Type IV phosphodiesterase activity specifically regulates cAMP-stimulated casein secretion in the rat mammary gland

Linda Pooley*

Hannah Research Institute, Hannah Research Park, Ayr, KA6 5HL Scotland, UK

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Abstract

This study investigates the regulation of cAMP-stimulated casein secretion in rat mammary explants by cAMP phosphodiesterase (cAMP-PDE) activity. cAMP-PDE activity of the lactating rat mammary gland is shown to be provided by three families, types II, III and IV. In mammary explants, general inhibition of the cAMP-PDE activity significantly increased the rate of cAMP-stimulated casein secretion. This effect could be mimicked using the type-IV specific inhibitor rolipram but not by the specific, or combined, inhibition of the type II and type III activity. Only type IV activity significantly affected intracellular accumulation of cAMP whereas all three cAMP-PDE activities were shown to influence the PKA activation ratio in cells. RT-PCR analysis showed that the mammary gland apparently expresses just three type IV isozymes, RNPDE4A5, RNPDE4A8 and RNPDE4D3. A specific role for type IV cAMP-PDE activity in the regulation of casein secretion is suggested and possible mechanisms for the effects of PDEIV activity discussed. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Mammary; Casein; Secretion; Phosphodiesterase; cAMP; PDEIV

1. Introduction

During lactation, mammary epithelial cells synthesise and secrete in a polarised manner, large amounts of the major milk proteins, caseins. Following synthesis, the caseins are transported through the secretory pathway, packaged into secretory vesicles and moved to the apical plasma membrane [1]. Here the majority of the vesicles are released by exocytosis though a small proportion is stored just beneath the plasma membrane for later release by a calcium-regulated process [2]. These two processes are referred to as basal constitutive secretion (calcium-independent) and triggered secretion (calcium-dependent). While we understand the fundamental principles of the basal (Ca^{2+}-independent) secretory pathway, much remains to be learned about the factors that regulate this constitutive passage of caseins through the mammary cell secretory apparatus.

Early in vitro work in rabbit mammary tissue did show acute regulation of the basal secretory pathway by prolactin [3]. It has also been suggested that oxytocin might increase constitutive casein secretion by causing contraction of the myoepithelial cells [4]. A role for the cyclic AMP signalling system was first suggested as early as 1975 and several reports using mammary tissue from different species show that cAMP-elevating agents stimulate the rate of casein secretion [5–7]. More recent work has significantly substantiated the evidence for a cAMP-mediated pathway showing that PKA mediates the tonic positive regulation of this process [8]. These authors showed that agents that caused the activation of PKA increased the rate of secretion of newly synthesised caseins and, conversely, inhibition of PKA activity slowed casein secretion.

In the cell, activation of PKA requires a critical threshold concentration of cAMP to be achieved. At any given time, the net intracellular concentration of cAMP is the consequence of the competing rates of its synthesis and its degradation. Cyclic AMP phosphodiesterases (cAMP-PDEs) provide the sole means of degradation of cAMP within the cell. Hence, cAMP-PDEs play a critical role in the regulation of intracellular cAMP concentrations and so may provide a point of regulation of cAMP-mediated processes. cAMP-PDEs are a complex family of enzymes and over 30 individual isozymes have now been identified [9]. These isozymes are expressed in a cell- and tissue-specific manner,

Abbreviations: IBMX, 3-isobutyl-1-methylxanthine; EHNA, erythro-9-(2-hydroxy-3-nonyl)-adenine; cAMP-PDE, cAMP phosphodiesterase; RNPDE4, Rattus norvegicus phosphodiesterase 4; CaM, calmodulin; PCA, perchloric acid

* Tel: +44-1292-674027; fax: +44-1292-674003.
E-mail address: pooley@hri.sari.ac.uk (L. Pooley).
differ in their catalytic and regulatory properties and may be targeted to discrete subcellular localisations [9,10]. This complexity is thought to allow for dynamic cell-specific controls on cAMP levels and hence cAMP-mediated processes [10]. Indeed, individual cAMP-PDE isozyme activities within a cell have been shown to be important in the control of a variety of physiological processes. These include PDE3 mediating the antilipolytic effect of insulin [11], PDE4 activity negatively regulating the secretion of gonadotropin-releasing hormone from neuronal cells [12], and in mesangial cells, compartmentalisation of the cAMP signalling pathway by PDE activity, such that PDE3 regulates apoptosis but PDE4 regulates superoxidation [13].

