Biochem. J. (1984) **224**, 195–200
Printed in Great Britain

Calcium-ion and calmodulin-dependent κ-casein kinase in rat mammary acini

Charles L. BROOKS* and Michael LANDT
Department of Pediatrics and Pathology, Washington University School of Medicine, and St. Louis Children's Hospital, St. Louis, MO 63110, U.S.A.

(*Received 6 June 1984/Accepted 2 August 1984*)

A Ca^{2+}- and calmodulin-dependent casein kinase specific for dephosphorylated bovine κ-casein was identified in a microsomal fraction of mammary acini prepared from rats in late lactation. This phosphorylation has an absolute requirement for Mg^{2+} for either the basal or the Ca^{2+}- and calmodulin-dependent activity. One-half of the maximal stimulation is achieved at a calmodulin concentration of 204 nM in the presence of Ca^{2+}. The Ca^{2+}- and calmodulin-dependent kinase activity (but not the basal activity) is inhibited by trifluoperazine. The casein kinase is associated with a microsomal fraction enriched in markers for plasma membrane and Golgi (5'-nucleotidase and galactosyltransferase respectively). The activity of this casein kinase remains relatively constant throughout lactation, but declines dramatically in 24h when rats are removed from their pups. This activity may represent the physiological activity responsible in part or whole for κ-casein phosphorylation occurring before micelle formation and milk secretion.

Caseins are the major proteins found in milk. This family of proteins is translated, phosphorylated and formed into micelles before being secreted by mammary epithelial cells. Casein phosphorylation occurs after translation has been completed (Turkington & Topper, 1966) but before micelle formation can occur (Bingham *et al.*, 1972*a*). Earlier work has identified a mammary casein kinase in rats able to phosphorylate several milk proteins in a Ca^{2+}- or Mg^{2+}-dependent manner (Bingham *et al.*, 1972*b*; Bingham & Farrell, 1974). These workers found that previous dephosphorylation of the substrate caseins enhanced the enzymic phosphorylation. This enzyme has been shown to be located in the Golgi apparatus and has been solubilized and characterized from bovine sources (MacKinlay *et al.*, 1977; Bingham & Grove, 1979; Szymanski & Farrell, 1982).

There has been a large number of other kinases described that use various caseins as substrates (Hathaway & Traugh, 1982). Most of the kinases are found in tissues other than the mammary and they often do not require previously dephosphorylated caseins to display maximal activity. There is no evidence that these kinases participate in the physiological phosphorylation of milk proteins.

During the past few years calmodulin, a Ca^{2+}-binding protein of approx. 16.8 kDa (Cheung, 1980), has been implicated in the control of a number of cellular processes, including control of secretion through phosphorylation (Means & Dedman, 1980). A number of Mg^{2+}-requiring Ca^{2+}- and calmodulin-dependent protein kinases have been described. Of these, the best-characterized kinase systems are myosin light-chain kinase and phosphorylase kinase (Walsh *et al.*, 1979; Cohen *et al.*, 1978). Other Ca^{2+}- and calmodulin-dependent kinases have been less well characterized and include a glycogen synthase kinase (Payne & Soderling, 1980) and a tubulin kinase (Burke & DeLorenzo, 1981).

As casein phosphorylation is a process integrated with micelle formation and secretion of milk, control of the protein kinases responsible for casein phosphorylation may be important in the regulation of these more distal processes in normal mammary function. Results of previous studies implicates calcium metabolism as a potential regulator of these processes (phosphorylation, micelle formation and secretion). A Ca^{2+}- and Mg^{2+}-dependent ATPase transporter has been identified in the Golgi apparatus of bovine (Baumrucker, 1978), rat (West, 1981) and mouse (Neville *et al.*, 1981) mammary tissue. This activity

* To whom all correspondence should be sent, at present address: Department of Veterinary Pathobiology, Ohio State University, 1925 Coffey Road, Columbus, OH 43210, U.S.A.

reflects the Ca^{2+}-uptake system that partitions this ion into the interior of the Golgi apparatus. The mammary casein kinases in rat and bovine Golgi membranes regulate the phosphorylation of milk proteins in a Ca^{2+}-dependent manner (Bingham *et al.*, 1972*b*; Bingham & Farrell, 1974; Bingham & Grove, 1979; MacKinlay *et al.*, 1977; Szymanski & Farrell, 1982). Farrell (1973) demonstrated phosphorylation of α_{S1}-casein to be required for normal micelle formation in the presence of κ-casein and Ca^{2+}. Finally, in tissues, including islets of Langerhans (Hedeskov, 1980), pancreatic acini (Hokin, 1966), pituitary cells (Tashjian *et al.*, 1978) and mammary acini (Smith *et al.*, 1982), Ca^{2+} has been demonstrated to be necessary during secretion. Because Ca^{2+} might regulate casein phosphorylation directly through a calmodulin-dependent kinase, we examined the effect of Ca^{2+} and calmodulin on the control of α_S-casein and κ-casein phosphorylation in preparations derived from mammary acini of lactating rats.

