Effects of adenosine 5'-triphosphate and growth hormone on cellular H⁺ transport and calcium ion concentrations in cloned bovine mammary epithelial cells

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Abstract

The present experiment was carried out to investigate the effects of exogenous adenosine 5'-triphosphate (ATP) and growth hormone (GH) on cellular H⁺ efflux rate (extracellular acidification rate) and Ca²⁺ concentration ([Ca²⁺]_c) in cloned bovine mammary epithelial cells (bMEC) raised from the mammary gland of a 26-day-pregnant Holstein heifer. Perfusion of 2-day cultured cells with a medium containing ATP (10, 100 and 1000 µmol/l) for 30 min caused a significant and concentration-dependent increase in the cellular H⁺ efflux rate. ATP application (100 µmol/l) caused a transient and large increase in [Ca²⁺]_c in all cells. In contrast, perfusion with a medium containing bovine GH at 10, 50 and 250 ng/ml for 30 min caused a significant decrease in the cellular H⁺ efflux rate in a concentration-dependent manner. However, bovine GH application (50 ng/ml) caused a small decrease followed by an increase, in some cases, in [Ca²⁺]_c. In bMEC treated with lactogenic hormones (1 µg/l prolactin, 1 nmol/ml dexamethasone and

5 µg/ml insulin) for 2 days, the increased H⁺ efflux rate induced by ATP was significantly reduced, whereas the negative response induced by GH was inversely and significantly changed to the positive. Treatment of the cells with lactogenic hormones reduced the increase in [Ca²⁺]_c induced by ATP stimulation, while it enhanced the increase in [Ca²⁺]_c induced by GH stimulation. Application of ATP or GH did not cause any significant changes in [pH]_c. Treatment with lactogenic hormones enhanced GH receptor (GHR) transcription that was determined by RT-PCR. From these results, we conclude that exogenous application of ATP and GH causes prompt and significant responses in H⁺ transport and [Ca²⁺]_c that were significantly changed in the opposite direction by the treatment with lactogenic hormones. The lactogenic hormone treatment also enhanced GHR transcription, which may change post-receptor signal transduction systems for both agents in the bMEC.

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Introduction

Growth hormone (GH), in collaboration with other hormones, is involved in the postnatal development and growth of the mammary gland in many species (Imagawa *et al.* 1990). Recently, there has been interest in the interaction between GH and an feedback inhibitory factor (FIL), which is assumed to act locally on the apical membrane of mammary epithelial cells (MEC) to inhibit milk production and cell proliferation (Knight *et al.* 1998). In ruminants, GH is also a galactopoietic hormone, that is, the systemic administration of GH to cows markedly increases milk production. Despite this, *in vitro*, the lactogenic complex causing the initiation of milk secretion is prolactin, insulin and cortisol in both rodent and ruminant MEC and GH does not appear to have a role (Etherton & Bauman 1998). *In vivo*, the action of GH appears to be indirect, via changes in metabolism or

insulin-like growth factor-I (IGF-I) system, as GH seems to cause extensive changes in intermediary metabolism for lactation rather than directly on the mammary gland (homeorhesis, see Etherton & Bauman 1998). Three lines of evidence have led to this conclusion. First, attempts to detect the existence of the GH receptor protein in mammary tissue have been unsuccessful (Gertler *et al.* 1983, Akers 1985, Keyes & Dijane 1988) except for one report (Glimm *et al.* 1990). Secondly, although the expression of the GH receptor mRNA is apparent in adult and fetal ruminant (Hauser *et al.* 1990, Jammes *et al.* 1991, Scott *et al.* 1992, Knabel *et al.* 1998) and rodent (Tiong & Herington 1991, Ilkbahar *et al.* 1995, 1999) mammary gland cells, the close arterial infusion of GH does not affect milk yield, while IGF-I causes a significant increase (McDowell *et al.* 1987, Prosser & Davis 1992, Prosser *et al.* 1990, 1994). Thirdly, the biological actions *in vitro* of GH on bovine and rat MEC are ill defined (Gerter *et al.* 1983,

Keyes *et al.* 1991, 1997, Walden *et al.* 1998) despite the exception that GH has been reported to increase the production of β -lactoglobulin from an ovine mammary heterogeneous cell population that contained epithelial, myoepithelial and fibroblast-like cells (Ilán *et al.* 1998).