In lactating rat mammary epithelial cells, early work indicated that the mammary gland contains at least two forms of cAMP-PDE, a cGMP-stimulated low-affinity cyclic nucleotide PDE and a high-affinity cAMP-specific PDE [14]. From in vitro experiments using acini, it was also suggested that the mammary gland contains cAMP-PDE activity subject to regulation by insulin [15].


difficult to identify. A specific role for the PDE4 family in the regulation of cAMP-stimulated casein secretion is reported. Of the PDE4 isozymes expressed in the rat mammary gland have been investigated using modern biochemical and molecular biological approaches, the individual PDE families and PDE isozymes expressed in the rat mammary gland have been identified. A specific role for the PDE4 family in the regulation of cAMP-stimulated casein secretion is reported.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all reagents were supplied by Sigma Chemical Company (Poole, Dorset, UK), BDH (Glasgow, Scotland, UK) and Fisher Scientific, (Loughborough, Leics, UK). Radiochemicals and incubation media were from ICN Pharmaceuticals (Basingstoke, Hants, UK). Except where indicated, all operations and experiments were performed at 37 °C under an atmosphere of 95%O2:5%CO2. All buffers and incubation media were pregassed for 15 min with O2/CO2 (95:5%). Shaking water baths were set at 180 strokes/min.

2.2. Animals

Wistar rats were maintained on a standard chow diet (Labsure irradiated CRM diet, Labsure, Poole, UK) and water ad libitum. All animals were in their first pregnancy and at day 10 or 11 of lactation. The number of pups per mother was adjusted to 10 within 24 h of birth. Ethical aspects of animal care were in compliance with applicable guidelines and licensing requirements administered by the UK Home Office.

2.3. Preparation of mammary explants and acini

On the day of the experiment, mammary tissue was routinely removed from the animal between 09:00 and 10:00 into 20 ml of KRBG buffer (KREBS buffer as specified by Krebs and Henseleit [20] modified to contain 5 mM glucose and 1.2 mM CaCl2). The tissue was coarsely cut, washed with KRBG and chopped into tiny explants using a multi-blade chopper. Acini were prepared by suspending explants in 30 ml of digestion medium (30 ml of KRBG containing 600 mg of dialysed fatty acid-free fraction V BSA, 1500 mg of dialysed Ficoll 400 and 40 mg of Collagenase (type CLS 3 from Worthington Biochemical Corporation, Lakewood, NJ, USA)) in a 250-ml conical flask for 60 min with shaking. The resulting digest was strained through a 400 μM nylon filter, centrifuged at 1000 × g for 15 s, washed three times in KRBG containing 2% Ficoll and finally suspended in KRBG containing 2% Ficoll and 4% BSA.

2.4. Metabolic pulse-chase labelling of newly synthesised protein and measurement of casein secretion

Explants prepared from approximately 3 g of tissue were washed, radiolabelled, and the labelling reaction quenched as described by Clegg et al. [8]. Radiolabelling was performed for 3 min using 500 μCi of TRAN35S-label™ in 10 ml RPMI-1640 (lacking methionine, cysteine and cystine). Unincorporated precursor was removed by washing and the explants suspended in 10 ml of RPMI-1640. Aliquots of 0.5 ml (typically containing 150–200 mg of explants) were then added to 3.5 ml of RPMI-1640, in 25-ml polycarbonate flasks in a shaking water bath. Pulse label was chased for 30 min in the presence or absence of putative effectors of secretion. This experimental time frame has previously been shown to allow passage of newly synthesised caseins through the mammary secretory apparatus [8,21]. The proportion of newly synthesised caseins secreted into the extracellular medium and retained in the mammary explant was assayed and calculated according to the method of Seddiki and Ollivier-Bousquet [22] as modified by Clegg et al. [8]. Briefly, explants and incubation medium were separated by gentle centrifugation. Incubation medium from
a single incubation was filtered through a 5.0 M filter while explants from a single incubation were weighed and homogenised into 1 ml of 0.2% SDS. Total secreted protein was then collected from the samples of filtered incubation media, and total cell-associated protein from the homogenised explants, by TCA precipitation. The precipitates were separated by SDS-PAGE (15% gels) and the position of the radiolabeled caseins detected by fluorography. The casein bands were cut from the gel and quantified by scintillation counting. For each incubation, the total amount of labelled precursor incorporated was calculated as Bq/mg of tissue by summing radioactive protein in the medium and in the precursor incorporated was calculated as a fraction of this total.

