γ -Glutamyl Transpeptidase Inhibition Suppresses Milk Protein Synthesis in Isolated Ovine Mammary Cells

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ABSTRACT

The membrane spanning enzyme γ -glutamyl transpeptidase (γ -GT; EC 2.3.2.2) catalyses the breakdown of the tripeptide glutathione and uses free amino acids (AA) to form γ -glutamyl transpeptidase (GT) AA that become transported into cells and converted back into free AA. γ -Glutamyl transpeptidase activity has been shown to be important for mammary AA uptake in rodent systems, and while γ -GT activity is high in lactating bovine mammary tissue, the role of this enzyme in milk protein synthesis of the ruminant has not been defined. The present study shows that γ -GT activity in the ovine mammary gland, like that of rodents, increases during pregnancy and peaks early in lactation. Acivicin, a well-known inhibitor of γ -GT, decreased γ -GT activity in acini isolated from the ovine mammary gland and did not have secondary toxicity effects on cell viability or the uptake of radiolabeled amino-isobutyric acid. Isolated ovine acini were incubated in the presence of radiolabeled leucine, and incorporation of label into secreted protein increased during incubation. Incubation of acini with acivicin decreased milk protein secretion by 75%, indicating that γ -GT plays an important role in milk protein production in the ruminant. Acivicin did not inhibit secretion of specific caseins but caused a global decrease in individual proteins suggesting that γ -GT may be responsible for providing a complement of AA for milk protein synthesis.

(Key words: acivicin, γ -glutamyl transpeptidase, isolated ovine mammary acini, milk protein)

Abbreviation key: ³H-AIB = α -amino-isobutyric acid [methyl-³H], DMEM = Dulbecco's modified Eagle's medium, DTT = dithiothreitol, FCS = fetal calf serum, IC₅₀ = constant at 50% inhibition, MeAIB = methylated

amino-isobutyric acid, γ -GT = γ -glutamyl transpeptidase, HUVE = human umbilical vein endothelial.

INTRODUCTION

The ability to manipulate yield and concentration of specific milk proteins through the cow's diet would lead the way in developing higher added value milk products and could potentially optimize processing of particular dairy protein products. However, the mechanisms controlling milk protein synthesis must be more clearly understood before nutritional strategies can be devised. Milk protein synthesis and secretion may be regulated at several levels including amino acid supply. Small increases in milk protein yield have been shown to be related to increases in uptake of mammary essential AA. Some AA have been identified to be potentially limiting for milk protein synthesis, but increased supply has only increased the uptake of some of these AA (Metcalf et al., 1996; Bequette et al., 2000). This indicates that substrate supply is not solely limiting for milk protein synthesis and there must be regulatory factors inside the gland downstream of transcription controlling uptake.

Several classical AA transport systems shown to be expressed by mammary epithelial cells could be rate-limiting for protein synthesis. These include the A system, which has affinity for short straight chain neutral AA as well as methylated amino-isobutyric acid (MeAIB), a nonmetabolizable AA that is often used in uptake studies. In addition to these conventional systems, a role has been suggested for the membrane-spanning enzyme γ -glutamyl transpeptidase (GT) in controlling AA uptake (Viña et al., 1989).

The role of γ -GT in AA transport has been criticized, but it is widely accepted that this enzyme catalyses catabolism of the tripeptide glutathione and uses free AA, particularly cystine to form γ -glutamyl AA (Meister and Anderson, 1983; Morita et al., 1994; Hanigan 1998; Wolff et al., 1998). γ -Glutamyl AA have been shown to enter mouse kidney cells where they can be converted to free AA and 5-oxoproline, and γ -GT inhibition in cultured human pancreatic cells (PaTu 8902) and hu-

Received June 7, 2003.

Accepted October 1, 2003.

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man umbilical vein endothelial (**HUVE**) cells has led to decreased cystine uptake (Griffith et al., 1978; Cotgreave and Schuppe-Koistinen, 1994; Sweiry et al., 1995). However, the transport mechanism for γ -glutamyl AA uptake by cells has not been defined. Most evidence suggests that γ -GT itself is not the transporter (Morita et al., 1994; Hanigan 1998; Wolff et al., 1998). Nevertheless, γ -GT may facilitate AA transport by forming γ -GT AA and thus may be important in regulating the supply of AA for protein synthesis.

