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Effects of Exogenous ATP on Calcium Mobilization and Cell Proliferation in C6 Glioma Cell

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To clarify the effect of extracellular ATP in cultured C6 glioma cells, ATP-induced cytosolic free calcium ($[Ca^{2+}]_i$) mobilization and cell proliferation were investigated. ATP-induced $[Ca^{2+}]_i$ increased in a dose-dependent manner (10^{-7} M~10^{-3} M). ATP-induced $[Ca^{2+}]_i$ increases were slightly slowed in extracellular calcium-free conditions especially in sustained phase. ATP-induced $[Ca^{2+}]_i$ increment was also inhibited by the pretreatment of U73122, a phospholipase C (PLC) inhibitor, in a time-dependent manner. Suramin, a putative P_{2Y} receptor antagonist, dose-dependently weakened ATP-induced $[Ca^{2+}]_i$ mobilization. Significant increases in cell proliferation were observed at 2, 3, and 4 days after ATP was added. Stimulated cell proliferation was also observed with adenosine at days 2 and 3. This cell proliferation was significantly inhibited by the treatment with suramin. Ionomycin also stimulated cell proliferation in a concentration-dependent manner. In conclusion, we suggest that extracellular ATP stimulates C6 glioma cell proliferation via intracellular free calcium mobilization mediated by purinoceptor.

Key Words: ATP, Purinoceptor, C6 glioma cell, Cytosolic free calcium, Cell proliferation

INTRODUCTION

Potent actions of purine nucleotides and nucleosides upon the mammalian heart were first reported over 60 years ago by Drury and Szent-Gyorgyi (1929). Cells possess complex mechanisms for the synthesis of ATP and for its control at the cytosolic level. ATP can be released during neurotransmission into the synaptic space or from 'dense granules' during blood platelet activation (El-Mostassim et al, 1992). In addition, stimulation with Ba^{2+} and high K^+ concentration stimulates the release of adenine nucleotides from a cultured sympathetic neuron (Neary & Norenberg, 1992). In addition, cell damage during tissue injury (i.e., vascular rupture and so on) may also lead to the release of ATP into the extracellular space. These forementioned findings suggest that when an extracellular ATP concentration level is high enough, extracellular ATP receptor stimulation reaches an active level. It is well-known that extracellular ATP mediates various physiological actions via specific receptors on the surface of cells, called purinergic receptors or purinoceptors.

Extracellular ATP was reported to stimulate phospholipase C (PLC) activity in various cells, with the resulting production of inositol phosphate and diacylglycerol (Neary et al, 1994). Also reported are the effects of exogenous ATP in glial cells. Extracellular ATP induces stellation and increases glial fibrillary acidic protein content and DNA synthesis. It also modulates cell proliferation in primarily cultured astrocytes of neonatal rat cortices and rat striatum (Dubyak, 1991). In addition, extracellular ATP induces intracellular free calcium mobilization in adult and immortalized Schwann cells (Anselin et al, 1997; Berti-Mattera et al, 1996; Linn & Chuang, 1993). However, the physiological effects of ATP receptors coupled with intracellular free calcium increase in C6 glioma cells have not been put to use

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satisfactorily to date.

This experiment was designed to investigate the physiological effects of ATP on intracellular free calcium mobilization and cell proliferation in cultured C6 glioma cells.

METHODS

Materials

The materials used were purchased from the following sources: ATP from Boehringer (Mannheim, Germany); U73122 from Research Biochemicals Inc, (Natic, USA); adenosine, ionomycin, Fura-2 and Fura-2/AM from Sigma Chemical (St. Louis, USA); Dulbecco's modified Eagle medium (DMEM), penicillin-streptomycin and fetal bovine serum from GIBCO (Grand Island, NY); suramin from Wako (Tokyo, Japan). All the other chemicals were of the highest grade available.