After incubation, the remaining medium was then collected from the samples of filtered incubation media, and total cell-associated protein from the homogenised explants, by TCA precipitation. The precipitates were separated by SDS-PAGE (15% gels) and the position of the radiolabeled caseins detected by fluorography. The casein bands were cut from the gel and quantified by scintillation counting. For each incubation, the total amount of labelled precursor incorporated was calculated as Bq/mg of tissue by summing radioactive protein in the medium and in the precursor incorporated was calculated as a fraction of this total.

Using radiolabelled explants from a single rat, experiments were designed such that controls and treatments were paired and replicated as indicated. For a given experimental condition, the resulting pairs of values obtained from multiple animals were then compared using Student’s t-test for paired samples.

2.5. Determination of PDE activities

PDE activity with 1 µM cAMP as substrate was assayed by a modification of the two-step procedure of Thomson and Appleman [23] and Rutten et al. [24] as described by Marchmont and Houslay [25]. The buffer used was 20 mM Tris/HCl, 10 mM Mg2+ supplemented with 2 mM EGTA [26]. The effects of Ca2+ /calmodulin (CaM), cGMP and the PDE inhibitors 3-isobutyl-1-methylxanthine (IBMX), erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) (a kind gift from Dr. Podzuweit, Max Planck Institute for Physiology and Clinical Research, Bad Neuheim, Germany), milrinone, cilostamide (Calbiochem, Nottingham, UK), and rolipram on PDE activity were all determined as described previously [26–28]. The PDE inhibitors IBMX, EHNA, cilostamide and rolipram were dissolved in 100% DMSO as stock solutions and diluted in 20 mM Tris/HCl pH 7.4 for use in the assay. The residual levels of DMSO were shown not to affect PDE activity at the concentration used in this study.

2.6. Measurement of intracellular cAMP concentrations and PKA activity in mammary acini

Acini suspensions (30 mg/ml) were prepared as above and dispensed into 25 ml polycarbonate flasks. These were incubated for 30 min with shaking in the presence and absence of putative effectors of intracellular cAMP concentrations and PKA activity. Determination of intracellular cAMP concentrations was as previously published [16] with the following minor modifications. For each 1 ml sample, the reaction was stopped using 90 µl of ice-cold 60% perchloric acid (PCA) (final concentration 5% w/v). All samples were processed immediately and neutralised by the addition of 5 M KOH containing 0.5 M triethanolamine. The concentration of cAMP in the samples was determined using a competitive radioligand binding assay [29] using cAMP binding protein prepared from bovine adrenals. For the determination of A-kinase activity, 1 ml of the acini suspension was removed after 15 min incubation to an eppendorf of known weight on ice and spun for 15 s at 1000 × g in a refrigerated microfuge. The supernatant was removed, the weight of acini determined and the samples snap frozen in liquid nitrogen. Samples were then stored at −70 °C until determination of the A-kinase activation ratio. Extracts for the assay of A-kinase were prepared as described by Clegg and Otey [30] and the A-kinase activity measured as previously [30,31].

2.7. Isolation of total RNA from mammary tissue

Total cellular RNA was isolated from mammary gland tissue using guanidinium isothiocyanate (Melfold Laboratories, Ipswich, Suffolk, UK), and CsCl centrifugation [32]. All preparations of RNA were resuspended in water and their RNA concentration determined according to UV absorbance measured at 260 nm. All preparations were assessed qualitatively on a 1.2% formaldehyde agarose gel [33].

2.8. RT-PCR

RT-PCR was performed using the Access RT-PCR system from Promega according to manufacturer’s instructions. Reactions routinely contained 1 µg of mammary RNA and 50 pmol of both upstream and downstream primer. Primers were designed as previously described [34] and supplied by Cruachem (Glasgow, Scotland, UK). Negative controls were prepared by replacing the RNA template with nuclease-free water, according to the manufacturer’s instructions. Positive controls were performed using RNA from tissue known to express the cAMP-PDE isozyme being investigated. Reaction products were resolved by electrophoresis on a 1.5% agarose gel and visualised with ethidium bromide under UV light.

