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Phosphorylation of Pig Epidermal Soluble Protein by Endogenous cAMP-Dependent Protein Kinase

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The distribution of adenosine 3′,5′-monophosphate (cAMP)-dependent protein kinase and its substrate proteins was analyzed using soluble and particulate fractions of pig epidermal homogenates. When histone was used as a substrate for this enzyme reaction, protein kinase activity was distributed almost equally between the soluble and particulate fractions. However, the effect of exogenously added cAMP was confined almost exclusively to the soluble enzyme. Endogenous protein phosphorylation in the absence of exogenous histone was higher in the particulate fraction than in the soluble fraction, but the stimulating effect of cAMP was observed only in the soluble fraction. These results indicate that cAMP-dependent protein kinase is predominantly localized in the soluble fraction and phosphorylates soluble epidermal proteins. The particulate fraction contains protein kinase which is cAMP-independent and phosphorylates particulate-bound proteins as well as histone. Based on these observations, the soluble fraction was incubated with [γ-^{32}P]-ATP in the presence or absence of cAMP, and phosphorylated protein was analyzed by SDS disc- or slab-gel electrophoresis followed by autoradiography. Among many proteins whose phosphorylation was slightly increased by cAMP, a protein with M_r ~45,000 was found which was markedly phosphorylated in the presence of cAMP. Although this protein corresponds to one of the richest proteins in the epidermal soluble fraction, an important physiologic role for this phosphorylation has not been clarified.

In a preceding report, we demonstrated the presence of adenosine 3′,5′-monophosphate (cAMP)-dependent protein kinase in pig epidermis, and activation of this enzyme by various agents that increased epidermal cAMP [1]. Although activation of this enzyme and subsequent phosphorylation of intracellular protein is thought to be essential for the effect of cAMP to appear, little is known about the epidermal protein that is phosphorylated by this enzyme. We now report that pig epidermis contains cAMP-dependent and -independent protein kinases which phosphorylate endogenous proteins. The endogenous substrate protein for the former kinase has been analyzed by electrophoresis.

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Abbreviations:
cAMP: adenosine 3′,5′-monophosphate
SDS: sodium dodecyl sulfate
TCA: trichloroacetic acid

MATERIALS AND METHODS

Epidermis was obtained from the backs of domestic pigs weighing 15–20 lb using a Castroviejo keratome set at 0.2 mm. It was homogenized in a conical glass homogenizer with 5 times the volume of 0.25 M sucrose and then centrifuged at 100,000 *g* for 1 h. The supernatant was dialyzed against 0.25 M sucrose for several hours and used as a supernatant fraction. The precipitate was resuspended, centrifuged, and used as a particulate fraction. These steps were carried out at 0°–4°C.

Protein kinase activity was measured in the reaction mixture containing 10 μmol of potassium phosphate buffer pH 6.8, 1 μmol of magnesium acetate, 25 μmol of EGTA, 1 μmol of NaF, 200 μg of mixed histone (from calf thymus, Sigma H_2S) and 2 nmol of ATP containing 0.5–2 μCi of [γ-^{32}P]-ATP (Amersham S.A., about 20 Ci/mmol) with or without 0.5 nmol of cAMP and up to 40 μl of enzyme in a total volume of 100 μl. The reaction was started by adding ATP, incubated at 30°C for 3–5 min, and terminated by addition of glacial acetic acid to a final concentration of 30%. Aliquots were spotted on a 2 × 2 mm phosphocellulose paper (Whatman P81), washed in tap water for 90 min, briefly dehydrated with ethanol and ether, and counted in 10 ml of counting media containing 4 g of Omnifluor (New England Nuclear) and 20 ml of Biosolv BBS3 (Beckman)/L of toluene. The details of this assay procedure had been reported previously [1].

In some experiments, cAMP-dependent protein kinase inhibitor protein up to 133 μg/assay was included in the reaction mixture. A crude inhibitor protein preparation was made by the method of Gilman [2]. When the phosphorylation of endogenous protein was studied, histone was omitted from the reaction mixture and the reaction was terminated by spotting the aliquots on the 2 × 2 cm piece of a filter paper (Whatman 3MM) and immediately immersing it into a large volume of 10% TCA solution, this was followed by washing with 2 changes of the TCA solution, rinsing with ethanol and ether, and counting as described above.