Experimental

Calmodulin was purified by a fluphenazine affinity column (Charbonneau & Cormier, 1979). α-Casein was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and was prepared by differential isoelectric precipitation (Warner, 1944). Analysis of this product by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Laemmli, 1970) or urea/polyacrylamide-gel electrophoresis revealed two major caseins, α_{S1} and κ. Other minor members of the α_S- casein family were also present. The identity of α_{S1}-casein as the upper band (Fig. 2, lane 2) on sodium dodecyl sulphate/polyacrylamide-gel electrophoretograms was determined by its molecular mass and its co-migration with pure α_{S1}-casein. κ-Casein was determined to be the lower band on sodium dodecyl sulphate/polyacrylamide-gel electrophoretograms by co-migration with pure κ-casein and its susceptibility to rennin treatment. (κ-Casein appeared on sodium dodecyl sulphate/polyacrylamide-gel electrophoretograms at a greater molecular mass than that indicated by sequence data. This is presumably due to the carbohydrate moiety attached to threonine-131.) κ-Casein was purified by the method of Thompson (1966). α-Casein or κ-casein (50mg) was dephosphorylated by treatment with 70 units (μmol/min) of alkaline phosphatase (bovine intestine; Sigma Chemical Co.) in 0.5ml of 10mM-Tris/HCl, pH10, for 7h at 37°C and chromatographed on Sephadex G-100 (Pharmacia, Piscataway, NJ, U.S.A.) in 5mM-ammonium bicarbonate buffer. Dephosphorylated casein was freeze-dried and stored at −20°C. [γ-^{32}P]ATP was purchased from New England Nuclear (Boston, MA, U.S.A.). Lactating female Sprague–Dawley rats were either purchased from Chappel Breeders (Bel Ridge, MO, U.S.A.) or obtained from the colony of the Department of Pediatrics.

Mammary acini were prepared by the method of Katz *et al.* (1974) by using collagenase digestion at 37°C followed by extensive washing in Ca^{2+}- and Mg^{2+}-free iso-osmotic buffer. All subsequent manipulations were performed at 4°C. The prepared acini were homogenized in TSE buffer (10mM-Tris/HCl buffer, pH7.0, containing 300mM-sucrose and 1mM-EGTA) at 10ml/g wet wt. of acini, followed by fractionation by differential centrifugation (Landt *et al.*, 1982). Briefly, homogenate was centrifuged at 600*g* for 5min, and the pellet was designated P_1; the supernatant was then centrifuged at 145000*g* for 10min, and the pellet was designated P_2; finally the supernatant was centrifuged at 145000*g* for 60 min, and the pellet and final supernatant were designated P_3 and S_3 respectively. The protein concentrations of all fractions were determined by the method of Lowry *et al.* (1951), with bovine albumin as standard, and they were stored at −70°C.

Enzyme data characterizing the subcellular fractions have established (C. L. Brooks & M. Landt, unpublished work) that P_1 contained the nuclei and unbroken cells, that P_2 was enriched for mitochondria, lysosomes, secretory vesicles and endoplasmic reticulum, and that P_3 was enriched for plasma membranes and Golgi membranes. S_3 was designated the cytosolic fraction. Homogenate or subcellular fractions were employed in casein kinase assays.