Endogenously released adenosine 5'-triphosphate (ATP) has recently been shown to cause an increase in the cellular calcium concentration ($[Ca^{2+}]_c$) and activate Ca^{2+} -dependent K channels in rodent and cancerous MEC as well as myoepithelial cells via P_2 -purinergic receptors, although the physiological significance of this is not fully understood (Enomoto *et al.* 1991, 1994, Furuya *et al.* 1989, 1993, Nakano *et al.* 1997). Adenosine 5'-triphosphate would make an interesting tool for the study of the cellular mechanisms of milk secretion, because until now no secretagogues have been available for MEC that cause a quick cellular response, and an increase in $[Ca^{2+}]_c$ is an inevitable step to increase exocrine secretion.

We recently cloned a bovine MEC from mammary tissue isolated from a 26-day-pregnant heifer. The cells differentiate to form extracellular matrix-dependent and lactogenic hormone-dependent mammospheres, into which MEC synthesizes and releases casein. The present experiment was conducted to elucidate the acute effects of ATP and GH on cellular H^+ transport and $[Ca^{2+}]_c$ in a cloned bovine MEC.

Materials and Methods

Cell preparation

Bovine MEC (bMEC) were raised from the mammary gland of a 26-day-pregnant Holstein heifer. The mammary tissue segments were digested by collagenase (500 U/ml) for 30 min at 37 °C, and the isolated cells were cloned by the limiting dilution method. They were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS) under 5% CO_2 and air at 37 °C. Immunocytochemical determination revealed that all of the cells were cytokeratin-positive. We also confirmed by staining with an antibody to casein that the cells differentiate to form extracellular matrix (Matrigel, Becton-Dickinson Microorganic Systems, Bedford, MA, USA)-dependent and lactogenic hormone (1 μ g/ml prolactin, 1 nmol/ml dexamethasone and 5 μ g/ml insulin)-dependent mammospheres, in which bMEC synthesizes and releases casein and triglycerides (T Komatsu & A Hagino, unpublished data). In the present experiment, the cells were between 10th and 15th passages.

Measurement of cellular H^+ efflux rate

The measurement of cellular H^+ efflux rate was carried out by measuring the rate of acidification of the medium around the cells (extracellular acidification rate) by the

microphysiometer (Cytosensor, Molecular Devices, Sunnyvale, CA, USA), being a similar method as previously reported for gastric gland cells (Thibodeau *et al.* 1994). In brief, the cells were cultured for 2 days on the microporous polycarbonate membrane of capsule cups in 12-well culture plate in DMEM under 5% CO_2 and air at 37 °C. After 2 days of culture, the cells were sandwiched between two membranes of the capsule cup and a capsule insert separated by a 50 μ m thick annular spacer. The capsule was placed in a flow-regulated chamber of the microphysiometer so that the lower membrane was in diffusive contact with the surface of a silicone-based light-addressable potentiometric sensor. The cells in a narrow space (3 μ l volume) made by an insertion of a plunger in the capsule insert were perfused with a low-buffered saline solution by a peristaltic tube pump (100 μ l/min) at 37 °C. The pump was automatically stopped for 40 s after a 110-s flow-on period to monitor the rate of pH decrease in the solution around the cells. An addition of amiloride (100 μ mol/l, Sigma, St Louis, MO, USA) to the medium decreased the acidification rate by 40% (not shown), suggesting that 40% of the H^+ efflux from bMEC depends on the H^+/Ca^{2+} and Na^+/H^+ anti-transporter systems in the resting state. Stimulation with ATP (Sigma) or bovine growth hormone (bGH) (NIDDK, Torrance, CA, USA) was performed by perfusing with a medium containing ATP or bGH at the concentrations defined in the text.

