Disruption of cell junctions induces apoptosis and reduces synthetic activity in lactating goat mammary gland

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ABSTRACT

Although it is known that disruption of the cell junctions in the mammary gland induces a decrease in milk yield, the cellular mechanisms involved in milk secretion reduction during mammary cell junction disruption are not well understood. The aim of this study was to investigate the cellular regulations taking place after cell junction disruption in the mammary gland of goats. We performed intramammary infusions of Ca chelators to induce cell junction disruption. In a first group of 5 goats, intramammary infusions of ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) in the right gland halves and saline as a control in the left gland halves were performed after 4 consecutive milkings. A second group of 4 goats received 4 intramammary infusions of citrate solution in the right gland halves and lactose solution as a control in the left halves. Intramammary infusion of EGTA and lactose induced a disruption of cell junctions, whereas citrate infusions failed to modify mammary epithelium integrity. The effect of the infused solutions was also tested *in vitro* via the measurement of transepithelial resistance, confirming mammary epithelium disruption by the EGTA, lactose, and citrate solutions at high concentrations. The disruption of mammary epithelium integrity by EGTA induced a decrease in the expression of the cell junction protein E-cadherin. Both the EGTA and lactose infusions induced a decrease in milk secretion that was accompanied by cellular modifications. We observed a decrease in milk casein, which was associated with a decrease in the mRNA level of κ -casein in the lactose-infused glands, and a decrease in milk lactose, which was associated with a downregulation

of α -lactalbumin transcripts in both the EGTA- and lactose-treated glands. Both the EGTA and lactose infusions increased terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate nick-end labeling (TUNEL) in the mammary tissue, indicating an induction of apoptosis. Lactose infusion increased the mRNA level of Bax, suggesting that apoptosis was regulated at the transcriptional level. The results obtained in these experiments suggest that disruption of mammary epithelium integrity was associated with both reduced synthetic activity and apoptosis induction in the mammary gland.

Key words: tight junction, apoptosis, mammary gland, lactation

INTRODUCTION

In most situations in which milk yield declines, a disruption of mammary epithelium integrity occurs, as was reported for extended lactation (Bertilsson et al., 1997), increased milking intervals (Stelwagen et al., 1994; Delamaire and Guinard-Flament, 2006), stressful situations (Stelwagen et al., 2000), and mastitis (Leitner et al., 2004). The integrity of the mammary epithelium is ensured by different cell junctions, including the tight and adherens junctions. These cellular structures not only separate the apical and basolateral sides of the cell (Niessen, 2007) but also are involved in cellular regulations by affecting gene expression (Balda and Matter, 2009). Some studies performed on different epithelia showed the involvement of cell junction constitutive proteins in cell proliferation and apoptosis (Matter et al., 2005).

Assessment of the contribution of the cell junction state to mammary gland function requires a method that directly induces cell junction disruption. Calcium is a constitutive element of cell junctions, because it ensures the linkage between some of their proteins, including E-cadherin (Rowlands et al., 2000). Calcium depletion is a phenomenon that seems to act on cell

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Table 1. Chemical composition and nutritive value of feed ingredients (DM basis)

Item	Feed component		
	English ryegrass hay	Whole-crop corn	Concentrate ¹
DM (%)	85.0	94	86.1
Ash (%)	9.0	3.8	5.8
Total N (N × 6.25) (%)	12.7	7.42	17.4
NDF (%)	59.1	40.2	17.7
ADF (%)	32.1	21.5	7.9
Starch (%)	—	34.6	—
Net energy ² (Mcal/kg)	1.36	1.55	1.60
PDIN ³ (g/kg)	85	45	115
PDIE ^{2,4} (g/kg)	91	81	97

¹Ingredients: 61.8% wheat, 14.4% colza, 7.5% fine wheat bran, 4.9% Amyplus (Tate & Lyle, Nestle, France), 3.7% sugar beet pulp, 3% alfalfa, 1.5% palm oil, 1.3% calcium carbonate, 1% glycerol, and 0.7% mineral and vitamin premix.

²Net energy was estimated from INRA feed tables (INRA, 2007).

³True protein digested in the small intestine according to N supply in the rumen (INRA, 2007).

⁴True protein digested in the small intestine according to energy supply (INRA, 2007).

junction structure and organization, given that changes in the localization of cell junction proteins were observed after Ca chelation (Rothen-Rutishauser et al., 2002). Therefore, one of the techniques used to study the effect of cell junction disruption is Ca chelation (Pitelka et al., 1983; Stelwagen et al., 1995).

Some *in vivo* studies performed in goats showed a decrease in milk yield because of the experimental disruption of mammary gland cell junctions (Neville and Peaker, 1981; Stelwagen et al., 1995). However, the cellular regulatory mechanisms linking tight junction disruption and milk secretion reduction are still unknown. The objective of our study was therefore to investigate the cellular regulatory pathways activated in the mammary gland after experimental cell junction disruption caused by intramammary infusions of Ca chelators. To confirm the results obtained *in vivo*, we used an *in vitro* model in which mammary epithelial cells were exposed to the substances infused *in vivo*, and the integrity of the cell monolayer was evaluated by transepithelial resistance measurements.

MATERIALS AND METHODS

In Vivo Experiments

All the procedures used on animals were approved by the animal care committee of the French Ministry of Agriculture in accordance with French regulations (Decree No. 2001-464, May 29, 2001).

Animals and Experimental Design. Nine multiparous Alpine goats at 70 ± 10 d of lactation and producing 3.2 ± 0.6 kg/d milk on average were selected because their milk yield and their lactose and protein contents were well balanced between the gland halves. The goats were housed at the La Motte experimental

dairy farm of the French National Institute of Agromomic Research (INRA), Unité mixte de recherche – Production du Lait (Le Rheu, France). During the trial, the goats were fed according to the INRA's recommendations (INRA, 2007). Offered ration consisted of *ad libitum* English ryegrass hay and 1 kg of dehydrated whole-crop corn and 800 g of concentrate per d (INRA goat GR VRAC, Cooperl-Hunaudaye, Lamballe, France). The chemical composition and nutritive value of feed ingredients are shown in Table 1. The goats were milked twice a day at 0700 and 1600 h in a double 12-stall parallel milking parlor. Milking was performed with a low line milk pipeline with a vacuum pressure of 38 kPa, a pulsation rate of 120 pulsations/min, and a pulsation ratio of 60:40.

