ATP and Purinergic Receptor Agonists Stimulate the Mitogen-Activated Protein Kinase Pathway and DNA Synthesis in Mouse Mammary Epithelial Cells

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ABSTRACT

The effects of adenosine 5'-triphosphate (ATP) and ATP analogs, P_{2y} purinoceptor agonists, on growth of normal mouse mammary epithelial cells (NMuMG) were examined. Cells were plated onto 24 well plates in DMEM supplemented with 10 % fetal calf serum. After serum starvation for 24 hours, ATP, P_{2y} purinoceptor agonists (AdoPP[NH]P, ATP-α-S, ATP-γ-S, β,γ-me-ATP and 2me-S-ATP), P_{2u} purinoceptor agonist (UTP) and P_{2y} purinoceptor antagonists (Reactive Blue 2, more selective to P_{2y} receptor than PPADS; PPADS) were added. DNA synthesis was estimated as incorporation of ^{3}H-thymidine into DNA (1 hour pulse with 1 μCi/ml, 18~19 hours after treatment). ATP, Adopp[NH]P, ATP-α-S or ATP-γ-S significantly increased DNA synthesis at 1, 10 and 100 μM concentrations with dose-dependency (P<0.05), and the maximum responses of ATP and ATP analogs were shown at 100 μM concentration (P<0.05). The potency order of DNA synthesis was ATP≥ ATP-γ-S>Adopp [NH]P>ATP-α-S. β,γ-me-ATP, 2me-S-ATP and UTP did not increase DNA synthesis. In autoradiographic analysis of percentage of S-phase cells, similar results were observed to those of DNA synthesis. Addition of 1, 10 or 100 μM Reactive Blue 2 or PPADS significantly decreased ATP (100 μM)-induced DNA synthesis, however, PPADS was less effective than Reactive Blue 2. In Elvax 40P implant experiment, ATP directly stimulated mammary endbud growth *in situ* suggesting the physiological regulator of ATP in mammary growth. ATP 100 μM rapidly increased MAPK activity, reaching a maximum at 5 min and then gradually decreasing to the base level in 30 min. ATP analogs, Adopp[NH]P and ATP-γ-S also increased MAPK activity, however, β,γ-me-ATP and 2me-S-ATP did not. The inhibitor of the upstream MAPK kinase (MEK), PD 98059 (25 μM), effectively reduced ATP (100 μM) or EGF(10 ng/ml, as positive control)-induced MAPK activity and DNA synthesis (P<0.05). These results indicate that ATP-induced DNA synthesis was prevented from the direct inhibition of MAPK kinase pathway. Overall results support the hypothesis that the stimulatory effects of normal mouse mammary epithelial growth by addition of ATP or ATP analogs are mediated through mammary tissue specific P_{2y} purinoceptor subtype, and MAPK activation is necessary for the ATP-induced cell growth.

(Key words : ATP, Purinergic receptor, MAPK, Epithelial cell, Mouse)

INTRODUCTION

There are evidences that extracellular ATP serve as a mediator of cell to cell communication by interacting with specific cell surface molecules known as P_2 purinergic receptors (Churchill and Ellis, 1993; Murgia et al., 1993; Kunapuli and Daniel, 1998). In the vascular system, aggregating platelets secrete ATP and ADP, which stimulate the release of nitric oxide and other vasodilators from the endothelium(Boeynaems and Pearson, 1990). In the immune system, ATP modulates lymphocyte function in stimulating DNA synthesis in bone marrow and thymus cells while inhibiting DNA synthesis in spleen and peripheral blood lymphocyte (Gordon, 1986), and mast cell degranulation (Osipchuk and Cahalan, 1992). In the human airway epithelium, ATP stimulates transepithelial ion transport(Mason et al., 1991), an effect that may underlie the therapeutic effect of ATP and UTP in the treatment of cystic fibroblast-related lung disease (Knowels et al., 1991). Current data support the existence of several classes, possibly six, P_2 receptor subclasses such as P_{2x}, P_{2y}, P_{2u}, P_{2D}, P_{2z} and P_{2T} receptors (Harden

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et al., 1995; Kunapuli and Daniel, 1998). P_{2y} receptors belong to the family of Ca^{2+}-mobilizing receptors, linked via G protein to release of Ca^{2+} from intracellular stores; P_{2x} receptors coincide with ion channels directly gated by the ligand; P_{2z} receptors have been identified with aqueous pores permeable to solutes of Mr up to 900; P_{2u} receptors share many features of P_{2y} receptors, except that the preferred agonist is reported to be UTP (Dubyak and El-Moatassim, 1993; Kunapuli and Daniel, 1998). Classification of P_2 receptors is still rudimentary, since lack of selective agonists/antagonists has prevented biochemical and molecular characterization. On the basis of a different response profile to ATP analogs, P_{2x} purinoceptor analogs may be listed in order of potency as follows: α,β-me-ATP $\geq$ β,γ-me-ATP > ATP ~ 2me-S-ATP=ADP(Dubyak and El- oatassim, 1993; Burnstock et al., 1994). For P_{2y} purinoceptors: agonists potency may be listed follows: 2me-S-ATP > ATP ≫ α ,β -me-ATP=β , γ -me-ATP (Manzini et al., 1986; Houston et al., 1987; Dubyak and El-Moatassium, 1993). In recent, the trypanoside suramin has been shown to be a specific antagonist for P_2-purinoceptors although not selective for the P_{2x}- and P_{2y}-subclasses (Hoyle et al., 1990), whereas the compound PPADS (pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid) appears to be more selective P_{2x} antagonist (Ziganshin et al., 1993). In addition, reactive blue 2 selectively inhibited the response via P_{2y}-purinoceptors, for which ATP is a more potent agonist than α , β -methylene ATP, but did not affect the response via P_{2x}-purinoceptors (Burnstock and Warland, 1987).