Measurement of changes in $[Ca^{2+}]_c$ and $[pH]_c$

The measurement of $[Ca^{2+}]_c$ and $[pH]_c$ was done using a confocal laser scanning microscope system (MRC-1024, Bio-Rad, Hercules, CA, USA) attached to an inverted microscope (Axiovert 100, Zeiss, Frankfurt, Germany) with a 40 $\times$ objective at room temperature (22–25 °C) as reported for goat anterior pituitary cells in a previous paper (Ohata *et al.* 1997). In brief, the cells were cultured on a collagen-coated, glass-bottomed well (35 mm o.d., Mat-Tek Corp., Boston, MA, USA) for 2 days in DMEM under 5% CO_2 and air at 37 °C. The cells were then incubated in HEPES-buffered saline (HBS) containing Fluo-3/AM for $[Ca^{2+}]_c$ or BCECF-AM for $[pH]_c$ at 10 μ mol/l (Dojin, Tokyo, Japan) for 90 min at room temperature (20–25 °C). The composition of the HBS used was (in mmol/l): 140.0 NaCl, 4.7 KCl, 1.1 $MgCl_2$, 10.0 HEPES, 2.0 $CaCl_2$ and 5.0 D-glucose (pH 7.4). The excitation and the emission wave lengths were 488 and 530 nm respectively. Adenosine triphosphate and bGH were added to the medium as 10 μ l aliquots of a 10-fold concentrated solution. Scanning images were taken every 2 s. The increase in $[Ca^{2+}]_c$ and $[pH]_c$ was expressed as the change in the intensity of the emitted fluorescent light directed to a photomultiplier through a detector pinhole. The addition of 10 μ l HBS alone did not cause a significant change in the intensity.

Figure 1 Concentration-dependent changes (A and B) and incremental areas (C and D) of cellular H⁺ efflux rate induced by stimulation with ATP (10, 100 and 1000 μmol/l) (A and C) or bGH (10, 50 and 250 ng/ml) (B and D) for 30 min in cloned bMEC. Bars shown in A and B represent the stimulation period. The results are represented as the mean ± s.e. (n=5). **P*<0.05 or ****P*<0.001 vs control.

Reverse transcript polymerase chain reaction (RT-PCR) for growth hormone receptor (GHR) mRNA

The principle for RT-PCR was based on the method described by Kölle *et al.* (1997). The primer used was 5'-ACC CAG TGG AAA ATG GAC CCT T-3' for the forward primer, and 5'-CTG TCT GTG TCT GAC CCT TCA GTC-3' for the reverse primer.

Total cellular RNA was prepared from 10 million cells using TRIzol reagent (GibcoBRL, Grand Island, NY, USA) according to the manufacturer's instructions. One microgram of RNA was denatured at 65 °C for 10 min, quick-cooled on ice and reverse transcribed in a final volume of 50 μl. The RT-PCR was performed using a commercial kit (Ready-To-Go RT-PCR Beads, Amersham Pharmacia Biotech, Piscataway, NY, USA) according to the manufacturer's instructions ('Two-step protocol for RT-PCR'). After being warmed at 42 °C for 30 min and then incubated at 95 °C for 5 min, the reaction was amplified by a thermal cycler (type 480J, Takara, Tokyo, Japan) on the condition: 94 °C denaturation for 1 min, 60 °C annealing for 2 min and 72 °C extension for 3 min. The procedure was repeated for a

total of 35 or 30 cycles for GHR and G3 PDH respectively. The PCR products were analyzed in a 2% agarose gel, stained with ethidium bromide and photographed. The intensity of GHR abundance was assigned as a ratio to that of G3 PDH abundance.

Statistics

The experiment for cellular H⁺ efflux rate was repeated five times on different days. The results are presented as the mean ± s.e. Statistical analysis was carried out by ANOVA, followed by Bonferroni's multiple range test, if necessary (Wallenstein *et al.* 1980). Experiments for [Ca²⁺]_c and GHR expression were repeated three times on different days.