In the mammary gland of rodents, γ -GT activity, in response to prolactin, increases during pregnancy, peaks early in lactation and then decreases at weaning. This implicates an important role for the enzyme in milk production in the rodent (Puente et al., 1979; Pocius et al., 1980). γ -Glutamyl transpeptidase has been shown to be primarily localized to the luminal surface of normal human breast cells rather than the basolateral surface (Fiala et al., 1980), but administration of γ -GT inhibitors in rats has been shown to affect the supply of AA to the mammary gland, suggesting that it may be present in the basolateral surface of lactating mammary cells (Viña et al., 1989). Species differences in hormonal regulation of lactation and milk composition discourage extrapolation of results from rodents to ruminants (Forsyth, 1984; Jenness, 1986; Kumar et al., 1994), but high levels of γ -GT activity in lactating bovine mammary tissue suggests that γ -GT also has a function in supporting milk protein synthesis in the ruminant (Baumrucker and Pocius, 1978). Unequivocal evidence supporting a role for γ -GT in protein synthesis in lactating ruminant mammary cells is required and may lead to understanding how supply of γ -GT AA regulates milk protein synthesis. Understanding the mechanisms involved in γ -GT AA supply may provide an avenue for manipulation of the differential synthesis of proteins in milk.

This paper sets out to show that γ -GT activity facilitates milk protein synthesis in the ruminant. γ -Glutamyl activity in the sheep was assayed during pregnancy and lactation for comparison with results of rodent studies, and the role of γ -GT in milk protein synthesis was studied using acini isolated from the sheep udder as a model for the ruminant mammary gland *in vivo*. Synthesis of secreted protein was followed by incorporation of ^3H -leucine added to the incubation media and the effects of γ -GT inhibition on total and individual milk protein secretion were examined by incubating acini in the presence of acivicin (L-(α S,5S)- α -amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid), a well-documented inhibitor of γ -GT. The effect of acivicin on the A transport system was also investigated.

MATERIALS AND METHODS

Ethical Approval and Animals

Ethical approval to undertake this study was obtained from the AgResearch Grasslands' Animal Ethics Committee (Palmerston North, New Zealand). Romney ewes used in the study were obtained from AgResearch Ltd., Ruakura Research Centre, or Massey University, Palmerston North, New Zealand.

Activity of γ -GT in Ovine Mammary Tissue and Acini

Mammary tissue was collected from 30 mixed age ewes at different stages of pregnancy and lactation and assayed for protein and γ -GT activity. The preparation of tissue and acini for γ -GT activity assay was modified from Baumrucker and Pocius (1978). Mammary tissue was frozen in liquid nitrogen and crushed using a modified French cell press. Approximately 2 g of powdered tissue was weighed and homogenized on ice using a Kinematica Polytron (Lucerne, Switzerland) in 5 volumes of enzyme extraction buffer (10 mM Tris-HCl pH 8.0, 80 mM MgCl_2). Acini (2 mL, 2×10^6 cell/mL, prepared as described below) were ground using an Eppendorf tissue grinder in 5 volumes of γ -GT assay extraction buffer on ice. Samples were sonicated for 15 min then 0.5 mL was weighed into 1.5-mL microfuge tubes. Triton X-100 (Riedel de Haën, Seelze, Germany) was added to approximately 0.5 g of homogenate to a final concentration of 2% (wt/vol) mixed very slowly for 1 h and then centrifuged ($9500 \times g$, 5 min). Supernatants were diluted 5-fold in extraction buffer prior to γ -GT assay. For protein assays (BCA Protein Assay Reagent Kit obtained from Pierce, Rockford, IL) supernatants were diluted 50-fold and 5-fold for tissue extracts and acini extracts respectively.

The assay for γ -GT activity monitored production of *p*-nitroanilide as described by Baumrucker and Davis (1980) and Pocius et al., (1980). Absorbance of samples and reagent blanks was monitored at 405 nm and 37°C every 2 min for 20 min. The appearance of 1 μmol of *p*-nitroanilide per minute was taken as 1 unit of enzyme activity and was calculated using a molar absorption coefficient for *p*-nitroanilide of $9.75 \text{ mM}^{-1}\cdot\text{cm}^{-1}$. Assays were carried out in triplicate.