In a first experiment, 3 h after milking, 5 dairy goats received a 250-mL intramammary infusion of 115 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA; Sigma-Aldrich, Lyon, France) in the right gland, leading to an active EGTA concentration of approximately 63 mM in the mammary alveolar lumen after taking into consideration the volume of milk present in the cistern at the time of infusion (estimated, on average, at 200 mL). An equal volume of physiological saline (Aguettant, Lyon, France) was infused into the left gland. The EGTA and physiological saline were infused using an intramammary metal cannula (10 cm, Alliance Pastorale, Montmorillon, France) and a peristaltic pump (OSI, Maurepas, France) at a flow rate of 30 mL/min. The infusions were repeated after 4 consecutive milkings.

In a second experiment, 4 other goats received a 32-mL infusion of a 50 mM citrate solution (Sigma-Aldrich) in the right gland and a 300 mM lactose solution (Sigma-Aldrich) in the left gland. The citrate and lactose solutions were infused via a sterilized stainless

steel intraductal cannula connected to a 35-mL syringe. As for EGTA-treated goats, the infusions were performed over 4 consecutive milkings.

The osmolarity of the different solutions used in this trial was measured using a 12/12 DR micro-osmometer (Hermann Roebling, Berlin, Germany). The osmolarity of the citrate solution was adjusted to 300 mosmol using the lactose solution described above. The pH of the infused solutions was adjusted to 6.8 using NaOH and HCl solutions. The citrate, lactose, and EGTA solutions were sterilized by filtration through a 0.22- μ m filter (Nalge Nunc International, Fisher Scientific Bioblock, Illkirch-Graffenstaden, France). After the infusions, the glands were massaged manually to facilitate the exposure of the alveoli to the infused solutions.

Sampling and measurements were performed similarly for both groups of goats and are described together in the following sections.

Milk Samples. Before the first infusion (M0) and at the following 3 milkings (M1, M2, and M3), milk yield was recorded, and representative milk samples were collected from both the right and left glands of each goat. A 50-mL volume of milk was collected and used for milk fat, true protein, and lactose analysis using an infrared method (Lillab, Châteaugiron, France). A volume of 200 mL of milk was collected and used for total N, true protein N (precipitation at pH 4.6 with TCA, then filtration), and casein (precipitation at pH 4.6 with 10% acetic acid and 1 M sodium acetate) analysis according to the Kjeldahl method (ISO 8968-1; IDF, 2001). A volume of 50 mL of milk was used for total milk Ca, Na, and K analysis by atomic absorption spectrophotometry (Spectra AA220, Varian, Les Ulis, France) as reported previously (Murthy and Rhea, 1967). Milk samples for Na, Ca, and K analysis were omitted for one goat in the EGTA experiment at each milking. An additional 5-mL milk sample was collected for milk serum albumin analysis by the bromocresol green method as described by Bouchard et al. (1999). A 50-mL volume of milk was defatted by centrifugation at $1,500 \times g$ at 4°C for 15 min and used for quantification of free Ca^{2+} . The free Ca^{2+} in milk was quantified using a Ca-sensitive electrode (Radiometer Analytical, Hach Lange, St-Malo, France) in skim milk samples. At M1, the milk sample collections for 1 and 2 goats were omitted for free calcium determination in the EGTA and citrate experiments, respectively.

Biopsies and Blood Samples. Mammary biopsies were collected before the milking following the fourth infusion (M4). Before tissue biopsying, local anesthesia was applied by s.c. injection of 40 mg of Xylocain 2% Adrenalin (Astra France, Rueil-Malmaison, France). The biopsies were performed using a 12G/10-cm, 22-mm Bard Monopty disposable core biopsy instrument

(Bard, Voisins-le-Bretonneux, France) as described previously (Caja et al., 2006). Three 20-mg biopsies were frozen in liquid nitrogen and stored at -80°C until they were used for RNA and protein extractions. Another biopsy sample was washed in PBS (Fisher Scientific Bioblock) and fixed for 24 h in 4% paraformaldehyde (Sigma-Aldrich) for immunohistochemistry. The fixed tissue was incubated for 48 h in a 40% sucrose solution (Sigma-Aldrich), embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek Europe, LaboNord, Templemars, France), frozen in a cooled bath of isopentane (Sigma-Aldrich), and stored at -80°C until use. Using heparin-coated Vacutainers (BD, Plymouth, UK), jugular blood samples were collected before the milking preceding the start of the intramammary infusions (M0) and before the biopsy (M4). Those blood samples were analyzed for lactose content by colorimetry (kit for lactose/D-galactose, Roche, Meylan, France) using a Kone Pro multi-parameter analyzer (Kone Instruments, Espoo, Finland).

RNA and Protein Extraction. Two 20-mg mammary biopsies were used for total RNA extraction with 1 mL of TRIzol (Invitrogen, Cergy Pontoise, France) according to the manufacturer's recommendations. The RNA pellet was suspended in RNase-free water. Total RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Labtech France, Palaiseau, France). The quality of the RNA was assessed with a 2100 Bioanalyzer (Agilent Technologies, Massy, France) using the RNA integrity number generated by the Agilent 2100 Expert Software, version B.02 (Agilent Technologies). Total proteins were extracted from 20 mg of mammary tissue using 1 mL of T-PER tissue protein extraction reagent (Pierce, Perbio Science, Brebières, France) according to the manufacturer's instructions. Protein concentrations were determined using the Lowry method with the DC Protein Assay Kit (Pierce) as described by the manufacturer.

Real-Time Reverse-Transcription PCR. Complementary DNA was obtained using the Reverse Transcription System kit from Promega (Charbonnières, France) according to the manufacturer's instructions. Real-time PCR was performed using the SYBR Green Master Mix (Applied Biosystems, Foster City, CA). The primers used for real-time PCR were described in previous studies (Boutinaud et al., 2008; Ben Chedly et al., 2009). The cDNA (12.5 ng) was mixed with 6 pmol of each forward and reverse primer and adjusted to 12.5 μL with SYBR Green Master Mix (2 $\times$) and DNase-free water. The PCR amplification protocol consisted of 2 min of incubation at 50°C , followed by 10 min at 95°C , 40 cycles of 15 s at 95°C , and 60 s at 60°C . The PCR reactions on each sample were performed in triplicate.

Quantification of mRNA. The relative method for mRNA quantification using the approximate number of amplified cDNA molecules was performed as described by Boutinaud et al. (2004) according to the formula

$$Nb\ Mol = 10^{\frac{(Ct-40)}{S}},$$

where *Nb Mol* is the approximate number of cDNA molecules, *Ct* is the average cycle threshold for the triplicate PCR samples of the gene under consideration, and *S* is the slope of the calibration curve generated using serial cDNA dilutions of a single sample.