To analyze the endogenous proteins being phosphorylated, the reaction was terminated by adding TCA to a final concentration of 10%. The protein was collected by centrifugation, and washed by dispersion with 10% TCA and recentrifugation. The pellet was rinsed with a small volume of acetone to remove TCA, and boiled for 2 min with 50 μl of 0.0625 M Tris-HCl buffer pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.001% bromphenol blue. A 25-μl aliquot was subjected to SDS-polyacrylamide slab-gel (8.5%) electrophoresis as described by Laemmli [3]. The gel was stained with Coomassie blue and then dried. The gel was autoradiographed using Kodak XRP film.

Alternatively, the protein precipitate from 200 μl of the reaction mixture was boiled with 200 μl of the solubilizing mixture and analyzed by SDS-polyacrylamide gel (10%) disc electrophoresis. The gel was cut into 1-mm thick pieces and counted with 10 ml of 3% Protosol (New England Nuclear) and 0.4% Omnifluor in toluene after overnight incubation at 37°C. The molecular size of the proteins was estimated using a low-molecular-weight standard protein mixture (Bio-Rad).

RESULTS

Fig 1 shows the time course of phosphorylation reaction with the epidermal soluble fraction without histone. The effect of cAMP is clearly seen. The reaction reached its maximum after 5-min incubation at 30°C. In the experiment shown on panel *A* of Fig 2, various amounts of soluble and particulate fractions were incubated in the presence or absence of cAMP using histone as phosphate acceptor. Protein kinase activity was located in both fractions, but the effect of cAMP was more marked in the soluble fraction. To rule out the possibility that protein kinase activity in the particulate fraction was due to any cAMP-independent form (i.e., the free catalytic subunit

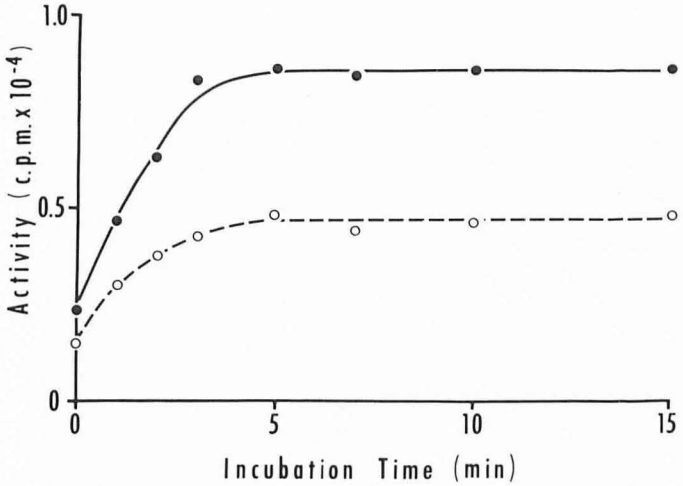

FIG 1. Time course of endogenous protein phosphorylation. Forty microliters of the supernatant was incubated with (●——●) or without (○-----○) cAMP. After the incubation 80 μl of the reaction mixture was spotted on 2 pieces of filter paper, washed with TCA solution, and counted.

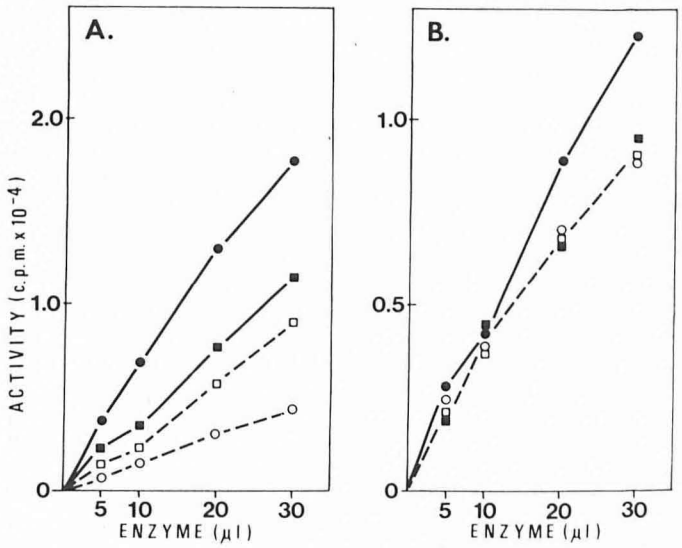

FIG 2. Protein kinase activity using histone as a substrate. *A*, Various amounts of the supernatant (●,○) or precipitate (■,□) were incubated in the presence (●——■) or absence (○-----□) of cAMP. *B*, Various amounts of the precipitate were incubated with (●,■) or without (○,□) cAMP in the presence (■,□) and absence (●,○) of protein kinase inhibitor protein (133 μg/tube). Incubation period: 4 min.

