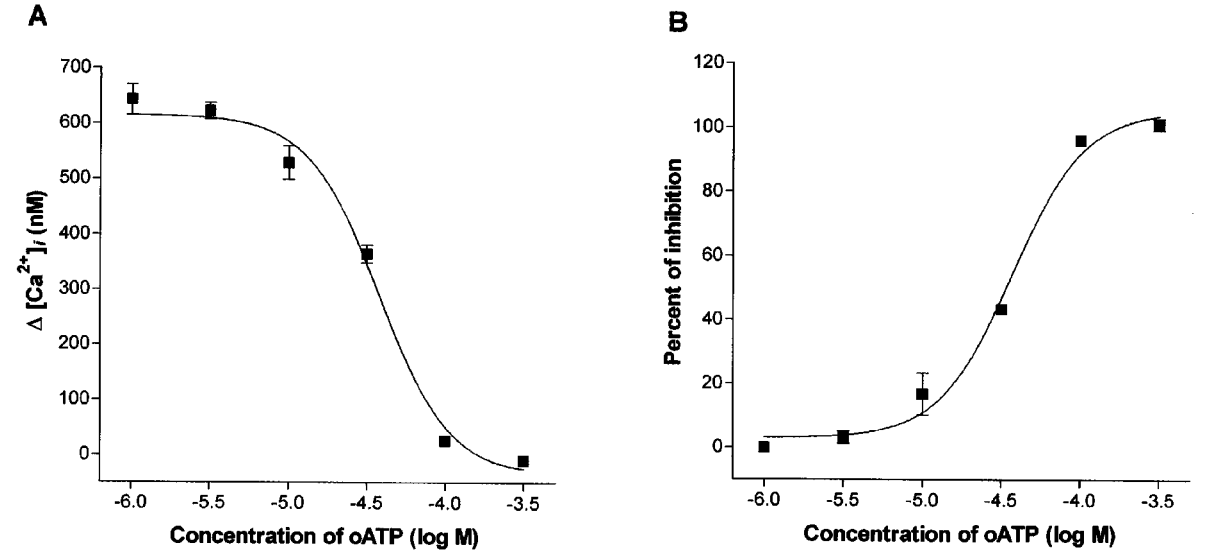

Figure 7 Preincubation with oATP inhibits BzATP-induced $[Ca^{2+}]_i$ increases. Fura-2 loaded GH3 cells were preincubated for 2 h with various concentrations of oATP before stimulation with 100 μM BzATP. (A) The relationship between the concentration of oATP and BzATP-induced $[Ca^{2+}]$ increase is shown. (B) Concentration-dependent inhibition of $[Ca^{2+}]_i$ increases by oATP is shown. The responses were converted to percentage of the control, the 100 μM BzATP-induced $[Ca^{2+}]_i$ increase without oATP pretreatment. Data are the mean ± s.e.mean from six separate experiments.

Ca^{2+} channels (VSCC) allowing further Ca^{2+} entry. Table 1 and Figure 2 show that three nucleotide agonists ATP, BzATP and 2-MeSATP, stimulate $[Ca^{2+}]_i$ increases in GH3 cells. In human lymphocytes, BzATP stimulated the influx of Ca^{2+} from the extracellular medium without triggering Ca^{2+} release from internal stores (Wiley *et al.*, 1994). This effect was attributed to a specific occupancy of $P2X_7$ (P2Z) receptors. It has been reported that BzATP was 10 fold more potent than ATP in stimulating Ca^{2+} influx *via* the $P2X_7$ (P2Z) receptor of rat parotid acinar cells (Soltoff *et al.*, 1992). Our study shows that EC_{50} values for BzATP and ATP were 18.37 μM and 651 μM, respectively and that BzATP is 35 fold more potent than ATP in eliciting a $[Ca^{2+}]_i$ increase (Figure 2).

At the present time, we could not rule out the existence of other subtypes of P2 purinoceptor in GH3 cells. We observed that most of intracellular calcium response by BzATP and ATP were largely attenuated by prior treatment of nimodipine and under the calcium-free condition (Figure 3). Also, prior treatments of caffeine (1 μM) did not significantly affect in calcium response by BzATP and ATP compared to the control (data not shown). Therefore, it is likely that calcium mobilization by a metabotropic P2 receptor cannot contribute in large proportion, but calcium influx could be the major route for intracellular calcium increase in response to the purinergic stimulation. Table 1 also shows that 2-MeSATP, a potent agonist for $P2Y_1$ and $P2X_{1-3}$ receptors (Ralevic & Burnstock, 1998), showed only moderate agonist activity in GH3 cells, the other P2 receptors agonists (α, β-methylene ATP, ADP and UTP) having no effect on $[Ca^{2+}]_i$ or membrane potential in GH3 cells. The ATP receptors in GH3 cells exhibit the following rank-order of agonist-induced $[Ca^{2+}]_i$ increase: BzATP > > ATP > 2-MeSATP. This pattern appears to fit best to the $P2X_7$ subtype (Dubyak & El-Moatassim, 1993; Surprenant *et al.*, 1996).