The calibration curves were generated for each target and housekeeping gene using serial dilutions of a mammary tissue cDNA sample (1:10, 1:20, 1:50, 1:100, 1:200, 1:1,000, and 1:2,000). A nontemplate negative control was incorporated into all PCR runs.

The mRNA levels of the genes under study were expressed relative to a reference gene. The cyclophilin, β -actin, r18S, GAPDH, and 60S acidic ribosomal protein P0 (**RPLP0**) genes were evaluated as potential reference genes. The BestKeeper (Robinson et al., 2007), geNorm (Vandesompele et al., 2002), and NormFinder (Andersen et al., 2004) software programs were used to assess the variability of the candidate reference genes. The analysis of the 5 candidate reference genes indicated that RPLP0 was the most stable gene in this experiment. As a result, it was used as the reference gene. For each target gene, the results are expressed as a ratio relative to the RPLP0 mRNA level.

Histochemistry. Apoptotic cells were quantified in mammary gland biopsies by the detection of DNA fragmentation using terminal deoxynucleotidyl transferase (TdT)-mediated 2'-deoxyuridine 5'-triphosphate (dUTP) nick-end labeling (**TUNEL**). Cryosections measuring 7 μ m thick and mounted on slides treated with 3-aminopropyltriethoxysilane (Sigma-Aldrich) were thawed and incubated for 30 min at 70°C in a 10 mM citrate sodium (Sigma-Aldrich) 0.1% triton solution (Fisher Scientific Bioblock), washed in PBS, and incubated for 30 min at 37°C in 200 ng/ μ L proteinase K solution (Promega). The tissue sections were incubated with DeadEnd Fluorometric TUNEL System (Promega) reagents according to the manufacturer's instructions.

After TUNEL staining, the sections were incubated for 3 min with 4',6-diamidino-2-phenylindole (**DAPI**; Sigma-Aldrich) at a concentration of 0.33 μ g/mL. All slides were mounted with Vectashield (Valbiotech, Paris, France) and examined under fluorescence using a Nikon Eclipse E400 microscope (Nikon France, Le Pallet, France). The images were captured with a DXM

1200 digital still camera (Nikon) and analyzed with the ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD). Eight microscopic fields (magnification, 200 $\times$; area, 0.14 mm² per microscopic field) were examined for both the right and left glands. The percentage of apoptotic cells in both glands was determined as a ratio of the TUNEL-labeled cells to the DAPI-counterstained nuclei.

Western Blot. Total protein extracts were mixed with a loading buffer containing 50 mM Tris-HCl at pH 6.8 (Bio-Rad, Marnes-la-Coquette, France), 2% SDS, 0.1% bromophenol blue (Sigma-Aldrich), 20% glycerol (Fisher Scientific Bioblock), and 5% β -mercaptoethanol (Sigma-Aldrich) and boiled for 5 min at 95°C. Total proteins (30 μ g) were separated on 10% SDS-polyacrylamide gels by electrophoresis at 90 V for 2.5 h at room temperature. The proteins thus separated were transferred onto polyvinylidene difluoride membranes (Amersham Biosciences, Orsay, France), which were incubated for 30 min with a blocking solution containing 5% dry skim milk dissolved in Tris-buffered saline Tween-20 (Bio-Rad), 50 mM Tris-HCl at pH 8.6 (Bio-Rad), 150 mM NaCl (Sigma-Aldrich), and 0.1% Tween (Sigma-Aldrich). The membranes were then incubated overnight at 4°C with one of the following: 1:2,500-diluted E-cadherin antibody (clone NCH-38, DakoCytomation, Trappes, France), 1:5,000-diluted β -actin antibody (A5441, Sigma-Aldrich), or 1:1,000-diluted occludin antibody (Zymed Laboratories, CliniSciences, Montrouge, France). After the primary antibody incubation, the membranes were washed in Tris-buffered saline Tween-20 and incubated with 1:2,500-diluted horseradish peroxidase-conjugated secondary antibody (31450, Pierce) for 1 h at room temperature. Peroxidase activity was detected using an enhanced chemiluminescence detection system (ECL system, Amersham Biosciences) as described by the manufacturer. Curix Ortho HT-G films (Agfa, Mortsel, Belgium) were exposed to the membranes, and the films obtained were digitized using a Canon D1250U2 scanner (Canon, Courbevoie, France). The images generated were calibrated to an optical density scale, and the optical density of different bands was quantified using the ImageJ software. The amount of E-cadherin and occludin proteins in each sample was normalized to β -actin.

In Vitro Experiment

Isolation of Epithelial Cells from Milk. Fresh milk (1.5 L) was collected from 2 Holstein cows in late lactation with no clinical signs of mastitis. Cell isolation was performed as described by Wellnitz et al. (2006) except that the cells isolated from each cow were

seeded in a 75-cm² tissue culture flask using 10 mL of Dulbecco's modified Eagle's medium/F12 medium containing 10% fetal bovine serum, 1,000 IU/mL penicillin, 1,000 µg/mL streptomycin, and 2.5 µg/mL amphotericin B (Wisent, Saint-Jean-Baptiste-de-Rouville, QC). After 1 week, the fetal bovine serum was reduced to 5%, and the concentrations of penicillin, streptomycin, and amphotericin B were reduced to 100 IU/mL, 100 µg/mL, and 0.25 µg/mL, respectively. At the third passage, the cell culture medium was exchanged for M199/F12 (Wisent) supplemented with 10% fetal bovine serum, 10 mM HEPES (Wisent), penicillin-streptomycin-amphotericin B (100 IU/mL, 100 µg/mL, and 0.25 µg/mL, respectively), 5 µg/mL insulin (Sigma-Aldrich, Oakville, ON, Canada), and 2 mM sodium acetate (2.6 mM final). The culture medium was changed every 2 or 3 d until the cells reached 90 to 95% confluency. The cells were then incubated for 5 min in a solution containing 0.25% trypsin, and 0.1% EDTA (Wisent) and seeded on 10-mm Nunc Anapore membrane inserts (pore size, 0.2 µm; VWR International, Mississauga, ON, Canada) at a density of 1.5×10^5 cells per insert. The same medium was used but supplemented with 5 µg/mL ovine prolactin (Sigma-Aldrich) and 2 µM dexamethasone (Sigma-Aldrich) to form the differentiation medium. The differentiation medium was changed every 1 or 2 d. The cells were cultured at 37°C in an atmosphere of 5% CO₂ and 95% air.

