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RESEARCH COMMUNICATION

Exocytosis from permeabilized lactating mouse mammary epithelial cells

Stimulation by Ca^{2+} and phorbol ester, but inhibition of regulated exocytosis by guanosine 5′-[γ-thio]triphosphate

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Lactating mouse mammary epithelial cells secrete large amounts of milk protein via constitutive or regulated exocytotic pathways. Secretion through both pathways was quantified by assaying the release of [^{35}S]methionine-labelled trichloroacetic acid-precipitable proteins from digitonin-permeabilized secretory acini isolated from mammary glands of 10-day-post-partum lactating mice. Protein secretion from the isolated permeabilized cells was either Ca^{2+}-dependent (regulated) or Ca^{2+}-independent (constitutive). In both cases there was a requirement for ATP. Addition of the phorbol ester phorbol 12-myristate 13-acetate (PMA) caused a marked increase in the percentage protein secretion from the cells in a Ca^{2+}-independent manner. However, the non-hydrolysable GTP analogue guanosine 5′-[γ-thio]triphosphate (GTP[S]) caused a partial inhibition of Ca^{2+}-dependent exocytosis, while having no significant effect on Ca^{2+}-independent exocytosis. Thus the GTP[S] is exerting its effect on the regulated pathway at a site subsequent to protein sorting and packaging into secretory vesicles at the trans-Golgi network.

INTRODUCTION

Protein secretion by exocytosis is a mechanism common to most cell types, and may be either constitutive or regulated [1]. Constitutive exocytosis is the secretion of protein immediately following synthesis and packaging into secretory vesicles, and this was the sole process by which lactating mammary epithelial cells were thought to secrete large quantities of caseins and other milk proteins [2–4]. However, recent results have shown that lactating mammary epithelial cells also possess a regulated exocytotic pathway, whereby stored secretory vesicles undergo exocytosis in response to a rise in the cytosolic free calcium concentration ($[Ca^{2+}]_i$) [5]. Whereas much work has centred on the effects of various hormones and growth factors on mammary cells and the induction of differentiation [6], relatively little recent work has been carried out to investigate the intracellular biochemical mechanisms which control exocytosis from these cells. Recent work has disclosed Ins(1,4,5)P_3-sensitive stores [7] and $[Ca^{2+}]_i$ oscillations modulated by mechanical stimulation [8]. These findings, coupled with the discovery of the Ca^{2+}-dependent regulated exocytotic pathway, give rise to the possibility of either a hormone-sensitive and/or a mechanically stimulated pathway for secretion.

One way to control precisely the ionic composition of the cell experimentally is to permeabilize the plasma membrane [9–12]. By using the plant glycoside digitonin we have been able both to manipulate the $[Ca^{2+}]_i$ to examine directly the presence of Ca^{2+}-regulated exocytosis, and also to investigate the control of the constitutive and regulated exocytotic pathways. We have paid particular attention to protein kinase C (PKC), which increases secretion in other cell types [13,14], and to the effect of activation of GTP-binding proteins by guanosine 5′-[γ-thio]triphosphate (GTP[S]), which has been shown to have stimulatory or inhibitory effects on the exocytotic pathway in many different cell types [15].

MATERIALS AND METHODS

Materials

Digitonin was obtained from Calbiochem; GTP[S] was from Boehringer. Unless stated otherwise, tissue culture media were obtained from Gibco and other reagents from Sigma.