Adenylate cyclase and protein kinase A activities increase during pregnancy in mice and rat mammary glands (Rillema, 1976; Sapag-Hagar and Greenbaum, 1974; Sharoni et al., 1984). Elevated these kinase activities are correlated with mammary growth at least during pregnancy (Sharoni et al., 1984). In addition, G-protein modulated bovine undifferentiated mammary epithelial cell growth *in vitro* (Shamay et al., 1990). As previously mentioned, extracellular nucleoside di- and triphosphates modulate a wide variety of physiological responses through activation of a family of G protein-coupled receptors, referred to P_{2y} receptor in a variety of tissues.

Critical molecules involved in the transduction of signaling for cell proliferation have been identified. Mitogen-activated protein kinases (MAPKs), which belong to a family of serine/threonine protein kinases, are activated during cell proliferation by a variety of stimuli (Davis, 1994; Gutkind, 1998). The stimulation of tyrosine kinases by some growth factors including epidermal growth factor elevates MAP kinase activity through multistep processes (Martinez-Lacaci, 2001). In addition, heterotrimeric G protein-coupled receptors such as bombesin, thrombin, pertussis toxin, and lysophosphatidic acid have induced MAPK activation (ERK1/2) through various types of G-protein subunits ($G_{\alpha s}$, $G_{\alpha i}$ or $G_{\beta\gamma}$) in variety of tissues and cultured cells (Faure et al., 1994; Wan and Huang, 1998; Gutkind, 1998), however, little known about the signaling process of the pathway by G protein-coupled purinergic receptor in mammary tissue.

In spite of the some feasibilities of G-protein-coupled receptor existence from the previous studies, the possibility that purinergic receptors are presented in mammary tissue and these receptor-mediated pathways are involved in mammary development has received little attention. Therefore, the objective of this study is to determine if purinergic receptor effector, ATP, alters growth of mammary epithelium and the growth stimulatory pathway by ATP is coupled to MAPK (ERK1/2) activation or not.

MATERIALS AND METHODS

Cells and Treatments

Normal mouse mammary epithelial cells (NMuMG line, Owens et al., 1974) were obtained from American Type Culture Collection (Rockville, MD, USA) and routinely cultured in DMEM supplemented with 5% fetal calf serum. For experiments, cells were plated (1×10^5 cells per well for 24 well plates, 1×10^4 cells per chamber for culture slides, and 4×10^5 cells for 12 well culture plates and 2×10^6 cells for 100 mm culture dish), allowed 24 hour attachment period and then serum deprived for 24 hours prior to treatment. Treatments were applied and measurements made as described for each experiment. Cells used in these studies ranged from passage 16~20. ATP, ATP-α -S and ATP-γ -S were purchased from Sigma Chemical Co., St. Louis, MO, USA. β , γ -imidoadenosine 5'-triphosphate (Adopp[NH]P), β , γ -methylene-L-adenosine 5'-triphosphate(β , γ -me-ATP), 2-methylthioadenosine triphosphate (2me-S-ATP), reactive blue 2 and pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid tetrasodium salt (PPADS) were purchased from Research Biochemicals Inc., Natick, MA, USA. Epidermal growth factor(EGF) was purchased from Upstate Biotechnology, Inc. NY, USA. All were added at the concentrations indicated in results, in DMEM.

DNA Synthesis

DNA synthesis was measured as incorporation of ^{3}H-thymidine (70~86 Ci/mmol, Dupont, Willmington, DE, USA) into DNA by 1 hour pulse of 1 μCi/ml beginning 18 hours after treatment. The radioactivity incorporated into trichloroacetic acid (TCA)-precipitable materials was measured as follows: cells were rinsed 3 times with Tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris, pH 7.5), three times with ice cold 10 % trichloroacetic acid (TCA) and two times with cold 100 % ethanol. Cells were dried, dissolved in 0.5 M NaOH

containing 0.1 % Triton X-100, neutralized with HCl and counted by liquid scintillation.

Autoradiography

For nuclear labeling studies, cells were plated on tissue culture slides (Lab-Tek, Miles Scientific, Naperville, IL, USA), and growth-arrested cells were stimulated. Incubation time and ^{3}H-thymidine incorporation into DNA were followed as previous treatment schemes except that ^{3}H-thymidine-pulsed cells were washed three times with Tris buffer (50 mM, pH 7.5) containing 150 mM NaCl, fixed with 10 % buffered formalin phosphate (30 min), washed with ethanol (70 %, 5 min) and distilled water (2× , 5 min), and allowed to dry. The dried cells were coated with Ilford K5 emulsion (Polyscience, Inc., Warrington, PA, USA), exposed at 5 ℃ for 9 days, developed in Kodak developer D-19, stained with hematoxylin, and observed under a light microscope. The percentage of labeled cells were calculated by dividing the number of cells that had incorporated ^{3}H-thymidine in a whole observable area with microscope by the total number cells in that area (approximately total 100~150 cells were observed).

Elvax 40P Implants and Surgical Implantation

A plastic implant material, ethylene vinyl acetate copolymer (Elvax 40P), which is capable of the slow-release of bioactive molecules in situ, was prepared and implanted into 5-week-old ovariectomized or intact virgin ICR mice. Briefly, Elvax 40P(Aldrich Chemical Co., WI, USA) was washed for two weeks in several changes of 95 % ethanol with continuous stirring, after which it was dissolved in methylene chloride to give a 20 % solution (wt/vol). Adenosine 5'-triphosphate (ATP) containing implants were prepared by dissolving the ATP in methylene chloride and mixing with liquid Elvax-40P to yield implants with the desired concentration (Silberstein and Daniel, 1987). The mixture was lyophilized and then the lyophilate was cut out 1x1x2 mm size of pieces.

Mice were anesthetized with pentobarbital (20 μg/ g body weight) and the forth mammary glands (inguinal mammary glands) exposed by pinning back the abdominal skin. Pockets were made in the gland and implants placed using forceps. Mammary glands were fixed in glacial acetic acid:ethanol (1:3) stained with carmine-alum, photographed and measured endbud diameter.