Cell culture

The C6 glioma cells were cultured in Dulbecco's modified Eagle's medium containing 10% FBS in 10 cm corning culture dish at 37°C with 5% carbon dioxide atmosphere until 70~80% confluency (3~5 days), For routine maintenance, confluent cells growing in DMEM with 10% FBS were harvested by treatment for 5 min with 0.25% trypsin with 1 mM EDTA. Cells were washed twice and reinoculated into 10 cm cultured dish at 5×10^5 cells/dish in 10 ml DMEM with 10% FBS.

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

The methods of $[Ca^{2+}]_i$ measurement have been previously described [10, 11]. Cells were harvested after trypsinization at five days before experiments, and seeded onto 22×22 mm cover glasses at the concentration of 10^5 cells/dish. The cover glass was attached to a 1 cm hole located at the bottom of 35 mm plastic culture dishes. Cells were then washed with modified Hanks' solution consisting of the following elements (in mM): NaCl, 127; 0.33 of each $MgSO_4$ and Na_2HPO_4; KH_2PO_4, 0.44; $MgCl_2$, 1; HEPES, 10; $CaCl_2$, 1 (pH 7.4); and loaded Fura-2/AM (10 μM) for 45 min at 37°C. Fluorescence-loaded cells were washed three times with the same solution for exclusion of unloaded Fura-2/AM. The fluorescence of C6 glioma cells were measured at room temperature using the InCaTM Imaging System manufactured by Intracellular Imaging Inc. (Cincinnati, OH, USA). For the calcium-free experiment, 10 μM (final concentration) of EGTA solution was added to the calcium-free bathing solution. The concentration of $[Ca^{2+}]_i$ was calculated from the standard curve generated in situ (Wahl et al, 1992).

Measurement of cell proliferation

Cells were seeded by 10^4 cells per 35 mm culture dish and put to rest for 1 day for the attachment of cells. After confirming the attachment of cells, agonists and antagonist at the concentrations of 10^{-5} M and 10^{-4} M, respectively were applied with the final concentration of 10^{-5} M and 10^{-4} M. To determine the cell proliferation, cells were detached by trypsin (0.25%)-EDTA (0.5 mM) solution, and the number of cell was counted by using trypan blue (0.2%).

Statistical analysis

All values are represented as means$\pm$SE. The statistical significance was determined by Student's t-test for a two-group comparison. A P value of less than 0.05 was considered to indicate a statistically significant difference.

RESULTS

The relationship between culture time and ATP-induced $[Ca^{2+}]_i$ response

To identify a best condition for ATP-induced $[Ca^{2+}]_i$ response, the relationship between culture time and ATP-induced $[Ca^{2+}]_i$ response was investigated. When the response to ATP-induced transient $[Ca^{2+}]_i$ increase was examined within 24 hr after plating, the cells showed a slight and an inconsistent response (data not shown). As shown in the Table 1, the number of cells reacting to ATP increased in a culture time-dependent manner. Since the maximum of calcium response and the number of cells reached the maximum at day 5, all subsequent experiments were performed at day 5 after seeding the cells.

ATP-induced intracellular free calcium mobilization

To investigate the effect of extracellular ATP, extracellular ATP-induced cytosolic free calcium levels were examined in the cultured C6 glioma cells. The basal level of $[Ca^{2+}]_i$ with 1 mM $CaCl_2$ in the bathing solution was 92.5±9 nM (mean±SE, n = 35). The significant effect of ATP on $[Ca^{2+}]_i$ increase was observed from the concentration of 10^{-7} M, and it was more conspicuous at higher concentrations in a concentration-dependent manner (Fig. 1). The $[Ca^{2+}]_i$ was eluted at the 10^{-7} M of ATP and saturated at the concentration of 10^{-5} M. The EC_{50} value of ATP for $[Ca^{2+}]_i$ increases was estimated at 1.1×10^{-6} M in cultured C6 glioma cells.

