Regulation of mammary tight junctions through parathyroid hormone-related peptide-induced activation of apical calcium channels

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Abstract

Tight junctions (TJs) play an essential role in cell–cell contact between mammary epithelial cells and, as such, play a critical role in cell function. Moreover, calcium (Ca) plays a crucial role in the formation and maintenance of mammary TJs. Given that parathyroid hormone-related peptide (PTHrP) is involved in cellular Ca homeostasis, we postulated a role for PTHrP in the regulation of mammary TJs. The effect of PTHrP(1–34) on TJs was studied in the mouse mammary cell line COMMA-1D by measuring transepithelial electrical resistance across cell monolayers and measuring the expression of TJ proteins. PTHrP stimulated TJ formation but only under conditions

where extracellular Ca was limiting. This effect of PTHrP appeared to be indirect and mediated via increased intracellular availability of Ca as a result of increased Ca-channel activity in the apical membrane. The changes in TJs were associated with altered expression of the TJ protein occludin, but expression of the TJ protein claudin-1 was not affected. The effects of PTHrP on mammary TJs are independent of prolactin. In conclusion, PTHrP enhances mammary TJ formation when extracellular Ca is limiting by maintaining intracellular Ca supplies.

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Introduction

Cell-to-cell contact between adjacent epithelial cells is critical to cell survival and functioning of the cell and is facilitated by the junctional complex, which consists of three components: desmosomes, intermediate or adherence junctions and tight junctions. The tight junction (TJ) surrounds the cell in a belt-like manner in close proximity to the apical (i.e. luminal) side of the cell and serves a dual function. In its 'fence' function it separates the plasma membrane of the cell into compositionally distinct basolateral and apical domains. In addition, the TJ acts as a barrier between adjacent epithelial cells and as such regulates the paracellular transport of ions and small molecules (Schneeberger & Lynch 1992, Stevenson & Keon 1998). Tight junctions have long been considered static structures of unknown composition, but during the past decade much insight has been gained into the molecular make-up of the TJ (Tsukita *et al.* 2001). The first true transmembrane TJ protein identified was occludin (Furuse *et al.* 1993) and, more recently, one other family of TJ proteins (claudins) has been isolated from junctional fractions (Furuse *et al.* 1998). These TJ molecules appear to be linked to the cytoskeleton of the cell through specialised proteins. Occludin is intimately connected to the cytoskeleton via the TJ-associated protein

ZO-1 (Fanning *et al.* 1998). The linkage of TJs to the cytoskeleton suggests that they play an important role in cell functioning. Indeed, the recent demonstration of a ZO-1-associated transcription factor provides direct evidence that TJs are involved in the regulation of gene expression (Balda & Matter 2000).

In the epithelium of the mammary gland TJ formation is a critical step in the differentiation cascade allowing cells to become polarised and to synthesise and secrete milk. TJs are formed during lactogenesis when the gland switches from a developing, non-lactating state to a lactating state (Linzell & Peaker 1974). The importance of the role that TJs play in maintaining milk synthesis is apparent from studies with goats and cows where an induced loss of the barrier function of mammary TJs, through chemical manipulation (Neville & Peaker 1981, Stelwagen *et al.* 1995) or decreasing the frequency of milk removal from twice to once daily (Stelwagen *et al.* 1994, 1997), resulted in a rapid reduction of milk secretion.

The mechanisms through which 'leaky' TJs affect milk secretion are not fully understood but endocrine factors, such as glucocorticoids, prolactin, and low levels or lack of progesterone are important for mammary TJ formation and maintenance (Zettl *et al.* 1992, Thompson 1996, Stelwagen *et al.* 1998, 1999, Nguyen *et al.* 2001). We recently proposed a role for parathyroid hormone-related