Preparation of mammary epithelial cell acini from the lactating mammary gland

Mammary tissue was removed from Tuck's No. 1 mice at mid-lactation (10 days post-partum) and dissociated by incubation with 0.14% collagenase (Worthington) for up to 90 min at 37 °C [5,16]. Digestion was stopped when the majority of acini were reduced to clumps of 10–30 cells, and the cell suspension was filtered through a 150 μm-pore nylon mesh (Lockertex). Cells were harvested by centrifugation at 80 *g* for 5 min and resuspended in Hanks' buffered saline solution supplemented with 5 μg of insulin/ml, 0.1 μg of cortisol/ml, 0.04 mg of DNAase I/ml (Boehringer) and 0.1 mg of trypsin inhibitor/ml. Cell pellets were washed by centrifugation and filtered through a 53 μm-pore nylon mesh. The filtrate was centrifuged as above and cells were resuspended in culture medium (50% M199, 50% Ham's F12, 5 μg of insulin/ml, 0.01 μg of prolactin/ml, 0.01 μg of epidermal growth factor/ml and 0.001 μg of tri-iodothyronine/ml. The cells were kept in tissue culture flasks for 1 h at 37 °C in an atmosphere containing 5% CO_2, to remove fibroblasts and macrophages which attach to the flasks. The resulting cell suspension showed 90–95% cell viability, as assessed by Trypan Blue exclusion, and approx. 85% of the cells were present in acini [5].

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; GTP[S], guanosine 5′-[γ-thio]triphosphate; $[Ca^{2+}]_i$, intracellular free calcium concentration; PKC, protein kinase C; CHO, Chinese hamster ovary.

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Incorporation of [^{35}S]methionine into cellular proteins, permeabilization of mammary epithelial cell acini, and assay of protein secretion

Freshly isolated acini were incubated in culture medium for 1 h with 25 μCi of L-[^{35}S]methionine/ml (cell labelling grade; specific radioactivity > 1300 Ci/mmol; Amersham) at a density of 6×10^6 cells per ml. The cell suspension was then centrifuged at 80 *g* for 5 min. The supernatant was discarded and the cell pellets were resuspended in ice-cold permeabilization buffer (139 mM-K^+ glutamate, 2 mM-ATP, 2 mM-$MgCl_2$, 5 mM-EGTA, 20 mM-Pipes, pH 6.5), and centrifuged for a further 5 min at 80 *g*. The supernatant was discarded and the cell pellets were resuspended in cold permeabilization buffer (lacking ATP or containing GTP[S] if so indicated) with appropriate amounts of $CaCl_2$ to give the calculated free calcium concentrations at pH 6.5. The cell suspension was supplemented with 10 μM-digitonin and cells were then transferred into 24-well tissue culture trays and incubated with or without drug additions for 1 h at 37 °C. Incubations were terminated by centrifugation at 3500 *g* in a Microfuge for 1 min. The incorporation of ^{35}S into cell-associated or released protein was determined by trichloroacetic acid precipitation and scintillation counting as previously described [5].

RESULTS

Freshly isolated intact lactating mammary epithelial cells were examined using Trypan Blue exclusion to determine the extent of permeabilization resulting from the addition of various concentrations of digitonin. More than 95% permeabilization was observed in the presence of 5 μM-digitonin, either in ice-cold permeabilization buffer or in permeabilization buffer at 37 °C. However, to ensure complete permeabilization of cells, 10 μM-digitonin was adopted as our working concentration.

Using the [^{35}S]methionine pulse–chase assay in conjunction with permeabilization by 10 μM-digitonin, we have begun to investigate the nature of exocytosis from lactating mammary epithelial cells. Table 1 shows the effect of incubating the digitonin-permeabilized cells in the presence or absence of 10 μM free Ca^{2+}. When 2 mM-ATP was present, secretion was greatly enhanced when the cells were incubated in 10 μM free Ca^{2+}. The data in Table 1 were taken from a single batch of cells, but in a series of experiments there was an 8.0 ± 1.7% ($n = 5$ cell batches) Ca^{2+}-dependent component of protein secretion in these circumstances. Table 1 also shows that, while secretion was elicited in the presence of 2 mM-ATP, in the absence of ATP Ca^{2+}-dependent and Ca^{2+}-independent secretion were both greatly decreased, indicating that protein release is likely to be due to exocytosis rather than to leakage from damaged vesicles.

We have studied the magnitude of protein secretion over a physiological range of Ca^{2+} concentrations, and investigated the action of the PKC-activating phorbol ester phorbol 12-myristate 13-acetate (PMA) over this range. Fig. 1 shows that, in both the presence and the absence of 100 nM-PMA, secretion was at a maximum at 10 μM free Ca^{2+}. Addition of PMA increased the percentage protein secretion at every Ca^{2+} concentration examined, even in the absence of added Ca^{2+}. Thus the action of PKC is Ca^{2+}-independent.