3. Results

3.1. Inhibition of cAMP-PDE activity stimulates the rate of cAMP-induced casein secretion in rat mammary explants

To assess the influence of cAMP-PDE activity on casein secretion, putative effectors of the cAMP system were added immediately after the pulse labelling of explants and cumulative casein secretion measured following the subsequent 30 min period of incubation (see Materials and methods). The results are shown in Table 1.

Freshly prepared rat mammary explants typically secreted about 20% of newly synthesised casein within the 30 min time course of the experiment. General inhibition of cAMP-PDE activity by IBMX failed to increase the amount of casein secreted. However, general inhibition of cAMP-PDE activity did increase the rate of cAMP-stimulated
Table 1
Effect of general cAMP-PDE inhibition on the rate of casein secretion from lactating rat mammary explants

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Effect</th>
<th>Percent casein secreted (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additions</td>
<td>Control</td>
<td>20.91 ± 1.40</td>
</tr>
<tr>
<td>1 mM IBMX</td>
<td>General inhibition of cAMP-PDE activity</td>
<td>18.55 ± 2.19</td>
</tr>
<tr>
<td>10 μM Forskolin</td>
<td>Elevation of intracellular cAMP levels</td>
<td>23.73 ± 0.83*</td>
</tr>
<tr>
<td>10 μM Forskolin and 1 mM IBMX</td>
<td>Elevation of intracellular cAMP levels</td>
<td>26.6 ± 0.95*</td>
</tr>
</tbody>
</table>

Casein secretion was measured as described in Materials and methods.
General cAMP-PDE inhibition using 1 mM IBMX had no effect on casein secretion. Forskolin significantly (P < 0.01) increased the rate of casein secretion compared to control, untreated samples (data from six separate animals: three experimental replicates per animal). General cAMP-PDE inhibition significantly increased the effects seen using forskolin (P < 0.01). Data from 15 separate animals (three replicates per animal) was shown to be significant by both paired Student’s t-test and ANOVA. All data is presented as the mean (± S.E.) rate of casein secretion.

* Denotes values that differ significantly from control.
† Denotes values that differ significantly from all others.

Full text:

3.2. Rat mammary gland expresses type II, type III and type IV cAMP-PDE activity

The cAMP-PDE activities expressed in the rat mammary gland (Table 2) were determined using experimental approaches previously described [26–28].

PDE1 activity can be selectively stimulated by the addition of calcium and CaM [35]. In these experiments, no increase in the total PDE activity of the homogenate was detected when calcium and CaM were included in the assay, indicating that PDE1 enzyme activity could not be detected in homogenates of rat mammary explants. PDE2 activity can be selectively inhibited by the compound EHNA (10 μM) [36], with maximum inhibition being achieved in the presence of maximally stimulating concentrations of cGMP [28]. In both the presence and absence of cGMP, approximately 17% of the total PDE activity of the rat mammary gland homogenate was inhibited by this compound. The PDE3 selective inhibitors, cilostamide (5 μM) and milrinone (5 μM) [37], were used to gauge the magnitude of PDE3 activity and showed that about 13% of the total activity could be attributed to type III activity. Rolipram (10 μM), a PDE4-selective inhibitor [9] inhibited some 70% of the total cAMP-PDE activity.

In experiments using 100 μM IBMX, only 82.1 ± 0.1% inhibition of cAMP-PDE activity was observed, suggesting that a small amount of PDE7 or PDE8 activity may be present [26,38,39]. As there are no specific inhibitors available for these two families, quantification of their activity is difficult. However, given that the combined activities of the types II, III and IV PDEs (26, 20.5 and 102 pmol/min/mg, respectively) equates with the total measured activity (144 ± 14 pmol/min/mg), and that the simultaneous inhibition of type II, III and IV PDEs using EHNA, milrinone and rolipram caused inhibition of a magnitude similar to that seen using 1 mM IBMX, it was assumed that any contribution of PDE7 or PDE8 activity to mammary gland cAMP-PDE activity is negligible. Furthermore, for the purpose of this study, the effects of general PDE inhibition on the rate of casein secretion can be completely mimicked by inhibition of PDE4 activity using rolipram at concentrations that do not inhibit PDE7 or PDE8 activity [38,39] (see later). Therefore, it was taken that any PDE7 or PDE8 activity has no effect on casein secretion. Consequently, the possibility of their expression in the mammary gland was not explored further.