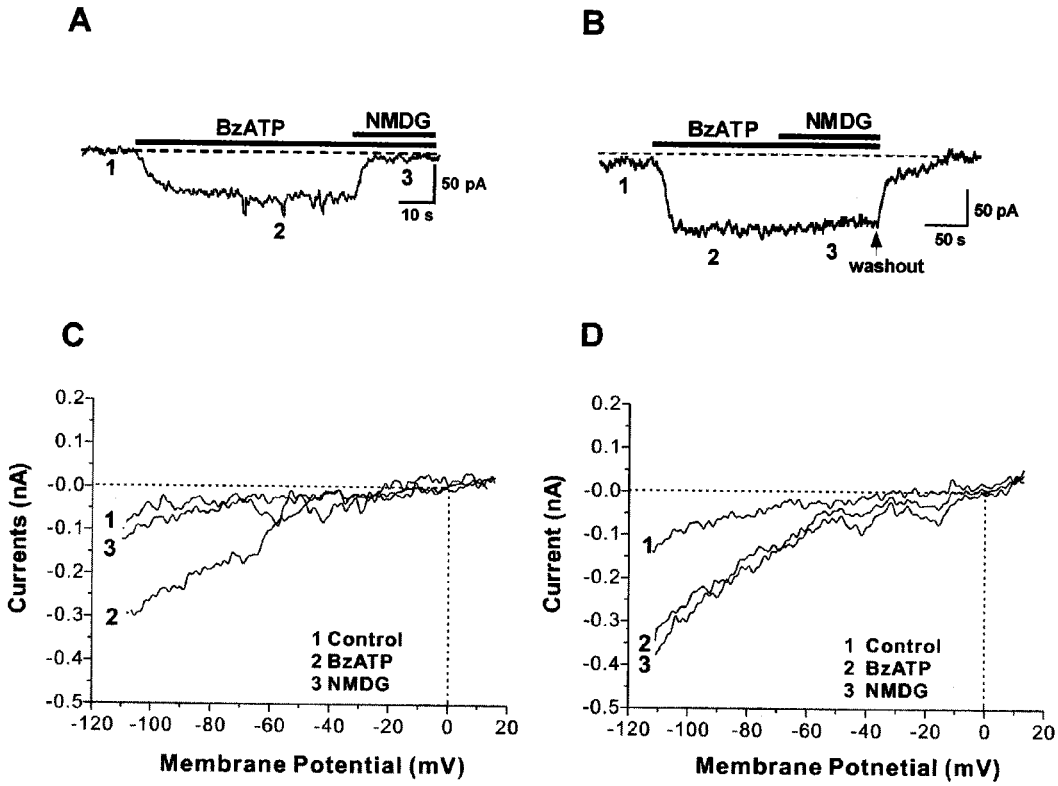

Figure 8 BzATP-induced currents in GH3 cells. The first (A) and the fifth (B) repetitive application of BzATP evoked the inward currents in solution containing 0.3 mM $CaCl_2$ and no magnesium (low divalents). The superfusing solution was changed from 154 mM NaCl to 154 mM NMDG (still in low concentrations of divalent cations). Current-voltage curves were obtained by ramp voltage commands at times 1 to 3 in an application of BzATP and NMDG (C,D). The bars above the current traces indicate the period of BzATP or NMDG application in low divalent solutions.