Measurement of MAPK Activity

NMuMG cells (2× 10^{6} cells/dish) were cultured in the 100 mm culture dish as described in routine cell culture method for 6 days to reach 80 % confluent state. Prior to stimulation, cells were serum-starved for 24 hours and then stimulated according to the experimental scheme. The reactions were stopped by placing culture dishes on the ice and by briefly washing cells with ice-cold PBS (pH 7.4). The cells were then lysed in lysis buffer {15 mM NaCl/50 mM Tris-HCl, pH 7.8/Nonidet P40 1 % (wt /vol)/leupeptin (1 μM)/aprotinin (0.1 μM)}. The lysates were collected with rubber policemen and clarified by centrifugation (10 min, 13,000 g, 4 ℃). The supernatant was resolved by 12 % SDS-polyacrylamide gel electrophoresis. Phosphorylation of p44/42 MAPK (Erk 1/2) was detected by protein immunoblotting using MAPK rabbit polyclonal phospho-antibody with anti-rabbit IgG HRP conjugate as secondary antibody (Promega, WI, USA). The membranes were then stripped by incubating in phosphate buffered saline-0.05 % tween-20 (PBST, pH 7.4) in a shaker, and re-probed with rabbit anti-MAPK antibody (Sigma, MO, USA) with anti-rabbit IgG HRP conjugate as second antibody (Promega WI, USA) to quantify the total MAPK loaded onto each lane. Quantification of phospho p44/42 phosphorylation was performed after development of membranes with Enhanced-chemiluminescence (ECL system, Amersham Pharmacia Biotech, UK) by scanning on Vilver Lourmat, image analysis system (France). The degree of p44/42 phosphorylation was normalized to total MAPK intensity within each treatment.

Statistical Analysis

Treatments were made in duplicate or triplicate, and repeated on three or four separate occasions. Data were analyzed by analysis of variance using a randomized complete block design model. Over all means were compared using planned comparison and mean differences of endbud diameters between of one lateral and the other lateral 4th mammary gland were compared by paired comparison (Snedecor and Cochran, 1987). Unless otherwise stated significance was set to $P<0.05$.

RESULTS

To determine the purinergic receptor subtype in mammary epithelial cell growth, the efficacy of different purinergic receptor agonists was examined. The known P_{2X} and P_{2y} purinergic receptor agonists, ATP, ATP-γ-S, ATP-α -S, Adopp[NH]P, β , γ-me-ATP and 2me-S-ATP were added with concentration range from 0 to 100 μM, and P_{2u} receptor agonist, UTP, was added and DNA synthesis was measured (Fig. 1). Addition of 10 μM ATP, ATP-γ-S, ATP-α -S or Adopp[NH]P increased 212.0, 180.1, 144.2 or 190.3% of DNA synthesis relative to that of control ($P<0.05$). In addition, 100 μM of ATP, ATP-γ -S, ATP-α -S or Adopp[NH]P increased 224.1, 252.3, 186.4 or 209.2% of DNA synthesis relative to that of control ($P<0.05$). The known P_{2X} and P_{2y} purinergic receptor agonists,β , γ-me-ATP and 2me-S-ATP or P_{2u}

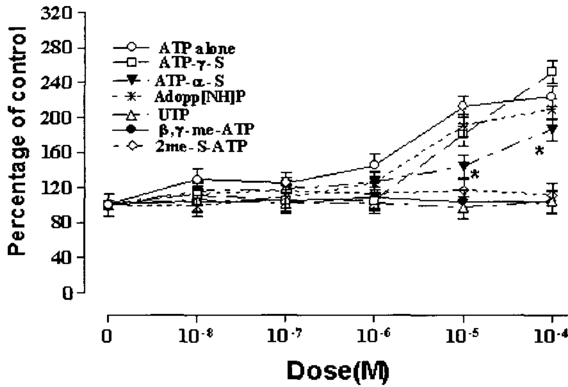

Fig. 1. Effect of ATP, ATP analogs(P_{2y} purinoceptor agonists, ATP-γ-S, ATP-α -S, Adopp[NH]P, β , γ-me-ATP and 2me-S-ATP), and UTP(P_{2u} purinoceptor agonist) on DNA synthesis in NMuMG cells. ^{3}H-thymidine incorporation into DNA was determined, as described in Materials and Methods, in synchronized cells. DNA synthesis was noted as percentage of control; DNA synthesis in the indicated group/DNA synthesis of control × 100(%). * = significantly different from 0 M dose ($P<0.05$).

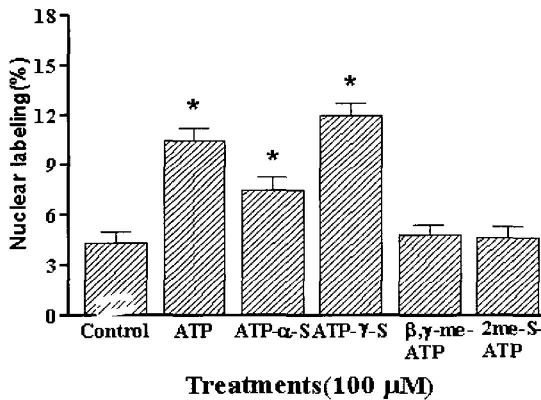

Fig. 2. Autoradiographic analysis of percent of ^{3}H-thymidine labeled NMuMG cells nuclei. ATP(100 μM) and ATP analogs (all 100 μM concentrations), ATP-α -S, ATP-γ-S, β , γ-me-ATP and 2me-S-ATP, were treated on eight chamber slides with the same culture conditions in Fig. 1, but cells were fixed on the slides with buffered formalin as detailed in Materials and Methods. Slides were immersed in photosensitive emulsion, exposed for 9 days at 4℃, and developed. The percentage of labeled cells were calculated by dividing the number of cells that had incorporated ^{3}H-thymidine in a whole observable area with microscope by the total number cells in that area(approximately total 100~150 cells were observed). * = significantly different from control (no treatment) ($P<0.05$).

receptor agonist, UTP, did not increase DNA synthesis ($P \geq 0.05$). Similar results were observed when DNA synthesis was measured by autoradiography (Fig. 2). Addition of 100 μM ATP, ATP-γ-S or ATP-α -S significantly increased percentage of S-phase cells from 4.3 % in control to 10.5 %, 12.0 % or 7.5 % ($P<0.05$), however, β , γ-me-ATP and 2me-S-ATP did not increase percentage of S-phase cells ($P \geq 0.05$). The potency order of DNA synthesis in mouse mammary epithelial cells was ATP≥ ATP-γ-S>Adopp(NH)P> ATP-α -S.