Results

Effects of ATP and bGH on cellular H⁺ efflux rate

Perfusion of the 2-day cultured cells with a medium containing ATP (10, 100 and 1000 μmol/l) for 30 min

Figure 2 Effects of treatment with lactogenic hormones (1 $\mu\text{g/ml}$ prolactin, 1 nmol/ml dexamethasone and 5 $\mu\text{g/ml}$ insulin) for 2 days on the time course (A and B) and incremental area (C and D) induced by stimulation with ATP (100 $\mu\text{mol/l}$) (A and C) or bGH (50 ng/ml) (B and D) on cellular H⁺ efflux rate in cloned bMEC. The data for control shown in A and B are the same as those used in Fig. 1A and B. Bars shown in A and B represent the stimulation period. The results are represented as the mean $\pm$ S.E. ($n=5$). *** $P<0.001$ vs control.

caused an increase in cellular H⁺ efflux rate in a concentration-dependent manner, the maximum value being obtained at 1000 $\mu\text{mol/l}$ ATP (Fig. 1A). The incremental area induced by ATP at 0, 10, 100 and 1000 $\mu\text{mol/l}$ over each pre-stimulation value (assigned as 100%) was 260.8 ± 58.7 , 1018.5 ± 214.4 , 3139.6 ± 568.7 and $7716.4 \pm 1571.4\%$ (min ($P<0.05$)) respectively, as shown in Fig. 1C.

In contrast, perfusion of the cells with a medium containing bGH at 10, 50 and 250 ng/ml for 30 min caused a decrease in cellular response in a concentration-dependent manner (Fig. 1B). The incremental area induced by bGH at 0, 10, 50 and 250 ng/ml was 260.8 ± 58.7 , -198.0 ± 81.7 , -273.9 ± 53.3 and $-527.7 \pm 47.3\%$ (min ($P<0.001$)) respectively, as depicted in Fig. 1D.

In bMEC cultured in a medium containing lactogenic hormones (1 $\mu\text{g/ml}$ prolactin, 1 nmol/ml dexamethasone and 5 $\mu\text{g/ml}$ insulin) for 2 days, the increase in ATP (100 $\mu\text{mol/l}$)-induced efflux rate was markedly reduced,

as shown in Fig. 2A, and the incremental area was significantly reduced from 3139.6 ± 568.7 to $131.5 \pm 87.5\%$ (min ($P<0.001$)), as shown in Fig. 2C. On the other hand, in the cells treated with lactogenic hormones, the bGH (50 ng/ml)-induced response was changed inversely, and the incremental area was significantly increased from -273.9 ± 53.3 to $306.7 \pm 67.9\%$ (min ($P<0.001$)), as shown in Fig. 2B and D.

Effects of ATP and bGH on $[\text{Ca}^{2+}]_c$ and $[\text{pH}]_c$

Stimulation with ATP (100 $\mu\text{mol/l}$) caused a transient and large increase in $[\text{Ca}^{2+}]_c$ in all cells, which was markedly reduced by treatment with lactogenic hormones (Fig. 3A and B). In contrast, the addition of bGH (50 ng/ml) caused a small decrease, followed by a slight increase in some cases, in $[\text{Ca}^{2+}]_c$ (Fig. 3C). Treatment with lactogenic hormones enhanced the GH-induced increase in $[\text{Ca}^{2+}]_c$ (Fig. 3D).

Figure 3 Effects of treatment of bMEC with lactogenic hormones (1 $\mu\text{g/ml}$ prolactin, 1 nmol/ml dexamethasone and 5 $\mu\text{g/ml}$ insulin) for 2 days on ATP- or bGH-induced changes in $[\text{Ca}^{2+}]_i$. Responses observed in individual cells are shown when cultured without (A and C) or with lactogenic hormones (B and D), and stimulated with ATP (100 $\mu\text{mol/l}$) (A and B) or bGH (50 ng/ml) (C and D). ATP or bGH was added at 60 s.

On the other hand, stimulation with ATP did not cause any significant changes in $[\text{pH}]_i$ in all cells although Na acetate (5 mmol/l) caused a significant reduction in $[\text{pH}]_i$ (Fig. 4).

