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Regulation Of Cyclic-amp Phosphodiesterase Of Rat Skeletal Myoblasts

Prem Kumar Seth

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COMPARISON OF THE PROPERTIES OF HOMOGENEOUS PHOSPHODIESTERASES FROM VARIOUS MAMMALIAN TISSUES

Tissue	Native Mr	Number of Sub-Units	Subunit Mr	Calmodulin requirement	Km for cyclic AMP
					μM
Pig liver	33,000	1	33,000	No	25[a]
Dog kidney	60,600	1	60,600	No	2.2
Bovine brain	126,000	2	59,000	Yes	150
Bovine brain	116,000	2	58,000	Yes	ND[b]
Bovine brain	135,000	3	61,000	Yes	100
			59,000		
			15,000		
Bovine heart	114,000	2	57,000	Yes	ND[b]
Bovine rod outer segments	170,000	3	88,000	No	>4,000[c]
			84,000		
			13,000		

[+]Taken from Helfman et al. (1981).

[a]The enzyme has a Km of 182 μM for cyclic CMP.

[b]ND, not determined.

[c]The enzyme has a Km of 150 μM for cyclic GMP.

REGULATION OF CYCLIC AMP PHOSPHODIESTERASE OF RAT SKELETAL MYOBLASTS

by

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ABSTRACT

The aim of the present investigation was to study the various regulatory mechanisms involved in the control of cAMP phosphodiesterases in rat skeletal myoblasts. In an effort to do this, cAMP phosphodiesterases present in rat skeletal myoblasts and adult muscle were first purified and characterized.

In rat skeletal myoblasts and adult muscle extracts, four forms of a high affinity cAMP phosphodiesterase are found in vitro. These are termed PDE I, PDE II, PDE III and PDE IV, and have approximate molecular weights of 1.5×10^6, 400,000, 120,000 and 60,000, respectively. Evidence is presented to show that there is only one primary form of phosphodiesterase in myoblasts, viz. PDE II, with the rest of the forms being derived from it. Various conditions are described under which PDE II can be aggregated to PDE I or degraded to PDE III and PDE IV. PDE I consists of only one type of subunit with a molecular weight of about 94,000; PDE III consists of one subunit of about 60,000 and PDE IV has two subunits of molecular weight 28,000 and 30,000. Among all the forms of phosphodiesterases, only PDE II is activated by proteases, NaSCN and α-tocopheryl phosphate.

In the presence of Bt_2cAMP or compounds which are able to augment *in vivo* concentrations of cAMP in the culture medium, the phosphodiesterase activity of skeletal myoblasts increases about 2-fold within 30 min of culture. PDE II is modified in some way which makes the enzyme nonresponsive to activating effect of snake venom. Activation is not affected by inhibitors of protein synthesis. Modification of PDE II can be demonstrated in cell-free extracts. Modification is entirely dependent on ATP and cAMP. The activation can be reversed by incubating extracts further with acid phosphatase. Evidence is presented to show that during *in vitro* activation of phosphodiesterase, PDE II is phosphorylated. Homogeneous preparations of PDE I are also phosphorylated *in vitro*. A 90,000 phosphoprotein was also partially purified after labelling the cells with ^{32}P-orthophosphoric acid.

When the myoblasts are exposed to Bt_2cAMP for 10-16 hours the activity of PDE III increases considerably. Leupeptin prevents the increase in the levels of PDE III suggesting that a protease *in vivo* may be responsible for the formation of PDE III from PDE II. Spontaneous or Rous Sarcoma virus-transformed myoblasts, however, show altered regulation of the two forms of PDE. In the presence of cAMP in the medium, unlike the non-transformed cells, the levels of PDE III do not increase

but the activity of PDE II rises. Simultaneously, PDE II becomes refractory to activation by proteases. The altered mode of PDE regulation in transformed cells is dominant in hybrids between normal and transformed myoblasts, which suggests that altered regulation is due to an 'acquisition' of some new property by transformed cells.

ACKNOWLEDGEMENTS

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NOMENCLATURE

ama^R	α-aminitin resistant
Az-2	8-azaguanine resistant
ACTH	adrenocorticotropic hormone
AMP-PNP	adenylyl imidodiphosphate
ATP	adenosine triphosphate
ATP-γ-S	adenosine 5'-0'(3-thio-triphosphate)
Bt_2cAMP	$N^6,0^{2'}$-dibutyryl cyclic adenosine monophosphate
Bt_2cGMP	$N^2,0^{2'}$-dibutyryl cyclic guanosine monophosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
DME	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
EGTA	ethylene glycol bis (β-amino-ethyl-ether) N,N,N',N'-tetra acetic acid
HAT	medium containing hypoxanthine, aminopterin and thymidine
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid
MEM	minimum essential medium
MIX	1-methylisobutyl xanthine
mRNA	messenger RNA
PBS	phosphate buffered saline
PDE	phosphodiesterase
PGE_1	prostaglandin E_1

RNA	ribonucleic acid
RSV	Rous Sarcoma Virus
SDS	sodium dodecyl sulfate
Tris	tris (hydroxymethyl) aminomethane
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethyl ethylenediamine
TSF	α-toluene sulfonyl fluoride

CHAPTER 1. INTRODUCTION

Since the discovery of cAMP by Rall and Sutherland (1958), several functions have been assigned to this nucleotide. In procaryotic cells cAMP has been shown to be a regulatory molecule for controlling gene expression (Pastan and Adhya, 1976). In eucaryotic cells cAMP has been suggested to be a second messenger of several hormones and neurotransmitters. (Robison et al., 1971; Greengard, 1978). Levels of cAMP have been correlated with cell shape and morphology (Sandoval and Cuatrecasas, 1976), Li et al., 1977), cell growth and transformation (Pastan et al., 1975; Puck, 1979) and differentiation (Rutter et al., 1973; McMohan, 1974; Willingham, 1976; Kessin, 1981; Knecht et al., 1981). Its role in myoblast differentiation is discussed below.

Though cAMP has been known to regulate several cellular functions, how cells regulate the levels of cAMP itself is not clearly understood. In eucaryotic cells, levels of cAMP are controlled mainly by the relative activities of adenylate cyclase (the enzyme which synthesizes cAMP) and phosphodiesterase (the enzyme which degrades cAMP). Regulation of activities of both these enzymes has been the subject of great interest in the past several years. Adenylate cyclase is a membrane bound enzyme and is stimulated by several

hormones and neurotransmitters (Rodbell et al., 1981, Sellinger and Cassel, 1981, Londos et al., 1981). Phosphodiesterase, on the other hand, is known to be located both in cytosolic and particulate fractions (Gain and Appleman, 1978). Its activity is known to be affected by several biologically relevant substances (see below), but the exact mechanisms of regulation have not been described (Vaughan et al., 1981). Paucity of information regarding the regulation of phosphodiesterases is due not only to the unstable nature of the enzyme, but also due to the presence of bewildering multiplicity of forms in different kinds of tissues (Strada and Thompson, 1978).

The bulk of studies on phosphodiesterases reported in the literature have been made on tissues, and since vertebrate tissues are made up of different kinds of cells, it seemed plausible to us that the variety of phosphodiesterases seen in whole tissues may simply be due to the contribution of one type of phosphodiesterase from each cell type. It appeared reasonable that if we were to succeed in studying the regulation of phosphodiesterases we should work with a cell type which does not have a large number of isozymic forms. The cells employed in the present investigation were rat skeletal myoblasts, which can be cultivated easily in the laboratory and can be grown under a variety of environmental conditions. These

cells have only one type of phosphodiesterase, viz., the low Km, calmodulin-independent form. Using the knowledge obtained about the low Km enzyme from from myoblasts, we have purified it from adult skeletal muscles and studied its structural and regulatory characteristics. In the following pages, I have summarized briefly information about the experimental material and whatever little is known about the nature and mode of regulation of phosphodiesterases.

1.1 Myogenesis

Myogenesis is the process whereby mononucleated myoblasts fuse to form multinucleated myotubes. The latter become striated and contract spontaneously. This process of myoblast differentiation involves several morphological and biochemical changes. Major morphological events are active cell division, alignment of cells in arrays leading to cell fusion. Biochemical changes involve increase in the activity of several enzymes required for muscle function (e.g. creatine phosphokinase, Ca^{2+} dependent ATPase); acetyl choline esterase receptors and muscle specific proteins like actin, myosin, etc. The subject has recently been reviewed by several workers (Buckingham, 1977; Merlie et al., 1977; Sanwal, 1979; Pearson, 1981).

The process of myoblast differentiation can be studied using both the primary culture (derived from

embryo muscle) and secondary cultured (i.e. permanent) cell lines, (Hauschka, 1972). Primary culture offers the advantage of being closer to the in vivo situation. In addition, these cells show a synchronized fusion. A difficulty in using such cultures is the inability to carry them for long term in culture. It is also usually quite difficult to obtain a homogeneous population of myoblasts in primary cultures. On the other hand, permanent cell lines can be cultured in the laboratory for indefinite periods of time. Moreover, since these cell lines are derived by cell cloning, they allow genetic manipulations often required to solve biochemical problems. In the work presented here we have chosen to employ two permanent cell lines of rat skeletal myoblasts L6, and L8, first isolated by Yaffe (1968).

The mechanism of myoblast differentiation is still an unresolved problem of biology. Basically two theories have been proposed. According to one, the process of differentiation does not require synthesis of DNA and is reversibly controlled by the components present in the medium in which the cells are cultured (Konigsberg, 1971). The other theory proposes the occurrence of an essential event during 'quantal mitosis' which produces one daughter cell irreversibly committed to differentiate. (Holtzer, 1970). Both these theories have some degree

of support. It is generally agreed that environmental factors do control the time course of differentiation. The major disagreement at present is about the exact point in the cell cycle at which the myoblasts become irreversibly committed to differentiation. (Buckingham, 1977; Merlie et al, 1977; Sanwal, 1979; Pearson, 1981; Levenson and Housman, 1981).

1.2 Role of cAMP in Myoblast Differentiation

One aspect of myogenesis which concerns this investigation is the role of cAMP in differentiation. Reporter (1972) found a drop in the levels of cAMP during the process of fusion. This was later ascribed to a fall in the activity of the enzyme adenylate cyclase (Wahrmann et al, 1973). However, Zalin and Montagne (1974) found that there is a transient rise in the levels of cAMP prior to the onset of fusion of chick myoblasts grown in primary culture. From the same laboratory it was reported that addition of prostaglandin (PGE_1), a compound which elevates the concentration of cAMP in the cells, caused a precocious burst of cell fusion (Zalin and Leaver, 1975; Zalin, 1977; Zalin, 1979). The onset of cell fusion was completely blocked when aspirin and indomethacin (inhibitors of prostaglandin synthesis) were added to the growth medium. However the inhibitory effect of these compounds was completely reversed by the addition

of PGE_1 (Zalin, 1977). Thus, these sets of experiments suggest that a transient rise of cAMP serves as a signal for myoblast differentiation. A similar sudden rise of cAMP levels followed by its decline has also been seen in the permanent cell line of L6 myoblasts (Ball and Sanwal, 1980), suggesting a role of cAMP in myoblast differentiation. Recently, Curtis and Zalin (1981) have shown that epinephrine and isoproterenol provoked primary chick myoblasts to initiate precocious cell fusion. Both the rise in intracellular cAMP and cell fusion generated by these effectors were prevented by propranolol, a specific blocker of β-adrenergic receptors. Propranolol had no effect on the cell fusion provoked by PGE_1 or on cell fusion in control cultures. The results support the idea that a rise in cAMP is the intracellular change responsible for initiating events that culminate in myoblast differentiation 4 to 5 hours later. The results also indicate that the hormone responsible for the regulation of myoblast differentiation in vitro is not acting through β-adrenergic receptor.

1.3 Multiple Forms of Phosphodiesterases

The presence of multiple forms of cAMP phosphodiesterases in mammalian tissues has been well documented (reviewed by Strada and Thompson, 1978).

These forms include those which have low affinity for cAMP and are activated by calmodulin, those with high affinity for cAMP and are unaffected by calmodulin, the forms capable of hydrolyzing both cAMP and cGMP as substrates and those which are capable of hydrolyzing both cCMP and cAMP as substrates. Recently, calmodulin-activated phosphodiesterase has been purified to homogeneity from bovine brain (Klee et al., 1979; Morrill et al., 1979 and Sharma et al., 1980), and from bovine heart (La Porte et al., 1979). So far there has been one report of purification of a high affinity cAMP phosphodiesterase, that from dog kidney (Thompson et al., 1979). Recently the enzyme form which degrades both cAMP and cGMP has also been purified from bovine adrenal and heart tissues (Martins et al., 1982). The enzyme form hydrolyzing cCMP and cAMP as substrates has also been purified from pig liver (Helfman and Kuo, 1982; Helfman et al., 1981).