Permeability of Cell Junctions In Vitro. On d 4 of culturing on inserts, the effects of sodium citrate, EGTA, lactose, or saline control solutions on the mammary epithelial permeability were evaluated by measuring the transepithelial electrical resistance (**TEER**). These substances were added to the differentiation medium on the apical side of the cells only. Sodium citrate was used at 2, 6, and 20 mM, EGTA at 20, 60, and 200 mM, and lactose at 15, 45, and 120 mM. The middle doses used were based on the concentration obtained in milk after the infusion. All reagents were purchased from Sigma-Aldrich. Inserts with (control) or without (blank) cells in differentiation medium on both sides were also included. The TEER was measured using an EVOM volt-ohmmeter and STX2 electrode (World Precision Instruments, Sarasota, FL) before the addition of the tested substances (0 h) and at 1, 4, 12, and 24 of the treatment. Before each measurement, the medium was replaced with fresh differentiation medium (on both sides), and after the readings, the apical medium was replaced with the test solutions. For each condition, 3 inserts were used, and the experiment was repeated once using cells from 2 different cows.

Statistical Analysis. For the in vivo experiments, milk data were corrected for dilution factors resulting from the EGTA, citrate, lactose, and saline infusions.

The yields and contents of milk lactose and protein were corrected as a function of the quantities of lactose and nitrogen infused into the mammary gland via the lactose, citrate, and EGTA solutions. The data from each experiment were analyzed separately. The data obtained at M0, corresponding to the observations before the start of the infusions, were tested for differences between the left and right glands by ANOVA using the MIXED procedure (SAS Institute, 1999), where the effects of goat and gland (left or right) were tested. For the EGTA experiment, because of missing data, the differences between the left and the right glands at M0 were tested on 3 goats only. Milk data collected during the intramammary infusions (M1, M2, and M3) were analyzed by ANOVA using the MIXED procedure with REPEATED statement (SAS Institute, 1999). Time was used as a repeated effect, and infusion (goat) was used as the subject. The effects of time, goat, infusion, and the infusion $\times$ time interaction were tested. Milk SCC data were log₁₀-transformed before analysis. The milk data obtained at M1, M2, and M3 were also compared with those obtained at M0 for both experiments by testing the H₀ hypothesis that there were no differences using Student's *t*-test.

Statistical analysis of real-time PCR data was performed on the ratio of the approximate number of cDNA molecules of the target gene to the number of cDNA molecules of the reference gene multiplied by 10⁴ and log₁₀-transformed using the MIXED procedure of SAS. Effects were considered significant at $P < 0.05$.

For the in vitro experiment, TEER data were corrected for the background reading (blank) and presented as log₁₀-inverted TEER values (expressed in ohm·cm²). The effects of the solutions under study on cell junction permeability were tested using log₁₀-transformed TEER data corresponding to h 0, 1, 4, 12, and 24. The TEER values were analyzed for each EGTA, citrate, and lactose treatment separately by ANOVA using the MIXED procedure of SAS (SAS Institute, 1999) with REPEATED statement. Time was used as the repeated effect and inserts (treatment) were used as the subject. The effects of the concentrations of the added solution, time, and the time $\times$ concentration interaction were tested. Means comparisons were performed using the Tukey's test.

RESULTS

In Vivo Experiment

Effect of Infusions on Milk Ca. As expected, the infusions of the EGTA solution in the right glands decreased milk Ca²⁺ concentration ($P < 0.001$) compared with the control glands (Table 2). Total milk Ca was also decreased by the EGTA infusions ($P < 0.01$). The

proportion of ionized Ca in total milk Ca (Ca^{2+} :total Ca ratio) decreased in the EGTA-infused glands ($P < 0.01$), as shown in Table 2.

In contrast to our expectations, we did not find a clear decrease in milk Ca^{2+} concentration in the citrate-infused glands compared with those infused with lactose (Table 3). Total milk Ca, milk Ca^{2+} , and the Ca^{2+} :total Ca ratio were similar in the lactose- and citrate-infused glands (Table 2).

Effect of Infusions on Milk Yield and Composition. Similar milk yields were observed between the left and right glands before the start of the infusions (at M0) in both experiments (Tables 2 and 3). The EGTA infusions decreased milk yield gradually ($P < 0.01$) to 75% of that of the saline-infused glands at the last 2 milkings (Table 2). Milk production was also affected in the second group of goats, but that effect was, surprisingly, caused by the lactose infusions ($P < 0.05$), as shown in Table 3.

The infusion of EGTA decreased milk lactose concentration ($P < 0.001$) and yield ($P < 0.01$; Table 2). Similarly, lactose infusion was associated with a decrease in milk lactose content ($P < 0.01$) and yield ($P < 0.01$), as shown in Table 3. The infusion of EGTA decreased milk fat content ($P < 0.05$) and milk fat yield ($P < 0.05$). Milk fat content and yield were lower in the lactose-infused glands after the second and third infusions ($P < 0.05$), as shown in Table 3.

Milk protein yield ($P < 0.01$) decreased in the EGTA-infused glands (Table 2). In the lactose-infused glands, there was a significant infusion $\times$ time interaction ($P < 0.05$) for milk protein yield. Thus, milk protein yield was lower only at M2 in the lactose-infused glands (Table 3). Milk casein content and yield decreased in the EGTA-infused ($P < 0.05$) and lactose-infused ($P < 0.05$) glands, as shown in Tables 2 and 3. Milk whey protein content increased in both the EGTA-treated ($P = 0.01$) and lactose-treated ($P = 0.02$) glands. Milk whey protein yield increased in the EGTA-infused glands ($P < 0.05$) and tended to increase in the lactose-infused glands ($P = 0.07$), as shown in Tables 2 and 3. The casein:whey protein ratio was lower in the milk from the glands infused with EGTA ($P < 0.05$) and lactose ($P < 0.01$).

Somatic cell count was higher in the milk from the EGTA-infused ($P < 0.01$) and lactose-infused ($P < 0.05$) glands compared with their contralateral glands. When compared with that at M0, the infusions of saline and citrate at M1, M2, and M3 did not affect milk SCC ($P > 0.10$).

Indicators of Cell Junction Opening In Vivo. Similar milk serum albumin, Na^+ , and K^+ concentrations were observed in the right and left glands before the start of the infusions (at M0) for both in vivo ex-

periments. A high Na^+ concentration was observed in saline-infused glands at M0 (Table 2), which was due to one goat with a high value. The infusions of EGTA increased serum albumin concentration in milk ($P < 0.01$), as shown in Table 2. Milk concentration of albumin tended to be higher in the lactose-infused glands at M2 and M3 ($P < 0.10$), as shown in Table 3.