To assess the effect of acivicin (in deionized water) (Sigma Chemical Company, St Louis, MO) on γ -GT activity in acini, 14.25 mL of suspension (prepared as described below) was incubated with acivicin (0.7 mM) or deionized water (control) for 5 min at room temperature. Acini were washed with supplemented Dulbecco's modified Eagle's medium (**DMEM**) containing 1% fetal calf serum (**FCS**) and centrifugation ($850 \times g$; 5 min

then $480 \times g$; 2 min $\times$ 3) then resuspended in supplemented DMEM excluding FCS and assayed for γ -GT activity as described for tissue above. Acini isolated from 2 sheep were used.

Preparation of Acini

Acini were isolated from tissue of the excised udder of 12 sheep at 3 wk of lactation using collagenase digestion as described by Wheeler and co-workers (1995). Briefly, 6 g of mammary tissue was injected with collagenase (Sigma Chemical Company, St Louis, MO) (586 U/mL) in DMEM (Life Technologies Inc., MD) supplemented with 20 mM hepes (Sigma Chemical Company), 2 mM NaHCO_3 , 8.3 mM sodium acetate, 4 mM glutamine, antibiotic/antimycotic (1 mL/L) (Life Technologies Inc.) and containing 10% FCS (Life Technologies Inc.), and incubated at 37°C with shaking for 80 min. The digest was crudely filtered through sterile nylon netting, and washed with supplemented DMEM containing 1% FCS and centrifuged ($850 \times g$, 5 min then $480 \times g$, 2 min $\times$ 2). The cell pellet was suspended (2×10^6 viable acinar cells/mL) in supplemented DMEM without FCS for assays and experiments.

Milk Protein Production and Amino-Isobutyric Acid Uptake by Acini

Acini suspensions, on Falcon 24-well flat-bottom multi plates coated in-house with Matrigel Basement Membrane Matrix (Becton Dickinson Labware, Bedford, MA), were incubated at 37°C in 5% CO_2 . To study milk protein secretion, acini were incubated for 15 min prior to addition of 0.2 GBq 3,4,5- ^3H -leucine (120 Ci/mmol) (American Radiolabelled Chemicals Ltd., St. Louis, MO) in 2% ethanol, to each well. Control treatments (deionized water) or acivicin (0.7 μM to 0.5 mM) were added in triplicate each with 3 background wells containing cycloheximide (25 μM) (Sigma Chemical Company). Media were harvested between 0 and 18 h after treatment was added. The effect of acivicin on the A AA transport system was determined after incubating acini with deionized water (control) or acivicin (6 μM) for 5 min before adding ^3H -amino-isobutyric acid ($^3\text{HAIB}$; 76 MBq/mol). Acini were harvested after 0, 10, and 20 min after the addition of ^3H -AIB.

At harvest, plates were placed on ice and medium was aliquoted into preweighed microfuge tubes and centrifuged ($10,000 \times g$, 5 min). The supernatant was aliquoted into fresh microfuge tubes and analyzed for lactose according to Davis et al. (1993) and secreted protein. The pellet was resuspended in ice-cold PBS (10 mM phosphate buffer pH 7.4, 1.7 mM KCl, and 0.137 M NaCl) washings from the incubation plates and cen-

trifuged twice ($10,000 \times g$, 5 min) and supernatant was discarded. For ^3H -AIB uptake analysis, cell pellets were resuspended in Starscint (Packard BioScience BV, Groningen, The Netherlands) and measured for total radioactivity (Packard Scintillation Counter).