The casein kinase assays were conducted in a total volume of 100μl buffered by 50mM-Pipes (1,4-piperazinediethanesulphonic acid)/NaOH, pH7.6, and containing 10mM-magnesium acetate. The homogenate or subcellular fraction in TSE buffer represented 40μl of the total volume and contained between 25 and 100μg of protein. The protein buffer provided a 0.4mM-EGTA concentration to the reaction mixture in order to minimize ionic Ca^{2+} contamination. Each reaction mixture contained 100μg of casein. Some of the tubes contained Ca^{2+} (1mM) and/or calmodulin (0.6–0.9μM unless otherwise stated. These ingredients were preincubated for 2min at 30°C and the reaction was begun by the addition of 20μl of [γ-^{32}P]ATP (80μM assay concentration, approx. 20μCi/tube). The reaction was run for 15s at 30°C and was stopped by the addition of 50μl of a solution containing 15% (v/v) glycerol, 9% (w/v) sodium dodecyl sulphate and 6% (v/v) 2-mercaptoethanol, immediately followed by immersion for 2min in boiling water.

Analyses of the reaction products were performed by separation of the phosphorylated proteins by sodium dodecyl sulphate/12%-polyacrylamide-slab-gel electrophoresis (Laemmli, 1970). After being stained and dried the gels were autoradiographed. The developed film was used as a guide to cut the labelled proteins from the gels. The small rectangles of the gel were rehydrated, dissolved and their ^{32}P radioactivities measured by scintillation spectrophotometry. The assay system was shown to incorporate ^{32}P into κ-casein at a constant rate for at least 1 min.

Results

Dephosphorylated α-casein were added to the P_3 fraction with or without Mg^{2+}, Ca^{2+}, calmodulin and the calmodulin antagonist trifluoperazine in order to determine the bivalent-cation-specificities and effect of calmodulin and its antagonist on casein kinase activities of mammary P_3 preparations. The phosphorylations of the two major dephosphorylated component proteins of α-casein, α_S-casein and κ-casein, were easily measured after electrophoresis of the reaction mixture on sodium dodecyl sulphate/polyacrylamide gels and autoradiography. Dephosphorylated α_S-casein was phosphorylated only in the presence of Mg^{2+} (10 mM). Ca^{2+} (1 mM) did not affect the dephosphorylated α_S-casein phosphorylation or substitute for Mg^{2+}. The further addition of calmodulin or its inhibitor trifluoperazine showed no effects on the phosphorylation of previously dephosphorylated α_S-casein phosphorylation.

Phosphorylation of previously dephosphorylated κ-casein required Mg^{2+} (10 mM) for basal activity (Fig. 1). Ca^{2+} alone did not substitute for Mg^{2+} or augment the Mg^{2+}-dependent basal activity, but when Ca^{2+} and calmodulin were simultaneously present in the reaction mixture phosphorylation of dephosphorylated κ-casein by the casein kinase was increased by an average of 238%. The Ca^{2+}- and calmodulin-dependent phosphorylation was attributed to a protein kinase specific for κ-casein. The lack of response to Ca^{2+} alone suggested that little calmodulin was present in the P_3 microsomal preparation. The presence of 50 μM-trifluoperazine significantly decreased the Ca^{2+}- and calmodulin-dependent casein kinase activity. The addition of cyclic AMP, cyclic GMP, AMP or GMP did not affect the basal or Ca^{2+}- and calmodulin-dependent activity (results not shown).

The phosphorylation of dephosphorylated and native casein was compared to determine if the Ca^{2+}- and calmodulin-dependent κ-casein kinase activity would phosphorylate casein at non-physiological sites, or required dephosphorylated casein for high rates of activity, implying phosphorylation at physiological sites. The κ-casein kinase activity was 0.8 ± 1.8 pmol/min per mg (mean ± S.D.) when native casein was used as substrate. In contrast, when dephosphorylated casein was the substrate the activity was 44.3 ± 24.0 pmol/min per mg (mean ± S.D.) (Fig. 2). Clearly these data demonstrate that removal of the native phosphate of κ-casein improves its ability to serve as a substrate. Dephosphorylation of α_S-casein did not alter the Ca^{2+}- and calmodulin-dependent ^{32}P incorporation in the subsequent kinase assay. Interestingly, the apparent molecular mass of α_S-casein on polyacrylamide-gel electrophoresis under denaturing conditions decreased after dephosphorylation. The 54 kDa protein substrate for a previously reported (Landt *et al.*, 1982) protein kinase can be seen as a small band under these assay conditions, and an additional Ca^{2+}- and calmodulin-dependent phosphorylation is present in the assays that used native α-casein as substrate (approx. 60 kDa, lane 5).