Table 1. The effect of ATP between culture time and calcium response

Culture time (day)	No. of responded cell/ No. of cells tested (ratio, %)
1	17/25 (68)
2	23/30 (77)
3	20/30 (67)
4	26/30 (87)
5	27/29 (93)

Concentration of ATP was 10^{-4} M.
Cells were seeded at 5×10^5 cells/35 mm culture dish.

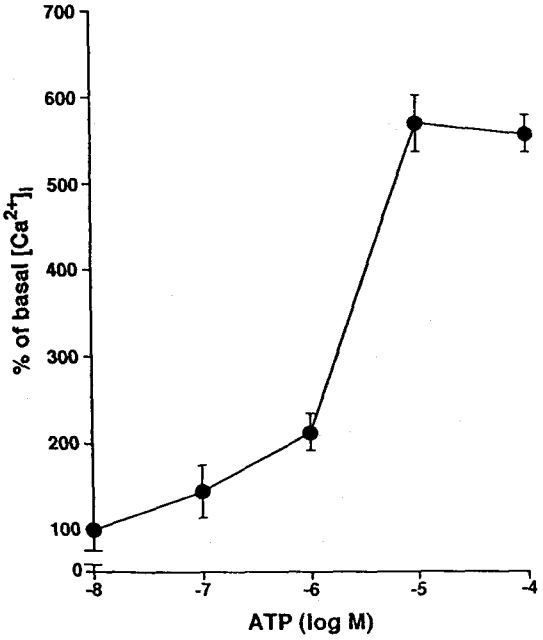

Fig. 1. Dose-response curve showing a transient increase in $[Ca^{2+}]_i$ which is caused by a function of ATP concentrations in cultured C6 glioma cells. ATP at each concentration was added to the medium from a stock solution to yield final agonist concentrations indicated. Basal $[Ca^{2+}]_i$ was 82.5±7.5 nM. Values are means±SE for 6 individual experiments.

Sources of ATP-mediated calcium

To determine whether the source of an ATP-induced increase in $[Ca^{2+}]_i$ was intracellular calcium stores or the extracellular milieu across plasma membranes, the effects of Ca^{2+} removal from the medium on ATP-evoked $[Ca^{2+}]_i$ transients were

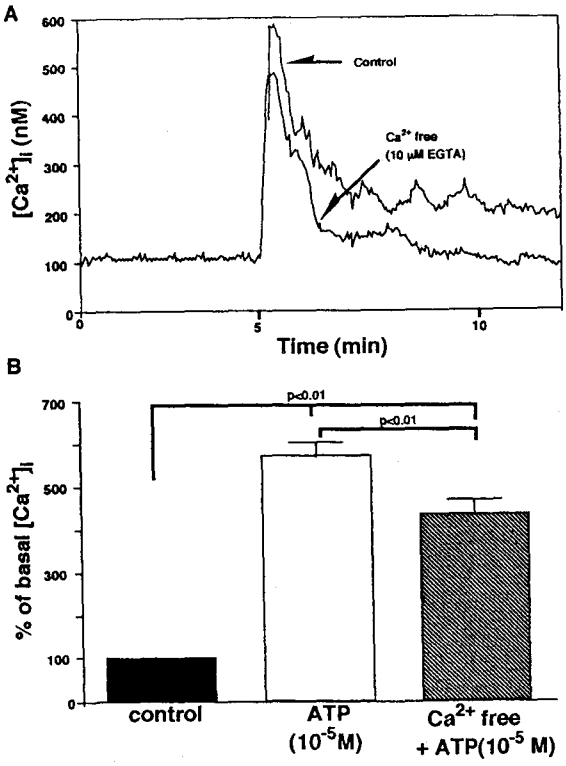

Fig. 2. A: Representative tracing of ATP-induced $[Ca^{2+}]_i$ increase when calcium is present in cultured C6 glioma cells. In order to confirm extracellular Ca^{2+} free conditions, 10 μM EGTA (final concentration)was added to the media without $CaCl_2$ 5 min before the addition of ATP. B: Effects of calcium removal from the medium on the peak of ATP-induced $[Ca^{2+}]_i$ transients in the C6 glioma cells. Each column represents the relative change in $[Ca^{2+}]_i$ with respect to the basal $[Ca^{2+}]_i$ in each sample. Values are mean±SE for 4 to 6 individual experiments.