In most tissues the different varieties of phosphodiesterases coexist. For instance, Gain and Appleman (1978) have reported the presence in adult rat skeletal muscle of both low and high affinity phosphodiesterases. Such is also the case in liver and several other tissues (Strada and Thompson, 1978). When a distinction between several phosphodiesterases within one cell type can be made on the basis of substrate affinity, substrate specificity, or modulation by effectors, it is reasonable

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to assume that the enzymes are probably structurally unrelated to each other. However, when multiple forms of phosphodiesterases are present in the same cell, which can not be distinguished from each other by simple kinetic or regulatory criteria, two questions of some significance arise. First, whether the various forms are interrelated or interconvertible, and second whether such interrelationships have any metabolic or regulatory significance.

1.4 Regulation of Phosphodiesterases

If the presence of multiple forms of phosphodiesterases in the same cell type has any physiological significance it is quite logical to assume that each of these forms is regulated by mechanisms independently of each other. If one goes through the literature one finds that this indeed is the case (Strada and Thompson, 1978). Since most of these forms are quite unrelated to each other and are not present in myoblasts (See Chapter 2), it is unnecessary to review the enormous amount of literature available in this area. In the following, therefore, only the pertinent information regarding the regulation of phosphodiesterase forms present in rat myoblasts is discussed. This form of phosphodiesterase has been termed as low Km phosphodiesterase and is regulated by the levels of cAMP in

the cells as well as by several hormones including insulin and by proteases.

1.4.1 Regulation of Phosphodiesterases by cAMP

The activity of low Km cAMP phosphodiesterases has been shown to be increased by cAMP or the agents which elevate the concentration of cAMP in the cells. There are two ways by which cAMP regulates phosphodiesterase activity. One is the short term activation which usually requires a brief exposure (1 min to 60 min) of cells to cAMP and does not require any de novo protein synthesis. The second is by the long term exposure of the cells to cAMP (sometimes up to 48 h). This process requires de novo protein synthesis. Both these mechanisms of regulation have been reported in several systems (Strada and Thompson, 1978; Vaughan et al., 1981).

Short term activation of phosphodiesterase by cAMP was reported about 10 years ago by Pawlson et al., (1974) who found that when rat fat cells were incubated with ACTH, epinephrine or theophylline for 2 to 10 min, the activity of low Km phosphodiesterase increased by about 35 to 50%. It was suggested that this increase in the activity of the enzyme was secondary to accumulation of cAMP, whether caused by stimulation of adenylate cyclase (ACTH, epinephrine) or inhibition of cAMP degradation (theophylline). Similar activation of phosphodiesterase by cAMP have been observed by Zinman and Hollenberg

(1974) using fat cells; by Allan and Sneyd (1975) and Loten et al. (1978) using rat hepatocytes and by Alvarez et al. (1981) using human platetets.

The mechanism of activation of phosphodiesterase by cAMP is still largely unknown. The activation of phosphodiesterase by cAMP has also been found in our system (See Chapter 3) and has been proposed to involve a protein phosphorylation step (Chapter 3 and Ball et al., 1980).

Induction of phosphodiesterases by cAMP has also been reported in several systems. d'Armiento (1972) found that in 3T3 cells phosphodiesterase was induced when the cells were exposed to Bt_2cAMP and MIX. Similar results were obtained by Manganiello and Vaughan (1972) using fibroblasts and by Bourne et al. (1973) using lymphoma S49 cells. Mutants of the S49 cell line, defective in protein kinase did not show induction of phosphodiesterase, suggesting the involvement of a protein kinase during induction. Ross et al. (1977) and Thompson et al. (1980) also observed an increase in the activity of phosphodiesterase when hepatoma cells or human lympocytes were exposed to Bt_2cAMP and MIX.

Induction of phosphodiesterase has also been observed in rat myoblasts (Ball et al., 1980 and Chapter 4 of this thesis). Although the exact mechanism of induction is still not known, involvement of a protease

has been indicated in the present work (See Chapter 4).

1.4.2 Regulation of Phosphodiesterase by Insulin

Several hormones (e.g. glucagon, PGE_1, epinephrine) raise cAMP levels in the cells by stimulating the membrane bound adenylate cyclase (Ross and Gilman, 1980). Such hormones therefore are capable of activating and/or inducing phosphodiesterases the way it has been described above (Strada and Thompson, 1978). It is believed that this type of regulation serves to remove cAMP from the cells to avoid any "toxic" effects. However, in the case of insulin another type of activation of phosphodiesterase has been observed. This hormone does not affect adenylate cyclase and does not use cAMP as messenger.

The interest in such studies began when Butcher et al.(1966) found that insulin rapidly lowered intracellular cAMP levels in adipose tissues. Such drop in cAMP levels was also observed in isolated liver cells (Pilkis et al,1975). Several investigators have shown increase in the activity of phosphodiesterase in response to insulin, using fat cells, (Loten and Sneyd, 1970; Manganiello and Vaughan,1973; Lovell-Smith et al,1977). In all these cases increase in the activity of the enzyme was observed in cell free extracts pretreated with insulin. Recently, Weber and Appleman (1982)

have shown increase in the activity of only one low Km form of phosphodiesterase when adipocytes were treated with insulin. This form of phosphodiesterase represents only one peak out of four resolved by ion exchange chromatography. In adipocytes, the effect of insulin on phosphodiesterase activation has been reported to require either metabolic energy or ATP (Zinman and Hollenberg, 1974; Kono et al,1975). The exact mechanism of insulin action is still not known in this system.

Reports of the effect of insulin on hepatic phosphodiesterase have also been published (Thompson et al,1973; Loten et al,1978). Insulin has also been shown to activate phosphodiesterase using partially purified membrane fractions of liver cells (House et al,1972). Marchmont and Houslay (1980) have reported the requirement of ATP and cAMP along with insulin for the activation of phosphodiesterase in crude rat liver plasma membrane fractions. It has also been shown by these investigators (Marchmont and Houslay, 1980; Marchmont and Houslay, 1981) that during this activation the enzyme is phosphorylated. Thus it seems that phosphodiesterase is amongst the large number of proteins which have been suggested to be regulated by phosphorylation-dephosphorylation mechanisms in response to insulin (Avruch et al,1978; Seals et al,1979a, Seals et al,1979b; Rosen et al,1981).

1.4.3 Regulation of Phosphodiesterases by Proteases

Effects of proteases on phosphodiesterase activity have also been described in the literature. For example, when rat liver extracts were treated with trypsin, a low Km phosphodiesterase activity appeared, This form of the enzyme was also produced when fresh extracts were stored at 4°C for 25 hr. (Russell et al., 1973). Thompson et al. (1976) reported that phosphodiesterase activity present in the extracts of human lymphocytes disappeared on storage and a kinetically different form appeared. This new form of phosphodiesterase had a lower apparent molecular weight than the enzyme present in the fresh extracts, indicating the involvement of an endogeneous protease. A similar conclusion was reached by Sakai et al. (1978) who found that freeze-thawing of rat liver caused activation of a new soluble phosphodiesterase. They were also able to demonstrate that freeze-thawing released a lysosomal protease which was responsible for activation of phosphodiesterase. Rat kidney particulate fractions also contain a similar factor (lysosomal protease) which can activate kidney supernatant phosphodiesterase (Strewler et al., 1978). Loten et al. (1980) could solubilize the activity of phosphodiesterase by treatment of a particulate fraction from liver with hypotonic buffers and have suggested the involvement of a proteolytic enzyme during this solubilization. The same conclusion was

reached by Makino et al (1980) using rat fat cells. Recently, Moreno et al.(1982) have also demonstrated that trypsin could cause the activation of phosphodiesterase present in the cell free extracts of Mucor rouxii. The treatment by trypsin resulted in a decrease of S20,w from 4 to 2.6.

Thus, on the basis of above survey, it seems that the activation of phosphodiesterases by endogeneous proteases is a common phenomenon among various cell types. Although, proteolytic conversions of phosphodiesterases explain many of the observations about phosphodiesterase, particularly the occurrence of multiple forms, the actual involvement of any protease in the physiological regulation still remains to be established.

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CHAPTER 2. REGULATION OF CYCLIC ADENOSINE 3':5'-MONOPHOSPHATE PHOSPHODIESTERASES. INTERRELATIONSHIP OF THE VARIOUS FORMS IN RAT SKELETAL MYOBLASTS AND ADULT MUSCLE

2.1 Introduction

Our laboratory has been interested for sometime (Sanwal, 1979) in the mechanism of differentiation of skeletal myoblasts and the role of cAMP in this process. This interest necessitated a study of phosphodiesterase which may conceivably control the levels of cAMP *in vivo*. Earlier studies (Ball *et al.*, 1979; Ball *et al.*, 1980) have shown that a permanent cell line of rat skeletal myoblasts, L6, exhibits multiple forms of a calmodulin-independent, high affinity cAMP phosphodiesterase. These forms have been designated PDE I, PDE II, PDE III and PDE IV depending upon the order of their emergence from a Bio-Gel A-1.5 M column (Ball *et al.*, 1980). In view of the existence of multiplicity of forms, the question arose whether there was any structural relationship between them. This information was also required before the molecular details of the control of the phosphodiesterases by a phosphorylation-dephosphorylation mechanism (Ball *et al.*, 1980) could be investigated.

This chapter describes the purification of PDE I and PDE IV from rat skeletal muscle and myoblasts, and a comparison of their kinetic properties and subunit structure. The evidence presented favors the view that myo-

blasts have only one primary form of phosphodiesterase (PDE II) which can be converted *in vitro* on one hand to PDE I by aggregation and on the other to PDE IV by proteolysis.

2.2 Materials and Methods

2.2.1 Chemicals

Radiochemicals were purchased from New England Nuclear (Boston, Mass.), Bio-Gel A-1.5 M and A-0.5 M were from Bio-Rad (Richmond, Ca.). QAE-A-25 Sephadex, Sephacryl S-200, and Sepharose 4B were from Pharmacia Fine Chemicals, Uppsala, Sweden. Powdered Dulbecco's Modified Eagle's Medium (DME) and horse serum were from Flow Laboratories (Rockville, Md.), gentamycin from Schering Corp. (Kenilworth, N.J.), ammonium sulphate from Schwarz-Mann (Orange-burg, N.Y.), and trypsin from Gibco (Grand Island, N.Y.). All other chemicals were from either Sigma Chemical Co. or from Fisher Scientific Co.

2.2.2 Cell Culture

Clones of rat myoblast cell lines, L6 (Yaffe, 1968) were used. The cells were usually plated at a density of 3500/cm^2 in a dish of the appropriate size in Dulbecco's Modified Eagle's Medium (supplemented with 10% horse serum and 5 µg/ml of gentamycin. At appropriate times (4 days old myoblasts or 8 days old myotubes), cells were harvested by scrapping them into a minimum volume of buffer

A (0.05 M potassium phosphate, 1 mM imidazole; 1 mM TSF, 5 mM EGTA, 0.1% 2-mercaptoethanol, pH 7.5). Homogenization was effected with a polytron homogenizer (Brinkman Instruments, Rexdale, Canada) for 15 s at a setting of 3. The mixture was centrifuged at 18,000 xg for 15 min at 4°C and supernatant was used for further purification or electrophoresis. Induction of phosphodiesterases in myoblasts or myotubes was achieved according to Ball et al.(1980). The cells were exposed to 0.7 mM Bt_2cAMP and 0.1 mM MIX for 16 h prior to their harvest.

2.2.3 Assay of cAMP Phosphodiesterases

The enzymes were assayed using the two step assay of d'Armiento et al,(1972) with minor modifications. In the first step the reaction mixture (0.2 ml) containing 40 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 30 µg BSA, (G-^{3}H) cAMP (200,000 cpm), unlabeled cAMP (1 µM), and an appropriate amount of enzyme was incubated at 30°C for the required amount of time (usually 10 min). The reaction was terminated by boiling in a water bath for 1 min. After cooling, 50 µg of snake venom was added in the second step and incubation continued for 10 min more at 30°C. At the end of this incubation period, one ml of a 1:3 slurry of AG 1 X 2 anion exchange resin containing 0.1 mM

adenosine was added and the mixture was centrifuged at 8000 xg for 2 min. A 0.5 ml aliquot of the supernatant was counted in a scintillation counter. The background radioactivity was routinely between 1-2%, except when high salt concentrations were used during the purification process. In this case, boiled enzyme aliquots were used as blanks to correct for the high background. One unit of enzyme is expressed as the amount of enzyme that hydrolyzes 1 pmole of cAMP per min, at substrate concentration of 1 μM.