We have investigated the effect of guanine nucleotide-binding proteins (G proteins) on exocytosis from permeabilized lactating mammary epithelial cells using GTP[S], a non-hydrolysable analogue of GTP. In the nominal absence of free Ca^{2+}, addition of 100 μM-GTP[S] had no significant effect on exocytosis. However, when 100 μM-GTP[S] was added to the cells at 10 μM free Ca^{2+}, Ca^{2+}-dependent exocytosis was partially inhibited. Data from one cell preparation are shown in Table 2. From data on three separate cell preparations, GTP[S] gave a 54.7 ± 6.5% inhibition. Our data thus show that GTP[S] inhibits the regulated pathway, but not the constitutive pathway.

Table 1. Effect of Ca^{2+} and ATP on protein secretion from permeabilized dissociated lactating mammary acini in a pulse–chase protocol

Mammary cells in suspension culture were incubated for 1 h with 25 μCi of [^{35}S]methionine/ml, washed in permeabilization buffer and 10 μM-digitonin was added. The cells were incubated for an additional 1 h in the presence or absence of 10 μM free Ca^{2+}, with or without 2 mM-ATP. The extent of protein secretion was determined by assaying the counts incorporated into cell-associated or medium trichloroacetic acid-precipitable protein. The data show the percentage of total counts that was secreted, and are means ± S.E.M. ($n = 4$). In the presence of ATP, increased secretion due to Ca^{2+} was statistically significant ($P < 0.02$). In this experiment 2369 c.p.m. were incorporated per 10^6 cells during the pulse-labelling.

Treatment	Radioactivity secreted (% of total of c.p.m. incorporated)
No Ca^{2+}, +ATP	7.9 ± 1.4
10 μM-Ca^{2+}, +ATP	13.8 ± 0.9
No Ca^{2+}, −ATP	4.2 ± 0.1
10 μM-Ca^{2+}, −ATP	0.9 ± 0.1

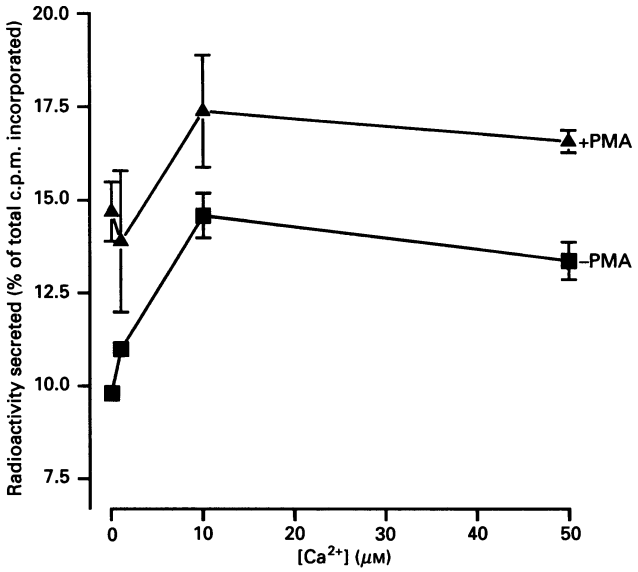

Fig. 1. Effect of Ca^{2+} concentration in presence or absence of PMA on protein secretion from permeabilized dissociated lactating mammary acini in a pulse–chase protocol

After labelling with 25 μCi of [^{35}S]methionine/ml for 1 h, cells were permeabilized with 10 μM-digitonin in permeabilization buffer containing 0, 1, 10 or 50 μM free Ca^{2+} with or without 100 nM-PMA and incubated for a further 1 h. The data show the percentage of total counts that was secreted and are means ± S.E.M. ($n = 4$). In this experiment, 19692 c.p.m. were incorporated per 10^6 cells during the pulse-labelling.