examined. ATP-induced $[Ca^{2+}]_i$ changes showed a transient increase to a peak between 5~15 sec after adding agonists. It was decreased to reach a steady level 5~7 min after the peak (Fig. 2-A). As shown in Figs. 2-A and -B, C6 cells were pretreated with the 10 μM EGTA in Ca^{2+}-free bathing solution for 5 min before the application of ATP. ATP (10^{-5} M)-mediated transient $[Ca^{2+}]_i$ increases was observed. The peak, however, was slightly lowered (ATP 10^{-5} M; 572±31%, Ca^{2+}-free; 437±33%). Moreover, the ATP-induced sustained increase in $[Ca^{2+}]_i$ was disappeared when the EGTA was pretreated to the C6 glioma cells.

Inhibitory effect of U73122 on ATP-induced $[Ca^{2+}]_i$ increase

To evaluate the possible involvement of phospholipase C in ATP-induced transient $[Ca^{2+}]_i$ increases, the effects of U73122 were investigated. The cultured C6 glioma cells were pretreated for 20 or 30 min with U73122 (5×10^{-6} M), a phospholipase C (PLC) inhibitor, before ATP was added. To exclude the time lag in responsiveness to ATP, ATP was treated 20 or 30 min after extracellular fura-2 was washed. There was no change in responsiveness to ATP (data not shown). As shown in Figs. 3-A and -B, ATP-induced transient $[Ca^{2+}]_i$ increases were time dependently inhibited by pretreatment with U73122. The resting $[Ca^{2+}]_i$ did not change by the pretreatment with U73122.

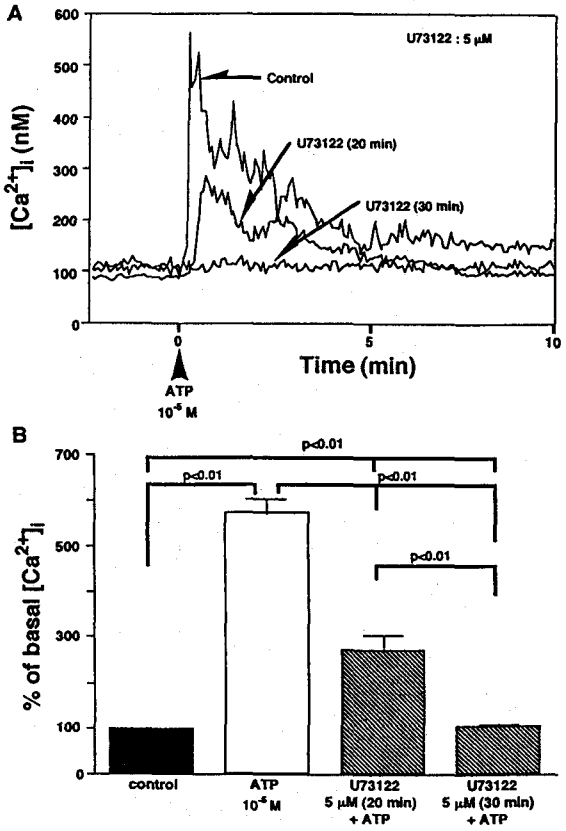

Fig. 3. A: Representative tracing showing effects of U73122 on ATP-induced $[Ca^{2+}]_i$ increase in cultured C6 glioma cells. B: Effects of U73122 on the peak of ATP-induced $[Ca^{2+}]_i$ transients in the C6 glioma cells. Each column represents the relative change in $[Ca^{2+}]_i$ with respect to the basal $[Ca^{2+}]_i$ in each sample. Values are mean±SE for 4 to 6 individual experiments.