Milk Na^+ concentration was higher in the EGTA-infused glands than in the saline-infused glands ($P = 0.01$) but did not differ between the citrate- and lactose-infused glands (Table 3). Milk K^+ concentration decreased in both the EGTA- and lactose-infused glands ($P < 0.05$), as shown in Tables 2 and 3. The EGTA infusions tended to increase the milk $\text{Na}^+:\text{K}^+$ ratio ($P = 0.07$). The milk concentrations of Na^+ , K^+ , and albumin were similar between the period before the infusions (M0) and the periods after the citrate infusions at M1, M2, and M3 ($P > 0.10$).

We observed an increase ($P < 0.05$) in blood lactose concentration between the start of the infusions (M0) and the period after the last infusion (M4) in both the saline/EGTA- and lactose/citrate-infused goats (Figure 1).

Gene Expression of Proteins Involved in Synthesis of Milk Constituents. We observed a lower α -LA mRNA level in the EGTA-infused glands than in the saline-infused ones ($P < 0.05$), as shown in Table 4. Similarly, the mRNA expression of α -LA was lower in the glands infused with lactose than in those infused with citrate ($P < 0.01$). κ -Casein gene expression was downregulated by the lactose infusions only ($P < 0.01$).

Gene Expression of Apoptosis-Related Proteins. Caspase-3 mRNA levels were similar in the lactose- and citrate-infused glands but tended to be lower ($P = 0.06$) in the mammary glands infused with EGTA than in those infused with saline (Table 4). The mRNA expression of Bax and Bcl-2 was similar between the EGTA- and saline-infused glands, as shown in Table 4. In contrast, the Bax mRNA level was higher in the lactose-infused glands ($P = 0.02$), whereas the Bcl-2 mRNA level was unaffected by this infusion. These variations led to an increase in the proapoptotic Bax:Bcl-2 ratio in the lactose-infused glands compared with the citrate-infused glands ($P < 0.05$).

Apoptosis in Mammary Tissue. The intramammary infusions of EGTA increased the percentage of apoptotic cells in the mammary tissue compared with the saline infusions ($P < 0.05$; Figure 2). Similarly, the percentage of apoptotic cells in the tissue from the lactose-infused glands was higher than in the tissue from the citrate-infused glands ($P = 0.01$).

Quantification of Cell Junction Constitutive Proteins and Their mRNA Levels. We observed a

Table 2. Effect of intramammary infusions¹ of ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) and saline on milk yield and composition in goats (n = 5)

Item	M0 ²		M1		M2		M3		SEM	<i>P</i> -value	
	Saline	EGTA	Saline	EGTA	Saline	EGTA	Saline	EGTA		Infusion	Infusion × time
Milk Ca ²⁺ (mmol/L)	5.1	4.9	2.7	0.5***	3.4	1.5***	2.6	0.5***	0.29	0.001	0.87
Milk total Ca ³ (mmol/L)	1,322	1,379	692	615	840	487***	647	281***	31.2	0.007	0.002
Ca ²⁺ :total Ca (10 ⁻³) ratio ⁴	3.6	3.4	3.8	0.1***	3.5	2.2***	3.8	1.3***	0.28	0.002	0.01
Milk yield (kg/milking)	1.14	1.13	0.55	0.59	0.96	0.74**	0.56	0.42†	0.05	0.006	0.09
Milk lactose											
g/kg	47.3	46.9	29.4	21.5**	33.9	15.6***	27.8	7.0***	1.49	<0.001	0.001
g/milking	59.1	56.1	17.5	12.8	33.5	13.2***	17.0	3.7***	2.17	0.006	0.001
Milk fat											
g/kg	31.9	32	26.5	24.0†	26.4	26.4	26.2	22.7*	0.91	0.04	0.2
g/milking	41.2	39	16.1	14.3	27.0	21.4*	16.3	11.1†	1.83	0.05	0.53
Milk protein											
g/kg	26	25.8	16.3	15.9	19.6	19.2	15.4	14.9	0.49	0.21	0.82
g/milking	32.9	31.1	9.6	9.4	19.4	15.2*	9.5	7.0	1.06	0.005	0.30
Milk casein ³											
g/kg	20.1	20	11.2	10.4	14.4	13.4	10.7	9.6†	0.40	0.02	0.92
g/milking	22.2	21.7	5.8	6.0	13.7	10.1*	6.0	3.7	1.10	0.03	0.35
Milk whey protein ³											
g/kg	4.7	4.4	2.4	2.8	3.1	4.6**	2.7	4.8***	0.27	0.01	0.02
g/milking	5	4.8	1.2	1.6	2.9	3.1	1.3	1.8	0.26	0.04	0.94
Casein:whey protein ratio ³	4.4	4.6	4.8	3.8†	4.7	3.0**	4.4	2.0***	0.37	0.02	0.16
Log ₁₀ SCC	4.7	5.1	5.7	6.4*	5.8	6.9**	5.8	6.8**	0.20	0.006	0.54
Milk albumin (mg/kg)	0.5	0.5	0.2	0.3	0.4	1.4***	0.3	1.4***	0.17	0.008	0.01
Milk Na ³ (g/kg)	29.8	15.4	18.2	35.8	16.8	54.1**	19.2	58.8**	8.31	0.01	0.43
Milk K ³ (g/kg)	46.1	46.6	29.9	24.2	36.3	20.7***	27.5	9.4***	2.51	0.02	0.008
Milk Na:K ratio ³	0.6	0.3	0.6	2.7	0.5	3.1†	0.7	7.0***	1.03	0.07	0.006

¹M0, M1, M2, and M3 represent, respectively, the milking before the start of the intramammary infusions and the milkings following the first, second, and third infusions. For each goat, 250 mL of a 115 mM EGTA solution was infused into the right gland, and an equal volume of saline was infused into the left gland. The infusions were performed 4 times 3 h after milkings.

²M0 data were analyzed separately from the data for the other milkings.

³The milk of 1 goat was not analyzed, resulting in n = 4.

⁴Because of missing values, the ratios presented are slightly differing from a ratio calculated on the above averages.