For the analysis of secreted milk protein, 100 μL of unlabelled leucine (76 mM) was added to dilute unincorporated label. Skim ovine milk (25 μL ; prepared by centrifugation ($3500 \times g$, 10 min) and removal of the fat layer), was added to the harvested incubation media to assist precipitation. Samples were precipitated in 7.5% trichloroacetic acid and centrifuged ($1600 \times g$, 15 min) and the pellet was resuspended in 300 μL of 6 M urea (Riedel de Haën, Seelze, Germany) twice and then in 400 μL of buffer containing 6 M guanidinium.HCl, 0.1 M bis-tris, 5.37 mM sodium citrate, and 1.95 mM dithiothreitol (DTT). A 100- μL aliquot was mixed with Starscint and analyzed for total radioactivity. A 50- μL sample was injected on to a Phenomenex Jupiter 250 4.6 mm $\times$ 5 μm C18 300 Å column plumbed into a Shimadzu LC10A HPLC system at 33°C. Individual caseins were eluted using 90% acetonitrile and 0.01% trifluoroacetic acid and detected by absorbance at 254 nm and radioactivity (cpm) using an online β -radioactivity counter (IN/US β -RAM, FL) after mixing with Starscint. Counting efficiency using this system was approximately 80%.

Assessment of Viability of Acini

Viability of cells in acini was determined qualitatively by trypan blue dye exclusion. A 50- μL aliquot of suspended cells from a fourth well treated as per test wells (acivicin, 0.5 mM or deionized water) was removed at harvest and mixed with 50 μL of trypan blue dye then viewed at 100 $\times$ magnification. Viable unstained cells were estimated as a percentage of the total number of cells. Acini from 2 sheep were used.

Statistical Analysis

For studies using acini, each experiment constituted acini from one sheep with treatments in triplicate, therefore, $n = 3$ unless one replicate was used for a different assay. The significance of treatment effects was determined using analysis of variance and least significant differences using GenStat 5 Release 4.2 (Fifth Edition), 2001 (VSN International Ltd., Oxford, UK).

RESULTS AND DISCUSSION

In the absence of a characterized transporter, it has often been assumed that as well as synthesizing γ -GT

Figure 1. γ -Glutamyl transpeptidase activity in pregnant and lactating sheep mammary tissue. Error bars indicate standard error of the means (d 0 n = 4; pregnant: d 90 n = 2, d 135 n = 6; lactating: d 14 n = 7, d 42 n = 11, where n is the number of sheep). The appearance of 1 μ mol of *p*-nitroanilide per min was taken as 1 unit of enzyme activity.

AA, γ -GT was also responsible for their cellular uptake. Despite a great deal of examination of the components of the γ -GT cycle, unequivocal evidence has not been obtained to support or disprove a role for γ -GT in AA transport. Research aimed to clarify this function of γ -GT appears to have stalled even though potential for this enzyme as a key control mechanism of protein synthesis exists. In the hope that this enzyme could provide a target for manipulating milk protein production for use in the dairy industry, we revisited the possible facilitative or regulatory role for γ -GT in AA transport. In the present experiment, we used collagenase to isolate ovine mammary acini from the gland, investigated their ability to take up labeled AA from and secrete labeled protein into the media, and looked at the ability of acivicin to inhibit milk protein production in order to scrutinize the relationship between γ -GT and milk protein synthesis of the ruminant.

Activity of γ -GT in Ovine Mammary Tissue and Acini

In rodent systems, γ -GT activity has clearly been shown to be associated with lactation (Pocius et al., 1980). In the present study, the specific activity of γ -GT increased towards parturition, and during early lactation it was 6 $\times$ higher than levels in nonpregnant, nonlactating sheep (Figure 1). This increase in the ru-

minant gland is consistent with reports in the rodent gland in which γ -GT activity was shown to respond to prolactin (Pocius et al., 1980). Ovine mammary tissue was not obtained at late lactation or involution, but in rodents, γ -GT activity in the mammary gland returns to pregnancy levels within 5 d of weaning the young (Puente et al., 1979; Pocius et al., 1980). Specific γ -GT activity of the ovine gland at 2 to 3 wk and 6 wk of lactation was 0.49 ± 0.05 and 0.54 ± 0.02 μ mol/min per mg of mammary protein, respectively. This was approximately 3-fold greater than the γ -GT activity reported previously for lactating mammary tissue from cows and rats (Baumrucker and Pocius, 1978; Puente et al., 1979; Pocius et al., 1980). The extraction buffer used in the present study was taken from Baumrucker and Pocius (1978), but assay results for γ -GT were low and variable until the buffer was modified by addition of Triton X-100 to samples during enzyme extraction. This probably increased the effectiveness of γ -GT extraction from the cell membrane resulting in the higher levels of activity in comparison with studies in cow and rat tissue in which Triton X-100 was not used (Baumrucker and Pocius, 1978; Puente et al., 1979; Pocius et al., 1980; Baumrucker et al., 1981).