DISCUSSION

Although much work has been conducted in order to understand the intracellular mechanisms operating in the exocytotic pathways of many cell types, surprisingly little has emerged on possible control mechanisms which might exist in lactating mammary epithelial cells. However, we recently demonstrated the occurrence of constitutive and regulated pathways for

Table 2. Effect of Ca^{2+} and GTP[S] on protein secretion from permeabilized dissociated lactating mammary acini in a pulse–chase protocol

After labelling with 25 μCi of [^{35}S]methionine/ml for 1 h, cells were permeabilized with 10 μM-digitonin in permeabilization buffer in the presence or absence of 10 μM free Ca^{2+}, with or without 100 μM-GTP[S], and incubated for a further 1 h. The data show the percentage of total counts that was secreted and are means ± S.E.M. ($n = 4$). The decrease in secretion due to GTP[S] in the presence of Ca^{2+} was statistically significant ($P < 0.01$). In this experiment 2020 c.p.m. were incorporated per 10^6 cells during the pulse-labelling.

Treatment	Radioactivity secreted (% of total of c.p.m. incorporated)
No Ca^{2+}	16.5 ± 1.2
10 μM-Ca^2	30.1 ± 0.2
No Ca^{2+} + GTP[S]	18.3 ± 1.0
10 μM-Ca^{2+} + GTP[S]	24.8 ± 1.3

secretion of milk proteins from these cells [5]. In order to define elements of the secretory pathways further, we have developed a permeabilized mammary cell system. This has enabled us to identify ATP as an intracellular requirement for exocytosis, a finding made originally with adrenal chromaffin cells [9] and subsequently in other secretory systems. With ATP present we have also been able to use this assay to further confirm our finding that there are indeed both Ca^{2+}-dependent and Ca^{2+}-independent exocytotic pathways [5]. As can be seen from Table 1, the presence of 10 μM free Ca^{2+} results in a virtual doubling of protein secretion, a result almost identical to that due to the addition of 10 μM-ionomycin to intact cells [5]. From the dose–response curves shown it can be seen that Ca^{2+}-dependent secretion is at a maximum at 10 μM free Ca^{2+} in both the presence and the absence of PMA, a Ca^{2+}-dependency similar to that seen in other cell types [10,12].

We found that PKC activation stimulated Ca^{2+}-independent exocytosis in permeabilized mammary cells. In most reports PKC-stimulated exocytosis is Ca^{2+}-requiring, but, as is the case in mammary cells, Ca^{2+}-independent secretion has been observed in the presence of PMA from the dense granules of permeabilized platelets [17]. One interesting possibility is that PKC might be stimulated by arachidonic acid [18]. Work on intact mammary cells has shown that protein secretion can be induced by arachidonic acid [19]. Thus it is possible that following the appropriate stimuli there is an elevation of arachidonic acid within the cell and that this leads to the subsequent activation of PKC. One possible way in which PKC and Ca^{2+} may act is via annexin II, a Ca^{2+}- and phospholipid-binding protein, which is a PKC substrate. Annexin II is specifically bound to both the apical membrane and the casein vesicles of lactating mammary epithelial cells [20], and has been implicated in exocytosis in chromaffin cells [21].

GTP-binding proteins have been implicated at several different sites of the secretory pathway in a wide range of secretory cells [15,22]. Such studies have revealed that, in cells which undergo constitutive secretion, non-hydrolysable GTP analogues block secretion at the stage of endoplasmic-reticulum-to-Golgi transport and transport between Golgi cisternae [23]. GTP[S] has also been shown to block transport from the trans-Golgi network to the plasma membrane in constitutively secreting permeabilized CHO cells [24,25]. The complex nature of the involvement of G proteins is illustrated by the fact that in some cells GTP[S] can have stimulatory or inhibitory effects on exocytosis [26,27]. Our data show that, in lactating mammary epithelial cells, it is the regulated pathway which is inhibited, whilst the constitutive pathway is apparently unaffected. This clearly implicates a site of action occurring after trans-Golgi network sorting, at a stage confined to the regulated pathway.