Within each milking, significant differences from saline are indicated as follows: †*P* < 0.10; **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Table 3. Effect of intramammary infusions¹ of lactose and citrate on milk yield and composition in goats (n = 4)

Item	M0 ²		M1		M2		M3		P-value		
	Lactose	Citrate	Lactose	Citrate	Lactose	Citrate	Lactose	Citrate	SEM	Infusion	Infusion × time
Milk Ca ²⁺ (mmol/L)	2.7	2.3	2.6	1.8	2.5	3.2†	1.3	1.8	0.34	0.50	0.09
Milk total Ca (mmol/L)	1,230	1,341	1,226	1,290	1,230	1,227	1,218	1,240	42.1	0.53	0.70
Ca ²⁺ :total Ca (10 ⁻³) ratio ³	2.2	1.8	2.1	1.6	2.1	2.6†	1.1	1.5	0.33	0.34	0.09
Milk yield (kg/milking)	1.08	1.06	0.77	0.77	0.73	1.04***	0.50	0.67**	0.04	0.01	0.005
Milk lactose											
g/kg	48.7	48.9	37.4	47.1*	38.2	46.9*	36.7	46.5*	2.69	0.006	0.98
g/milking	52.5	51.4	29.6	36.2	27.9	48.6***	18.5	30.8**	3.07	0.003	0.12
Milk fat											
g/kg	24.2	23.7	34.5	34.3	23.3	28.8*	28.2	36.2**	2.10	0.07	0.11
g/milking	25.8	24.7	27.9	27.1	17.2	29.7**	14.3	24.1*	2.84	0.05	0.04
Milk protein											
g/kg	27.1	27.3	26.9	25.6	27.8	24.2**	27.6	25.5†	1.02	0.05	0.44
g/milking	29	28.5	21.4	19.8	20.3	25.0*	13.9	16.9†	1.41	0.20	0.04
Milk casein											
g/kg	21.6	21.6	17.6	19.8**	19.0	18.7	17.6	19.1*	0.48	0.02	0.10
g/milking	23.1	22.6	13.5	15.3	13.8	19.3***	8.8	12.7**	0.79	0.01	0.09
Milk whey protein											
g/kg	5.3	5.3	10.0	4.9***	8.2	4.9*	9.0	5.6**	0.81	0.02	0.26
g/milking	5.7	5.6	7.7	3.8***	6.0	5.1	4.5	3.7	0.63	0.07	0.01
Casein:whey protein ratio	4.1	4.1	1.9	4.1***	2.4	4.0***	2.1	3.4***	0.20	0.002	0.12
Log ₁₀ SCC	4.9	5.1†	6.6	5.8†	7.0	5.6**	6.9	6.3	0.33	0.03	0.49
Milk albumin (mg/kg)	0.5	0.5	1.8	0.5†	2.0	0.7†	1.9	0.6	0.51	0.12	0.98
Milk Na (g/kg)	18.1	12.6	24.8	18.2	36.2	16.1†	26.1	21.4	7.09	0.30	0.25
Milk K (g/kg)	50.7	49.8	38.3	47.7***	33.1	45.1**	39.3	40.7	2.13	0.03	0.06
Milk Na:K ratio	0.4	0.3	0.7	0.4	1.1	0.4*	0.8	0.5	0.25	0.22	0.25

¹M0, M1, M2, and M3 represent, respectively, the milking before the start of the intramammary infusions and the milkings following the first, second, and third infusions. For each goat, 32 mL of a 50 mM citrate solution was infused into the right gland and an equal volume of 300 mM lactose was infused into the left gland. The infusions were performed 4 times 3 h after milkings.

²M0 data were analyzed separately from the data for the other milkings.

³Because of missing values, the ratios presented are slightly differing from a ratio calculated on the above averages.

Within each milking, significant differences from lactose are indicated as follows: † $P < 0.10$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 1. Blood lactose concentration in goats given intramammary infusions of saline/ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) and lactose/citrate: before the first infusion M0 (□) and after the last infusion M4 (■). **P* < 0.05. M0 and M4 represent the milking before the start of the intramammary infusions and the milking following the fourth infusion, respectively. The data presented are adjusted means ± SEM.

decrease in E-cadherin levels (*P* < 0.05) in the EGTA-infused glands compared with the saline-infused glands, as shown in Figure 3. At the same time, the levels of occludin tended to be lower (*P* = 0.10; Figure 3) in the tissue from the EGTA-infused glands. The E-cadherin and occludin levels were similar between the lactose- and citrate-infused glands, as shown in Figure 3. The mRNA levels of E-cadherin and occludin were similar in the EGTA- and saline-infused glands as well as in the lactose- and citrate-infused glands, as shown in Table 4.

In Vitro Experiment

The mammary epithelial cells recovered from milk and cultured in the medium described above were able to form cell junctions, given that a TEER value averaging 720 ohm·cm² was measured. The addition of the EGTA solution to the culture medium induced a quick and lasting drop in TEER values (*P* < 0.001; Figure 4A). The different EGTA concentrations tested gave similar values. The addition of the citrate solution to the culture medium induced a decrease in TEER values, but only at the 20 mM concentration (*P* < 0.01; Figure 4C). The addition of lactose solution to the culture medium induced a decrease in TEER, but only at the 120 mM concentration (*P* < 0.005), as shown in Figure 4B.

DISCUSSION

The preliminary step of our study was to verify the ability of EGTA infusions as Ca chelator to disrupt mammary epithelium integrity. We found that intramammary EGTA infusions induced a decrease in K⁺ concentration and an increase in Na⁺ and serum albumin concentrations in milk, as well as an increase in blood lactose concentration. This indicates that the EGTA infusions disrupted mammary epithelium integrity. The effect of EGTA on the cell junctions in mammary tissue was previously reported by Neville and Peaker (1981) and Stelwagen et al. (1995) in goats. Accordingly, we observed a decrease in TEER values in the mammary epithelial cells cultured in a medium containing EGTA. A low TEER value represents low electrical resistance. Thus, the TEER results showed that EGTA induced a loss of epithelium integrity in the cultured cell monolayer. The results obtained in vitro

Table 4. Log₁₀ of target gene mRNA levels expressed as a ratio to RPLP0 mRNA level data in the mammary gland for the saline/ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) and lactose/citrate goat groups