2.2.4 Polyacrylamide Gel Electrophoresis

Electrophoresis was done as described by Davis (1964) for the analysis of column fractions. Subunit molecular weights were determined using Laemmli's slab gels (1970) in 0.1% sodium dodecyl sulfate. To analyze for phosphodiesterase activity in gel fractions, electrophoresis was performed in cylindrical tubes (0.5 x 9 cm) using 7.2% acrylamide in 0.5 M Tris phosphate, 1 mM imidazole, 30% ethylene glycol, 5 mM EGTA, pH 7.5 (Buffer B). The gel was polymerized with 0.575% (v/v) TEMED and 0.07% (w/v) ammonium persulfate. Electrophoresis was performed using buffer B at 4°C with 2 mAmps per gel in the absence of a stacking gel. The gels were prerun for 30 min prior to the application of samples in an equal volume of

80% sucrose containing 2.5 µg bromophenol of blue. Following electrophoresis, the gel was cut at the dye marker and sliced longitudinally in half. One half was stained with coomassie blue while the other half was cross-sectioned into 1 mm slices. Each slice was eluted with 100 µl of a buffer containing 0.05 M potassium phosphate, 30% ethylene glycol, 0.03% (w/v) BSA, 1 mM imidazole, 1 mM TSF, 5 mM EGTA, 0.1% 2-mercaptoethanol, pH 7.5, at 4°C for 16 h in a shaker. 40 µl of the supernatant was assayed for cAMP phosphodiesterase activity following the standard procedure except that the incubation time was extended from 10 min to between 30 and 120 min. Rf's were calculated by dividing the distance from the top of the gel by the total distance traversed by the bromophenol blue dye marker.

Proteins were cross-linked by dimethylsuberimidate at pH 8.5 as described by Davies and Stark (1970). Protein concentration was determined by the method of Lowry et al.,(1951).

2.2.5 Amino Acid Analysis

Amino acid analyses were done using a Beckman model 119 CL micro-single column analyzer after hydrolysis in 6N HCl. NH_2 terminal analyses were performed according to the method of Gray (1972).

2.2.6 Preparation of MIX-Sepharose

Hexanediamine Sepharose 4B was prepared by coupling hexanediamine to Sepharose 4B by the cyanogen bromide method of March et al. (1974). The 7-acetyl derivative of MIX was prepared according to the method described by Mohindru et al. (1978) except that the final product was acid-precipitated 3 times instead of being recrystallized in ethanol. The 7-acetyl derivative of MIX was coupled to hexanediamine Sepharose by using carbodiimide. The resultant derivative had approximately 8 mol of MIX bound per ml of packed gel as estimated by the difference between the amount of MIX added to the coupling reaction and that recovered in the wash.

2.3 Results

2.3.1 Phosphodiesterase Activity in Crude Extracts of Muscle Tissue and Myotubes

We had shown earlier (Ball et al., 1979 and Ball et al., 1980) that freshly prepared extracts of cultured rat myoblasts gave three peaks of activity of Bio-Gel

A-1.5 M columns; PDE I emerged in the void volume, and PDE II and PDE III at a ve/vo ratio of 1.3 and 1.6, respectively; PDE II was the only form activated 3-4 fold by snake venom proteases. Essentially similar results are obtained when fresh extracts of adult rat muscle are chromatographed on Bio-Gel A-1.5 M (Fig. 1A), except that the peak of PDE I is much larger than that found in myoblast extracts. When muscle extracts are prepared in buffer containing 5 mM EGTA or 6 µg/ml of the protease inhibitor, leupeptin, only forms I and II are predominant (Fig. 1B). Incubation of crude extracts with 2.5 mM $CaCl_2$ for brief periods results in the disappearance of all forms and the appearance of a new one, PDE IV with a ve/vo ratio of about 1.9 (Fig. 2). Prolonged incubation (more than 48 hr) of cell extracts at 4°C with calcium does not produce forms smaller than PDE IV. This form like PDE I and PDE III was not activatable by snake venom or other proteases.

Since muscle tissue contains a calcium activated neutral protease (Meyer et al, 1964), it seems plausible that PDE IV arises from proteolytic breakdown of the various forms of phosphodiesterases. Activation of the protease by calcium would be necessary to produce PDE IV because the calcium-activatable protease in muscle is inactive due to the presence of an inhibitor

FIGURE 1

GEL EXCLUSION CHROMATOGRAPHY OF cAMP PHOSPHODIESTERASE FROM RAT MUSCLE EXTRACTS AFTER VARIOUS TREATMENTS

Crude supernatant from fresh rat skeletal muscle extracts were prepared in buffer A without EGTA (A) or with 5 mM EGTA (B). Solid ammonium sulphate was added after incubation of the supernatant at 30°C for 30 min to give 60% saturation, and the resulting precipitates were dissolved and applied onto a Bio-Gel A-1.5 M column (2.6 x 90 cm). The column was equilibrated and eluted with the same buffer for preparation of extract. In A, about 700 mg of protein, and in B, about 350 mg of protein were applied to the columns. Fractions of 5 ml were collected and assayed for phosphodiesterase activity in the presence (o) and absence (●) of snake venom.

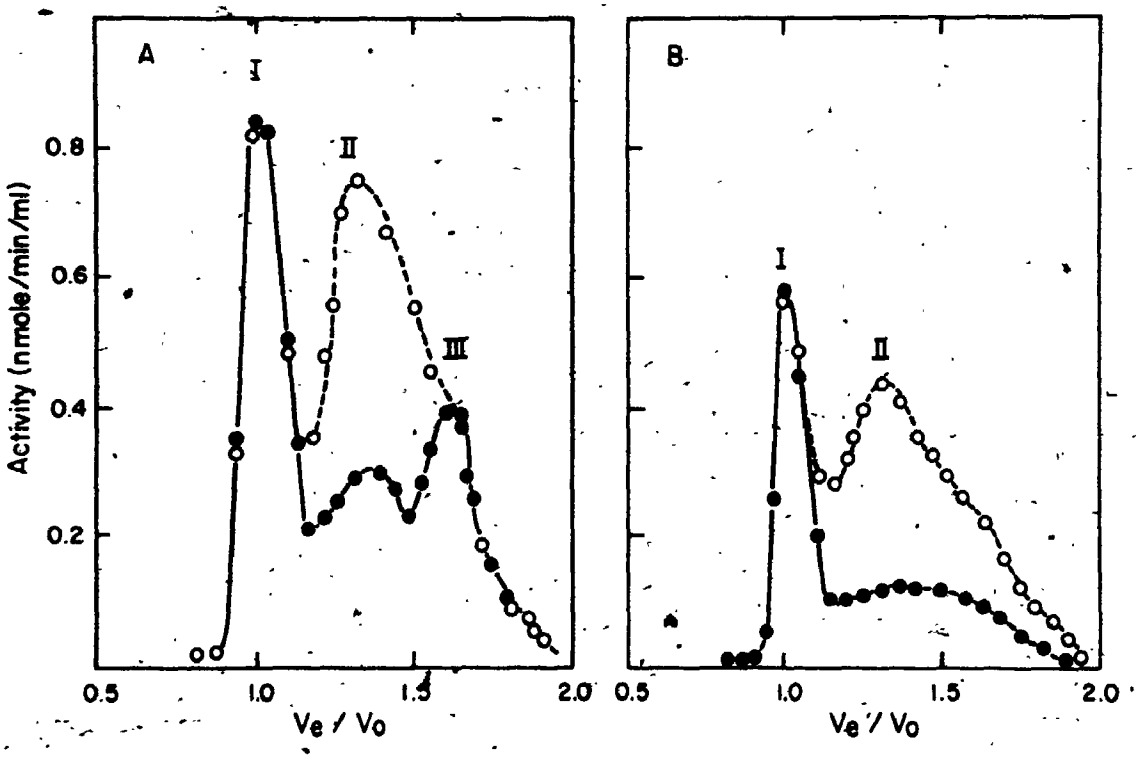
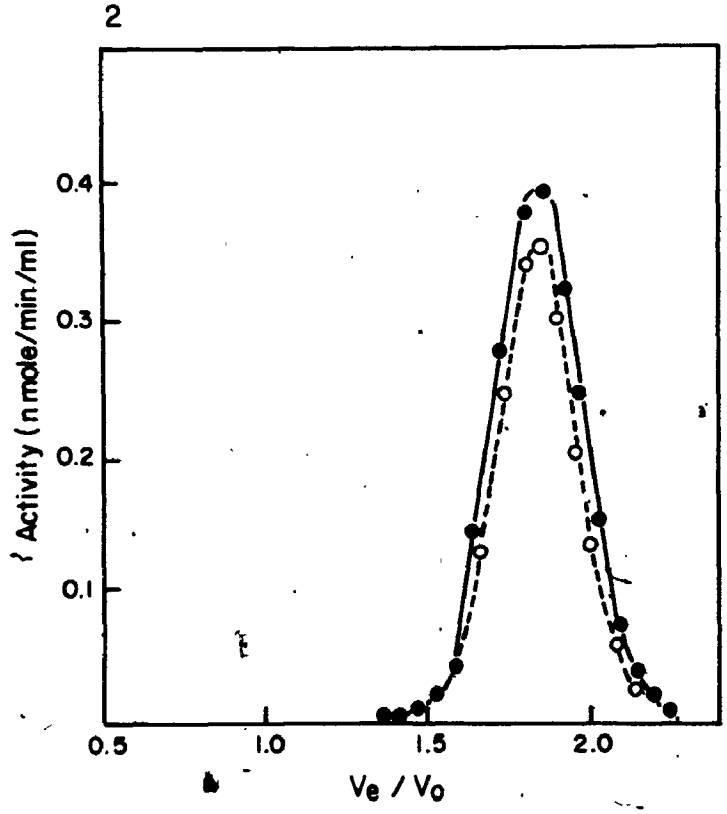

A
B
I
II
III
Activity (nmole/min/ml)
0.8
0.6
0.4
0.2
0.5
1.0
1.5
2.0
Ve / Vo

FIGURE 2

GEL EXCLUSION CHROMATOGRAPHY OF cAMP PHOSPHODIESTERASE FROM RAT MUSCLE EXTRACT IN THE PRESENCE OF $CaCl_2$

Rat muscle extract was prepared in 50 mM-Tris-HCl, pH 7.5, 0.1 mM TSF, 1 mM imidazole, 0.1% 2-mercapto-ethanol and 2.5 mM $CaCl_2$, and was then incubated at 30°C for 15 min prior to precipitation by 60% ammonium sulphate. The precipitate was dissolved and about 350 mg of proteins was applied to a Bio-Gel A-1.5 M column (2.6 x 90 cm). Fractions of 5 ml were collected and assayed for phosphodiesterase activity in the presence (o) and absence (●) of snake venom.


2
0.4
0.3
0.2
0.1
Activity (n mole/min/ml)
0.5
1.0
1.5
2.0
Ve / Vo

(Croll et al, 1978). Since this inhibitor is absent from post mitotic myoblasts as shown by Kaur and Sanwal (1981), we used these cells as a source of material in preference to adult muscle to test our hypothesis. The results are presented in Fig. 3. When fresh extracts from myotubes were electrophoresed in polyacrylamide gels, protease insensitive phosphodiesterase form I, III (trace amounts) and IV with Rf values of 0.09, 0.33 and 0.41, respectively are resolved. In addition instead of the expected one form of protease-activatable PDE II, two bands-termed IIa and IIb with Rf values of 0.18 and 0.26 respectively are also separated (Fig. 3A). When the extract is kept for more than 12 hr at 4°C in the presence of calcium and then electrophoresed, PDE IV is the predominant form obtained (Fig. 3B). Crude extracts made in EGTA (5 mM) containing buffer do not show the presence of PDE IV, although this form does appear after prolonged storage.