To characterize further the purinoceptor subtype mediating ATP responses in mammary epithelial cells, the putative P_{2y} receptor antagonists Reactive Blue 2

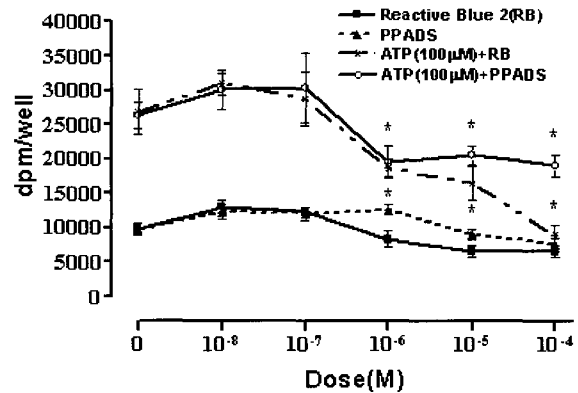

Fig. 3. Effect of Reactive Blue 2(P_{2y} purinoceptor antagonist) and PPADS(P_{2x} or P_{2y} purinoceptor antagonist) on basal and ATP(100 μM)-induced DNA synthesis in NMuMG cells. Cells were serum starved for 24 hr, purinoceptor antagonists were added at the indicated concentration with or without 100 μM ATP, and DNA synthesis was measured 18 hr later as dpm ^{3}H-thymidine incorporated into acid insoluble material. * = significantly different from ATP(100 M) with no antagonist ($P<0.05$).

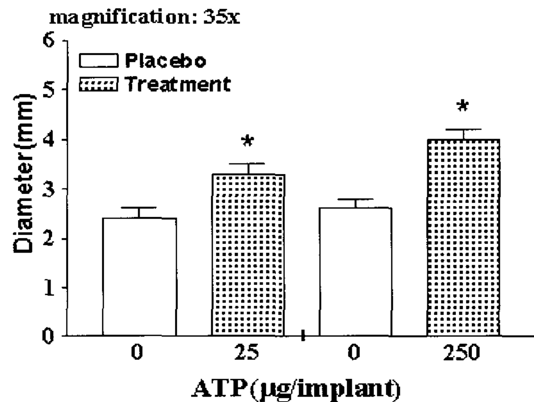

Fig. 4. Influence of Elvax 40P implants containing ATP on mammary gland endbud diameter in intact ICR mice. ATP treated-glands were compared to contralateral glands containing blank implants (Placebo) by paired comparison. * = significant difference between placebo and treatment at a given concentration of ATP/implant in intact.

(Burnstock and Kennedy, 1985; Houston et al., 1987) and PPADS (Lambrecht et al., 1992; Ziganshin et al., 1993) were added(Fig. 3). Addition of 100 μM ATP alone significantly increased DNA synthesis(2.7 fold increase to no addition). Addition of 0, 0.01 or 0.1 μM Reactive Blue 2 or PPADS into 100 μM ATP did not change the response of 100 μM ATP, however, addition of 1, 10 or 100 μM those of P_{2y} receptor antagonists significantly decreased DNA synthesis of 100 μM ATP. Reactive Blue 2 completely inhibited the response of 100 μM ATP at 100 μM concentration, however, PPADS was not effective as much as Reactive 2 in inhibition.

Ethylene vinyl acetate copolymer(Elvax 40P) is capable of the sustained release of bioactive molecules to local regions of the developing gland, thus allowing primary effects to be observed in a natural structural and chemical milieu, greatly reducing or eliminating the confounding effects of systemic reactions(Silberstein and Daniel, 1987). To observe the direct effect of ATP *in situ* of mammary tissue, we implanted Elvax 40P containing ATP in mammary glands(Fig. 4). One lateral gland received only Elvax 40P as placebo, and the contralateral gland received Elvax 40P containing ATP(25 or 250 μg /implant). Implants containing ATP significantly increased local endbud diameter of intact mice($P<0.05$).

Many G_i, G_q, and G_s-coupled receptors have induced MAPK activation(ERK1/2) in variety of tissues and cultured cells, however, little known about the signaling process of the pathway by purinergic receptor in mammary tissue. For this reason, we tried to determine if exogenous ATP-stimulated pathway coupled to ERK1/2 activation in mammary epithelial cells or not. In our experiment, addition of ATP(100 μM) rapidly increased MAPK activity, reaching a maximum at 5min and then gradually decreasing to the base level in 30 min(Fig. 5). The activation of MAPK by ATP(100 μM) or EGF(10

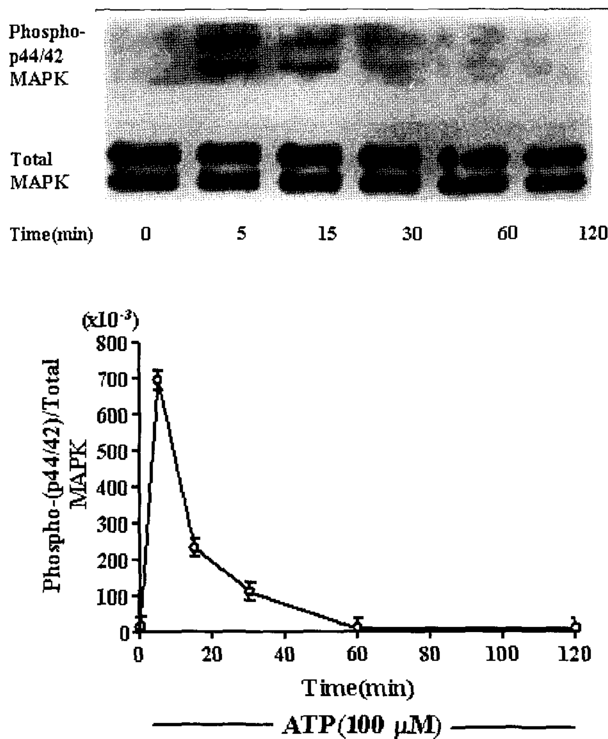

Fig. 5. Time courses of ATP-stimulated MAPK activation in NMuMG cells. NMuMG cells were grown to 80 % confluent in DMEM with 10 % FCS on 100-mm culture dish. After 24 hour serum starvation, cells were stimulated with ATP(100 μM) for 0, 5, 15, 30, 60 , or 120 min. Protein separation by SDS-PAGE, immunoblotting, MAPK activity, and densitometry scanning were described in Materials and Methods. The amount of phospho-p44/42 MAPK was normalized by total MAPK expression. The ratio of phospho-p44/42 MAPK to total MAPK was calculated with arbitrary optical density units. The photograph shows one of two same experiments.