Item	Saline/EGTA experiment ¹				Lactose/citrate experiment ²			
	Saline	EGTA	SEM	<i>P</i> -value	Lactose	Citrate	SEM	<i>P</i> -value
α-Lactalbumin	6.70	6.31	0.07	0.02	6.65	7.10	0.03	0.002
κ-Casein	6.82	6.64	0.06	0.11	6.40	6.63	0.02	0.004
Caspase-3	1.90	1.72	0.05	0.06	1.91	2.08	0.06	0.13
Bax	2.40	2.24	0.10	0.31	3.08	2.81	0.04	0.02
Bcl-2	1.00	0.85	0.07	0.18	1.48	1.51	0.03	0.41
Ratio ³ Bax/Bcl-2	1.35	1.44	0.56	0.97	1.60	1.30	0.06	0.03
E-cadherin	4.13	3.88	0.17	0.35	4.76	4.37	0.17	0.21
Occludin	2.68	2.53	0.08	0.27	3.34	3.20	0.14	0.53

¹In the saline/EGTA experiment, the mammary biopsies were performed after the fourth intramammary infusion of the saline and EGTA solutions in the left and right glands, respectively.
²In the lactose/citrate experiment, the mammary biopsies were performed after the fourth intramammary infusion of the lactose and citrate solutions in the left and right glands, respectively.
³The Bax/Bcl-2 ratio was calculated by dividing the number of RNA molecules of Bax by those of Bcl-2 and was not log₁₀-transformed.

Figure 2. Quantification of the percentage of mammary apoptotic cells by terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate nick-end labeling (TUNEL) for both experiments after the fourth intramammary infusion of the tested solutions. For the first experiment ($n = 5$), the glands were infused with saline ($\square$) or ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) ($\blacksquare$); for the second experiment ($n = 4$), the glands were infused with lactose ($\square$) or citrate ($\blacksquare$). * $P < 0.05$. The data presented are adjusted means $\pm$ SEM (A). Representative fluorescent micrographs of mammary tissue stained with DAPI (4',6-diamidino-2-phenylindole; dark gray) and TUNEL labeling (white) for saline-infused gland (B), EGTA-infused gland (C), lactose-infused gland (D), and citrate-infused gland (E). Color version available in the online PDF.

confirmed that EGTA did indeed induce cell junction disruption in mammary tissue.

In the second group of goats, the effect of the infused solutions was different from our expectations. We expected that the citrate infusions would induce Ca chelation and, as a consequence, disrupt the cell junctions in the mammary gland. However, the Na^+ , K^+ , and serum albumin concentrations in milk observed in the citrate-infused glands did not indicate that this treatment induced a disruption of mammary epithelium integrity when compared with M0. Nevertheless, Neville and Peaker (1981) observed a disruption of cell junction after infusion of a citrate solution into the mammary gland. This difference in results might be attributable to the lower amount of citrate used in our experiment, a possibility supported by the observations of Neville and Peaker (1981) that milk Ca^{2+} was not affected by the addition of low concentrations of citrate in vitro. We also observed that citrate induced a decrease in TEER, but only at the highest concentration, which was 20 mM. We calculated that the citrate concentration in milk after the infusions reached just 13 mM. This probably explains the lack of effect of citrate infusion on cell junction disruption indicators. Further investigations into the effect of citrate concentration on mammary epithelium integrity in vivo are needed before any conclusions can be reached on that matter.

In this experiment, we obtained a surprising effect of lactose infusion on the integrity of the mammary

epithelium. In fact, we observed an increase in the milk $\text{Na}^+:\text{K}^+$ ratio and a tendency toward an increase in milk serum albumin concentration after the second lactose infusion compared with the citrate infusion, suggesting a disruption of the mammary epithelium. Although this observation was surprising, the effect of lactose infusion on mammary epithelium integrity was confirmed by the in vitro experiment, which showed a decrease in the TEER of cultured cells treated with lactose. Data on the effect of lactose on epithelium tissue integrity are lacking. However, a previous study already showed a disruption of hepatocyte cell junction integrity after infusion of a 0.5 M lactose solution (Goodenough and Gilula, 1974). Based on the results obtained both in vivo and in vitro in the present study, we can conclude that lactose did indeed induce cell junction disruption in the mammary epithelium.

The cell junction disruption observed in the EGTA-infused glands can be attributed to the chelation of Ca. Our study confirms that EGTA infusion is able to bind Ca in vivo and deplete Ca in milk, because milk Ca^{2+} concentration and the proportion of ionized Ca were lower in the EGTA-infused glands. It was shown previously that Ca deprivation on the apical side of the mouse mammary cell line COMMA-D induced epithelium integrity disruption (Stelwagen and Callaghan, 2003). It has been proposed that Ca depletion in Madin-Darby canine kidney cells can induce an unhooking of Ca^{2+} from extracellular loops of cell

Figure 3. Western blot quantification of occludin (panel A) and E-cadherin (panel B) protein levels expressed in the mammary gland relative to β -actin for both experiments after the fourth intramammary infusion of the tested solutions. For the first experiment ($n = 5$), the glands were infused with saline ($\square$) or ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) ($\blacksquare$); for the second experiment ($n = 4$), the glands were infused with lactose ($\square$) or citrate ($\blacksquare$). NS = nonsignificant; $\dagger P < 0.10$; $*P < 0.05$. The data presented are adjusted means $\pm$ SEM. Representative pictures of Western blot of occludin (panel C) and E-cadherin (panel D).

junction proteins such as E-cadherin, which can cause conformational or localization changes or both in these proteins (Citi, 1992; Rothen-Rutishauser et al., 2002). Thus, after EGTA treatment, the adhesive function of this protein seems to be abolished. In our experiment, a decrease in the levels of the cell junction constitutive proteins E-cadherin and occludin was associated with disruption of mammary epithelium integrity in the EGTA-infused glands. This last result raises the question of whether the decreases in E-cadherin and occludin are responsible for the disruption of cell junction integrity or whether they are a consequence of Ca chelation. Whatever the case may be, we can attribute

the effect of EGTA on the disruption of the mammary epithelium to Ca chelation.

The disruption of cell junctions by lactose is a surprising phenomenon, given that lactose is a constituent of milk. We think that the lactose effect on mammary epithelium integrity does not involve a free Ca depletion pathway. Despite the fact that Charley and Saltman (1963) reported Ca chelation by lactose, we did not observe such an effect on milk *in vitro* (data not shown) or *in vivo*, suggesting that lactose causes disruption of mammary epithelium integrity through a Ca-independent pathway. Lactose infusion did not affect the levels of cell junction constitutive proteins, further suggesting

Figure 4. Effect of ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) (A), lactose (B), and citrate (C) solutions on the transepithelial electrical resistance (TEER) of milk mammary epithelial cells in vitro ($n = 6$). The substances were added to the differentiation media on the apical side of the cells only. The substances were added at h 0, and the TEER was measured in triplicate at h 1, 4, 12, and 24. The experiment was repeated once using cells from 2 different cows.

that EGTA and lactose disrupt mammary epithelium integrity through different mechanisms.