2.3.2. Formation of PDE I From PDE II

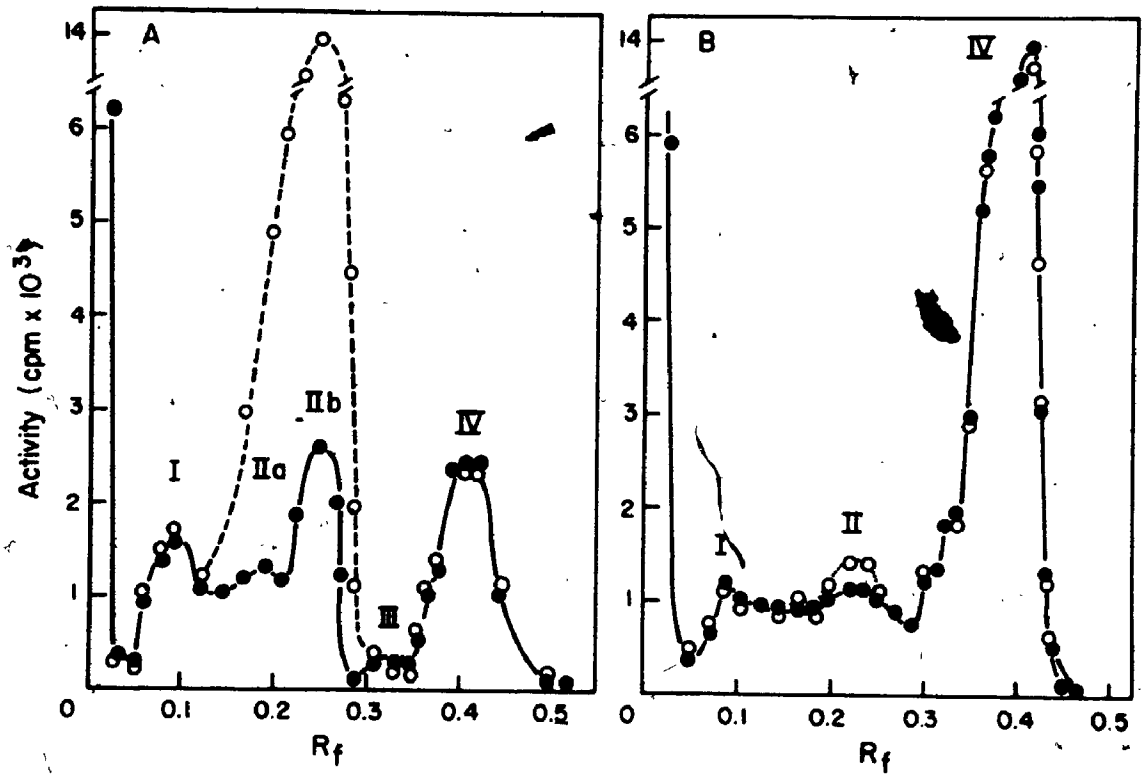
The results reported above suggest that a protease, very probably calcium-activated protease is involved in the genesis of the PDE IV form. The question then arises as to the identity of the phosphodiesterase form which serves as a precursor for PDE IV and the other

FIGURE 3

NONDENATURING GEL ELECTROPHORESIS

PROFILES OF EXTRACT FROM MYOTUBES

Electrophoresis in 7.2% acrylamide was carried out immediately after extraction (A) or after the extract was kept at 4°C for 12 h in the presence of $CaCl_2$ (B). Gels were sliced, eluted and assayed for phosphodiesterase activity in the presence (o) and absence (●) of snake venom as described in the text.


A
B
I
IIa
IIb
III
IV
II
Activity (cpm x 10³)
R_f
0
0.1
0.2
0.3
0.4
0.5
1
2
3
4
5
6
14

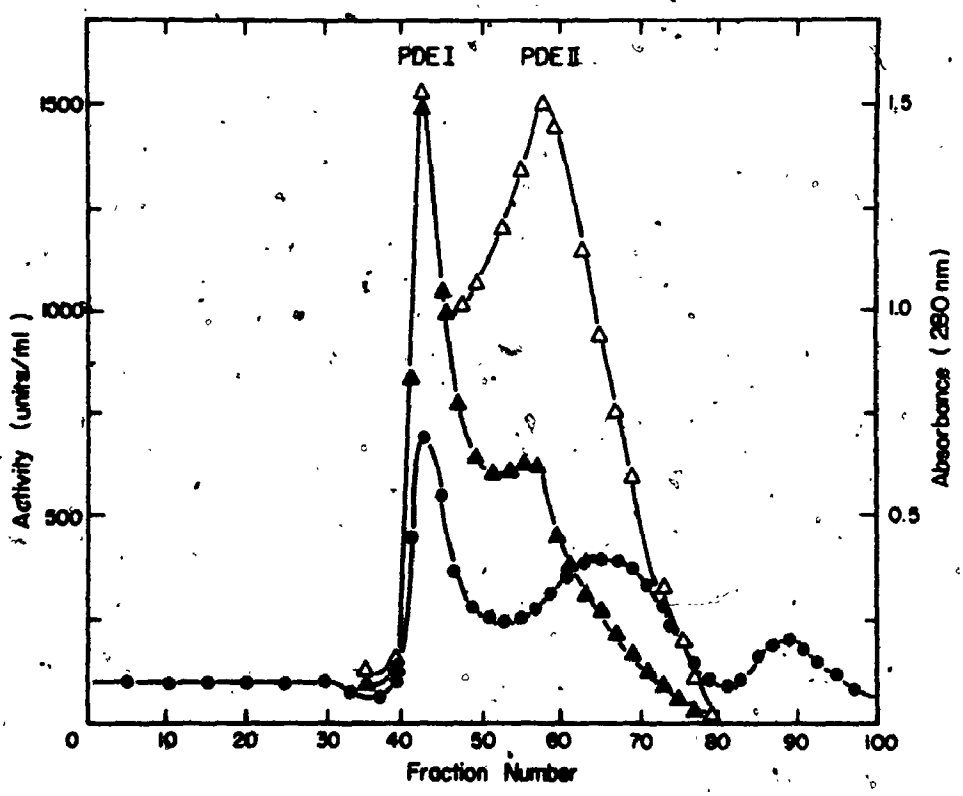
forms separable both on the basis of molecular weights (Fig. 1 and 2) and charge (Fig. 3). Since our earlier experiments had suggested that PDE II is the form susceptible to regulation by various means such as phosphorylation and proteolysis (Ball et al, 1980), it is possible that this is the precursor form. Accordingly, we attempted to purify PDE II in order to examine whether by appropriate experimental manipulations it could be converted to other phosphodiesterase forms in vitro. Freshly dissected rat leg muscle was homogenized in 4 times its volume of buffer A for 1 min. After centrifugation at 18,000 xg for 30 min, the supernatant was withdrawn and filtered through glass wool. Solid ammonium sulphate was then added to give 35% saturation. The precipitate was collected by centrifugation, dissolved in buffer A, and dialyzed overnight against the same buffer. The enzyme preparation was then subjected to polyethylene glycol precipitation and batchwise DEAE-cellulose absorption exactly as described in the scheme for purification of PDE IV (later in this chapter). The enzyme (about 50-60 fold purified) was then applied to a column of Bio-Gel A-1.5 M (2.6 x 90 cm) which was equilibrated with buffer C (see PDE IV purification). The enzyme was eluted with the same buffer. The elution profile for the Bio Gel column is shown in Fig. 4. The

FIGURE 4

CHROMATOGRAPHY OF CRUDE PDE II

FRACTIONS FROM MUSCLE ON BIO-GEL A-1.5 M

A partially purified PDE II fraction from muscle (described in the text) was applied onto Bio-Gel A-1.5 M column (2.6 x 90 cm) equilibrated with buffer C. Fractions of 5 ml were collected and assayed for phosphodiesterase activity in the presence (Δ) and absence (▲) of snake venom. The closed circles in the figure denote absorbance of 280 nm.


PDE I
PDE II
1500
1000
500
0
10
20
30
40
50
60
70
80
90
100
Activity (units/ml)
Absorbance (280 nm)
1.5
1.0
0.5
Fraction Number

phosphodiesterase activity appeared in two peaks. PDE I was eluted in the void volume, while PDE II appeared at the expected ve/vo ratio of 1.3. The latter was activatable by snake venom while the former was not. Significantly, the purification resulted in the absence of PDE III and IV. However, when the peak fraction (Fig. 4) of PDE II was electrophoresed under nondenaturing conditions, it was resolved into forms IIa and IIb, both activatable by snake venom (Fig. 5A). Very little of PDE I was present in the PDE II fraction (Fig. 5A). When the PDE II fraction, almost free of PDE I, was stored at 4°C for several days and re-electrophoresed, a new peak of activity with a Rf of 0.09 corresponding to PDE I, appeared with a corresponding decrease in the amount of PDE IIa and IIb (Fig. 5B). Thus it seems that PDE I is a polymerized form of PDE II.

We have now found several ways in which PDE II can be converted to PDE I, all suggesting that hydrophobic interactions are probably primarily involved in the aggregation of PDE II. One procedure involves treatment of the enzyme with a gradually increasing concentration of ammonium sulphate. In a typical experiment, 200 µgm of partially purified PDE II was dialyzed overnight at 4°C against 100 volumes of buffer C saturated with ammonium sulphate. The enzyme

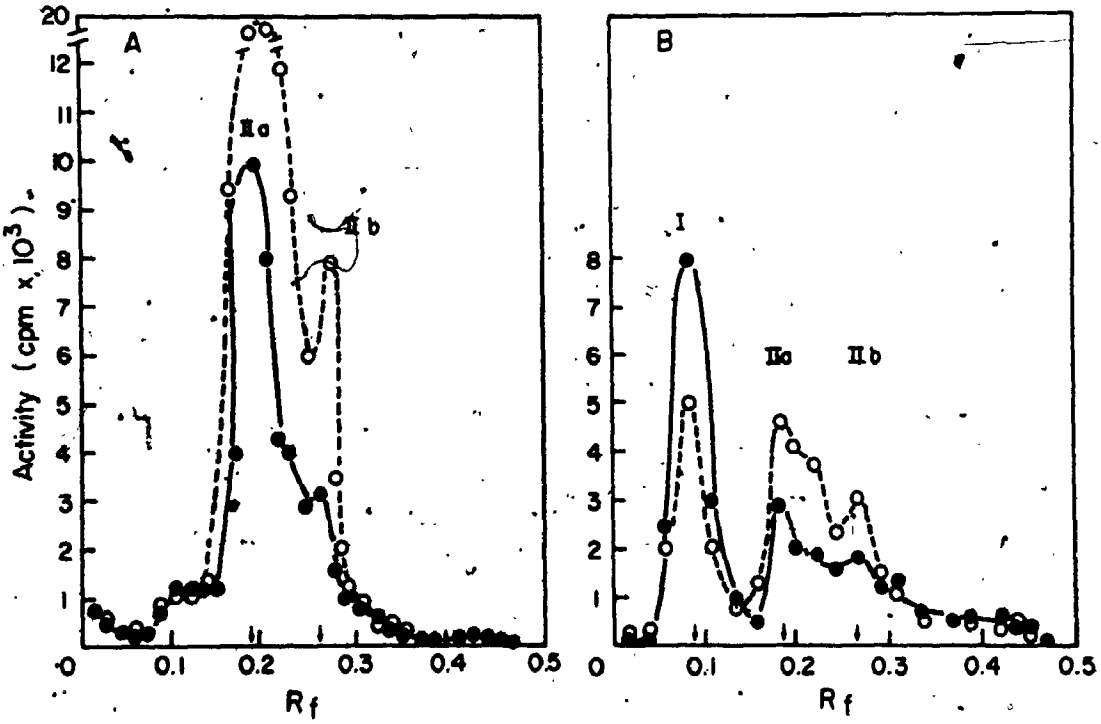
FIGURE 5

NONDENATURING GEL ELECTROPHORESIS

PROFILES OF SEMI-PURIFIED RAT

MUSCLE PDE II

Electrophoresis in 7.2% acrylamide of the peak fraction of PDE obtained from Bio-Gel A-1.5 M column (Fig. 4) was carried out immediately (A) or after storage at 4°C for 7 days (B). Gels were sliced, eluted, and assayed in the presence (o) and absence (●) of snake venom. Arrows indicate Rf values of the peaks.


A
B
IIa
IIb
I
Activity (cpm x 10^3)
R_f
0
0.1
0.2
0.3
0.4
0.5
1
2
3
4
5
6
7
8
9
10
11
12
20

was then redialyzed for 24 h against two changes of buffer C in the absence of ammonium sulphate. About 80-90% of PDE II is converted by this procedure into PDE I as analyzed by gel exclusion chromatography using Bio-Gel A-1.5 M. PDE I appears in the void volume (Fig. 6) and as expected, becomes non activatable by snake venom. Another procedure for the conversion of PDE II to PDE I is by treatment of the partially purified enzyme with 0.1 mM α-tocopheryl phosphate. When PDE II is passed through a Bio-Gel A-1.5 M column equilibrated and eluted with buffer C containing 0.1 mM α-tocopheryl phosphate, almost all of the phosphodiesterase activity appears in the void volume. As shown later, α-tocopheryl phosphate enhances PDE II activity about 4 fold.