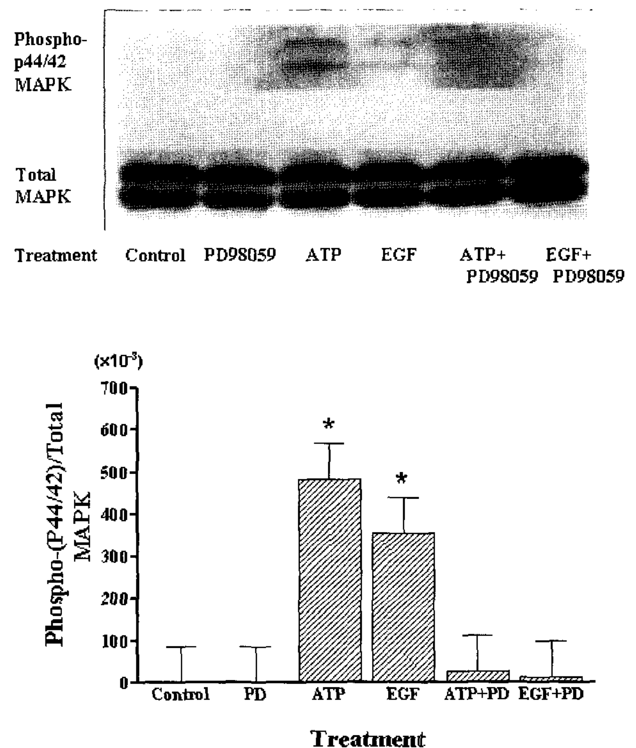

Fig. 6. The effect of PD98059 (MEK inhibitor) on MAPK activation in response to ATP or EGF(as positive control in MAPK activation). The cells were pretreated with or without PD98059(25 μM) for 30 min before stimulation with ATP(100 μM) or EGF(100 ng/ml) for 5 min. Protein separation by SDS-PAGE, immunoblotting, MAPK activity, and densitometry scanning were described in Materials and Methods. The amount of phospho-p44/42 MAPK was normalized by total MAPK expression. The ratio of phospho-p44/42 MAPK to total MAPK was calculated with arbitrary optical density units. The photograph shows one of two same experiments.

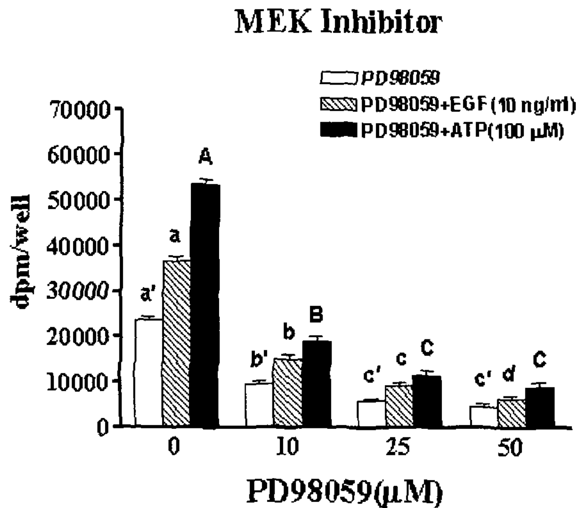

Fig. 7. The effect of PD98059(MEK inhibitor) on DNA synthesis in response to ATP(100 μM) or EGF(10 ng/ml). DNA synthesis rates with different superscripts within same type bars are significantly different, $P<0.05$.

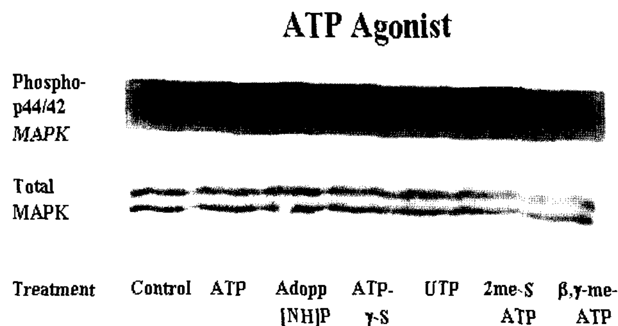

Fig. 8. Effect of ATP, ATP analogs (P_2 purinoceptor agonists, ATP-γ-S, Adopp[NH]P, β, γ-me-ATP, 2me-S-ATP), and UTP(P_{2u} purinoceptor agonist) on MAPK activation in NMuMG cells. The cells were treated with 100 μM ATP, ATP analogs or UTP for 5 min. Protein separation by SDS-PAGE, immunoblotting, and MAPK activity were described in Materials and Methods. The amount of phospho-p44/42 MAPK was normalized by total MAPK expression.

ng/ml, as positive control) was effectively reduced by the inhibitor of the upstream MAPK kinase(MEK), PD98059 25 μM(Fig. 6). PD98059 25 μM also showed dosedependent attenuation of the ATP (100 μM)- or EGF (10 ng/ml)- induced DNA synthesis (Fig. 7, $P<0.05$). These results indicate that ATP induced DNA synthesis was prevented from the direct inhibition of MAPK kinase pathway. These results support the hypothesis that MAPK activation is necessary for the signaling process in the ATP induced mammary epithelial cell growth.