The relationship of α_S-casein and κ-casein during the kinase reaction was explored by

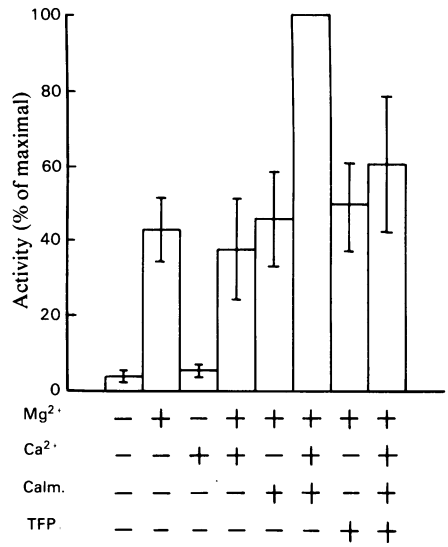

Fig. 1. *Effects of Mg^{2+}, Ca^{2+}, calmodulin and trifluoperazine on κ-casein phosphorylation*

Fraction P_3 was assayed for its ability to phosphorylate previously dephosphorylated α-casein in a 50 mM-Pipes buffer, pH 7.6, containing 0.4 mM-EGTA with additions as indicated: Mg^{2+} (10 mM), Ca^{2+} (1 mM), calmodulin (Calm.) (0.63 μM) and trifluoperazine (TFP) (50 μM). The results are from three experiments where the data were normalized to the maximal (plus Mg^{2+}, Ca^{2+} and calmodulin) activity. The vertical bars represent the standard deviations for the three experiments.

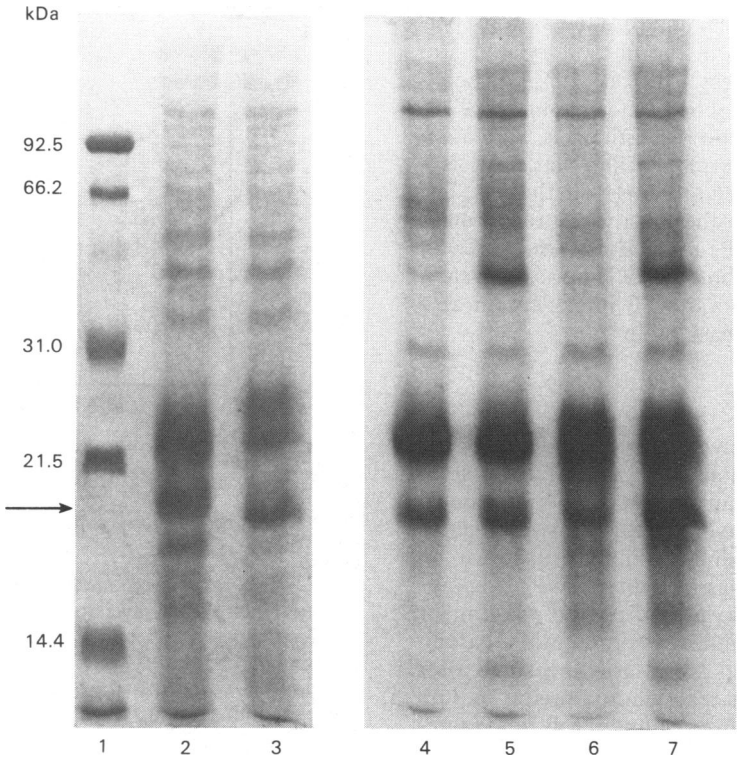

Fig. 2. *Phosphorylation of native and dephosphorylated κ-casein*

Membranes of mammary acini of lactating rats were assayed for their Ca^{2+} and calmodulin-dependent kinase activity in reactions containing either native (lanes 2, 4 and 5) or dephosphorylated (lanes 3, 6 and 7) α-casein. Lanes 1–3 are the Coomassie Blue staining pattern: 1, molecular-mass standards; 2, reaction mixture containing native casein; 3, reaction mixture containing dephosphorylated casein. Lanes 4–7 are autoradiographs of reactions containing: 4, native casein; 5, native casein plus Ca^{2+} and calmodulin; 6, dephosphorylated casein; 7, dephosphorylated casein plus Ca^{2+} and calmodulin. Arrow indicates κ-casein as determined by sensitivity to rennin. Reactions were conducted and analysed as described in the Experimental section.

comparing the phosphorylations of dephosphorylated κ-casein when dephosphorylated α_S-casein was present in or absent from the assay mixture. The κ-casein kinase was equally active whether or not α_S-casein was present (results not shown). These results suggest that native α-casein structure is not needed for phosphorylation and that κ-casein phosphorylation does not require its association with α_S-casein, or that a micelle-like association before phosphorylation does not impede the enzyme. The $K_{0.5}$ of calmodulin for the κ-casein kinase was $3.48 \pm 0.17\,\mu g/ml$ ($n = 3$; mean ± S.D.) (204 nM). Calmodulin activation increased rapidly at low concentrations but saturated at high concentrations (Fig. 3).