The procedure of choice, which results both in the aggregation of PDE II as well as complete purification of the resulting PDE I is affinity chromatography on a MIX-Sepharose column (Fig. 7). The PDE II peak from a Bio-Gel column (See Fig. 4) is applied to an affinity column (1.5 x 20 cm) equilibrated with buffer C containing 2 µg/ml of leupeptin. The column is washed with 3 bed volumes of equilibration buffer and is eluted with a gradient of 150 ml each of 0.1 M to 0.7 M potassium phosphate in buffer C containing 2 µg/ml leupeptin. The column is further washed with 100 ml of 0.7 M phosphate in buffer C, followed by

FIGURE 6

GEL EXCLUSION CHROMATOGRAPHY

OF PDE II AND PDE I PRODUCED FROM PDE II

200 µgm of partially purified PDE II was dialyzed overnight at 4°C against 100 volumes of buffer C saturated with ammonium sulphate. The enzyme was then redialyzed for 24 hr against two changes of buffer C in the absence of ammonium sulphate and chromatographed on Bio-Gel A-1.5 M columns. The phosphodiesterase activity was assayed in the absence (●) and presence (o) of snake venom. Panel A shows the activity profile before conversion to PDE I and Panel B shows the profile of PDE I produced from PDE II.

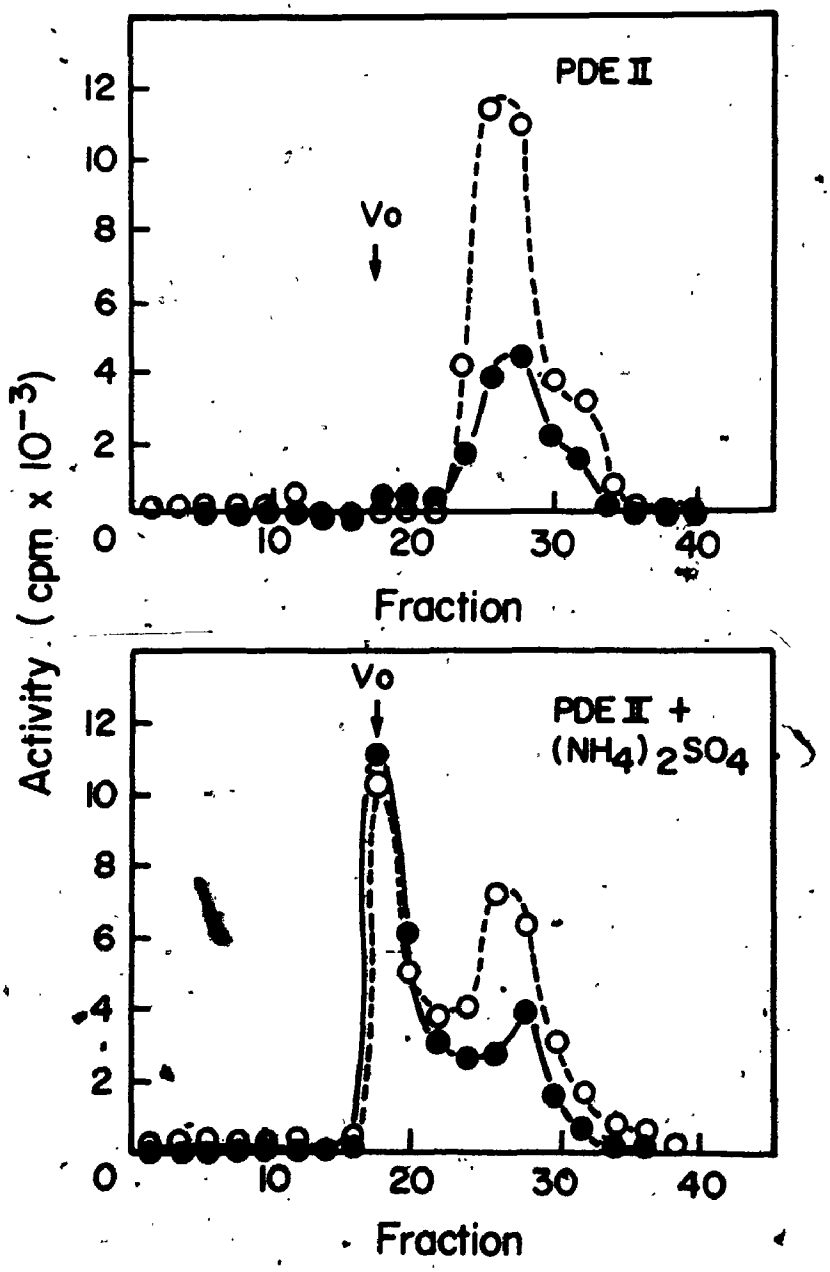
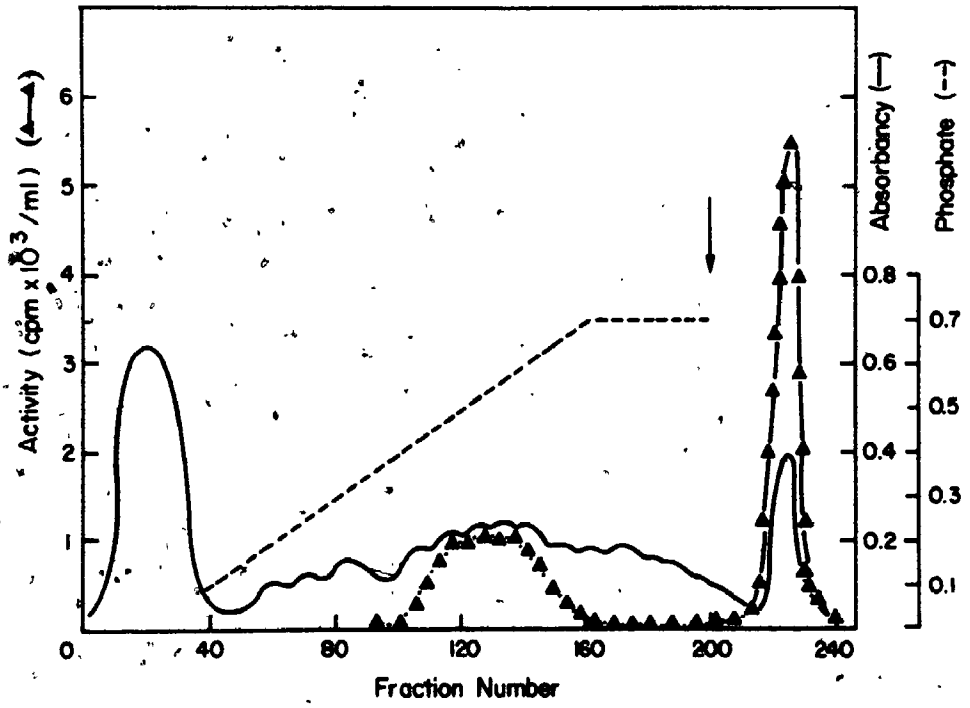

PDE II
Vo
Activity (cpm x 10^{-3})
Fraction
PDE II + $(NH_4)_2SO_4$
Vo
Fraction

FIGURE 7

AFFINITY CHROMATOGRAPHY OF PDE II

PDE II peak fractions of Bio-Gel A-1.5 M (Fig. 4) were pooled and applied onto a MIX-Sepharose column (1.5 x 20 cm). The column was eluted with a gradient of 150 ml each of 0.1 and 0.7 M potassium phosphate in buffer C. The arrow indicates the initiation of elution by 1 M KCl, 0.1 M potassium phosphate, and 0.1 mM cAMP in buffer C.


Activity (cpm x 10^3/ml) (▲—▲)
Absorbancy (—)
Phosphate (- -)
Fraction Number
0
40
80
120
160
200
240
1
2
3
4
5
6
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8

100 ml of buffer C containing 0.1 M phosphate, 1 M KCl, 0.1 mM cAMP and 2μm/ml of leupeptin. PDE I is eluted as a single, sharp peak (Fig. 7). The partially purified PDE I from Bio Gel column (Fig. 4) can also be further purified on the affinity column exactly as described above.

The protein in the peak eluted by KCl (Fig. 7) when electrophoresed under nondenaturing conditions, surprisingly gave multiple bands, (all enzymatically active) which also included bands with Rf of 0.09 (PDE I) and Rf 0.18 - 0.20 (PDE II). Some of the enzyme activity also remained on top of the gel (Fig. 8A) suggesting the presence of highly aggregated protein in the preparation. The presence of multiple bands, however, was not due to impurities in the enzyme preparation, since only one band of protein, corresponding to a molecular weight of about 94,000 was discernible (Fig. 8B) when the same preparation was electrophoresed on SDS-polyacrylamide gel.

If PDE I is a polymerized form of PDE II, as the experiments reported earlier suggest, it should be possible to dissociate PDE I completely into PDE II. However, we have not been able to do so by various procedures tried, such as dissolution in low ionic strength buffer at several pH values, treatment with trypsin for short periods of time, and inclusion of

FIGURE 8

DISC GEL ELECTROPHORESIS

OF PURIFIED PDE I

A, gel electrophoresis under non denaturing conditions in 6% acrylamide at pH 8.8. The arrows indicate the position of the band which was cut out to test for enzyme activity. B, gel electrophoresis in the presence of 0.1% sodium dodecyl sulphate in 7.5% acrylamide.


B

parallel experiments (not shown) demonstrate, all PDE II to be converted to PDE IV. As expected from a proteolytic process, in the presence of low concentrations of chymotrypsin, increase in enzyme activity (and conversion to PDE IV, presumably) occurs much more slowly. It is clear from Fig. 10 that a 2-fold activation occurs during the conversion of PDE II to PDE IV.

2.3.5 Purification of PDE IV

In order to trace structural relationship between the various phosphodiesterase forms, it became essential to purify PDE IV. We found that freezing and subsequent thawing of the leg muscle tissue of rat resulted in the formation of PDE IV as the preponderant form of phosphodiesterase. Frozen muscle tissue was, therefore, used as the starting material, in contrast to the use of fresh tissue in the purification of PDE I and PDE II. Following is the detailed procedure used for the purification of phosphodiesterase IV from rat skeletal muscle.

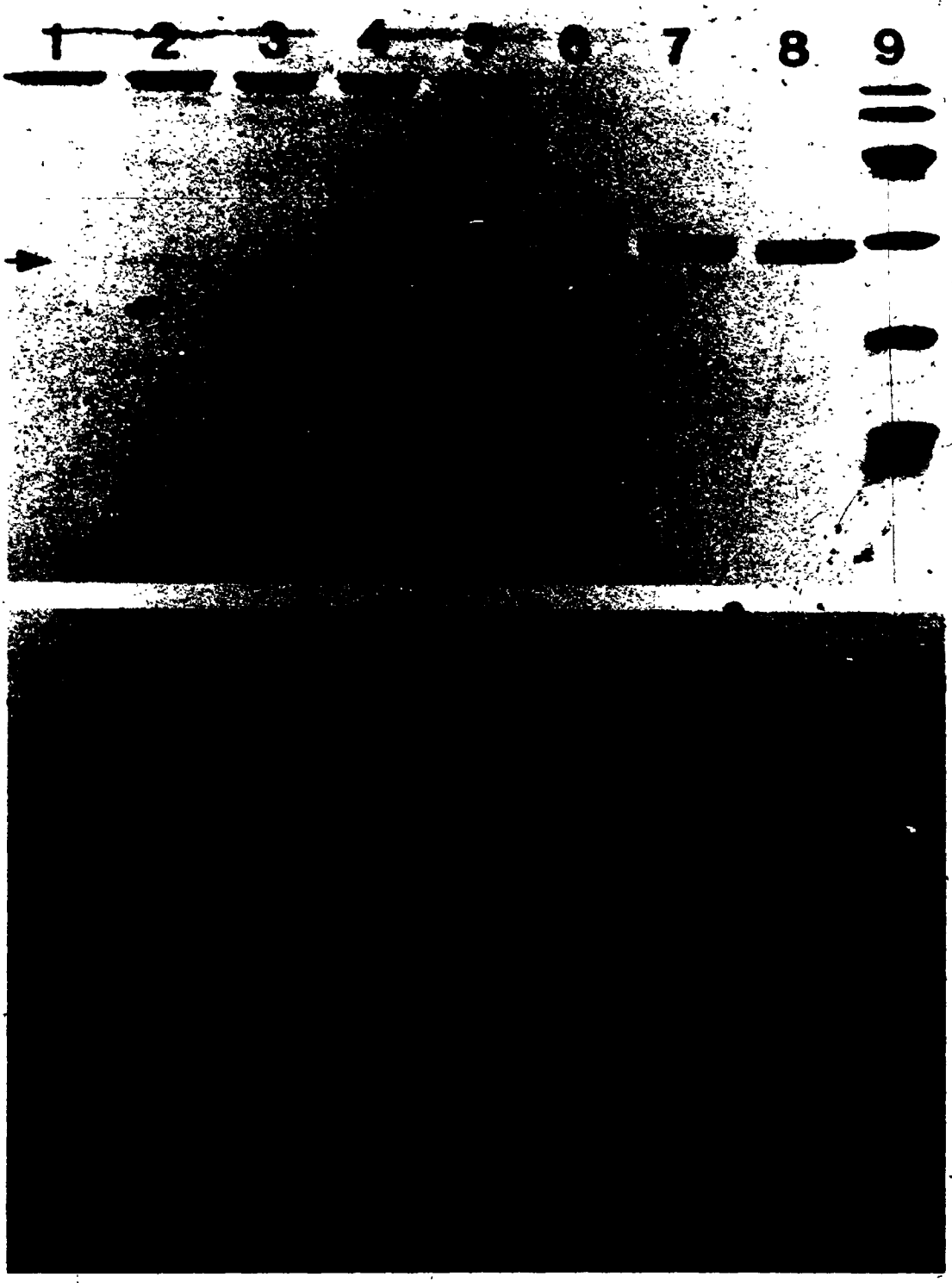
Step 1: Tissue Homogenization and Ammonium Sulfate Fractionation