In Fig. 8, 100 μM of ATP, Adopp[NH]P, ATP-γ-S and UTP increased MAPK activity, however, β, γ-me-ATP and 2me-S-ATP did not. In previous data, ATP, Adopp [NH]P and ATP-γ-S significantly increased DNA synthesis rate except UTP, 2me-S-ATP or β, γ-me-ATP. The results of MAPK activity were highly compatible to those of DNA synthesis rate except UTP.

DISCUSSION

Previously, a wide variety of roles have been proposed for extracellular ATP and related nucleotides. In present study, ATP, ATP-γ-S, ATP-α-S and Adopp [NH]P significantly increased DNA synthesis in mammary epithelium and Elvax 40P containing ATP directly stimulated *in situ* endbud growth. However, the known P_{2y} purinoceptor agonists, α,β,-me-ATP(result not shown), β, γ-me-ATP and 2me-S-ATP did not stimulate DNA synthesis of mammary epithelial cells. In addition, P_{2u} receptor agonist UTP also did not stimulate DNA synthesis. UTP, however, increased MAPK activity in mammary epithelial cells. To characterize further the receptor subtype mediating ATP responses in mammary epithelial cells, the known putative P_{2y} purinoceptor antagonist Reactive Blue 2(Inoue et al., 1991) and P_{2x} and P_{2y} receptor antagonist PPADS (Lambrecht et al., 1992) were incubated in the absence and presence of ATP. In Figure 3, the known putative P_{2y} receptor antagonists Reactive Blue 2 and PPADS inhibited ATP-stimulated DNA synthesis of mammary epithelial cells. It was reported that Reactive Blue 2 selectively inhibited the response via P_{2y} receptors, for which ATP is more potent agonist than α,β me-ATP or β, γ-me-ATP, but did not affect the response via P_{2x}-purinoceptors, for which α,β me-ATP or β, γ-me-ATP is a more potent agonist than ATP, in vascular smooth muscles (Burnstock and Warland, 1987). The effect of Reactive Blue 2 in this report should be due to its antagonism to P_{2y} receptors rather than P_{2x} receptors because of the efficacy of Reactive Blue 2 against ATP response in mammary epithelial cells. PPADS antagonized selectively ATP, α, β me-ATP or β, γ-me-ATP-induced tension responses in rabbit vas deferens (Lambrecht et al., 1992), and PPADS efficiently antagonized agonists of P_{2x} receptor family agonists rather than those of P_{2y} receptors (Abbracchio and Burnstock, 1994). In our experiment, PPADS blocked ATP-induced mitogenic response in mammary epithelial cells but did not block effectively. These results suggest that mitogenic response of ATP should be mediated through P_{2y} rather than P_{2x} purinoceptors. The agonist specificity profile in mammary tissue is somewhat different from that reported in other systems. To date, eleven subtypes of P_{2y} receptors have been identified (Communi et al., 1997; Kunapuli and Daniel, 1998; Ray et al., 2002). Although some of the classical pharmacological P_2 purinoceptor subtypes could be identified as a specific molecular subtype, most appear to be a mixture of molecular P_2 purinoceptor subtypes. The classification of P_{2y} purino-

ceptor subtypes is not still clear, since lack of selective agonists/antagonists has prevented biochemical and molecular characterization and the various P_2 purinoceptor subtypes are found with tissue specificity. Many of these subtypes, identified by sequence identity, still need to be demonstrated to be functional nucleotide receptors.

Adenosine nucleotides have been reported to exhibit optimal levels in sharply affecting in DNA replication in isolated nuclei(Hershey, 1977; Enomoto et al., 1981; Rapaport, 1983). In otherwise, high ATP levels, as well as high ATP/ADP ratios, lead to marked reduction in the rate of DNA synthesis, in both intact cells and isolated nuclei(Rapaport, 1983). In our experiments(data not shown), the increased endogenous ATP analogs(ATP-γ-S and α,β-me-ATP) levels by addition of these reagents did not affect on the DNA synthesis rate in mammary epithelial. These results indicated that the stimulatory effect of exogenous ATP and ATP analogs should not be due to the increase of endogenous ATP. In addition, with the use of Elvax 40P pellets, specifically designed to provide a slow and sustained release of an array of bioactive ATP *in situ*, significant growth stimulatory effect of endbud was found in the presence of ovarian function. This result indicates that ATP directly stimulates mammary endbud growth and should be physiological regulator in mammary tissue in the presence of extracellular ATP. Extracellular ATP has been shown to stimulate cell proliferation, including Swiss 3T3, A431, vascular smooth muscle cells and renal mesangial cells, and P_{2y} receptors mediated these effects(Huang et al., 1989; Huang et al., 1991; Huwiler and Pfeilschifter, 1994; Yu et al., 1998).

In Figure 5, 6, and 8, exogenous ATP and ATP analogs activated MAPK(ERK1/2) at 5 min after stimulation and its activity was blocked by MEK inhibitor(PD 98059). In addition, the increased MAPK activities by ATP and ATP analogs were compatible with the increased DNA synthesis rate by those treatments. In previous experiments, EGF induced DNA synthesis in normal mouse mammary epithelial cells(Johnson et al., 1996) and expression of heparin-binding EGF-like growth factor gene in the transformed human mammary epithelial cells(Martinez-Lacaci, 2001) through *ras* and MAPK pathway. In the present study, PD 98059 attenuated the induction of EGF or ATP induced DNA synthesis and MAPK activity in mammary epithelia. These results indicate that the signaling pathways of mammary epithelia growth by ATP at least partially involved the same downstream of MEK pathways of EGF. In previous results, P_{2u} receptor agonist UTP did not stimulate DNA synthesis, but increased MAPK activity in mammary epithelial cells. One possible explanation to the discrepancy between DNA synthesis rate and MAPK activity would be due to the increase of thymidine nucleotide pool size resulted from the novo synthesis of thymidine nucleotide from the UTP. Thus DNA synthesis rate was not notably measured. Another possible explanation is that mammary tissue contains P_{2u} receptors but the physiological role of its receptors in mammary tissue do not involve in cell growth. UTP couples to most P_{2y} subtype receptors and increases in intracellular Ca^{2+}, rapid Ca^{2+} influx and phospholipase C activation in other tissues(Kunapuli and Daniel, 1998).