Effect of suramin on ATP-induced $[Ca^{2+}]_i$ change

To characterize the receptor subtype mediating ATP responses in cultured C6 glioma cells, we utilized the putative P_{2Y} receptor antagonists, suramin. As shown in Figure 4, the pretreatment of C6 glioma cells with suramin (10^{-5} and 10^{-4} M) for 3 min significantly inhibited ATP-induced $[Ca^{2+}]_i$ increases in a concentration-dependent manner. There was no change in resting $[Ca^{2+}]_i$ by the pretreatment with suramin.

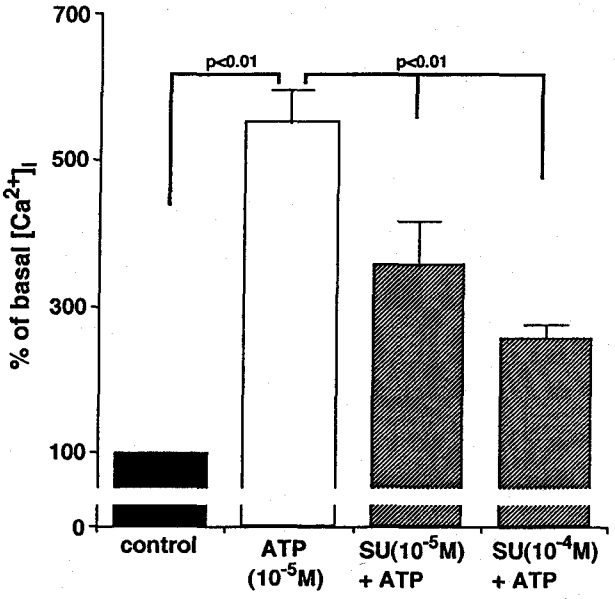

Fig. 4. Effects of suramin on ATP-induced $[Ca^{2+}]_i$ increase in cultured C6 glioma cells. Each samples were pretreated with suramin for 3 min before the addition of ATP. SU: suramin.

Effect of ATP and ionomycin on cell proliferation

To investigate a physiological role of extracellular ATP, we studied ATP-stimulated cell proliferation. ATP (10^{-5} M)-stimulated cell proliferation was observed at day 1, 2, and 3 after the application of agonists. Cell proliferation by adenosine was also detected at day 2 and 3. However adenosine-stimulated cell proliferation was significantly slower than that by ATP (Fig. 5). This ATP-stimulated cell proliferation was significantly inhibited by the treatment with 10^{-4} M of suramin (Fig. 6). There was no single effect of suramin on cell proliferation. To determine the effect of extracellular Ca^{2+} influx on cell proliferation, we utilized the ionomycin which was a specific ionophore for calcium divalent ion to make a calcium channel in plasma membrane. Ionomycin also stimulated cell proliferation in a concentration-dependent manners. The maximal cell proliferation was observed at the concentration of 10^{-6} M.

DISCUSSION

The cytoplasmic ATP concentration is assumed to be over 5 mM in most cells, and a significant proportion of the cytoplasmic ATP can be released into the extracellular space as a neurocotransmitter or as a result of sudden cell death; thus, the concentrations of pericellular ATP could easily reach a high enough concentration to stimulate the P_2-receptors. The local concentrations of ATP may depend on the amount of ATP released, the distributed fluid volume in the extracellular space, and the levels of activity of the catabolic enzymes, especially the ectonucleotidases present on adjacent cells. Teleological reasoning suggests that this possibility for increases in the extracellular ATP concentrations may explain the wide distribution range of ectonucleotidase