RT-PCR for GHR mRNA

Treatment with lactogenic hormones for 2 days enhanced the GHR mRNA abundance in bMEC. A typical example is depicted in Fig. 5, showing that GHR mRNA abundance was raised by the lactogenic hormone treatment from 0.61 to 0.97 units. Similar results were also confirmed in another experiments.

Discussion

This is the first report to show that bovine MEC are able to respond promptly to stimulation with exogenous ATP and bGH in terms of the cellular H^+ efflux rate and

Figure 4 Effects of ATP (100 $\mu\text{mol/l}$) or Na acetate (5 mmol/l) on $[\text{pH}]_i$. The bars represents the stimulation periods.

Figure 5 Effects of treatment of bMEC with lactogenic hormones (1 $\mu\text{g/ml}$ prolactin, 1 nmol/ml dexamethasone and 5 $\mu\text{g/ml}$ insulin) for 2 days on GHR mRNA transcription. (A) Detection of bovine GHR mRNA and G3 PDH mRNA. C, mRNA abundance for bMEC cultured without lactogenic hormones; T, mRNA abundance for bMEC cultured with lactogenic hormones. (B) GHR mRNA abundance assigned as a ratio to G3 PDH mRNA.

$[\text{Ca}^{2+}]_c$. The response for ATP was the opposite to that of GH and, additionally, these responses were oppositely modulated by lactogenic hormone treatment.

As the morphogenesis and functions of the mammary gland are influenced by their surrounding adipocytes, it would be interesting to elucidate the interaction between adipocytes and the mammary epithelial cells with respect to adenosine/ATP (purinergic) receptors. Adipose tissues are known to produce locally and release physiological factors such as adenosine and prostaglandin E into the extracellular space to suppress lipolysis (Schwabe *et al.* 1975). Additionally, ATP is reported to be secreted at 1 $\mu\text{mol/l}$ in response to the mechanical stimulation of repeated pipetting of mammary tumor cells, and to cause increased cellular $[\text{Ca}^{2+}]_c$ in tumor and normal mouse MEC (Enomoto *et al.* 1991, Furuya *et al.* 1993, Enomoto *et al.* 1994) and myoepithelial cells (Nakano *et al.* 1997). Long-term or chronic exposure of mouse mammary explants and MEC to adenosine analogues has been shown to suppress casein synthesis through A_1 -receptors

(Hom *et al.* 1996). This subclass of adenosine receptors is known to be responsive to ATP (Burnstock 1990). However, the action of purinergic receptors was probably not caused by cellular cyclic AMP because PIA (N^6 -phenylisopropyladenosine), an anti-lipolytic agonist, increased (Hom *et al.* 1996), while adenosine suppressed (Saggerson 1980, Vernon *et al.* 1983, 1991), the level of cellular cyclic AMP and this anti-lipolytic action was not mimicked by a cyclic AMP derivative in adipocytes (Hom *et al.* 1996). This is also likely to be the case in the mammary epithelial cells of the present experiment because of the swiftness of the onsets of cellular responses to ATP and GH.

On ATP (purinergic) receptors in mammary epithelial cells, Sundow & Burgoyne (1997) stated in the discussion session that ATP application did not elicit an increase in $[\text{Ca}^{2+}]_c$ in mouse lactating acini. This result seems consistent with our present data (Fig. 3) showing that treatment of cells with lactogenic hormone drastically suppressed ATP-induced responses. The authors had used lactogenic hormones to culture acini before measuring $[\text{Ca}^{2+}]_c$.