peptide (PTHrP) in the regulation of mammary TJs (Stelwagen 2001). PTHrP, reviewed by Philbrick *et al.* (1996), was originally isolated from tumour cells but since then it has been found in several non-malignant tissues, including the mammary gland. It plays several complex physiological roles in the regulation of smooth muscle tone, in tissue development and differentiation, and in Ca transport and homeostasis. It is this latter function of PTHrP that potentially allows it to play a role in the maintenance and regulation of mammary TJs. In an experiment with goats that were milked unilaterally once or four times a day, Thompson *et al.* (1994) showed that PTHrP production and its rate of secretion into milk were significantly reduced in the once-daily milked gland compared with the contralateral gland from which milk was removed four times daily. We also know that reducing the frequency of milk removal to once a day rapidly impairs the barrier function of mammary TJs in goats and cows (Stelwagen *et al.* 1994, 1997). Furthermore, from both *in vitro* and *in vivo* experiments it is clear that maintaining adequate extracellular Ca levels is critical to the integrity of mammary TJs (Pitelka *et al.* 1983, Stelwagen *et al.* 1995) and PTHrP appears to be an important hormone in maintaining cellular Ca homeostasis and epithelial Ca transport in various tissues, including the mammary gland (Seitz *et al.* 1993, Philbrick *et al.* 1996, Friedman 2000, Lafond *et al.* 2001).

The first objective of the present study was, therefore, to examine whether PTHrP is involved in the regulation of TJs in the mammary epithelium. Prolactin is necessary for full barrier function of TJs (Stelwagen *et al.* 1999) and it also appears to play a role in regulating PTHrP expression in mammary tissue (Thiede 1989). Hence, the second objective of the study was to determine if prolactin is involved in mediating a potential PTHrP effect of TJs.

Materials and Methods

Reagents

Cell culture medium, Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM-F12), and fetal calf serum (FCS) were obtained from Gibco BRL (Life Technologies, Auckland, New Zealand). Dexamethasone (DEX), ovine prolactin (PRL), PTHrP(1–34), murine submaxillary gland epidermal growth factor, bovine pancreatic insulin, penicillin-streptomycin, BSA, Bay K-8644, Nifedipine, EGTA and the secondary antibody were all purchased from Sigma Chemical Co. (St Louis, MO, USA). Primary antibodies for the TJ proteins occludin and claudin-1 were obtained from Zymed Laboratories Inc (Innovative Sciences Ltd, Christchurch, New Zealand). Cell culture inserts (Nunc Anopore, 10 mm, 0.2 µm pore size) were from Life Technologies. The BCA Protein Assay Kit was from Pierce (Rockford, IL, USA). Protease inhibitor tablets were purchased from

Roche (Auckland, New Zealand). Cell lysis buffer 5X was from Promega (Madison, WI, USA). All other reagents were of the highest available purity.

Cell culture

The mouse mammary cell line COMMA-1D (Danielson *et al.* 1984) was used in all experiments. This cell line has the ability to form TJs (Stelwagen *et al.* 1999). Cells were cultured as previously described (Stelwagen *et al.* 1999). Briefly, COMMA-1D cells were seeded on Nunc Anopore membranes at a density of 1.8×10^5 cells per insert. Cells were grown to confluence in growth medium (DMEM-F12), supplemented with 2% FCS, BSA, (300 ng/ml), penicillin-streptomycin, insulin (5 µg/ml) and epidermal growth factor (5 ng/ml). Once cells became confluent they were maintained for 24 h in priming medium (same as growth medium, but without epidermal growth factor). Cells were then kept in differentiation medium, i.e. priming medium supplemented with PRL (5 µg/ml) and DEX (1 µM). The medium was changed every 24 h, and cells were cultured at 37 °C in an atmosphere of 5% CO₂ and 95% air. PTHrP was added only to the apical side of the cells.