γ -Glutamyl activity was also assayed in acinar cells isolated by collagenase digest from the lactating mammary gland of sheep. The specific γ -GT activity in isolated ovine acini (Table 1) was much greater than that previously reported (Baumrucker et al., 1981) for bovine acini (0.182 ± 0.031 μ mol/min per mg of protein), probably resulting from the use of Triton X-100 in the extraction buffer.

The Effect of Acivicin on γ -GT Activity

To define the role of γ -GT in milk protein production we used acivicin, a well-documented inhibitor of γ -GT activity. Acivicin has been used as an inhibitor of γ -GT in isolated enzyme studies (Stole et al., 1994; Smith et al., 1995), in cell culture studies (Cotgreave and Schuppe-Koistinen, 1994; Sweiry et al., 1995), and in whole animal (McGovren et al., 1988; Viña et al., 1990) and human studies (McGovren et al., 1985; Eisenhauer et al., 1987). In the present study acivicin (0.7 mM) was added directly to the media of isolated acini. Unbound inhibitor was removed by a series of centrifugation and washing steps before assaying for γ -GT activity. The γ -GT activity was shown to be inhibited as acini treated with acivicin retained only 28% of the specific γ -GT activity observed in untreated acini in experiments using acini isolated from 2 sheep (Table 1). A similar decrease in γ -GT activity with acivicin was reported for a human pancreatic cell line (Pa Tu 8902) where acivi-

Table 1. γ -Glutamyl transpeptidase activity of isolated ovine mammary cells. Cells were incubated with deionized water (control) or acivicin (0.7 mM) (sheep 1 n = 3; sheep 2 control n = 1, acivicin n = 3 where n is the number of wells).

	Sheep 1		Sheep 2	
	Control	Acivicin	Control	Acivicin
Total activity ¹ $\pm$ SEM (units ² per g wet weight)	927 $\pm$ 13	188 $\pm$ 4.8	1532	334 $\pm$ 7.0
Percentage of control		20.2		21.8
Specific activity $\pm$ SEM (units ² per mg protein)	38.9 $\pm$ 0.55	7.6 $\pm$ 0.20	38.3	13.8 $\pm$ 0.29
Percentage of control		19.6		36.1

¹Total activity per g wet weight of fresh tissue.

²The appearance of 1 μ mol of *p*-nitroanilide per minute.

cin (0.5 mM) treated cells retained 40% of the activity of untreated cells (Sweiry et al., 1995).

The Effect of Acivicin on Milk Synthesis and Secretion by Isolated Ovine Acini

The level of radiolabeled endogenous protein increased during the first 2 h of incubation before reaching a plateau and was taken as being newly synthesised protein (Figure 2a). In a separate experiment, the level of radiolabeled protein in the media increased after 2 h of incubation (Figure 2b). Together these results indicate that acini continued to synthesize and secrete milk protein after isolation from the gland. In isolated lactating rat mammary acini a significant amount of newly synthesized milk protein is not secreted within the first 3 h of incubation (Geursen et al., 1987). A similar retention of newly synthesized milk protein in ovine acini seems to account for the lag in secretion of milk protein seen in the first 2 h in the present study.

The incubation of acini with acivicin resulted in decreased milk protein synthesis and secretion (Figure 2a and b). Although the results shown in Figure 2a and b are from separate experiments, they do indicate that synthesis does not recover from acivicin treatment during 18 h of incubation. In the presence of acivicin, after an initial increase at 2 h, radiolabeled endogenous protein falls to background levels (Figure 2a). As the level of secreted protein in the media tends to increase in the presence of acivicin this must be due to continued secretion of protein synthesized within the first 2 h of incubation indicating that acivicin does not affect protein secretion (Figure 2b). These results indicate that in isolated acini, either γ -GT activity is required for milk protein synthesis, or acivicin affects milk protein synthesis through a mechanism independent of γ -GT, or that acivicin has some negative secondary effect on mammary cell metabolism.