Table 2
Effect of selective PDE inhibitors and activators on PDE activity of mammary gland explants taken from rats at mid-lactation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activity determined</th>
<th>Calculated PDE activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additions</td>
<td>Total</td>
<td>144.3 ± 14.0</td>
</tr>
<tr>
<td>Ca2+/CaM-stimulated component</td>
<td>Type I</td>
<td>not detected</td>
</tr>
<tr>
<td>EHNA-inhibited component</td>
<td>Type II</td>
<td>25.6 ± 5.8</td>
</tr>
<tr>
<td>Cilostamide-inhibited component</td>
<td>Type III</td>
<td>21.4 ± 8.1</td>
</tr>
<tr>
<td>Milrinone-inhibited component</td>
<td>Type III</td>
<td>19.4 ± 6.7</td>
</tr>
<tr>
<td>Rolipram-inhibited component</td>
<td>Type IV</td>
<td>102.2 ± 11.4</td>
</tr>
<tr>
<td>IBMX (1 mM)-inhibited component</td>
<td></td>
<td>135.8 ± 14.1</td>
</tr>
<tr>
<td>IBMX (100 μM)-inhibited component</td>
<td></td>
<td>116.1 ± 11.8</td>
</tr>
<tr>
<td>EHNA + milrinone + rolipram-inhibited component</td>
<td></td>
<td>133.5 ± 0.87</td>
</tr>
</tbody>
</table>

Mammary explants were harvested as described in Materials and methods. Explants (approximately 100 mg) were homogenised into 1 ml 20 mM Tris/HCl pH 7.4 containing protease inhibitors by three 5-s bursts of an Ultra-Turrax T25 homogeniser (Scientific Laboratory Supplies, Nottingham, UK). Cellular debris was removed by centrifugation at 1000 g for 5 min at 4 °C. The supernatant was removed and designated “homogenate”. Total activity was determined using 1 μM cAMP as substrate with no additions. Type II activity (EHNA-inhibited component) was calculated as the difference between this total activity and the activity measured in samples treated with EHNA. Similarly, Type III and Type IV activities were calculated as the difference in measured activity between this total activity and samples treated with cilostamide/milrinone and rolipram, respectively. All activities are expressed as pmol cAMP hydrolysed/min/mg homogenate and shown as the mean ± S.E.
Table 3
Effect of selective PDE inhibitors on the rate of forskolin-stimulated casein secretion from rat mammary explants

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative rate of casein secretion</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 μM Forskolin</td>
<td>100*</td>
</tr>
<tr>
<td>10 μM Forskolin + 1 mM IBMX</td>
<td>116.7 ± 4.1 b</td>
</tr>
<tr>
<td>10 μM Forskolin + 10 μM EHNA</td>
<td>104 ± 3.4*</td>
</tr>
<tr>
<td>10 μM Forskolin + 10 μM milrinone</td>
<td>104 ± 5.0*</td>
</tr>
<tr>
<td>10 μM Forskolin + 10 μM milrinone</td>
<td>99.87 ± 8.4*</td>
</tr>
<tr>
<td>and 10 μM EHNA</td>
<td></td>
</tr>
<tr>
<td>10 μM Forskolin + 10 μM rolipram</td>
<td>122 ± 7.0b</td>
</tr>
</tbody>
</table>

Casein secretion was measured as described in Materials and methods. The mean rate of casein secretion is expressed as a percentage relative to that seen in explants (+ S.E.) treated with 10 μM forskolin, which is arbitrarily set as being 100%. The data shown is from at least three separate animals. For each animal, four paired experimental samples were analysed for each of the conditions. Values with different superscript differ significantly (P<0.05) from each other.

3.3. Type IV cAMP-PDE activity specifically regulates casein secretion

Specific inhibitors were used to assess the relative contribution of each individual cAMP-PDE family to the control of cAMP-stimulated casein secretion. For each individual inhibitor, cumulative forskolin-stimulated casein secretion in the presence of the inhibitor was compared to the cumulative casein secretion from explants treated with forskolin alone. The results (Table 3) are expressed as previously [8] as the rate of secretion in the presence of the inhibitor relative to casein secretion in the presence of 10 μM forskolin only, which is arbitrarily set as being 100%.