TJ formation

Formation and permeability of TJs were assessed by measuring transepithelial electrical resistance (TER) across cell monolayers as described previously (Stelwagen *et al.* 1999). Briefly, inserts containing a cell monolayer are temporarily placed inside an Endohm-12 chamber (World Precision Instruments, Sarasota, FL, USA) containing the same medium in which cells are cultured. The bottom of the chamber, containing one electrode is positioned towards the basolateral side of the cells and the lid of the chamber containing the top electrode is positioned towards the apical side of the cells. The TER is measured across the monolayer using a Millicell-ERS voltohmmeter (Millipore Corp., Bedford, MA, USA). An increase in TER represents increased TJ formation and a corresponding decrease in TJ permeability. TER was measured across cell monolayers every 24 h and TER values were corrected for background readings (i.e. inserts without cells and medium only), and membrane surface area (i.e. 0.6 cm² per insert). TER is expressed as Ω.cm².

Ca-switch model

To assess the effect of PTHrP and Ca on TJs, the Ca-switch model was used (Cereijido *et al.* 1994). In this model, DMEM-F12 medium that has a low Ca content (i.e. 2 µM; commercially available Ca-free medium supplemented with 2 µM Ca) is used instead of normal DMEM-F12 medium which contains 1.8 mM Ca. Briefly, 3 days prior to the start of an experiment cells

were switched to the low-Ca medium and switched back to normal-Ca medium at the start of the experiment at 0 h or kept in the low Ca-medium throughout the experiment, as indicated for each experiment.

Ca-channel regulation

The effect of PTHrP on Ca-channels was examined using the Ca-channel activator Bay K-8644 and the Ca-channel blocker Nifedipine as described in Bacskai and Friedman (1990). Briefly, cells were cultured with or without Bay K-8644 at a final concentration of 50 μ M, or with or without Nifedipine also at a final concentration of 50 μ M. Both Bay K-8644 and Nifedipine were dissolved in ethanol and 2 μ l were added to the 500 μ l medium in the apical chamber of the culture inserts. This small amount of ethanol did not affect cell appearance or TER values as determined in separate experiments (data not shown).

Western blot analyses for occludin and claudin-1

Cells were grown in 6-well culture plates (Nunc, Life Technologies) under the same conditions as described earlier. Following treatment, cells were harvested in lysis buffer and the protein content of cell lysates was determined using the BCA assay. Sample lysates, normalised for protein content were subjected to 12% (occludin) or 15% (claudin-1) SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (1970). Following electrophoresis, separated proteins were transferred (1 h at 0.8 mA/cm²) to nitrocellulose membranes (BioTrace-NT, Pall Co., Ann Arbor, MI, USA). Membranes were then probed with either polyclonal rabbit anti-human occludin (1:300 000) or a polyclonal rabbit anti-human claudin-1 (1:200 000) antibody. The secondary antibody was goat anti-rabbit IgG (1:10 000) conjugated to horseradish peroxidase; signals were detected by chemiluminescence (ECL).

Statistical analyses

Data were analysed by ANOVA (SAS Systems for Windows, Release 6.11 1996, SAS Institute Inc., Cary, NC, USA). Differences between means were considered significant at $P < 0.05$. A Fisher protected least significant differences test was performed to compare means. When appropriate, TER values over the 24 h immediately preceding the start of an experiment at 0 h were used as covariates in the analyses. All values are expressed as means $\pm$ S.E.M.

Results

Effect of Ca on TER

The first experiment showed that in cells exposed to normal-Ca medium on both sides rapid TJ formation

Figure 1 Effects of apical and basolateral exposure of cells to normal-Ca medium (1.8 mM Ca) and/or low-Ca medium (2 μ M Ca) on mammary TJ permeability. AN, normal-Ca on apical side; AL, low-Ca medium on apical side; BN, normal-Ca on basolateral side; BL, low-Ca medium on basolateral side. Means represent the average of $n=5$ inserts per treatment. Means with different letters per time point differ at $P < 0.05$.

Figure 2 Effect of PTHrP on mammary TJ formation. The differentiation medium, containing a normal-Ca concentration of 1.8 mM, was supplemented with 0, 10, or 100 nM PTHrP for 48 h.

occurred (Fig. 1). Basolateral exposure to low-Ca medium had no effect on this pattern of TJ formation. In contrast, low-Ca medium on the apical side nearly prevented TJ formation during the entire experiment, regardless of the type of medium on the basolateral side (Fig. 1).