Frozen rat hind legs were thawed, muscle was dissected relatively free of fat and connective tissues

FIGURE 9

TRYPTIC DIGESTION OF PDE I AND PDE IV

A, homogeneous preparations of PDE I (0.23 mg/ml) or PDE IV (0.2 mg/ml) were incubated with 10 µg/ml trypsin at 30°C. Aliquots (100 µl) were withdrawn at various time intervals and digestion was stopped by adding α-toluene sulfonyl fluoride to a final concentration of 10 mM. The samples were analyzed by SDS-polyacrylamide gel electrophoresis (15% gels) as described in the text. Lanes 1-5 show PDE I treated for 0,10,20,30, and 60 min, respectively. Lanes 6-8 show PDE IV treated for 0,30, and 60 min, respectively. Lane 9 contains molecular weight markers (from top to bottom, phosphorylase b, 94,000; bovine serum albumin 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soyabean trypsin inhibitor 20,100; α-lactalbumin, 14,400). The arrow marks the position of trypsin used in the digestion. B, PDE I (0.25 mg/ml) or PDE IV (0.25 mg/ml) were incubated with 50 µg/ml trypsin at 30°C. Samples were withdrawn and treated as described in A. Lanes 1-5 show PDE I treated for 0,10,20,30 and 60 min. Lanes 6-8 show PDE IV treated for 0,30 and 60 min. Lane 9 shows the same molecular weight markers as in A. The arrow marks the position of trypsin.


1
2
3
4
5
6
7
8
9

in the same way. Treatment with low or high concentrations of trypsin does not alter the Mr of the 30,000 and 28,000 subunits of PDE IV (Fig. 9A and B). Activity of the enzyme, however is gradually lost on incubation with trypsin. At a concentration of 10 µg of trypsin/ml, the half life of PDE IV activity is about 30 min, and at 50 µg/ml it is only 10 min. PDE I, as mentioned earlier, does not lose activity on incubation with low concentration of trypsin, but at higher concentrations activity are lost with a half life of about 30 min (data not shown). Since substantial amounts of enzyme activity are still discernible (about 80%) 10 min after treatment with trypsin, when all the protein is present as fragments of Mr = 60,000 and 28,000 (Fig. 9B), it is very likely that the 60,000-dalton form is also enzymically active.

Since we have shown earlier that PDE I probably arises from PDE II by aggregation, it should be possible, in view of the findings reported above, to demonstrate that PDE II and PDE III (which we have earlier suggested is probably derived from PDE II) also can serve as precursors of PDE IV. To probe into this aspect of the problem, we partially purified PDE II from myoblasts, which had been exposed to 16-18 hr to Bt_2cAMP, as outlined under "Materials and Methods." We had shown previously (Bell et al,1980 and

chapter 4) that in such myoblasts, high levels of PDE III are present. Myoblast extracts in buffer A were passed through a DEAE-Bio Gel column. After washing with two column volumes, a linear gradient of 0-100 mM NaCl was applied and passed through a Bio-Gel A-1.5 M column as described in the legend to Fig. 4, except that the buffer composition was 25 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM imidazole, 10 mM 2-mercaptoethanol, and 150 mM NaCl. The peak corresponding to PDE III (ve/vo ratio of 1.6) was rechromatographed on Bio-Gel A-1.5 M. Only one peak of phosphodiesterase activity (non activatable by snake venom) was obtained at the expected position free from PDE II or PDE IV. PDE II free from other forms of phosphodiesterase was partially purified as described elsewhere in this chapter. To both PDE II and PDE III preparations (about 200 µg of protein) bovine serum albumin (1 mg/ml) was added (to protect enzyme activity) and each was treated separately with 10 µg/ml of chymotrypsin for 30 min at 37°C. The treated preparations were immediately chromatographed on Bio-Gel A-1.5 M columns. In both cases, only one peak of activity corresponding to PDE IV (ve/vo ratio of 1.9) was discernible. Treatment of the enzymes with 1 µg/ml of trypsin or 50 µg/ml of snake venom produced the same effect as chymotrypsin, i.e. both

PDE II and PDE III were reduced to PDE IV. The effect of chymotrypsin, trypsin and snake venom on the phosphodiesterases could be entirely eliminated if they were pretreated with TSF, soybean trypsin inhibitor, or a mixture of EGTA and cysteine (1 mM each), respectively. This suggests that proteolysis was involved in the conversion of both PDE II and PDE III to the smaller molecular weight form, PDE IV. In non-denaturing polyacrylamide gels, PDE IV produced both from PDE II and PDE III migrated with an Rf value of 0.41, exactly as did a sample of homogeneously purified preparation of PDE IV.

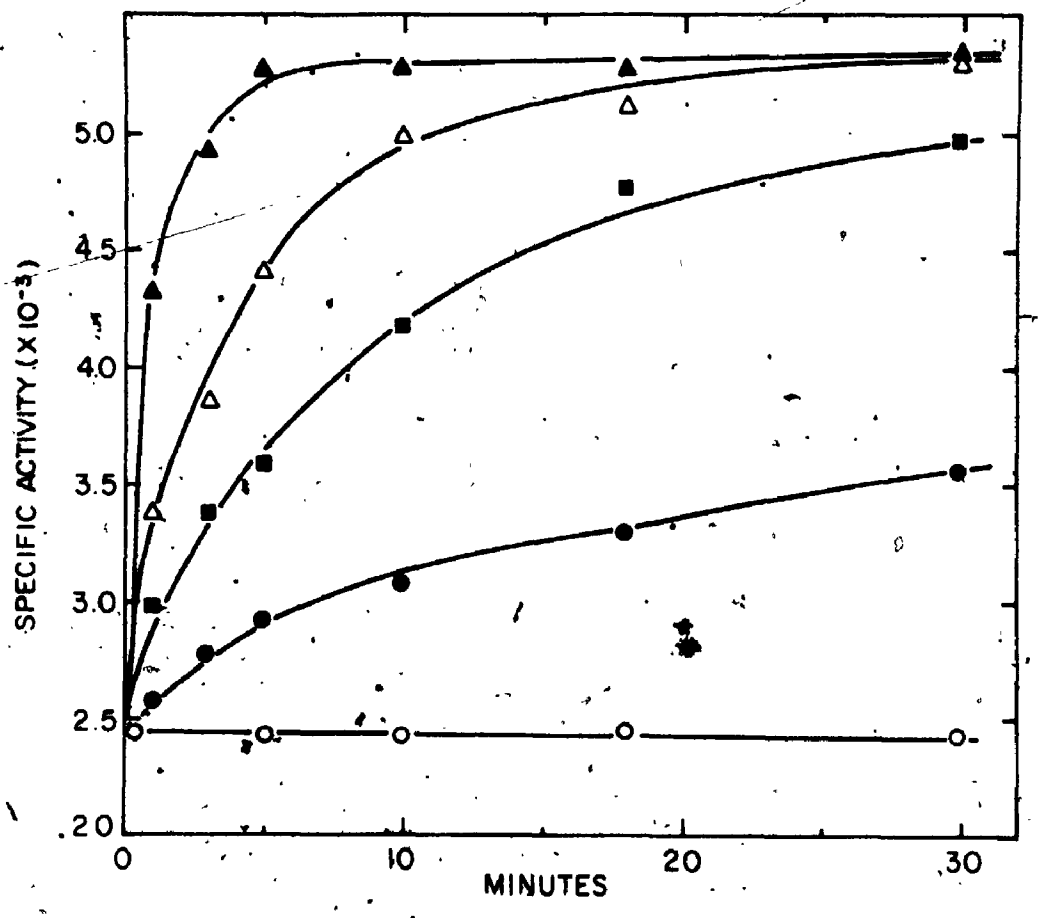
2.3.4 Stimulation of Phosphodiesterase Activity During Formation of PDE IV

Just like the conversion of PDE II to PDE I as described earlier, which results in a 2-fold activation and simultaneous loss of protease activatability, the conversion of PDE II to PDE IV also leads to activation and protease insensitivity. The time course of activation is shown in Fig. 10. Incubation of a partially purified preparation of PDE II (devoid of other phosphodiesterase forms) with chymotrypsin leads to a time-dependent increase in enzyme activity (Fig. 10). At high concentrations of chymotrypsin (5 µg/ml), maximum activation occurs in 10 min, at which time,

FIGURE 10

ACTIVATION OF PDE II BY CHYMOTRYPSIN

Partially purified (about 60-fold) PDE II was incubated at 30°C with various concentrations of chymotrypsin in 1-ml volume. Protein concentration was 15.7 µg/ml. At indicated time intervals 50 µl aliquots were pipetted, into tubes containing 50 µl of 5 mM TSF to stop proteolysis, and enzyme activity was measured. Specific activity is pico-moles of cAMP utilized/min/mg of protein. The concentrations of chymotrypsin used were: o——o control; ●——● 0.5 µg/ml; ■——■ 1.25 µg/ml; Δ——Δ, 2.5 µg/ml; ▲——▲, 5 µg/ml.


SPECIFIC ACTIVITY (X 10⁻³)
5.0
4.5
4.0
3.5
3.0
2.5
.20
0
10
20
30
MINUTES

parallel experiments (not shown) demonstrate, all PDE II to be converted to PDE IV. As expected from a proteolytic process, in the presence of low concentrations of chymotrypsin, increase in enzyme activity (and conversion to PDE IV, presumably) occurs much more slowly. It is clear from Fig. 10 that a 2-fold activation occurs during the conversion of PDE II to PDE IV.

2.3.5 Purification of PDE IV

In order to trace structural relationship between the various phosphodiesterase forms, it became essential to purify PDE IV. We found that freezing and subsequent thawing of the leg muscle tissue of rat resulted in the formation of PDE IV as the preponderant form of phosphodiesterase. Frozen muscle tissue was, therefore, used as the starting material, in contrast to the use of fresh tissue in the purification of PDE I and PDE II. Following is the detailed procedure used for the purification of phosphodiesterase IV from rat skeletal muscle.

Step 1: Tissue Homogenization and Ammonium Sulfate Fractionation

Frozen rat hind legs were thawed, muscle was dissected relatively free of fat and connective tissues

and diced. The muscle was homogenized in a waring blender at 4°C in 4 volumes of 50 mM Tris-HCl (pH 7.5), 0.1 mM TSF, 0.1% 2-mercaptoethanol, 1 mM imidazole and 2.5 mM $CaCl_2$ for 1 min. The homogenate was centrifuged at 18,000 xg for 30 min. The resultant supernatant was filtered through glass wool and incubated at 30°C for 15 min. Following incubation, solid ammonium sulfate was added to 35% saturation. The mixture was stirred at 4°C for 30 min and centrifuged at 18,000 xg for 30 min. After centrifugation, the pellet was discarded and more ammonium sulphate was added to 60% saturation. After 30 min stirring, the precipitate was collected by centrifugation, dissolved in homogenizing buffer and dialyzed overnight against the same buffer.