ATP induced Raf-1 and MAPK activation which was triggered by activation of G protein coupled P_{2y} receptors leading eventually to DNA synthesis and cell proliferation in vascular smooth muscle cells(Yu et al., 1996). Recently identified subtypes of P_{2y} purinoceptors such as P_{2y2} and P_{2y4}, have been proposed to mediate cell proliferation through activation of the MAPK pathway (Harper et al., 1998). In addition, both of adenosine A_2 receptors and P_{2y2} receptors which are G protein coupled purinergic receptors, are simultaneously activated MAPK pathways in human embryonic kidney-293 cells(Gao et al., 1999). These results suggest that ATP induced mitogenic effects in most cells are mediated through the signal transduction pathways at least G protein-coupled MAPK activation mechanism. The physiological relevance of purinoceptor mediated stimulatory pathways in mammary growth is unclear at present time. This experiments, however, partially indicate the increase of DNA synthesis of mammary epithelial cells and the local growth of endbuds by ATP or P_{2y} purinoceptor agonists are associated with mammary specific P_{2y} purinoceptor subtypes and these effects are processed through the MAPK dependent pathway.

ATP and other nucleotides release from viable cells of several cell types such as platelets, neurons, fibroblasts, smooth muscle cells and vascular endothelial cells by exocytosis, and they interact with their own specific receptors on the surface of many different cells and plays potential regulatory role for the tissue specific function (Gordon, 1986). Cytoplasmic ATP in most cells is over 5 mM in concentration and significant proportion can be released; thus, the concentrations of pericellular ATP could easily reach the high micromolar range. The mammary tissue consists of ductular and secretory alveolar epithelial cells embraced in a heterogenous matrix of other cell types, including myoepithelial cells, adipocytes, fibroblasts, and smooth muscle. In addition, leukocytes, lymphocytes, cells associated with the vascular system, and neurons are found in the mammary tissue. Thus, it is conceivable that the heterogenous matrix of mammary tissue could be a source of ATP, and locally produced extra- and intracellular ATP can regulate mammary development at least during mitogenic period.

Overall results indicate that the increase of DNA synthesis of mammary epithelial cells and the local growth of endbuds by ATP or P_{2y} purinoceptor agonists are associated with mammary specific P_{2y} purinoceptor subtypes and these effects are processed through the

MAPK dependent pathway.

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REFERENCES

1. Abbracchio MP, Burnstock G (1994): Purinoceptors: are there families of P_{2x} and P_{2y} purinoceptors ? Pharmacol Ther 64:445-475.
2. Boeynaems JM, Pearson JD (1990): P_2-Purinoceptors on vascular endothelial cells: physiological significance and transduction mechanisms. Trends Pharmacol Sci 11:34-37.
3. Burnstock G, Fischer B, Hoyle CHV., Maillard M, Ziganshin AU, Brizzolara AL, Von Isacovics A, Boyer JL, Kendall-Harden T, Jacobson KA (1994): Structure activity relationships for derivatives of adenosine-5'-triphosphate as agonists at P_2 purinoceptors: heterogeneity within P_{2x} and P_{2y} subtypes. Drug Develop Res 31:206-219.
4. Burnstock G, Kennedy C (1985): Is there a basis for distinguishing two types of P_2-purinoceptor ? Gen Pharmac 16:433-440.
5. Burnstock G, Warland JJ (1987): P_2-purinoceptors of two subtypes in the rabbit mesenteric artery: reactive blue 2 selectively inhibits responses mediated via the P_{2y}-but not the P_{2x}-purinoceptor. Br J Pharmacol 90: 383-391.
6. Churchill PC, Ellis VR (1993): Pharmacological characterization of the renovascular P_2 purinergic receptors. The J of Pharmacol and Exper Thera 265: 334-338.
7. Communin D, Govaerts C, Parmentier M, Boeynaems JM (1997): Cloning of a human purinergic P_{2Y} receptor coupled to phospholipase C and adenylyl cyclase. J Biol Chem 272:31969-31973.
8. Davis RJ (1994): MAPKs: new JNK expands the group. Trends Biochem Sci 19:470-473.
9. Dubyak GR, El-Moatassium C (1993): Signal transduction via P_2-purinergic receptors for extracellular ATP and other nucleotides. American J of Physiol 265:c577-606.
10. Enomoto T, Tanuma S, Yamada M (1981): ATP requirement for the process of DNA replication in isolated HeLa nuclei. J Med Chem 20:907-911.
11. Faure M, Voyno-Yasenetskaya TA, Bourne HR (1994): cAMP and $\beta\ \gamma$ subunits of heterotrimeric G proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells. J Biol Chem 269:7851-7854.
12. Gao Z, Chen T, Weber MJ, Linden J (1999): A_{2B} adenosine and P_{2y2} receptors stimulate mitogen-activated protein kinase in human embryonic kidney-293 cells. J Biol Chem 274:5972-5980.
13. Gordon L (1986): Extracellular ATP: effects, sources and fate. Biochem J 233:309-319.
14. Gutkind JS (1998): The pathways connecting G-protein-coupled receptors to the nucleus through divergent mitogen-activated protein kinase cascades. J Biol Chem 1839-1842.
15. Harden TK, Boyer J, Nicholas RA (1995): P_2- purinoceptors: subtype-associated signaling responses and structure. Annu Rev Pharmacol Toxicol 35:541-579.
16. Harper S, Webb TE, Charlton SJ, Ng LL, Boarder M R (1998): Evidence that P_2Y_4 nucleotide receptors are involved in the regulation of rat aortic smooth muscle cells by UTP and ATP. Br J Pharmacol 124:703-710.
17. Hershey HV (1977): Effect of ATP analogs on DNA synthesis in isolated nuclei. Biochem Biophys Acta 479:256-264.
18. Hoyle CH, Knight CE, Burnstock G (1990): Suramin antagonizes responses to P_2-purinoceptor agonists and purinergic nerve stimulation in the guinea-pig urinary bladder and taenia coli. Br J Pharmacol 99: 617-621.
19. Houston DA, Burnstock G, Vanhoutte PM (1987): Different P_2-purinergic receptor subtypes of endothelium and smooth muscle in canine blood vessels. J Pharmac Exp Ther 241:501-506.
20. Huang NN, Wang DJ, Heppel LA (1989): Extracellular ATP is a mitogen for 3T3, 3T6 and A431 cells and acts synergistically with other growth factors. Proc Natl Acad Sci USA 86:7904-7908.
21. Huang NN, Wang DJ, Gonzalez FA, Heppel LA (1991): Multiple signal transduction pathways lead to extracellular ATP-stimulated mitogenesis in mammalian cells. II. A pathway involving arachidonic acid release, prostaglandin synthesis, and cyclic AMP accumulation. J Cell Physiol 146:483-494.
22. Huwiler A, Pfeilschifter J (1994): Stimulation by extracellular ATP and UTP of the mitogen-activated protein kinase cascade and proliferation of rat renal mesangial cells. Br J Pharmacol 113:1455-1463.
23. Inoue K, Nakazawa K, Ohara-Imaizumi M, Obama T, Fujimori K, Takanaka A (1991): Antagonism by reactive blue 2 but not by brilliant blue G of extracellular ATP-evoked responses in PC12 phaeochromocytoma cells. Br J Pharmacol 102:851-854.
24. Johnson JL, Fenton S, Sheffield LG (1996): Prolactin inhibits epidermal growth factor-induced Ras-MAPK