In experiments using acini from 6 animals, at each harvest time point after 2 h acivicin caused a decrease

Figure 2. Acivicin inhibition of protein synthesis. (a) Endogenous radiolabeled (3 H-leucine) protein of ovine mammary acini isolated from one sheep incubated for 18 h in the absence and presence of acivicin (0.5 mM). Error bars represent standard error of the means (n = 3 wells). (b) Radiolabeled (3 H-leucine) protein secreted of ovine mammary acini isolated from one sheep incubated for 18 h in the absence and presence of acivicin (0.5 mM). Error bars represent standard error of the means (n = 3 wells).

Table 2. Acivicin inhibition of milk protein secretion in 6 sheep. Radiolabeled (^3H -leucine) protein (kBq/g media extract) secreted from isolated ovine mammary acini incubated in the absence and presence of acivicin (0.5 mM).

Time	Number of sheep	n ¹	Control	Acivicin	LSD
0	6	18	0.01 ^a	0.03 ^a	0.109
2	2	6	0.41 ^a	0.00 ^a	0.426
3	1	3	1.4 ^b	0.19 ^a	0.878
4	4	12	2.8 ^b	0.71 ^a	1.475
5	2	6	2.2 ^b	0.70 ^a	0.627
8	6	18	5.0 ^b	1.4 ^a	1.22
10	1	3	5.5 ^b	1.9 ^a	1.20
18	5	15	11.4 ^b	2.1 ^a	3.69

¹n = Number of wells.

^{a,b}Means in the same row without a common superscript differ ($P < 0.05$).

in secreted protein of on average 75% compared with the controls ($P < 0.05$) (Table 2). This is consistent with the decrease in γ -GT activity observed in acivicin-treated acini (Table 1) and strongly supports the suggestion that γ -GT inhibition is responsible for the decrease in secretion of milk protein.

Acini from 3 sheep were incubated in the presence of different concentrations of acivicin. Figure 3 shows

Figure 3. Inhibition of protein synthesis at different acivicin concentrations. Concentration dependent acivicin inhibition of radiolabeled (^3H -leucine) protein in medium extract of isolated ovine mammary cells from 3 sheep. The amount of protein secreted with each concentration of acivicin was expressed as a percentage of the protein secreted by acini incubated with deionized water. Error bars indicate standard error of the means (0 μM n = 7; 0.7 μM n = 3; 1.4 μM n = 7; 2.7 μM n = 4; 6.1 μM n = 4; 10.4 μM n = 3). Data have been fitted to a first-order exponential decay curve giving estimated IC_{50} (concentration of inhibitor at 50% inhibition) of (—) 2.7 μM .

that the level of protein secreted is dependent on acivicin dose. Assuming that decreased milk protein secretion is a direct response to γ -GT inhibition by acivicin, the constant for 50% inhibition (IC_{50}) under the conditions used in this study was estimated to be 2.7 μM .

The IC_{50} estimated for isolated ovine mammary cells under the conditions used in the present study is lower than that reported for PaTu 8902 pancreatic adenocarcinoma cells (0.3 mM) and that for HL-60 promyelocytic leukemia cells (25 μM) for γ -GT activity assays in the presence of acivicin (Sweiry et al., 1995; Antczak et al., 2001). As acivicin binds γ -GT in equimolar amounts (Smith et al., 1995), this suggests that PaTu 8902 and HL-60 cells contain more γ -GT than mammary cells. However, tissue from lactating bovine mammary tissue has been shown to have greater γ -GT activity than pancreas (Baumrucker and Pocius, 1978). It is possible that acivicin has effects secondary to γ -GT inhibition causing a decrease in milk protein secretion. Cystine transport in PaTu 8902 cells was found to decrease by 30% in the presence of approximately 440 μM acivicin, but the concentration of acivicin required to inhibit γ -GT by 30% was much greater (approximately 1.3 mM) (Sweiry et al., 1995). This indicates that acivicin may affect some other mechanism of cysteine uptake or may negatively affect cell function. Therefore, decreased milk protein secretion in isolated ovine mammary cells as a result of acivicin administration may result from other factors apart from γ -GT inhibition and the IC_{50} estimated here must be accepted as a constant for milk protein secretion inhibition rather than specifically for inhibition of γ -GT activity.