bound to the particulate fraction) of cAMP-dependent kinase, the reaction with the particulate fraction was carried out in the presence of cAMP-dependent kinase inhibitor protein (panel *B* of Fig 2). The slight stimulatory effect of cAMP was abolished by the inhibitor, but a further decrease of cAMP-independent activity was not seen. These experiments indicate that protein kinase activity is localized in both the soluble and particulate fractions, but that these activities are due to different enzymes. The cAMP-dependent enzyme is mostly located in the soluble fraction while the particulate enzyme is cAMP-independent. In the experiments shown in Fig 3, the soluble and the particulate fractions were incubated without histone to study phosphorylation of endogenous protein. Again, the effect of cAMP was clearly seen in the soluble fraction (panel *A*), while no effect of cAMP was seen in the particulate protein. It should be noted that despite the lack of effect of cAMP, protein phosphorylation was higher in the precipitate than in the supernatant (panel *B*).

The soluble and particulate fractions were mixed in different proportions and the effect of cAMP studied in these preparations. The results suggested that no phosphorylation of particulate protein by a cAMP-dependent enzyme in the soluble fraction was occurring (data not shown). The epidermal soluble protein which was phosphorylated by cAMP-dependent protein kinase was analyzed using SDS-polyacrylamide disc gel electrophoresis. Fig 4 shows the radioactivity of ^{32}P incorporated into various proteins. Many proteins were phosphorylated and showed an increase with cAMP (*solid line*), suggesting the presence of a wide variety of proteins that are substrates of this enzyme. There was one peak of remarkable ^{32}P incorporation that occurred only in the presence of cAMP in the M_r range slightly larger than 43,000 (about 45,000d).

To see the relationship of this phosphorylation and the protein bands more directly, the reaction mixture was subjected to SDS-polyacrylamide slab gel electrophoresis followed by protein staining and autoradiography (Fig 5). The band phos-

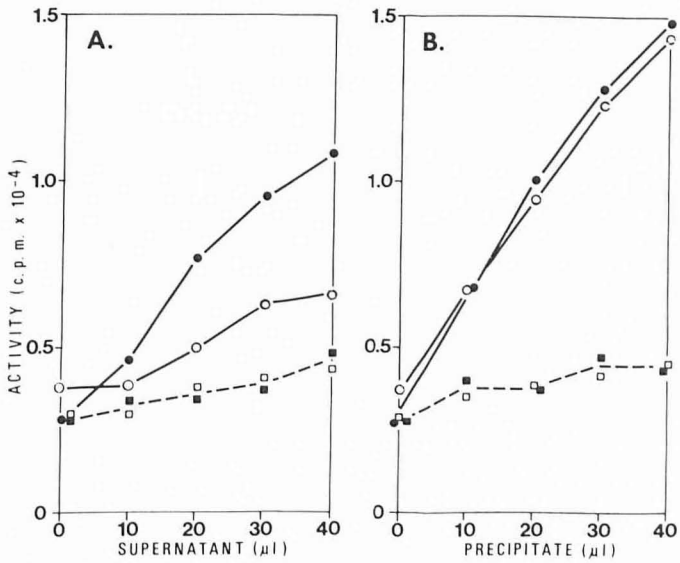

FIG 3. Phosphorylation of endogenous protein. Cyclic AMP was included in (●,■) or omitted from (○,□) the reaction mixture. Samples were incubated for 4 min (●——○) or 0 min as a control (■-----□).