Step 2: Polyethylene Glycol Precipitation

The dialyzed enzyme was made to 8% polyethylene glycol by the gradual addition of an appropriate amount of 50% polyethylene glycol, at 4°C. After stirring for 30 min, the mixture was centrifuged at 20,000 xg for 30 min. The pellet was discarded and the supernatant was saved for the next step.

Step 3: Batchwise DEAE-Cellulose Treatment

The supernatant from the polyethylene glycol

precipitation step was added to 250 ml of packed DEAE-cellulose, previously equilibrated with buffer A. The slurry was gently stirred for 30 min, filtered and washed batchwise with 2 l of buffer A. The DEAE-cellulose containing the phosphodiesterase was further washed with 1 l of buffer A, containing 0.125 M phosphate, and the combined wash was discarded. Finally, phosphodiesterase was eluted with 300-400 ml of buffer A containing 0.35 M phosphate, concentrated to a final volume of approximately 100 ml using Sephadex G-25 and precipitated by the addition of ammonium sulfate to 70% saturation. The precipitate was collected by centrifugation and the pellet was dissolved in a small volume of a buffer containing 0.1 M potassium phosphate, 1 mM imidazole, 1 mM TSF, 5 mM EGTA, 0.1% 2-mercaptoethanol and 30% ethylene glycol (buffer C).

Step 4: Bio-Gel A-0.5 M Column Chromatography

Bio-Gel A-0.5 M was packed in a 2.6 x 90 cm column equilibrated with buffer C. Enzyme from the DEAE-celluose step was chromatographed in buffer C at a flow rate of 40 ml/h. Five ml fractions were collected.

Step 5: QAE-A-25 Sephadex Column Chromatography

Fractions containing phosphodiesterase from the Bio Gel column were pooled and applied to a column (1.5 x 40 cm) of QAE-A-25 Sephadex equilibrated with buffer C. The column was washed with 2-3 bed volumes of the starting buffer and the enzyme was eluted using a gradient composed of 250 ml each of 0.1 and 0.7 M potassium phosphate in buffer C.

Step 6: Affinity Chromatography on MIX-Sepharose

Fractions containing enzyme activity from QAE-Sephadex columns were concentrated, dialyzed against buffer C and applied to a column (1.5 x 20 cm) of MIX-Sepharose. The column was washed with 3 bed volumes of the starting buffer and the enzyme was eluted with a gradient of 150 ml each of 0.1 M to 0.7 M phosphate in buffer C. The flow rate was 0.6 ml/min and 2.5 ml fractions were collected. The elution profile on MIX-Sepharose is shown in Fig. 11. In some preparations, a second MIX-Sepharose step was necessary to remove the remaining contaminants. The results of a typical purification are summarized in Table 1.

2.3.6 Criteria of Purity

The final enzyme preparation gave an unusual pattern when analyzed on nondenaturing polyacrylamide

FIGURE 11

AFFINITY CHROMATOGRAPHY OF PDE IV

PDE IV fractions obtained from QAE-Sephadex A-25 column were dialyzed against buffer C and applied to a methylisobutyl xanthine-Sepharose column (1.5 x 20 cm). The column was eluted with a gradient of 150 ml each of 0.1 M to 0.7 M potassium phosphate in buffer C. Fractions of 2.5 ml were collected and assayed for phosphodiesterase activity. Fractions containing enzyme activity were electrophoresed in 7% acrylamide under nondenaturing conditions. Protein patterns on gels are shown in inset.

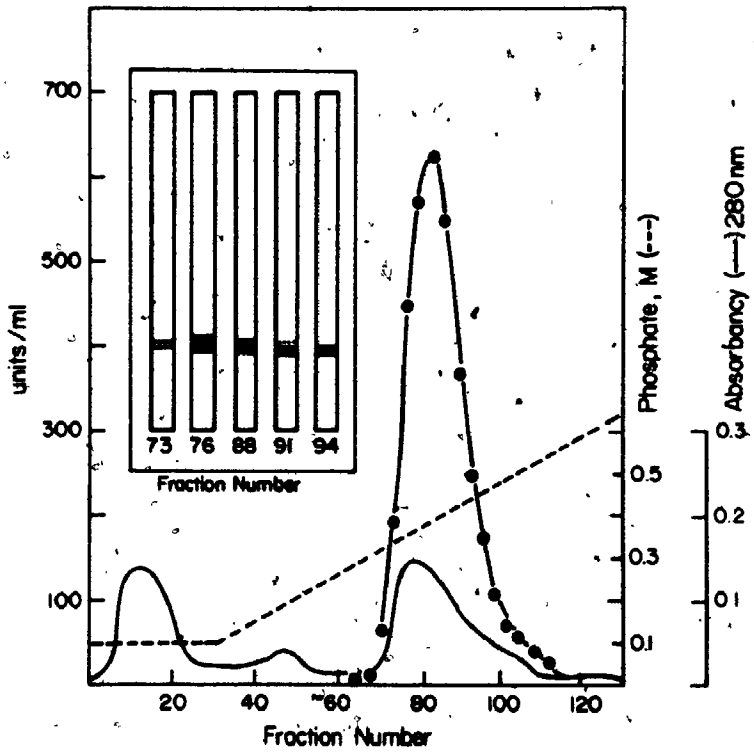

700
500
300
100
units/ml
73 76 88 91 94
Fraction Number
Phosphate, M (---)
Absorbancy (—)280nm
0.5
0.3
0.1
0.3
0.2
0.1
20
40
~60
80
100
120
Fraction Number

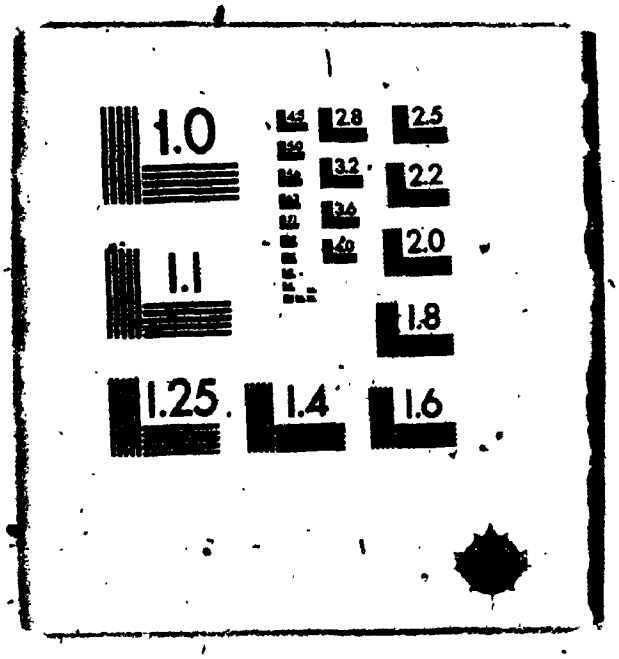
TABLE 1

PURIFICATION OF RAT SKELETAL MUSCLE

PHOSPHODIESTERASE IV

Step	Total Activity (pmoles/min)	Total Protein (mg)	Specific Activity	Fold Purification
Crude Extract	2.8×10^6	59,840	47.6	1
Polyethylene glycol supernatant	2×10^6	13,650	154	3
DEAE-celluose eluate	7.6×10^5	282	2689	56
Bio-Gel A-0.5 M	3.7×10^5	144	2554	54
QAE-A-25 Sephadex	1.1×10^5	34	3040	64
MIX-Sepharose	3.5×10^4	6.4	6164	129

2



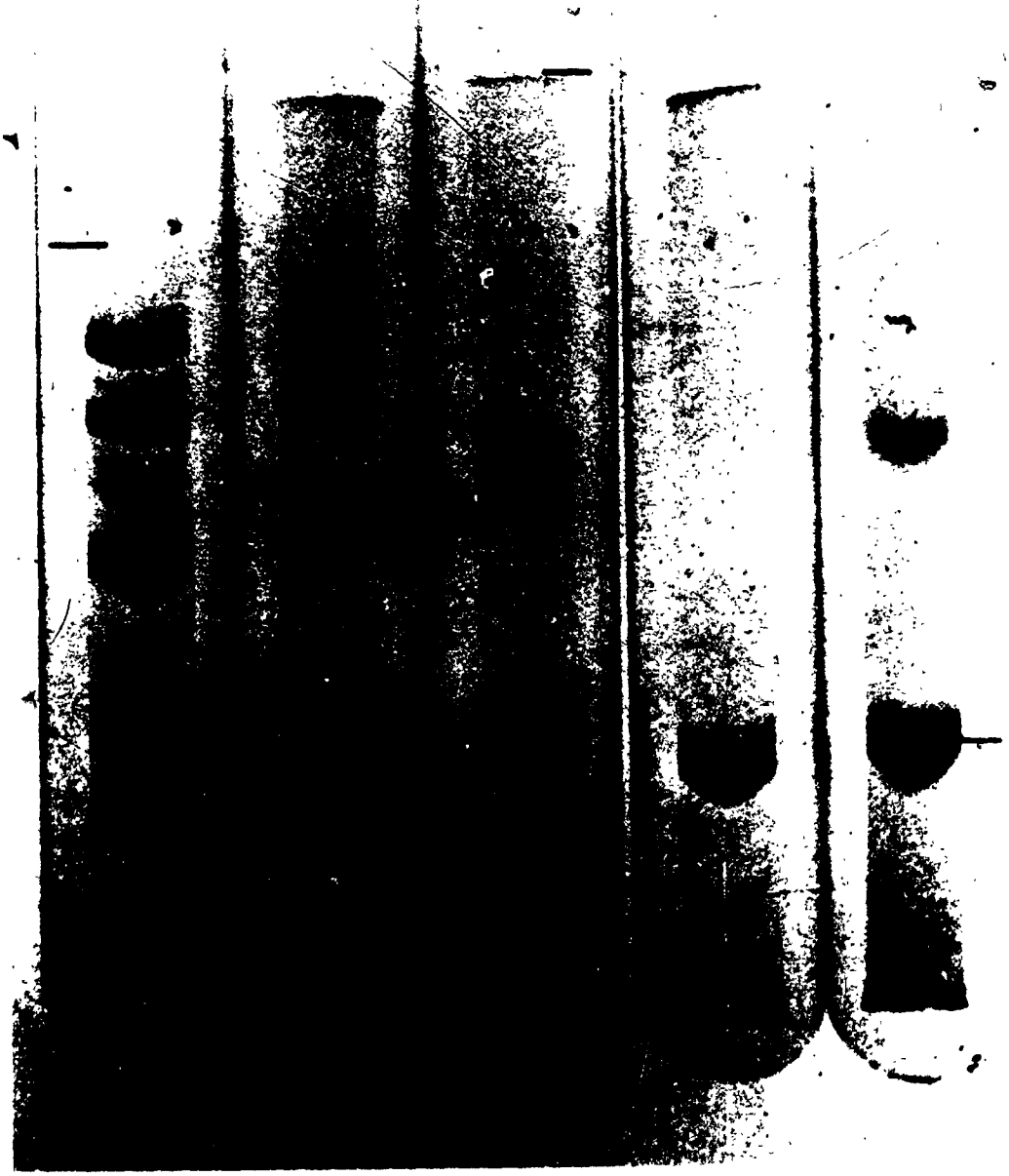
gel electrophoresis. As shown in Fig. 11 (inset), the protein bands at the leading edge of the activity peak eluted from the MIX-Sepharose column appeared as a doublet with equal intensity. However, the protein profile at the trailing edge shows the top band gradually diminishing until only the lower band remains. Repetition of some chromatographic steps did not change the pattern of protein bands. More interestingly, when a doublet containing fraction and a singlet containing fraction were analyzed by SDS-polyacrylamide gel electrophoresis, both gave rise to the same pattern, viz., two dissimilar subunits with a molecular weight of 30,000 and 28,000 daltons (Fig. 12). Furthermore, when the single band and double band enzymes were cross-linked with dimethyl-suberimidate and analyzed by SDS gel electrophoresis, both showed similar protein profiles consisting of 2 non cross-linked subunits and an additional cross-linked product with molecular weight about 60,000 (Fig. 12). These results suggest that the fractions containing single and double bands are both the same enzyme. The presence of 2 bands on nondenaturing polyacrylamide gel electrophoresis may be due to charge isomers possibly resulting from different degrees of phosphorylation (See Chapter 3).