signaling in mammary epithelial cells. J Biol Chem 271(35):21574-21578.
25. Kunapuli SP, Daniel JL (1998): P_2 receptor subtypes in the cardiovascular system. Biochem J 336:513-523.
26. Knowles MR, Clarke LL, Boucher RC (1991): Activation by extracellular nucleotides of chloride secretion in the airway epithelia of patients with cystic fibrosis. N Engl J Med 325:533-538.
27. Lambrecht G, Friebe T, Grimm U, Windscheif EB, Hildebrandt C, Baumert HG, SpatzKumbel G, Mutschler E (1992): PPADS, a novel functionally selective antagonist of P_2 purinoreceptor-mediated responses. Eur J Pharmacol 217:217-219.
28. Manzini S, Hoyle CHV, Burnstock G (1986): An electrophysiological analysis of the effect of relative blue 2, a putative P_2-puriniceptor antagonist, on inhibitory junction potentials of rat caecum. Eur J Pharmacol 127:197-204.
29. Martinez-Lacaci I, De Santis M, Kannan S, Bianco C, Kim N, Wallace-Jones B, Wechselberger C, Ebert AD, Salomon DS (2002): Regulation of heparin-binding EGF-like growth factor expression in Ha-ras transformed human mammary epithelial cells. J of Cell Physiol 186(2):233-242.
30. Mason SJ, Paradiso AM, Boucher RC (1991): Regulation of transepithelial ion transport and intracellular calcium by extracellular ATP in human normal and cystic fibrosis airway epithelium. Br J Pharmacol 103:1649-1656.
31. Murgia M, Hanau S, Pizzo P, Rippa M, Virgilio FD (1993): Oxidized ATP: An irreversible inhibitor of the macrophage purinergic P_{2z} receptor. The J of Biol Chem 268:8199-8203.
32. Osipchuk Y, Cahalan M (1992): Cell-to-cell spread of calcium signals mediated by ATP receptors in mast cells. Nature Lond 359:241-244.
33. Owens RB, Smith HS, Hackett AJ (1974): Epithelial cell cultures from normal glandular tissue of mice. J Natl Cancer Instit 53:261-265.
34. Rapaport E (1983): Treatment of human tumor cells with ADP or ATP yields arrest of growth in the S phase of the cell cycle. J Cell Physiol 114:279-283.
35. Ray FR, Huang W, Slater M, Barden JA (2002): Purinergic receptor distribution in endothelial cells in blood vessels: a basis for selection of coronary artery grafts. Atherosclerosis 162:55-61.
36. Rillema JA (1976): Cyclic nucleotides, adenylate cyclase, and cyclic AMP phosphodiesterase in mammary glands from pregnant and lactating mice. Proc Soc Exp Biol Med 151:748-751.
37. Sapag-Hagar M, Greenbaum AL (1973): Changes of the activities of adenylate cyclase and cAMP-phosphodiesterase and of the level of 3',5' cyclic adenosine monophosphate in rat mammary gland during pregnancy and lactation. Biochem Biophys Res Com 53:982-987.
38. Shamay A, Pines M, Waksman M, Gertler A (1990): Proliferation of bovine undifferentiated mammary epithelial cells *in vitro* is modulated by G-proteins. Mol Cell Endocrinol 69:217-226.
39. Sharoni Y, Feldman B, Teuerstein L, Levy J (1984): Protein kinase activity in the rat mammary gland during pregnancy, lactation and weaning: A correlation with growth but not with progesterone receptor levels. Endocrinology 115:1918-1924.
40. Silberstein GB, Daniel CW (1987): Investigation of mouse mammary ductal growth regulation using slow-release plastic implants. J Dairy Sci 70:1981-1990.
41. Snedecor GW, Cochran WG (1980): In Statistical Methods, 7th ed., The Iowa State University Press, Ames.
42. Wan, Y, Huang XY (1998): Ananlysis of the Gs/mitogen-activated protein kinase pathway in mutant S49 cells. J Biol Chem 273:14533-14537.
43. Yu SM, Chen SF, Lau YT, Yang CM, Chen JC (1996): Mechanism of extracellular ATP-induced proliferation of vascular smooth muscle cells. Molecular Pharmacol 50:1000-1009.
44. Ziganshin AU, Hoyle CHV, Xuenong B, Lambrecht G, Mutschler E, Baumert HG, Burnstock G (1993): PPADS selectively antagonizes P_{2X}-purinoreceptor-mediated responses in the rabbit urinary bladder. Br J Pharmacol 110:1491-1495.

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