Acinar starting material appeared to be almost exclusively epithelial cells, as determined by light-microscopy. We used differential centrifugation to prepare several fractions from these cells, with the P_3 fraction particularly enriched for Golgi and plasma membranes (C. L. Brooks & M. Landt, unpublished work). The average activity in fraction P_3 of κ-casein kinase is 34.9 ± 1.0 pmol/min per mg (mean ± S.D.), more than 30-fold enriched from the homogenate and several-fold more active than other cellular fractions. A large standard deviation in fraction P_1 activity resulted from a large variation in the efficiency of homogenization. We have previously shown fraction P_3 to be enriched 2.5-fold for Golgi membranes and 2-fold for plasma membranes.

Fraction P_3 from lactating rats that were 0, 7, 14 and 20 days *post partum* were assayed to discern if the activity of κ-casein kinase modulated during lactation. Analysis of variance demonstrated (Sokol & Rohlf, 1969) no significant differences between enzyme activities at various times of lactation. No relationship between the number of pups nursed and the kinase activity was discerned.

Ca^{2+}- and calmodulin-dependent κ-casein kin-

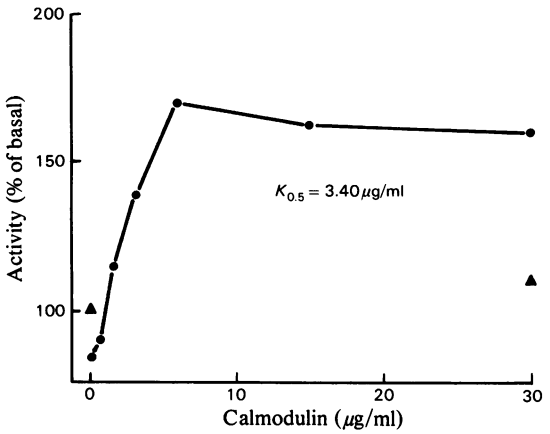

Fig. 3. *Calmodulin titration of Ca^{2+}-dependent casein kinase activity*

Casein kinase assays were performed as described in the Experimental section with increasing concentrations of calmodulin. ●, Assays with Ca^{2+} and between 0 and 30 μg of calmodulin/ml. ▲, Assays in which Ca^{2+} was absent with either 0 or 30 μg of calmodulin/ml. The $K_{0.5}$ is the calmodulin concentration required for half-maximal activation of the casein kinase activity in the presence of Ca^{2+}. The Figure represents the results of one experiment of the three performed.

ase activity was determined in fraction P_3 from mothers in late lactation who had been removed from their pups 24 h earlier. The enzyme activity in a control group (not weaned) was 10.8 ± 4.3 pmol/min per mg (mean ± S.D.), whereas that of the weaned mothers was 1.1 ± 1.7 pmol/min per mg (mean ± S.D.). Analysis by the Mann–Whitney U-test revealed a significant decrease ($P = 0.05$) in enzyme activity associated with removal of the pups (Goldstein, 1964). These results were not due to a redistribution of enzyme into other subcellular fractions (results not shown). The qualitative appearance of the protein staining patterns after electrophoresis was not visibly altered by weaning for 24 h.

Discussion

Our present work has described a new Ca^{2+}- and calmodulin-dependent protein kinase present in lactating rat mammary acini that may be physiologically important in the phosphorylation of κ-casein. The activity may be associated with the Golgi apparatus and/or the plasma membrane. Concentrations of casein kinase are significantly lowered by a 24 h period of pup removal, indicating that the kinase activity is acutely regulated, perhaps by the same mechanism that regulates secretion of milk proteins. In concert with the findings for other calmodulin-dependent activities, the full kinase activity requires the simultaneous presence of both Ca^{2+} and calmodulin, Ca^{2+} cannot replace the requirement for Mg^{2+} for basal activity, and trifluoperazine, a compound that binds to calmodulin (Klevit *et al.*, 1981) and blocks its actions, inhibits the Ca^{2+}- and calmodulin-dependent stimulation of κ-casein kinase. Perhaps the basal activity is a second enzyme, the Ca^{2+}- or Mg^{2+}-dependent activity early described by Bingham *et al.* (1972*b*).