Growth hormone is a galactopoietic hormone in cows because of the marked increase in milk production when administrated (Etherton & Bauman 1998). The effect of GH on milk synthesis is also confirmed in rat mammary glands (Feldman *et al.* 1993, Flint & Gardner 1994). However, the effect of GH is thought to be indirect in the rat (Flint & Gardner 1994). The action of GH has been thought to increase lipolysis in adipocytes by an increased response and sensitivity to lipolytic (β -adrenergic) agonists and a decreased response and sensitivity to antilipolytic factors such as adenosine and prostaglandin E (Doris *et al.* 1998). Although a direct action of GH on mammary epithelial cells is questionable because of the scarcity of GH receptor proteins, a line of evidence suggesting the expression of GH receptor mRNA is accumulating in bovine (Hauser *et al.* 1990, Knabel *et al.* 1998), mouse (Ilkbahar *et al.* 1995), rabbit (Jammes *et al.* 1991) and rat (Lincoln *et al.* 1990, Tiong & Herington 1991) mammary gland cells. As shown in Fig. 5, GHR mRNA exists in bMEC, and treatment of the cells with lactogenic hormones enhanced the GHR transcription. These results imply that the transcription of GHR may be increased by the presence of lactogenic hormones, and also that the enhanced transcription of GHR may change the post-receptor responses to GH stimulation in bMEC.

In the present study, GH exerted prompt effects on cellular H^+ efflux rate and $[\text{Ca}^{2+}]_c$. We also challenged recombinant bGH (bST) (Monsanto, St Louis, MO, USA and LG, Seoul, Korea) and confirmed that bST caused the same cellular responses for Ca^{2+} and pH as pituitary GH did (not shown). However, a commercially available bST contains α -tocopherol acetate as an anti-oxidant. That is the reason why we employed pituitary GH, but not

bST, through the present experiment. The treatment of bMEC with lactogenic hormones reversed GH-induced H^+ efflux rate from negative to positive levels, which seems consistent with the action of GH on an increased milk yield. The precise mechanism for the prompt action of GH on mammary epithelial cells remains to be clarified, although it is known that the inhibition of lipolysis by PIA, which was attenuated by a long-term (24-h) exposure to GH, is due to an impairment of Gi protein in ovine adipocytes (Doris *et al.* 1998). There is only one report showing that an ovine mammary epithelial cell line (NISH), a heterogeneous cell population, dramatically increased β -lactoglobulin secretion in response to GH stimulation (1 μ g/ml) in the presence of lactogenic hormones and in an extracellular matrix-dependent manner (Ilan *et al.* 1998). It also needs to be clarified whether the effects of bGH is not due to the activation of prolactin or other hormone receptors because human GH is known to bind to prolactin receptors (Hughes & Friesen 1985).

Although it is not known whether or not cellular H^+ transport and $[Ca^{2+}]_c$ are related to milk synthesis, an increase in $[Ca^{2+}]_c$ is likely for exocrine secretion because an increased $[Ca^{2+}]_c$ and followed ion channel activation are essential for fluid secretion in exocrine glands (Petersen 1992). On the basis of this concept, treatment with lactogenic hormones seems preferable for GH-induced, but not for ATP-induced, increases in $[Ca^{2+}]_c$. However, ATP, which may suppress the lactogenic response in mammary epithelial cells, exerts an important role for milk ejection because ATP may cause contraction of the myoepithelial cells by an increased $[Ca^{2+}]_c$ through P_2 receptors as indicated in mouse mammary glands (Nakano *et al.* 1997).

Figure 4 implies that the enhanced H^+ efflux rate induced by ATP or GH application may be caused by an enhanced cellular metabolism because ATP application did not cause a marked change in $[pH]_c$ as depicted in Fig. 4. Furthermore, this finding also suggests that the massive change in $[Ca^{2+}]_c$ in response to ATP or GH application was not the indirect response induced by reduced $[pH]_c$.

Lower experimental temperature than the physiological may have enhanced cellular Na^+ accumulation by reducing Na^+/K^+ pump activity. That may have reduced H^+ transport due to suppressed Na^+ -dependent H^+ efflux, but raised $[Ca^{2+}]_c$ due to suppressed Na^+ -dependent Ca^{2+} efflux.

Taken together, the results of the present experiment indicate that ATP and GH caused significant and prompt responses in H^+ efflux rate and $[Ca^{2+}]_c$ in an opposite manner and that the treatment of the cells with lactogenic hormones significantly changed both cellular parameters induced by both agents in bMEC, although the precise cellular mechanisms remain to be clarified.

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