Effect of PTHrP on mammary TJ formation

Subsequent experiments examined the effect of 0, 10 and 100 nM PTHrP on TJ formation. PTHrP did not appear to have an effect on TJ when cells were cultured in normal-Ca medium (Fig. 2). However, under limiting Ca conditions PTHrP caused an increase in TER, with the highest dose resulting in the biggest increase (Fig. 3). When cells were kept initially in low-Ca medium TJ formation was suppressed but when the medium was replaced with normal-Ca medium at 0 h and Ca was no

Figure 3 Effect of PTHrP on mammary TJs under normal and low-Ca conditions. Low-Ca conditions were introduced 3 days prior to 0 h and continued as indicated. An asterisk indicates cells maintained in low-Ca medium during the 3 days prior to 0 h and then switched to normal-Ca medium at 0 h. Normal-Ca and low-Ca media contain respectively 1.8 mM and 2 μ M Ca. Means represent the average of $n=5$ inserts per treatment. Means with different letters per time point differ at $P<0.05$.

longer limiting from then on, TER increased rapidly and within 24 h was no different from that in control cells maintained in normal-Ca medium throughout the experiment. Moreover, in this case, there was no longer a PTHrP effect (Fig. 3).

Effect of PTHrP on TJs mediated through apical Ca-channels

Experiment 1 indicated that Ca-channels in the apical membrane are important for TJ formation. This, and the effect of PTHrP on TJs when extracellular Ca is limiting (Fig. 3), prompted us to study the role of PTHrP on apical Ca-channels and how this may effect TJs in mammary cells. Figure 4 demonstrates that when extracellular Ca was limiting TER does not increase (control) but that addition of PTHrP increased TER within 24 h ($P<0.08$) and as such confirms the data in Fig. 3, but, when after 24 h, the Ca-channel activator Bay K-8644 was added a further increase in TER was observed at 48 h. At this stage TER values were not statistically different from those where Bay K-8644 was added at 0 h. In contrast, Bay K-8644 by itself, without PTHrP, did not increase TJ formation.

To study further the Ca-channel-mediated effect of PTHrP on TJ we next investigated the effect of Nifedipine, a Ca-channel blocker. Consistent with earlier findings, PTHrP caused a significant increase in TER after 24 h, but when Nifedipine was added after 24 h TER significantly dropped (Fig. 5). Furthermore, there was no difference in TER between cells maintained in the presence of Nifedipine alone or Nifedipine and PTHrP combined from 0 h onwards, and TER decreased to close to zero within 48 h.

Figure 4 Effect of PTHrP (100 nM) and the Ca-channel activator Bay K-8644 (Bay K) on mammary TJ formation. Cells were maintained in low-Ca medium (2 μ M Ca) throughout the experiment and starting 3 days prior to 0 h. PTHrP was added at 0 h and Bay K-8644 was added at 0 h or at 24 h as indicated. Means represent the average of $n=5$ inserts per treatment. Means with different letters per time point differ at $P<0.05$.

Effect of PTHrP on TJ protein expression

Occludin and claudin are the major known true integral transmembrane TJ proteins. To understand further how the PTHrP effects on TJs are mediated we examined the expression of these TJ proteins in the presence or absence of PTHrP. Figure 6 shows the protein expression data for occludin. Under normal-Ca conditions there was a low level of occludin expression (lane 1), whereas expression was highest when TJs were challenged by low-Ca conditions (lane 2). PTHrP reduced occludin expression in

Figure 5 Effect of PTHrP (100 nM) and the Ca-channel blocker Nifedipine (Nif) on mammary TJ formation. Cells were maintained in low-Ca medium (2 μ M Ca) throughout the experiment and starting 3 days prior to 0 h. PTHrP was added at 0 h and Nifedipine was added at 0 h or at 24 h as indicated. Means represent the average of $n=5$ inserts per treatment. Means with different letters per time point differ at $P<0.05$.

a dose-dependent fashion (lanes 3 and 4). Claudin-1 was expressed in COMMA-1D mammary cells but the level of expression was not affected by PTHrP (data not shown).