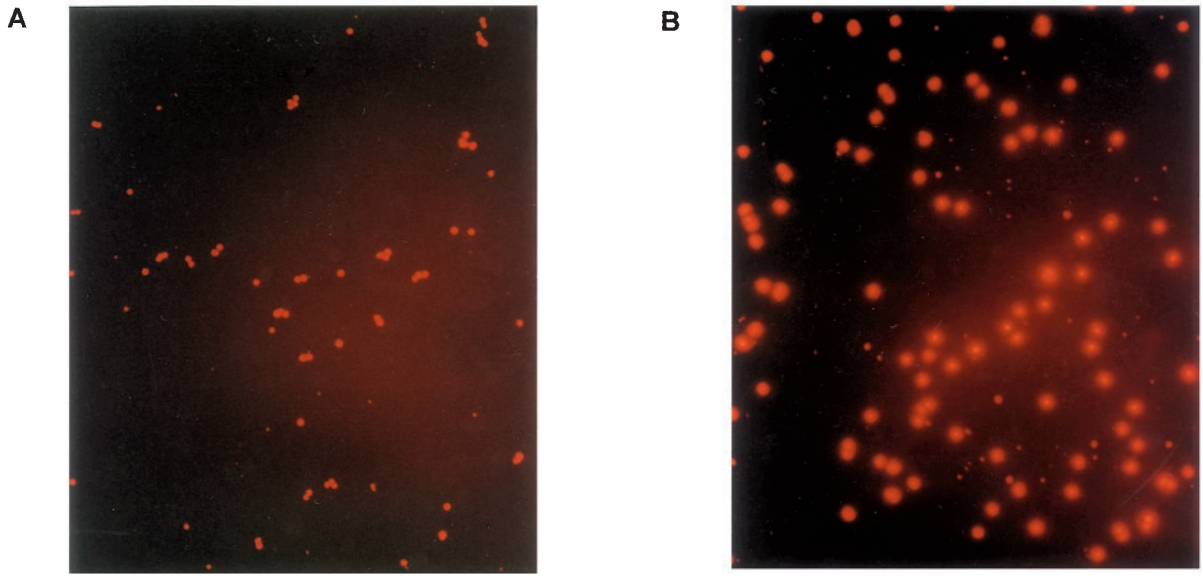

Figure 9 BzATP permeabilizes the plasma membrane of GH3 cells to ethidium bromide. (A) Fluorescence micrographs of GH3 cells treated with 20 μM ethidium bromide, without BzATP. (B) Represents cell from the same experiment incubated with 300 μM BzATP and ethidium bromide. Scale bar in 33 μm.

In this study, GH3 cells responded with a sustained depolarization, as shown in Figure 5. This effect was abolished when pretreated for 2 h with oxidized ATP (300 μM) (data not shown). The irreversible inhibition of ATP and BzATP-induced $[Ca^{2+}]_i$ increase by oxidized ATP confirmed the proposal of Murgia *et al.* (1993).

Taking our results together, BzATP and ATP initially increased nonselective cationic conductance in non-inactivating manner, followed by large calcium influx which was activated by L-type calcium channels. L-type calcium channels readily inactivate with time and in response to calcium itself, whereas depolarization by BzATP and ATP persisted, explaining the

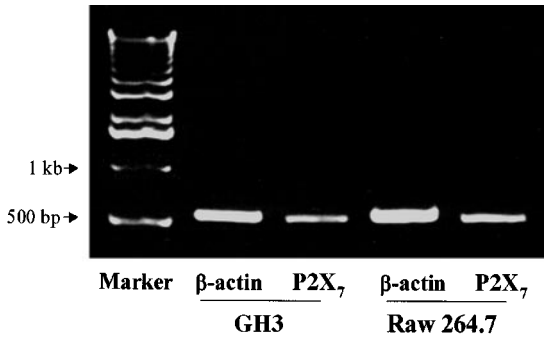

Figure 10 Detection of $P2X_7$ receptor in agarose gel electrophoresis. Total RNAs were isolated from GH3 cells and RAW 264.7 cells and the steady-state mRNA levels of $P2X_7$ receptor (519 bp) and β-actin (540 bp) were determined by RT–PCR. PCR products were analysed on a 1.1% agarose gel stained with ethidium bromide.

transient increase in intracelluar calcium which peaked at 10 ~ 30 s and declined to a steady-state value thereafter.

At the present time, we can not explain the relationship between the increase in intracellular calcium and channel-to-pore conversion. Fernando *et al*. (1999) suggested that ATP-stimulated influx of divalent cations activated intracellular PLD activity. The sustained activation of intracellular PLD might be involved in a delayed permeability lesion. It has been shown that various type of cells, including macrophages, can be permeabilized by high concentrations of ATP (Steinberg *et al*., 1987; Ferrari *et al*., 1996). This permeabilization can result in uptake or release of large molecules such as ethidium bromide (M.W. 394; Ferrari *et al*., 1996; Chessell *et al*., 1997). In our studies, a 30 min application of BzATP in low divalent solution caused the uptake of ethidium bromide in approximately 90% of cells, while very few fluorescent cells were observed in the absence of BzATP. BzATP (100 μM) evoked non-desensitizing inward currents which reversed at ~0 mV, suggesting activation of a non-selective cation channel (Figure 8C,D). Repetitive application of BzATP increased the peak currents and the cation channel became permeable even to NMDG (Figure 8A,B). These characteristics are consistent with the electrophysiological properties of native and expressed $P2X_7$ receptors (Surprenant *et al*., 1996; Chessell *et al*., 1997; Michel *et al*., 1998). Thus, the findings in our study would seem to indicate that BzATP-induced permeabilization is mediated *via* $P2X_7$ receptors. In the RT–PCR studies, $P2X_7$ receptor expression of the GH3 cells are similar to that in macrophages, which has been shown to have $P2X_7$ receptor (Suprenant *et al*., 1996, Chessell *et al*., 1998). It is suggested that GH3 cells express the endogenous $P2X_7$ receptor. These observations provide strong evidence that the responses of ATP and BzATP are mediated by $P2X_7$ receptors.

In conclusion, these data indicate that the responses to ATP and BzATP in GH3 cells are mediated by a single class of receptor, pharmacologically characterized as the $P2X_7$ receptor. These purinergic regulations may play an important role in neuroendocrine modulation on GH3 cells.

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