The type II PDE inhibitor, EHNA (10 μM), had no effect on the rate of forskolin-stimulated casein secretion. Although a slight increase in the average rate of casein secretion relative to that seen using 10 μM forskolin alone was observed, this rise was not statistically significant. The influence of type III cAMP-PDE activity in the rat mammary gland was similarly assessed using 10 μM milrinone. Again, no significant increase in the rate of forskolin-stimulated casein secretion was observed. Indeed, even when 10 μM EHNA and 10 μM milrinone were administered simultaneously, together inhibiting about one third of the total cAMP-PDE activity in the mammary explants, still no significant increase in the rate of forskolin-stimulated casein secretion could be detected. Specific inhibition of the Type IV cAMP-PDE activity by rolipram (10 μM) in the presence of forskolin increased casein secretion by a significant amount compared to that seen using just forskolin. The rate of forskolin-stimulated secretion in the presence of rolipram...
was the same as that seen in the presence of the general PDE inhibitor IBMX. These results suggest that in mammary epithelial cells, the rate of cAMP-stimulated casein secretion is specifically regulated by type IV cAMP-PDE activity.

3.4. Regulation of casein secretion by type IV cAMP-PDE is accompanied by specific changes in intracellular cAMP and activation of PKA but does not require ongoing transcription

Stimulation of casein secretion by forskolin and rolipram was significantly reduced by the cell-permeant PKA inhibitor myr PKI (a kind gift from Dr. Clegg, Hannah Research Institute, Scotland, UK) [8] (see Table 4). It was thought, therefore, that cAMP-PDE activity may affect casein secretion by regulating intracellular levels of cAMP and PKA activation in the secretory epithelial cells and this possibility was explored in acini. Acini were chosen for this experimental work as they are enriched in the secretory epithelial cells, making measurement of changes in cAMP more easily detectable.

The increase in the rate of forskolin-stimulated casein secretion by application of the general PDE inhibitor IBMX was accompanied by a rapid increase in the accumulation of intracellular cAMP to some five-fold higher than that seen in acini treated with forskolin alone (Fig. 1a). An increase of similar magnitude compared to forskolin treatment alone could be seen using the PDE4 specific inhibitor rolipram. Again, this increase was observed within 5 min of application (Fig. 1a). In contrast, application of the PDE2-selective inhibitor EHNA with forskolin caused a slower accumulation of intracellular cAMP (Fig. 1b). The maximum increase in intracellular cAMP levels that could be achieved using EHNA was just 1.5-fold over a period of 30 min. Inhibition of PDE3 activity using milrinone caused only a very small increase in the levels of intracellular cAMP over and above those achieved using only forskolin (Fig. 1c). Concurrent administration of EHNA and milrinone with forskolin increased cAMP some two-fold (Fig. 1d).

The increases in intracellular cAMP levels were mirrored by alterations in the PKA activation ratio in mammary acini (Table 5). By itself, forskolin caused just a two-fold increase in the PKA activation ratio whereas the inclusion of IBMX with forskolin brought about an increase of some seven-fold. The PKA activation ratio elicited by forskolin could also be increased by inhibition of each of the cAMP-PDE families. The maximum increase in the PKA activation ratio (five-fold) was seen in acini in which PDE4 activity was inhibited (acini treated with forskolin and rolipram). In comparison, the maximum stimulation achieved by inhibition of both PDE2 and PDE3 together was just some three-fold and individually, these PDE families had even less effect on the PKA activation ratio.