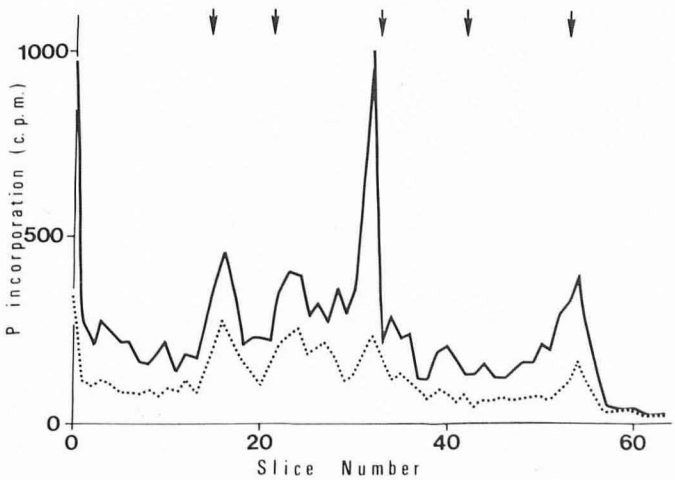

FIG 4. Analysis of phosphorylated protein by disc-gel electrophoresis. Incubation was done with (——) or without (-----) cAMP. After electrophoresis, gels were cut into 1-mm thick pieces and counted. *Arrows* indicate positions of marker proteins which are (from right to left): soybean trypsin inhibitor, 21,000; carbonic anhydrase, 30,000; ovalbumin, 43,000; bovine serum albumin, 68,000; and phosphorylase B, 94,000.

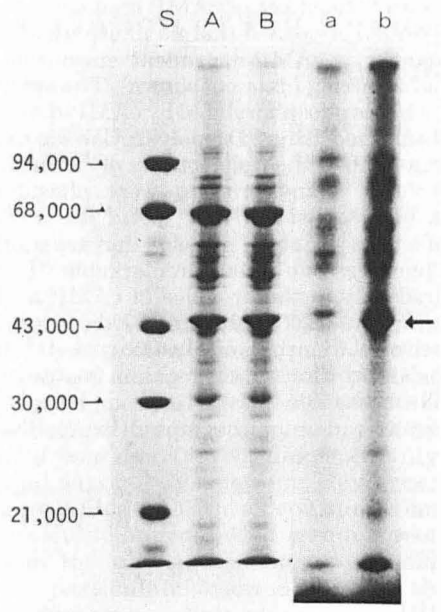

FIG 5. Autoradiographic analysis of phosphorylated soluble proteins of epidermis (soluble fraction). *A* and *B*, Protein staining, without and with cyclic AMP, respectively. *a* and *b*, Autoradiogram, without and with cyclic AMP, respectively. *S*, Marker proteins as shown in Fig 4. An *arrow* indicates the 45K epidermal protein.

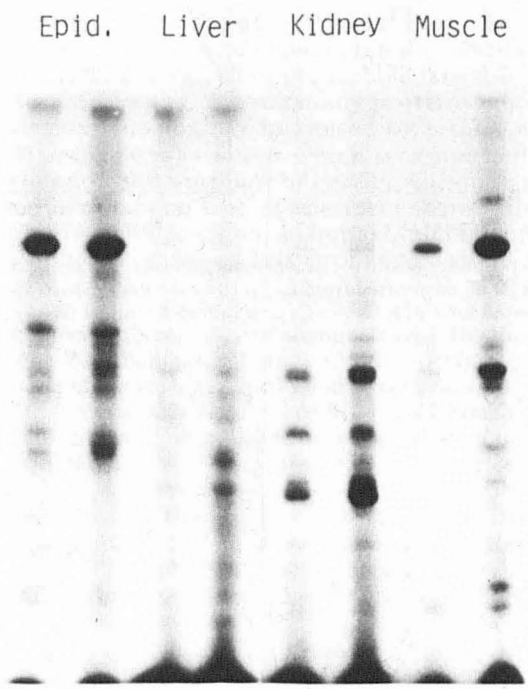

FIG 6. Autoradiographic analysis of phosphorylated proteins of epidermis, liver, kidney, and muscle. Samples from different organs were prepared and treated simultaneously as described in text. The incubation condition was the same as that in Fig 5. (*Right*, without cAMP; *left*, with cAMP.)

phorylated in the presence of cAMP corresponded to one of the major proteins in the epidermal soluble fraction. The M_r of this protein was estimated to be about 45,000 based on the motility of the marker proteins electrophoresed simultaneously.

Fig 6 shows an autoradiograph of supernatant fractions from epidermis, liver, kidney, and muscle, which had been prepared and incubated with [^{32}P]-ATP simultaneously as described in Fig 5. It is clear that the cAMP-dependent phosphorylation of the 45K protein is unique to the epidermal preparation.