Secondary Effects of Acivicin on Isolated Ovine Acini

To check the possibility that acivicin was affecting cell function by mechanisms other than γ -GT activity, acini were incubated in the presence of acivicin and with labeled ^3H -AIB, a non-metabolizable AA, which is

Table 3. Secondary effects of acivicin. Effect of incubation with acivicin on the normal activity of isolated ovine mammary cells from 2 sheep as monitored by lactose secretion, uptake of ^3H - α -amino-isobutyric acid (^3H -AIB), and cell viability as indicated by trypan blue stain exclusion; $n = 6$ wells (see Methods section).

Incubation time	Control	SEM	Acivicin	SEM
Cell viability (%)				
0–4 h	90		90	
4–8 h	80–90		80–90	
8–18 h	80		80	
Lactose (fmol/cell per h)				
0–4 h	25 ^a	1.1	17 ^b	1.1
4–8 h	27 ^a	3.6	18 ^a	3.4
^3H -AIB uptake (kBq/g wet weight cells)				
0 min	75 ^a	12.8	92 ^a	9.2
10 min	108 ^a	9.5	141 ^a	18.1
20 min	95 ^a	16.5	119 ^a	18.0

^{a,b}Means in the same row without a common superscript differ ($P < 0.05$).

known to be transported specifically by the A system (Lee et al., 1996). The uptake of ^3H -AIB was unaffected by acivicin, indicating that acivicin does not exert its effect through inhibition of the A transport system (Table 3). Secondary effects of acivicin were investigated further by monitoring cell viability, assessed by trypan blue dye exclusion, and lactose secretion (Table 3). Mammary cells isolated from the gland die when their association with other cells is lost, so acini could not be broken up to count individual cells. Therefore only a qualitative assessment of viability can be made. Acivicin has been shown to induce apoptosis in γ -GT deficient cells (Aberkane et al., 2001), but in the present study the viability of acivicin treated acini appeared no different from untreated acini. Lactose secretion over the 0- to 4-h period of incubation in the presence of acivicin decreased by 32% ($P < 0.05$). This action by acivicin on lactose secretion probably results from decreased α -LA synthesis rather than from adverse effects on cell viability. Together with galactosyl transferase, the whey protein α -LA forms lactose synthase, the enzyme that catalyses lactose production. Decreased supply of AA by acivicin inhibition of γ -GT would result in decreased AA availability for synthesis of the enzymes that catalyse lactose formation and hence would decrease the lactose concentration in the medium of acivicin treated cells. In the present study, we have been able to show that acivicin does not affect cell viability as assessed by trypan blue exclusion and by the ability of cells to continue the energy-requiring process of lactose synthesis (albeit at a slower rate probably due to decreased enzyme activity). We have also shown that transport systems involved in AIB uptake by acini cells were not affected by acivicin. However, acivicin has been shown to negatively affect glutamine dependent enzymes and cause apoptosis (Tso et al., 1980; Aberkane et al., 2001). Although, in the present study, acivicin has been shown to inhibit γ -GT, secondary effects

of acivicin on mammary cell metabolism cannot be excluded as the mechanism behind the decrease in milk protein synthesis.

The Effect of Acivicin on the Secretion of Individual Milk Proteins

Secreted proteins were separated by HPLC to determine if acivicin affected the secretion of specific proteins. In addition to the major caseins shown in Table 4, we also observed that acivicin retarded the secretion of α -LA to approximately 20% of the control values at 5 (control: 0.33; acivicin 0.07 kBq/g medium extract) and 8 h (control: 0.48; acivicin 0.09 kBq/g medium extract). As discussed above, this probably accounts for the decrease in lactose synthesis observed in this study.