PTHrP effects on TJ are independent of prolactin

To examine if prolactin was involved in mediating the PTHrP effect on TJ an experiment was conducted in which the effects of PTHrP were studied in the presence or absence of prolactin under low Ca conditions (Fig. 7). Prolactin was added at a concentration of 5 μ g/ml, which was shown previously to elicit the maximum effect on TJs (Stelwagen *et al.* 1999). Although the cells treated with both prolactin and PTHrP showed the highest increase in TER at 24 h, by 48 h the TER in all cells receiving PTHrP was significantly elevated. The presence of prolactin did not result in a significant additional increase in TER.

Figure 6 Effect of low-Ca conditions and PTHrP on mammary TJ protein expression. (A) Representative Western analyses of occludin. (B) Average band densities of six occludin analyses. Cells were exposed to normal Ca without PTHrP (lane 1) or to low Ca conditions with 0 nM (lane 2), 10 nM (lane 3), or 100 nM (lane 4) PTHrP. In B, means with different letters differ at $P<0.05$. arb. units, arbitrary units.

Figure 7 Effect of PTHrP (100 nM) with or without prolactin (PRL) on mammary TJ formation. Cells were maintained in low-Ca medium (2 µM Ca) throughout the experiment and starting 3 days prior to 0 h. Means with different letters per time point differ at *P* < 0.05.

Discussion

Reducing the frequency of milk removal from the mammary gland to only once daily induces a rapid loss of TJ barrier function (Stelwagen *et al.* 1994, 1997) and also coincides with a significant reduction in mammary PTHrP production (Thompson *et al.* 1994). Moreover, maintaining extracellular Ca levels, presumably to facilitate adequate intracellular levels of Ca, is a prerequisite for preventing mammary TJ breakdown *in vitro* and *in vivo* (Neville & Peaker 1981, Pitelka *et al.* 1983, Stelwagen *et al.* 1995). The role of PTHrP in maintaining cellular Ca homeostasis and Ca transport in epithelia, including that of the mammary gland, is well recognised (Seitz *et al.* 1993, Philbrick *et al.* 1996, Friedman 2000, Lafond *et al.* 2001). Taken together, these data led us to speculate that PTHrP may be involved in the regulation of the mammary TJ barrier function.

According to Bacskai and Friedman (1990) in polarised epithelia extracellular Ca enters the cell via Ca-channels in the apical membrane and is extruded via the basolateral membrane. The mammary epithelium is also a polarised epithelium and in the first experiment we confirmed that in mammary cells Ca sensitivity is indeed mediated only via the apical membrane. Moreover, we also confirmed earlier studies showing that extracellular Ca levels can influence TJ status (Neville & Peaker 1981, Pitelka *et al.* 1983, Stelwagen *et al.* 1995). Thus, for PTHrP to have an effect on mammary TJs it would have to act on the apical side of the cell. It was, therefore, surprising to see that

PTHrP (≤ 100 nM) administered apically did not affect TJs. However, when cells were exposed to PTHrP under Ca-limiting conditions, which led to a rapid loss of TJ barrier function, it did cause an increase in TJ formation. Taken together these data indicate that PTHrP does affect TJs, but only when extracellular Ca is limiting and presumably intracellular Ca pools are not replenished. In this situation PTHrP may increase Ca-channel activity or activate additional Ca-channels to utilise the limited amount of available extracellular Ca more efficiently. This is supported by the work of Bacskai & Friedman (1990) showing that PTHrP is effective in raising intracellular Ca concentrations in renal epithelia.