An important aspect of the κ-casein kinase activity in rat mammary acini is the requirement for dephosphorylated substrate. κ-Casein is phosphorylated *in vivo* only on serine-149 (Mercier *et al.*, 1973). The use of native rather than dephosphorylated κ-casein as substrate decreased the kinase activity by 98%. These results indicate that the specific availability of serine-149 for phosphorylation is a requirement for kinase activity. This fact, and the observation that the κ-casein kinase activity was rapidly lost once the pups were weaned, suggests that the Ca^{2+}- and calmodulin-dependent κ-casein kinase may participate in κ-casein phosphorylation and secretion *in vivo*.

There are several similarities between our activity and that reported in rats by Bingham & Farrell (1974) and Bingham *et al.* (1972*b*). Both activities can be found in preparations enriched in Golgi membranes, and both are more active when a dephosphorylated substrate is added. Cyclic AMP did not affect either activity. There are also several important differences. First, the Ca^{2+}- and calmodulin-dependent κ-casein kinase requires Mg^{2+} for basal activity, whereas the other enzyme was fully active in the presence of either Mg^{2+} or Ca^{2+}; secondly, the κ-casein kinase activity is dependent on Ca^{2+} and calmodulin for full stimulation; thirdly, this calmodulin-dependent kinase activity uses only dephosphorylated κ-casein as a substrate, as no calmodulin-dependent stimulation of α_S-casein phosphorylation was detected. We also noted an absolute requirement for Mg^{2+} in our α_S-casein kinase activity, which is inconsistent with the observations made by Bingham & Farrell (1974) but consistent with those made by MacKinlay *et al.* (1977) in bovine tissue. Assay differences could conceivably account for the discrepancies between these data, in that our tissue preparations contained EGTA to eliminate any Ca^{2+} carried into the assay by our tissues. We conclude from these observations that the Ca^{2+}- and calmodulin-dependent κ-casein kinase, as described here, is a distinct enzyme from the Ca^{2+}-requiring enzyme previously reported.

Calmodulin-dependent phosphorylation of casein by other kinase activities is not new. DePaoli-Roach *et al.* (1981) demonstrated that phosphoryl-

ase kinase from rabbit muscle can phosphorylate κ-casein in a Ca^{2+}- and calmodulin-dependent manner, and Fukunaga *et al.* (1982) found a brain calmodulin-dependent activity that phosphorylated casein. Since these activities were measured with native (non-dephosphorylated) casein, phosphate was presumably incorporated at non-physiological sites, and these activities are not likely to be involved in casein phosphorylation *in vivo*.

The affinity of calmodulin for various protein kinases shows a large variance. Our κ-casein kinase had a $K_{0.5}$ of 204nM. This value is near the median for a group of protein kinases found by our group in several rat tissues (Landt *et al.*, 1982). The affinity is similar also to the K_m of rat brain tubulin kinase of 238nM (Burke & DeLorenzo, 1981). Several kinases display a greater affinity for calmodulin. These kinases include a calmodulin-dependent glycogen synthase kinase in rabbit liver (100nM; Payne *et al.*, 1983) and in rabbit skeletal muscle (27nM; Woodgett *et al.*, 1982). Walsh *et al.* (1979) reported an extremely high affinity of calmodulin and myosin light-chain kinase from bovine cardiac muscle of 1.3nM. The significance of the wide variance in affinity for calmodulin remains to be verified by use of purified kinase preparations.

One point of caution must be discussed. The kinase activity that we describe is in rat tissues and we have used bovine caseins as substrates. It remains to be established whether a protein in rat milk would be phosphorylated in a similar manner. A κ-casein type protein has been described in rat milk by Hirose *et al.* (1981). Perhaps this protein endogenous to the rat, and which shares many properties with the bovine κ-casein (solubility in Ca^{2+} and rennin-sensitivities), is the physiological substrate in rats. Additionally, the existence of a Ca^{2+}- and calmodulin-dependent κ-casein kinase needs to be established in bovine tissues.