PKA elicits a cellular response by phosphorylating target substrate molecules. A possible target of PKA action in this system would be an increase in gene transcription by the translocation of PKA to the nucleus and its binding to CREB/CREM sites. To see if such a mechanism was involved in the cAMP-stimulation of casein secretion, the need for ongoing transcription was signalled with effectors of the cAMP signalling pathway

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated)</td>
<td>0.049 ± 0.011</td>
</tr>
<tr>
<td>10 μM Forskolin</td>
<td>0.098 ± 0.013</td>
</tr>
<tr>
<td>10 μM Forskolin + 1 μM IBMX</td>
<td>0.341 ± 0.023</td>
</tr>
<tr>
<td>10 μM Forskolin + 10 μM EHNA</td>
<td>0.161 ± 0.031</td>
</tr>
<tr>
<td>10 μM Forskolin + 10 μM milrinone</td>
<td>0.123 ± 0.034</td>
</tr>
<tr>
<td>10 μM Forskolin + 10 μM EHNA + 10 μM milrinone</td>
<td>0.171 ± 0.016</td>
</tr>
<tr>
<td>10 μM Forskolin + 10 μM rolipram</td>
<td>0.251 ± 0.014</td>
</tr>
</tbody>
</table>

Mammary acini were prepared and treated as described in Materials and methods. The results shown are from three separate animals. For each treatment, three separate determinations were made. All data are presented as mean ± S.E.
transcription in this system was investigated. Casein secretion from rat mammary explants was measured as previously described in both control (untreated) samples and samples stimulated to increase their rate of casein secretion by the addition of forskolin and rolipram. Transcription was inhibited by the inclusion of 800 nM actinomycin-D (concentrations previously shown to inhibit transcription in mammary explants) [40]. Inhibition of transcription had no effect on the rate of casein secretion (Table 4). This suggests that CREB is not the target of PKA activity in the regulation of casein secretion.

3.5. Rat mammary gland specifically expresses three isoforms of the PDE4 family: RNPDE4A5, RNPDE4A8 and RNPDE4D3

The type 4 family of PDEs is encoded by four separate genes, 4A, 4B, 4C and 4D. Within each of these families, alternative splicing gives rise to multiple isoforms. To assess which members of the type 4 family are expressed in the rat mammary gland and hence which isoforms may regulate casein secretion, RT-PCR studies were undertaken as previously described [36]. Of the four genes known to be expressed in this family, transcripts for only two, 4A and 4D, were detected. No transcripts could be found for either the 4B or 4C families (Fig. 2). In the rat, the 4A gene encodes at least three different splice products RNPDE4A1, RNPDE4A5 and RNPDE4A8 [41] and the 4D gene five different products, RNPDE4D1, RNPDE4D2, RNPDE4D3, RNPDE4D4 and RNPDE4D5 [42]. Using available genetic sequences and splice-variant-specific primers, only transcripts for two of the 4A splice variants RNPDE4A5 and RNPDE4A8 could be detected. Using the genetic sequences available for the rat PDE4D splice variants, only RNPDE4D3 could be detected (Fig. 3). To the author’s knowledge, this data represents the first full characterisation of the type IV cAMP-PDE isoforms expressed in lactating rat mammary tissue.

4. Discussion

This study clearly demonstrates that cAMP-PDE activity negatively regulates the rate of constitutive casein secretion in lactating rat mammary gland. Most interestingly, although the gland contains multiple forms of cAMP-PDE activity, the effects on casein secretion are specifically modulated by PDEIV. This data demonstrates for the first time, a potential physiological role for CAMP phosphodiesterase activity in the mammary gland. Furthermore, it adds to the growing body of evidence that individual PDE activities perform specific cellular functions. The biochemical and RT-PCR data reported represents the first complete characterisation of the cAMP-PDE isoforms of the lactating rat mammary gland.

Previous work has suggested that PKA activity is required to maintain basal levels of constitutive casein secretion [8]. Perturbations of PKA activity act to regulate the rate of casein secretion such that inhibition of PKA decreases secretion whereas elevation of cAMP, and hence PKA activity, increases the rate of secretion. The data presented here confirms these observations and extends them showing that the cAMP-PDE activity of mammary cells also contributes to the regulation of the system. The data suggests that cAMP-PDE activity constrains the extent of PKA activation, and hence in vivo, may limit the amount by which casein secretion can be increased using this signalling pathway.

The relative importance of this regulation in vivo remains to be determined. Previously, much consideration has been given to the calcium-regulated, triggered release of casein [2]. However, given that milk protein secretion is considered largely constitutive rather than triggered, any potential mechanisms of regulation of the Ca^{2+}-independent, basal-constitutive pathway are of interest. Evidence presented here, coupled with previous findings [8], indicate that the cAMP-signalling pathway may be important in this respect. Physiologically, this pathway may contribute to the mechanisms used by the mother to control milk output.