Despite the fact that the EGTA and lactose infusions used different pathways to induce cell junction disruption, both treatments decreased milk yield. Our results agree with previous observations showing a decrease in milk secretion after disruption of mammary epithelium integrity (Neville and Peaker, 1981; Stelwagen et al., 1995). The second step of our study was to evaluate the cellular regulations involved in the decrease in milk secretion after the experimental induction of mammary cell junction disruption. We studied the mechanisms acting on synthetic activity and cell death in the mammary gland.

The synthetic activity of the mammary epithelial cells was affected by cell junction disruption after EGTA and lactose infusions. In both treatments, milk lactose yield and content were decreased. This phenomenon can be attributed to the passage of a part of the synthesized lactose into the blood through disturbed cell junctions, which was confirmed by the increase in blood lactose concentration after the mammary infusions. The decrease in the secretion of milk lactose can also be attributed to a decrease in its synthesis. The decrease in lactose synthesis was supported by a downregulation of α -LA transcripts, the co-enzymes of lactose synthase. The involvement of α -LA transcripts in the regulation of lactose synthesis was previously reported (Boutinaud et al., 2008). The decrease in milk lactose can partly explain the decrease in milk volume, because lactose is the milk component with the greatest osmotic capability.

Milk protein secretion was also affected by disruption of mammary epithelium integrity in both groups of goats. Milk protein and casein secretions decreased after both the EGTA and lactose treatments. Only milk whey protein content was higher, as a consequence of the transfer of proteins from the blood through the disrupted mammary epithelium. This was supported by the increase in milk serum albumin content. For lactose infusion, we demonstrated that the decrease in casein synthesis involved the downregulation of the κ -CN mRNA level. The effect of EGTA on the κ -CN mRNA level was not significant. We do not have any explanation for the effect of lactose on milk casein synthesis. In contrast, the effect of EGTA may be the result of Ca depletion. Indeed, previous in vitro studies reported a decrease in casein synthesis by mammary epithelial cells after Ca^{2+} depletion in mice (Duncan and Burgoyne, 1996) and rabbits (Wilde et al., 1981). Our results showed that cell junction disruption was associated with the decrease in casein synthesis.

The decrease in milk secretion after the disruption of mammary epithelium integrity seems to involve the

modulation of cell numbers in the mammary gland as well. We observed an increase in the percentage of apoptotic cells in the mammary gland in both the EGTA- and lactose-infused glands. The apoptotic percentages observed in this experiment are lower than those reported in previous papers for goat mammary gland (around 0.7%; Wareski et al., 2001; Caja et al., 2006). However, the presented results of apoptotic percentage are higher than some of our previous results (0.15%; Ben Chedly et al., 2009). The level of apoptosis in mammary gland during lactation detected by the TUNEL assay is quite different between published reports in cows—it can vary from 0.07% (Capuco et al., 2001) and 0.16% (Nørgaard et al., 2005) to 2.4% (Wilde et al., 1997). These variations could result from variations in the procedure to treat the mammary sections before labeling (e.g., citrate, triton, or H₂O₂) and also from the ability to accurately detect the entire population of normal mammary cells, because the level of apoptosis is a ratio between apoptotic cells and the total number of cells. This can depend on the thickness of mammary sections. For Caja et al. (2006), the mammary sections were thinner (3 μ m vs. 7 μ m in our study); thus, a lower total number of cells was likely detected in that study compared with ours; consequently, a higher rate of apoptosis was obtained by Caja et al. (2006).

The occurrence of cell death in EGTA- and lactose-infused glands could be a consequence of the disruption of epithelium integrity. Cells disconnected from others could enter into programmed cell death. However, different mechanisms seem to be involved in cell death induction after infusion of EGTA or lactose.

The induction of apoptosis observed in the EGTA-infused glands may involve different regulatory pathways. One of the pathways may be a direct effect on cell junction constitutive proteins such as E-cadherin. Indeed, we observed a decrease in E-cadherin levels, which could be considered an apoptosis inducer signal. The decrease in E-cadherin expression was shown to induce apoptosis in the mouse mammary gland (Bous-sadia et al., 2002) and in other cell types (Peluso et al., 2001). The second regulatory pathway responsible for apoptosis induction after EGTA treatment could be linked to Ca chelation. To our knowledge, there are no available data concerning the apoptotic effect of Ca chelation on mammary epithelial cells. However, apoptosis has been induced in astrocytes cultivated in a medium depleted in Ca (Chiesa et al., 1998) and in cells cultivated with Ca chelators (Kluck et al., 1994; Peluso et al., 2001). In our experiment, we noticed a tendency toward downregulation of proapoptotic transcripts of caspase-3 in the EGTA-infused glands. This could be considered a compensatory mechanism aimed at reducing apoptosis induction. Thus, our results indicate that

the induction of apoptosis in the mammary gland after EGTA infusion could involve a decrease in E-cadherin expression or Ca deprivation or both.

The disruption of mammary epithelium integrity after lactose infusion also induced apoptosis in the mammary gland. As far as we know, there are no data reporting an apoptotic effect of lactose on mammary epithelial cells. The induction of mammary epithelial cell apoptosis after lactose infusion seems to be the consequence of the disruption of cell junctions. Unlike in the EGTA-treated glands, apoptosis induction in the lactose-treated glands was associated with an upregulation of proapoptotic Bax transcripts, leading to an increase in the Bax:Bcl-2 ratio. This result suggests that apoptosis induction after EGTA or lactose infusion involves different regulation pathways at the transcriptional level.

CONCLUSIONS

As demonstrated *in vivo* and *in vitro*, EGTA and lactose are 2 agents that induce mammary epithelium disruption. The induction of mammary epithelium disruption by intramammary EGTA infusion was associated with a decrease in the expression of cell junction constitutive proteins. The disruption of cell junctions in the mammary gland by both lactose and EGTA infusion induced a decrease in milk yield. That decrease was associated with 2 phenomena. The first one is a decrease in mammary epithelial cell activity, which contributes to the decrease in milk secretion. The regulation of mammary epithelial cell synthetic activity involved transcriptional regulations affecting lactose and casein synthesis. The second phenomenon consists of apoptosis induction in mammary epithelial cells, which leads to the decrease in the number of milk secretory cells. The regulation of mammary epithelial cell dynamics after cell junction disruption may involve different transcriptional pathways depending on the inducing agent.

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