References

Baumrucker, C. R. (1978) in *Lactation: A Comprehensive Treatise* (Larson, B., ed.), vol. 4, pp. 463–474, Academic Press, New York

Bingham, E. W. & Farrell, H. M. (1974) *J. Biol. Chem.* **249**, 3647–3651

Bingham, E. W. & Grove, M. L. (1979) *J. Biol. Chem.* **254**, 4510–4515

Bingham, E. W., Farrell, H. M. & Carroll, R. J. (1972*a*) *Biochemistry* **13**, 2450–2454

Bingham, E. W., Farrell, H. M. & Basch, J. J. (1972*b*) *J. Biol. Chem.* **247**, 8193–8194

Burke, B. E. & DeLorenzo, R. J. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 991–995

Charbonneau, H. & Cormier, M. J. (1979) *Biochem. Biophys. Res. Commun.* **90**, 1039–1047

Cheung, W. Y. (1980) *Science* **207**, 19–27

Cohen, P., Burchell, A., Foulkes, J. G., Cohen, P. T. W., Vanaman, T. C. & Nairn, A. C. (1978) *FEBS Lett.* **92**, 287–293

DePaoli-Roach, A. A., Bingham, E. W. & Roach, P. J. (1981) *Arch. Biochem. Biophys.* **212**, 229–236

Farrell, H. M. (1973) *J. Dairy Sci.* **56**, 1195–1206

Fukunaga, K., Yamamoto, H., Matsui, K., Higashi, K. & Miyamoto, E. (1982) *J. Neurochem.* **39**, 1607–1617

Goldstein, A. (1964) *Biostatistics*, p. 55, Macmillan Publishing Co., New York

Hathaway, G. M. & Traugh, J. A. (1982) *Curr. Top. Cell. Regul.* **21**, 101–127

Hedeskov, C. J. (1980) *Physiol. Rev.* **60**, 442–509

Hirose, M., Kato, T., Omori, K., Maki, M., Yoshikawa, M., Sasaki, R. & Chiba, H. (1981) *Biochim. Biophys. Acta* **667**, 309–320

Hokin, L. E. (1966) *Biochim. Biophys. Acta* **115**, 219–230

Katz, J., Wals, P. A. & Van de Velde, R. L. (1974) *J. Biol. Chem.* **249**, 7348–7357

Klevit, R. E., Levine, B. A. & Williams, R. J. P. (1981) *FEBS Lett.* **123**, 25–29

Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

Landt, M., Kloepper, R. F., Miller, B. E., Brooks, C. L. & McDonald, J. M. (1982) *Comp. Biochem. Physiol. B* **73**, 509–516

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

MacKinlay, A. G., West, D. W. & Manson, W. (1977) *Eur. J. Biochem.* **76**, 233–243

Means, A. R. & Dedman, J. R. (1980) *Mol. Cell. Endocrinol.* **19**, 215–227

Mercier, J.-C., Brignon, G. & Ribadeau-Duman, B. (1973) *Eur. J. Biochem.* **35**, 222–235

Neville, M. C., Selker, F., Semple, K. & Watters, C. (1981) *J. Membr. Biol.* **61**, 97–105

Payne, M. E. & Soderling, T. R. (1980) *J. Biol. Chem.* **255**, 8054–8056

Payne, M. E., Schworer, C. M. & Soderling, T. R. (1983) *J. Biol. Chem.* **258**, 2376–2382

Smith, J. J., Park, C. S. & Keenan, T. W. (1982) *Int. J. Biochem.* **14**, 573–576

Sokol, R. R. & Rohlf, F. J. (1969) *Biometry*, p. 204, W. H. Freeman and Co., San Francisco

Szymanski, E. S. & Farrell, H. M. (1982) *Biochim. Biophys. Acta* **702**, 163–172

Tashjian, A. H., Lomedico, M. E. & Maina, D. (1978) *Biochem. Biophys. Res. Commun.* **81**, 798–805

Thompson, M. P. (1966) *J. Dairy Sci.* **49**, 792–795

Turkington, R. W. & Topper, Y. J. (1966) *Biochim. Biophys. Acta* **127**, 366–372

Walsh, M. P., Vallet, B., Autric, F. & Demaille, J. G. (1979) *J. Biol. Chem.* **254**, 12136–12144

Warner, R. C. (1944) *J. Am. Chem. Soc.* **66**, 1725–1730

West, D. W. (1981) *Biochim. Biophys. Acta* **673**, 374–386

Woodgett, J. R., Tonks, N. K. & Cohen, P. (1982) *FEBS Lett.* **148**, 5–11