γ -Glutamyl has a high affinity for cystine (Thompson and Meister, 1977) and γ -GT inhibition has been shown to decrease cystine uptake in cultured human umbilical vein endothelial cells and PaTu 8902 cells indicating that this enzyme in some way facilitates the transport of cystine into these types of cells (Cotgreave and Schuppe-Koistinen, 1994; Sweiry et al., 1995). Cystine is converted to cyst(e)ine inside the cell. Therefore, acivicin inhibition of γ -GT was expected to specifically decrease cysteine containing proteins. Ovine κ -CN contain one to two cysteine residues, while ovine α - and β -caseins do not contain cysteine. β -Casein was found to be the predominant casein secreted by isolated ovine mammary cells in this study (Table 4). After 8 h of incubation in the presence of acivicin α -casein was reduced to 40% of control values, β -casein to 31% and κ -casein to 21% of control values ($P < 0.05$). The inactivation of γ -GT by acivicin impaired secretion of all caseins, with κ -casein reduced by the greatest amount. Because γ -GT inhibition by acivicin decreased the synthesis of different types of caseins by different amounts, a targeted increase in γ -GT activity may increase synthesis

Table 4. Acivicin inhibition of casein synthesis. Radiolabeled (^3H -leucine) casein (kBq/g medium extract $\pm$ SEM) secreted into the medium of isolated ovine mammary cells from 2 sheep incubated for up to 8 h in the absence and presence of acivicin (0.5 mM) (t_2 n = 3 wells, $t_{4,8}$ n = 6 wells).

	Time (h)	Control	Acivicin
α -casein	2	0.11 ^a $\pm$ 0.021	0.030 ^a $\pm$ 0.003
	4	0.20 ^a $\pm$ 0.013	0.027 ^b $\pm$ 0.012
	8	0.27 ^a $\pm$ 0.023	0.12 ^b $\pm$ 0.016
β -casein	2	0.48 ^a $\pm$ 0.030	0.096 ^b $\pm$ 0.005
	4	0.37 ^a $\pm$ 0.017	0.060 ^b $\pm$ 0.027
	8	1.40 ^a $\pm$ 0.021	0.41 ^b $\pm$ 0.021
κ -casein	2	0.042 ^a $\pm$ 0.008	0.030 ^b $\pm$ 0.033
	4	0.14 ^a $\pm$ 0.029	0.014 ^b $\pm$ 0.006
	8	0.33 ^a $\pm$ 0.071	0.074 ^b $\pm$ 0.012

^{a,b}Means in the same row without a common superscript differ ($P < 0.05$).

of some casein over others. That noncysteine containing proteins were affected by γ -GT inhibition indicates that γ -GT has a role in supply of other AA in addition to cysteine for milk protein synthesis.

Experiments with γ -GT knockout mice have shown that supplying cysteine in the form of N-acetylcysteine can overcome the effects of having no γ -GT (Lieberman et al., 1996). This indicates that γ -GT has an important role in modulating the supply of cysteine in the mouse. Although γ -GT has a strong affinity for cystine the enzyme also has specificity for most other AA (Thompson and Meister, 1977). A direct role for γ -GT in AA transport has not been agreed upon, but products of γ -GT action have been shown to enter cells (Griffith et al., 1978; Baumrucker et al., 1981). γ -GT degrades glutathione and forms γ -glutamyl AA (Griffith et al., 1978), but it may also function in protein synthesis by supplying a specific transporter with γ -glutamyl AA. The identity and mechanisms of this proposed γ -glutamyl AA carrier need to be confirmed and characterized.

In the present study, inhibition of γ -GT in acini isolated from ovine mammary gland led to decreased milk protein synthesis, indicating that γ -GT may have a role in the supply of a range of AA for milk protein synthesis. This role may be in providing γ -glutamyl AA for uptake by transporter(s) yet to be characterized. Understanding the mechanism of uptake for γ -glutamyl AA may allow manipulation of AA supply to mammary cells and provide a way of differentially altering particular milk proteins. The use of acivicin in the present study suggests that it may be possible, through targeting the mechanism acted on by acivicin, for the dairy industry to alter the ratio of proteins rather than create an overall increase, and this may provide options for the manufacture of specific dairy products. Further work on the interaction between γ -GT activity and milk protein synthesis is required but the current study supports at least a facilitative role for γ -GT in the synthesis of milk proteins in the ruminant, and points to the poten-

tial for regulating milk protein production through modulating the activity of γ -GT in the mammary gland.

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