The cAMP-PDE activity specifically affects cAMP-stimulated casein secretion. The failure of general cAMP-PDE inhibition in the absence of a cAMP-elevating agent to affect casein secretion may be due to the inability of IBMX by itself to significantly increase levels of intracellular
cAMP in mammary cells (as previously shown [16]). Similar effects have been well-documented in hepatocytes (as summarised in Ref. [10]). In hepatocytes, inhibition of cAMP-PDE activity in the absence of additional effectors has no effect on the activity of PKA (attributable to a low basal level of adenylate cyclase activity). However, once adenylate cyclase and PKA are activated, cAMP-PDE activity can have a profound influence on the magnitude and duration of that activation [10]. Previous data [16] and observations made here suggest the same scenario is also apparently true in mammary epithelial cells. The nature and activity of the adenylate cyclase forms expressed in the mammary gland has not been fully explored or reported.

The lactating rat mammary gland is shown to express just three cAMP-PDE activities, type II, III and IV. The type II and type IV activity measured represents that previously reported [14]. Using selective inhibitors the presence of a third activity, PDE3 is confirmed. Type III PDE activity can be stimulated by insulin [11] and this PDE is presumably the target of the insulin effects in mammary acini previously reported by Aitchison et al. [15]. A fuller investigation of the type IV activity using RtpCR showed just three type IV isozymes, RNPDE4A5, RNPDE4A8 and RNPDE4D3 are expressed. The apparent expression of RNPDE4A8 is particularly noteworthy as it was previously thought that this isoform was expressed predominantly if not exclusively in testes [43]. Notably though, in that paper, the authors did not screen lactating mammary tissue. Together these results present the fullest characterisation of cAMP-PDE activity of lactating mammary gland to date.

Given the number of cAMP-PDE activities in the gland, it is particularly interesting that casein secretion is specifically regulated by type IV activity. Clearly, the type IV activity has the most profound effect on intracellular cAMP levels. In addition, considering the PKA activation ratio data, although inhibition of PDE4 does not achieve the same magnitude of activation seen using IBMX, it has a far greater influence than PDE2 and PDE3 activities. These differences may possibly partly explain the specific regulation of casein secretion by PDE4 activity. However, although inhibition of PDE2 and PDE3 do not affect cAMP and PKA to the same extent as PDE4, both do have some influence on these parameters. Why then, do they not affect casein secretion?

It is hypothesised that the coordination in both space and time of the components of the cAMP signalling system allows the cell to generate compartmentalised cAMP responses within a single cell [10]. This results in localised concentration gradients of cAMP and specific pools of PKA activity. Depending upon their location and activity within the cell, individual PDE activities may regulate the different pools of cAMP and PKA. In this context, it is worth noting that the isozymes expressed in the rat mammary gland have previously been shown to be able to be targeted to specific intracellular locations [41–43]. Such targeting gives them the potential to participate in compartmentalised intracellular responses to cAMP as discussed. Also critical to this model is the location of the downstream target of PKA. It has previously been suggested that a phosphorylation event, possibly associated with the Golgi apparatus, may be important in the PKA-mediated regulation of casein secretion [8]. The finding that ongoing transcription is not required for PKA-mediated increases in casein secretion lends weight to the hypothesis that a phosphorylation event is critical in this process.

Using compartmentalisation as a working model, the data presented here would suggest that PDE4 activity influences the pool of cAMP and PKA activity associated with casein secretion but that PDE2 and PDE3 activities do not. Further work to characterise the subcellular localisations of the components of the cAMP-signalling system and the nature and location of the target of PKA phosphorylation may support this hypothesis. It may also provide an insight into which individual isoforms are involved in the regulation of casein secretion. In this respect, the role of PDE4D3 would be particularly interesting as this isozyme has previously been implicated in the negative regulation of secretory processes [12]. Unfortunately, such questions could not be addressed using the methods employed in this study, as no gene-family specific inhibitors are yet available. Similarly, an antisense-approach to specifically ablate one activity could not be applied to this system as the tissue and secretory activity can only be maintained for a short time in culture. Indeed, no robust, transfactable, cell system that secretes casein at physiological levels exists. The development of such an in vitro system would greatly aid such experiments and also allow for real-time intracellular imaging of key components of the cAMP-signalling system.

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