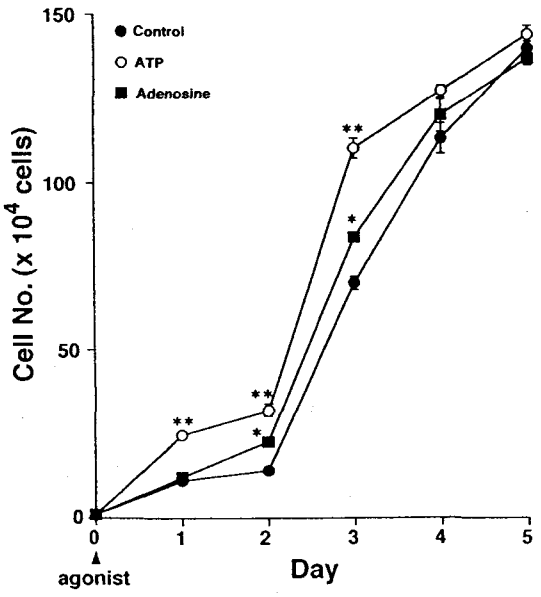

Fig. 5. The effects of ATP (10^{-5} M) or adenosine (10^{-5} M) on purinoceptor-mediated cell proliferations. The results are presented as the mean±SE of triplication from three separate experiments. *: $p<0.05$ and **: $p<0.01$ vs. control.

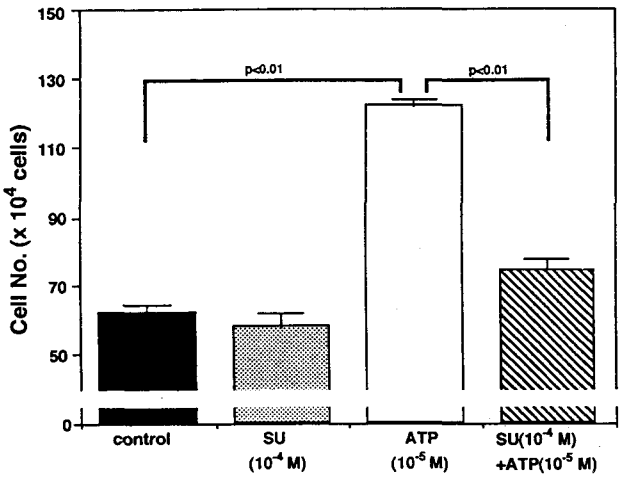

Fig. 6. The effect of suramin (SU) on ATP-stimulated cell proliferation. The results are presented as the mean± SE of triplication from three separate experiments.

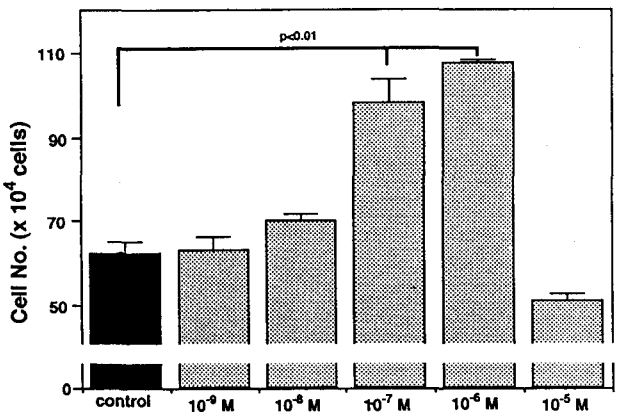

Fig. 7. The effect of ionomycin on cell proliferation. The results are presented as the mean±SE of triplication from three separate experiments.

(Umemura et al, 1992).

Recently, Valera et al (1994) have reported that molecular cloning data on a new class of ligand-gated ion channel could define the P_{2X} receptor for extracellular ATP. Several molecular cloning investigations have revealed that P_{2U}-, P_{2Y}-, and P_{2Y1}-purinoceptors from mouse neuroblastoma cells, mouse and rat insulinoma, and the embryonic chick whole brain cDNA library by hybridization screening, respectively, are coupled with G-proteins which have seven transmembrane spanning domains (Lustig et al, 1993; Webb et al, 1993; Tokuyama et al, 1995).