DISCUSSION

The presence of cAMP-dependent protein kinase has been demonstrated in monkey and human epidermal tissues [4–6]. In the preceding report [1] we have also demonstrated this enzyme activity using pig epidermal slices. The activity was activated by incubation of the tissue with various agents known to increase epidermal cAMP. This enzyme activity, like those in other mammalian tissue, can be separated into two isoenzymes, i.e., kinase I and II by DEAE-cellulose column chromatography [7]. Phosphorylation of various proteins by this enzyme is the only known mechanism of action of cAMP. The wide range of substrate proteins identified so far in mammalian tissues (phosphorylase kinase, glycogen synthetase, pyruvate kinase, hormone-sensitive lipase, tyrosine hydroxylase, histone, protamine, plasma membrane proteins, ribosomal proteins, etc.) suggests a very important role for this enzyme. In regard to the endogenous substrate protein for the epidermal enzyme, the data presented here show that many proteins in the epidermal soluble fraction can be phosphorylated by this enzyme (Fig 4). The most remarkable peak of ^{32}P incorporation was seen in the M_r range of about 45,000, and this peak seems to correspond to one of the major proteins in the epidermal soluble fraction (Fig 5). Although the molecular sizes of known substrate proteins in pig epidermis are not readily available for comparison, data from other mammalian tissues suggest that those are either larger or smaller than 45,000, and at the present time, the nature and function of this particular protein is not clear.

Freedberg and coworkers have also reported phosphorylation of epidermal protein by prelabeling cultured cells from humans and mice with inorganic phosphate [8]. They found marked incorporation of phosphate into protein with M_r about 46,000, 54,000, and 65,000. However, these proteins are different from those we observed, because they are soluble only in the presence of SDS and 2-mercaptoethanol. The 52K protein phosphorylated in the presence of epidermal growth factor which was observed by Aoyagi et al [9] is not readily comparable with this one either because of the differences in the experimental procedures. Recently Dale and coworkers isolated a precursor of stratum corneum basic protein from newborn rat epidermis [10]. This protein, extractable with 1 M potassium phosphate buffer pH 7.0, contained covalently bound phosphate and had a M_r of 53,000. Although it is tempting to correlate our protein with this precursor protein, we have not yet obtained direct evidence to support the concept. Further study of the 45K protein is required to determine any possible relationship to epidermal differentiation.

Another possible endogenous substrate for epidermal protein kinase(s) is a regulatory subunit of protein kinase, particularly the type II regulatory subunit (R_{II}), which has been reported to be phosphorylated [11]. The M_r of this subunit in SDS-gel electrophoresis is 55,000 [11] as compared with our protein being 45,000. This possibility that the 45K protein may be the R_{II} subunit appears to be unlikely for the following reasons: (1) The 45K phosphorylation is rather unique to the epidermal preparation and is not seen in other organs, as shown in Fig 6 (M_r of the R subunit appears to be the same in various organs [12]). (2) The addition of Zn^{++} in the incubation medium should inhibit the cAMP-dependent phosphorylation of the R_{II} subunit, but not the basal phosphorylation level [13]. Our additional experiments (data not shown) indicate that the addition of Zn^{++} caused strong inhibition of phosphorylation of the 45K protein both in the presence and in the absence of cAMP.

REFERENCES

1. Yoshikawa K, Takeda J, Nemoto O, Halprin KM, Adachi K: Activation of cAMP-dependent protein kinase in epidermis by the compounds which increase epidermal cAMP. J Invest Dermatol 77:397–401, 1981