To summarize, we have directly demonstrated a Ca^{2+}-activated exocytotic pathway in permeabilized lactating mammary epithelial cells and shown that ATP is required for both constitutive and regulated secretion. We have also shown that PMA stimulates secretion, and that GTP[S] inhibits the regulated pathway only.

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REFERENCES

1. Burgess, T. L. & Kelly, R. B. (1987) Annu. Rev. Cell Biol. **3**, 142–193
2. Franke, W. W., Luder, M. R., Kartenbeck, J., Zerban, H. & Keenan, T. W. (1976) J. Cell Biol. **69**, 173–195
3. Linzell, J. L. & Peaker, M. (1971) Physiol. Rev. **51**, 564–597
4. Saacke, R. G. & Heald, C. W. (1974) in Lactation: A Comprehensive Treatise, vol. 2 (Larson, B. L. & Smith, V. R., eds.), pp. 147–198, Academic Press, New York
5. Turner, M. D., Rennison, M. E., Handel, S. E., Wilde, C. J. & Burgoyne, R. D. (1992) J. Cell Biol. **117**, 269–278
6. Howlett, A. R. & Bissell, M. J. (1990) Protoplasma **159**, 85–95
7. Yoshimoto, A., Nakanishi, K., Anzai, T. & Komine, S. (1990) Cell Biochem. Funct. **8**, 191–198
8. Furuya, K. & Enomoto, K.-I. (1990) Brain Res. Bull. **25**, 779–781
9. Baker, P. F. & Knight, D. E. (1978) Nature (London) **276**, 620–622
10. Lindau, M. & Gomperts, B. D. (1991) Biochim. Biophys. Acta **1071**, 429–471
11. Dunn, L. A. & Holz, R. W. (1983) J. Biol. Chem. **258**, 4989–4993
12. Burgoyne, R. D. (1991) Biochim. Biophys. Acta **1071**, 174–202
13. Rink, T. J., Sanchez, A. & Hallam, T. J. (1983) Nature (London) **305**, 317–319
14. TerBush, D. R., Bittner, M. A. & Holz, R. W. (1988) J. Biol. Chem. **263**, 18873–18879
15. Gomperts, B. D. (1990) Annu. Rev. Physiol. **52**, 591–606
16. Rennison, M. E., Handel, S. E., Wilde, C. J. & Burgoyne, R. D. (1992) J. Cell Sci., in press.
17. Coorssen, J. R., Davidson, M. M. L. & Haslam, R. J. (1990) Cell Regul. **1**, 1027–1041
18. Shearman, M. S., Naor, Z., Sekiguchi, K., Kishimoto, A. & Nishizuka, Y. (1989) FEBS Lett. **243**, 177–182
19. Seddiki, T. & Olliver-Bousquet, M. (1991) Eur. J. Cell Biol. **55**, 60–70
20. Handel, S. E., Rennison, M. E., Wilde, C. J. & Burgoyne, R. D. (1991) Cell Tissue Res. **264**, 549–554
21. Ali, S. M., Geisow, M. J. & Burgoyne, R. D. (1989) Nature (London) **340**, 313–315
22. Burgoyne, R. D. (1992) Trends Biochem. Sci. **17**, 87–88
23. Melancon, P., Glick, B. S., Malhotra, V., Weidman, P. J., Serafini, T., Gleason, M. L., Orci, L. & Rothman, J. E. (1987) Cell **51**, 1053–1062
24. Miller, S. G. & Moore, H.-P. H. (1991) J. Cell Biol. **112**, 39–54
25. Helms, J. B., Karrenbauer, A., Wirtz, K. W. A., Rothman, J. E. & Wieland, F. T. (1990) J. Biol. Chem. **265**, 20027–20032
26. Sikdar, S. K., Zorec, R., Brown, D. & Mason, W. T. (1989) FEBS Lett. **253**, 88–92
27. De Matleis, M. A., Di Tullio, G., Buccione, R. & Luini, A. (1991) J. Biol. Chem. **266**, 10452–10460

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