The main role of glial cells is to support the function of neurons. Because it is well known that ATP induces $[Ca^{2+}]_i$ mobilization in some types of glial cells (Linn & Chuang, 1993; Berti-Mattera et al, 1996; Anselin et al, 1997), our present study provides several lines of evidence for the presence of ATP-responsive receptor subtypes in cultured C6 glioma cells. At first, we demonstrated that ATP receptors are present in C6 glioma cells. The minimal effective concentration of ATP required for $[Ca^{2+}]_i$ increase was 10^{-7} M. Although plasma ATP concentrations may vary, these concentrations of ATP ranging from $10^{-8} \sim 10^{-5}$ M are likely to exist under physiological and/or pathological conditions.

The source of calcium mobilized by ATP was investigated under conditions whereby no calcium was supplied in the extracellular medium. Our results suggest that a large portion of the calcium increased phase of the initial $[Ca^{2+}]_i$ by ATP originates from intracellular stores in the C6 glioma cells (Fig. 2-A).

In some tissues, ATP has mediated the opening of receptor-operated Ca^{2+} channels, although this may be a secondary effect of the increased cytosolic Ca^{2+} concentration. PLC/Ca^{2+}, as a possible second messenger system, has been demonstrated to be activated by binding to P_2-purinoceptors in the C6 glioma cells. The results using U73122 clearly showed that the P_2 receptor in C6 glioma cells was coupled with PLC (Fig. 3).

Recently, however, there have been some changes in terminology; P_{2Y}, P_{2U}, and P_{2Z} are now referred to as P_{2Y1}, P_{2Y2}, and P_{2X7}, respectively (Surprenant et al, 1996). The P_{2U}-purinoceptor, which is activated by ATP and other nucleotides, such as UTP, has also been found on reported. Studies using other tissues have shown that α, β-Me-ATP is 10 times more potent than ATP in stimulating the P_{2X}-purinoceptor and is equally or less potent in stimulating the P_{2Y}-purinoceptor.

From the experimental data of suramin (Cha et al, 1995), this compound was found to inhibit ATP-induced transient $[Ca^{2+}]_i$ increment in a dose-dependent manner. Therefore, the putative purinoceptor subtype existing in the C6 glioma cells was P_{2Y} type. To confirmation of the receptor subtype, it is necessary to conduct a molecular cloning experiment.

As a physiological role of ATP, cell proliferation was studied. It is well known that calcium influx from extracellular fluid and the increase of intracellular calcium concentration influence the cell proliferation and differentiation in some cells. In vascular smooth muscle cells, calcium influx modulates DNA synthesis (Sperti & Colucci, 1991). The degree of proliferation and terminal differentiation of cultured keratinocytes is dependent on the concentration of extracellular calcium (Boyce & Ham, 1983; Stanley & Yuspa, 1983; Al-Ani et al, 1988), which in turn directly influences intracellular free calcium levels directly (Hennings et al, 1989). In addition, extracellular Ca^{2+} and HCO_3^- influence the epidermal growth factor-induced DNA synthesis in cultured rat hepatocytes (Zhang & Farrel, 1995). In order to confirm the relationship between calcium influx and cell proliferation, the effect of ionomycin-induced cell proliferation was observed. The treatment of ionophore induced cell proliferation in a concentration-dependent manners. The maximal cell proliferation was observed at the concentration of 10^{-6} M. ATP-induced $[Ca^{2+}]_i$ registered an initial transient increase and entered into a relatively long sustained phase. Given the fact that the sustained phase disappeared in extracellular calcium-free conditions, this sustained phase of $[Ca^{2+}]_i$ increase proves the influx of extracellular calcium through the plasma membrane. These data agrees with the results of Lin & Chuang's investigation (1993). In light of thes, it can be assumed that the ATP-induced cell proliferation was related to purinoceptor-mediated calcium mobilization. Interestingly, adenosine also stimulate cell proliferation, which means to relate the P_1-purinoceptor in glial cells.

In summary, the present study demonstrates that ATP mediates an increase in $[Ca^{2+}]_i$ cells, and ATP-induced $[Ca^{2+}]_i$ affects the early stages of cell proliferation in cultured C6 glioma.

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