The results of the experiments in which the Ca-channel activator and blocker were used clearly demonstrated that the effects of PTHrP on TJs are, indeed, mediated through up-regulating apical Ca-channel activity, since the activator significantly enhanced the PTHrP effect while it had no effect on its own and, more importantly, Nifedipine blocked any PTHrP effect. Moreover, when Ca was not limiting, and there would be no need to restore intracellular Ca pools, PTHrP did not have any effect on mammary TJs. It should be pointed out that cells had been kept for 3 days in the low-Ca medium prior to the start of the experiment and that intracellular Ca pools would likely have been partly depleted at the time treatments were applied. Hence the rapid decline in TER when Ca-channels were blocked for a further 48 h. On the other hand when PTHrP, alone or in combination with Bay K-8644, was added, utilisation of the limited amount of

available Ca in the low-Ca medium was enhanced. Taken together, these results clearly demonstrate that PTHrP acts on TJs via up-regulation of Ca-channels in the apical membrane. Such an effect is consistent with the effect of PTH facilitating intracellular Ca replenishment through up-regulation of apical Ca-channels in renal cells (Bacskai & Friedman 1990).

Ca is an important factor in intracellular signalling events and as such is believed to be important for TJs (Cereijido *et al.* 1994, Jovov *et al.* 1994). Our results suggest that the effect of PTHrP on mammary TJs is not a direct one, but instead is indirectly mediated through ensuring that intracellular Ca levels remain sufficient to allow TJ formation. Exactly how changes in intracellular Ca concentration affect TJ barrier function is not fully understood and is beyond the scope of the present study.

To further understand the effect of PTHrP on mammary TJs we also looked at its effect on the expression of occludin and claudin, the two main integral membrane TJ proteins. Expression of occludin was changed by PTHrP, indicating that its effect on TJ barrier function, albeit an indirect effect, is at the level of the TJ itself rather than on cytoplasmic TJ-associated and/or scaffolding proteins that link the TJ to the cytoskeleton (Fanning *et al.* 1996, 1998). Interestingly, the expression of occludin was highest with 0 nM PTHrP and lowest with the 100 nM dose, approaching that observed in cells grown in normal-Ca medium. This suggests that with a low-Ca challenge there is enhanced TJ synthesis (repair), but that there is less synthesis occurring as the level of PTHrP increases, which increasingly facilitates a replenishment and/or maintenance of intracellular Ca stores and thus negates the necessity for excessive levels of TJ protein synthesis. The effects of PTHrP on occludin expression therefore corroborates the TER data. Claudins make up a family of over 20 genes that are expressed in tissue-specific patterns. Claudin-1, however appears to be expressed in most epithelial and endothelial tissues (Tsukita *et al.* 2001) including normal human mammary tissue (Hoewel *et al.* 2002). In our study we showed that claudin-1 is also expressed in murine mammary epithelial cells. However, the claudin-1 expression pattern was unaffected by PTHrP suggesting that claudin-1 expression is not dependent on intracellular Ca levels. This agrees with the finding that in transfected L-fibroblasts claudin-1 exhibited Ca-independent cell adhesion activity (Kubota *et al.* 1999).

We showed previously that the lactogenic hormone, prolactin, is necessary to fully establish the barrier function of mammary TJs (Stelwagen *et al.* 1999). Prolactin also regulates mammary gland PTHrP expression. In rats, a suckling-induced surge in prolactin induced PTHrP mRNA expression, whereas cessation of suckling, which will lead to loss of mammary TJ barrier function, resulted in a sharp decline in prolactin and subsequently in mammary PTHrP mRNA expression (Thiede 1989). This information was instrumental in developing a proposed

model of regulation of mammary TJs, in which a decrease in prolactin brings about a decrease in PTHrP, resulting ultimately in a loss of TJ barrier function (Stelwagen 2001). Results from the current study, however, do not indicate a need for prolactin to be present for PTHrP to assert its effects on mammary TJs and suggest that the effects on TJs of both prolactin and PTHrP are independent of each other.

In conclusion, when Ca is limiting, PTHrP enhances mammary TJ formation indirectly by maintaining intracellular Ca supplies which, in turn, results in enhanced TJ protein expression. In addition, prolactin is not involved in the PTHrP-mediated effects on mammary TJs.

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