2. Gilman AG: A protein binding assay for adenosine 3′5′-cyclic monophosphate. Proc Natl Acad Sci USA 67:305–312, 1970
3. Laemmli UK: Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227:680–685, 1970
4. Kumar R, Tao M, Piotrowski R, Solomon L: Multiple forms of adenosine 3′,5′-cyclic monophosphate-dependent protein kinase from monkey skin. Biochim Biophys Acta 315:66–72, 1973
5. Kumar R, Tao M, Solomon LM: Cyclic 3′,5′-adenosine monophosphate-stimulated protein kinase from human skin. J Invest Dermatol 57:312–315, 1971
6. Yoshikawa K, Adachi K: Protein kinase in the hair follicles of the human scalp and beard. Biochim Biophys Acta 315:333–342, 1973
7. Nemoto O, Adachi K, Takeda J, Yoshikawa K, Halprin KM, Levine V, Woodyard C: Cyclic AMP-dependent protein kinase isozymes of pig and human skin from normal and psoriatic subjects. J Invest Dermatol 80:111–115, 1983
8. Gilmartin ME, Culberson VB, Freedberg IM: Phosphorylation of epidermal keratin. J Invest Dermatol 75:211–216, 1980
9. Aoyagi T, Adachi K, Halprin KM, Levine V: Stimulation of protein phosphorylation by epidermal growth factor in pig skin (epidermis). J Invest Dermatol 74:421–424, 1980
10. Lonsdale-Eccles JD, Haugen JA, Dale BA: A phosphorylated keratohyalin derived precursor of epidermal stratum corneum basic protein. J Biol Chem 255:2235–2238, 1980
11. Zoller MJ, Kerlavage AR, Taylor SS: Structural comparisons of cAMP-dependent protein kinase I and II from porcine skeletal muscle. J Biol Chem 254:2413–2418, 1979
12. Walter U, Uno I, Liu AYC, Greengard P: Study of autophosphorylation of isoenzymes of cyclic AMP-dependent protein kinases. J Biol Chem 252:6588–6590, 1977
13. Walter U, Kanof P, Schulman H, Greengard P: Adenosine 3′:5′-monophosphate receptor proteins in mammalian brain. J Biol Chem 253:6275–6280, 1978

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Cyclic AMP-Dependent Protein Kinase Isozymes of Pig Skin and Human Skin from Normal and Psoriatic Subjects

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Cyclic AMP-dependent protein kinase isozymes of pig and human skin (epidermis) were separated by DEAE-cellulose column chromatography after micromodification for small biopsy samples. Clear-cut separations of type I and type II isozymes, which were of about equal amounts, could be obtained only when the ischemia effect was avoided by in vivo freezing of skin and homogenization for less than 10 s. Intradermal injections of epinephrine caused dose-dependent activation of type I isozyme, but not of type II. Injections of other skin adenylate cyclase stimulators such as histamine, adenosine, and prostaglandin E_2 elevated the local cyclic AMP levels to not more than 5 pmol/mg protein and also stimulated only the type I isozyme.

Incubation of keratome-sliced pig skin under various conditions caused both activation by dissociation and inactivation by reassociation of the subunits, which appeared to be dependent on the cyclic AMP content. Epinephrine added to the incubation medium led to complete activation of both type I and type II isozymes (the intraepidermal cyclic AMP contents ranged from 20–50 pmol/mg protein).

The isozymes of normal skin and involved skin of psoriatics showed identical peaks of type I and type II isozymes of equal amounts. The data indicate that protein kinase in the involved skin is not in an activated (by cyclic AMP) state.

Activation of the cyclic AMP system by hormones or other chemicals is followed by a specific phosphorylation process mediated by cyclic AMP-dependent protein kinase. Since the physiologic effects of an increase in cyclic AMP are solely dependent on this activation process, cyclic AMP-dependent protein kinase activity may be more important than the increase in cyclic AMP level itself. Cyclic AMP-dependent protein kinase has 2 regulatory and 2 catalytic subunits [1]. The inactive holoenzyme dissociates in the presence of cyclic AMP into the active catalytic subunit and the regulatory subunit–cyclic AMP complex [2]. Since Reimann et al [3] first demonstrated two different types of cAMP-dependent protein kinase, these isozymes (types I and II) have been found in most mammalian tissues [2–7]. These two isozymes have the same catalytic subunit, although the regulatory subunits are different [8]. Type I and type II isozymes can be separated by DEAE-cellulose column chromatography [1,4], i.e., a free catalytic subunit can be eluted by 10 mM potassium phosphate buffer, and type I and type II isozymes by 0.1 and 0.2 M of NaCl, respectively. Type I kinase can be rapidly dissociated into the free regulatory and catalytic subunits by salt or substrate. These subunits will slowly reassociate by removing the dissociating agents. In contrast, type II kinase can be only slowly dissociated but will rapidly reassociate [2,9].

It appears that these isozymes have specific functions in some tissues. Byus et al [10] showed that glycogenolysis in rat hepatocytes was regulated by the activation of type I kinase. Costa et al [11] reported that type I kinase activity was high in mitosis while type II kinase activity was associated with the S phase of

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Abbreviations:
cyclic AMP: cyclic adenosine 3′,5′-monophosphate
EGTA: ethyleneglycol-*bis*-(β-aminoethyl ether)-N,N′-tetraacetic acid
IBMX: isobutylmethylxanthine
PGE_2: prostaglandin E_2