FIGURE 12

SDS-GEL ELECTROPHORESIS OF PURIFIED PDE IV IN THE PRESENCE AND ABSENCE OF CROSS-LINKING REAGENT

25 µg of proteins were incubated with dimethyl-suberimidate (3 mg/ml) in 0.2 M triethanolamine, pH 8.5) for cross-linking, or in 0.2 M triethanolamine (control) at room temp. for 18 hr. The proteins were then electrophoresed in 10% acrylamide gel in the presence of 0.1% sodium dodecyl sulfate. A: protein standards, from the top: phosphorylase b (Mr = 94,000); bovine serum albumin (Mr 67,000); ovalbumin (Mr 43,000); carbonic anhydrase (Mr 30,000); soybean trypsin inhibitor (Mr 20,000) and α-lactalbumin (Mr 14,400). Band C; control and cross-linked enzyme from fraction 94 of MIX Sepharose (Fig. 11, 1 band enzyme; D and E: control and cross-linked enzyme from fraction 76 of MIX-Sepharose column (Fig. 11, 2 band enzyme).

A B C D E



2.3.7 Comments on Purification of PDE IV

500 gm of muscle gave us a yield of about 6 mg of pure PDE IV with a specific activity of 6100 units per mg protein. The purified PDE IV was stable for at least 2-3 months when stored in 0.1 M potassium phosphate in buffer C at 4°C. It should be also mentioned that the above scheme has also been successfully used to purify PDE III to homogeneity (Fig. 13). On SDS-gel electrophoresis, PDE II shows a single subunit of molecular weight about 60,000 (Fig. 13,B).

2.3.8 Amino Acid Composition

Amino acid analysis of PDE IV and PDE I is presented in Table 2. There is a certain amount of similarity in the mole percent amino acid composition of the two forms of the enzymes but surprisingly PDE I has less tyrosine residues as compared to PDE IV. The N-terminal amino acid of PDE IV was found to be aspartic acid.

2.3.9 Molecular Weights of the Different Forms of Phosphodiesterases

Data regarding the subunit structure and molecular weights of the various phosphodiesterases are summarized in Table 3. PDE I, PDE III and PDE IV were homogenous preparations and PDE II was partially purified as described earlier.

FIGURE 13

DISC GEL ELECTROPHORESIS

OF PURIFIED PDE III

A, gel electrophoresis under nondenaturing conditions in 7.5% acrylamide as described in Materials and Methods.

B, gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate in 7.5% acrylamide.

C, gel electrophoresis of molecular weight standards.

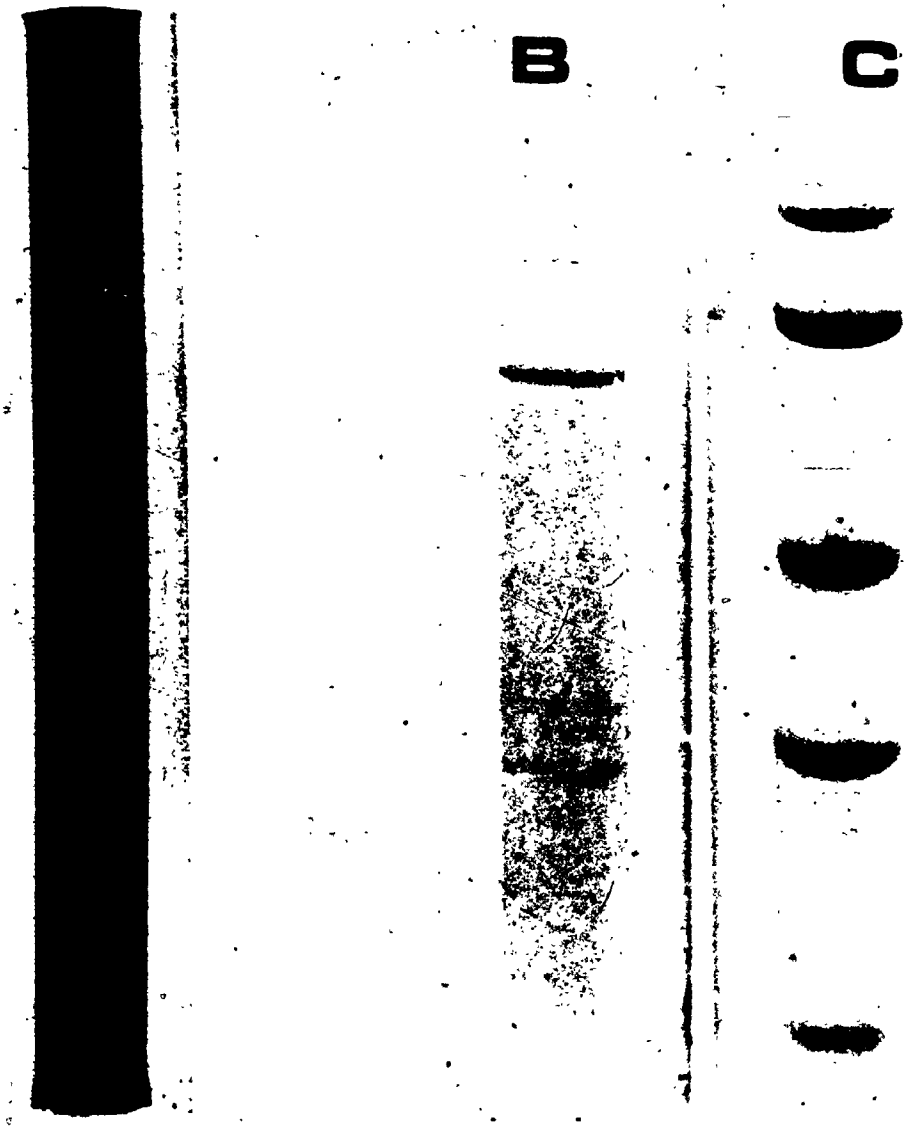

B
C

TABLE 2

AMINO ACID COMPOSITION OF PDE I AND PDE IV

Amino Acid	PDE I		PDE IV	
	Residues/mol[a]	nmole percent	Residues/mol[b]	nmol percent
Asp	110	13	33	13
Thr	37	4	10	4
Ser	61	7	16	6
Glu	145	17	41	16
Pro	30	3	6	2
Gly	87	10	12	5
Ala	74	9	25	9
Val	46	5	15	5
Met	17	2	7	3
Isoleu	40	5	13	5
Leu	71	8	29	11
Tyr	9	1	12	5
Phe	23	3	6	2
His	18	2	6	2
Lys	41	5	20	8
Arg	55	6	13	5

[a] Mr = 94,000

[b] Mr = 30,000

TABLE 3

MOLECULAR WEIGHTS OF VARIOUS FORMS OF PHOSPHODIESTERASES

Form of Enzyme	Molecular Weight of Native Form[a]	Subunit Molecular Weight	Probable Degree of Polymerization
PDE I	1,500K[a]	92-94 K[c]	variable
PDE II	400-450 K[a]	92-94 K[d]	Tetramer
PDE III	120-140 K[a]	60-65 K[c]	Dimer
PDE IV	60-80 K[b]	30 K[c] 28 K[c]	Dimer

[a]Determined by gel exclusion chromatography on Bio-Gel A-1.5 M column. The column was calibrated with the following proteins: Ferritin (M_r 800K), lactate dehydrogenase (M_r 140K), pyruvate kinase (M_r 237K), creatine phosphophokinase (M_r 81K), hemoglobin (M_r 65K).

[b]Determined by gel giltration through a calibrated Sephacryl S-200 column.

[c]Determined by SDS-polyacrylamide gel electrophoresis.

[d]Assumed value (see the text).

2.3.10 Thermostability of Various Forms

PDE II and the rest of the three forms can be distinguished from each other by their temperature sensitivity at 45°C. The half life of PDE II is about 30 min while that of PDE III is 3 min. Highly purified PDE I and PDE IV give half lives of 2.5 and 2 min, respectively (data not shown). Addition of snake venom to PDE II preparation reduces its half life to 2.5 min, but has little effect, if any, on the half lives of PDE III, PDE I or PDE IV.

2.3.11 Kinetics and Regulatory Properties of the Phosphodiesterases

All of the four forms of phosphodiesterases yield the same Km, 2-3 μM with cAMP as the variable substrate. They are unaffected by calmodulin or calcium singly, or in combination only PDE II, and none of the other forms, is activated by thiocyanate (Table 4). Activation probably results from the chaotropic activity of thiocyanate as is shown by the fact that $NaClO_4$ also is able to activate the enzyme at elevated concentrations (0.4 M). α-tocopheryl phosphate also activates only PDE II. Similarly, only the PDE II form is activated by proteases, like chymotrypsin, trypsin and snake venom protease. In each case activation is due to an increase in Vmax of 2-3 fold.

TABLE 4

SOME REGULATORY PROPERTIES OF

PHOSPODIESTERASE II

Phosphodiesterase II was partially purified from rat myoblasts as outlined in the text. The various substances tested were added simultaneously with the substrate in the assay medium. The concentration of the activators given is the amount which produces maximum effect. Activity of the enzyme in the absence of additions is considered as 100 percent.

Additions	Concentration	Percent Activation
NaSCN	0.2 M	275
Chymotrypsin	5 µg/ml	335
NaSCN + chymotrypsin		280
α-tocopherylphosphate (α-TP)	0.1 mM	360
α-TP + chymotrypsin		320

The Km essentially remains unchanged. When maximum activation has been achieved by α-tocopheryl phosphate or NaScN, proteases are unable to further activate the enzyme. Very likely this is due to a polymerization of PDE II to PDE I in the presence of these reagents. We have shown earlier that PDE I is not activated by trypsin or chymotrypsin, in contrast to PDE II.

2.3.12 Interconversion of Phosphodiesterases in Myoblasts

It should be mentioned in passing that the conversion of PDE II to PDE I, PDE III and PDE IV in rat skeletal muscle as discussed in the above section, was also found to occur exactly the same way in rat skeletal myoblasts. The results are not shown here to avoid the repetition.

2.4 Discussion

The evidence obtained in this work makes it very plausible that rat myoblasts contain only one primary phosphodiesterase, viz. PDE II. The rest of the forms of the enzyme, PDE I, III and IV are probably derived from PDE II. The evidence for the precursor role of PDE II is strongest in regard to the genesis of PDE I. Thus, when PDE II is dialyzed against ammonium sulphate,

stored at 4°C, treated with α-tocopheryl phosphate, or passed through methylisbutyl xanthine-Sepharose column, it gets converted into the aggregated form, PDE I. This form can be recognized not only by its electrophoretic properties and molecular weight but also its insensitivity to proteolytic activation. The fortuitous circumstance that PDE II can be polymerized into the PDE I form provided, a means of purifying the enzyme to homogeneity and determining the native subunit molecular weight of PDE II unambiguously and to infer the mode of the origin of the low molecular weight form, PDE IV. Clearly, if the subunit (monomer) molecular weight of PDE II is 94,000, as determined by SDS-gel electrophoresis, the native enzyme with a molecular weight of about 400,000 (determined by gel exclusion chromatography) is probably a tetramer. It may also be remarked that the subunit molecular weight of 94,000 is probably an approximate value, because, given the susceptibility of PDE II to proteolysis, it would be hard to ascertain whether the subunit has not suffered proteolytic attack during the preparation of cell free extracts. Indeed, electrophoresis of fresh extracts has consistently revealed the presence of two differently charged forms of PDE II, one of which probably arises by proteolysis (Fig. 5). However, the proteolytic modification of PDE II must remain quite limited for

it to polymerize to PDE I, since we have shown that extensively degraded forms such as PDE III and PDE IV (see below) are unable to polymerize into PDE I by treatments which are effective for the conversion of PDE II to PDE I. The only puzzling feature of PDE II to PDE I conversion is that once formed, PDE I can not be easily dissociated into PDE II. Polymerized forms are known in the case of calmodulin-sensitive, low affinity phosphodiesterase from rat liver and human platelets (Pichard and Cheung, 1976), but in these cases the polymerization is freely reversible. It is possible that very strong hydrophobic interactions occur in the formation of PDE I, although the polypeptide itself does not have any unusual preponderance of hydrophobic amino acid residues.

There is a good evidence that PDE IV is produced by proteolysis from PDE II. PDE III is probably an intermediate in this conversion, because a form resembling PDE IV in many characteristics can be shown to arise by proteolysis from PDE III.

If the subunit of PDE IV and PDE II are compared, a relationship between the two forms of the enzyme is not immediately obvious. Firstly, PDE I seems to have only one type of subunit while PDE IV appears to have two dissimilar subunits. Secondly, the monomer molecular weight of PDE II is 94,000 while that of PDE IV is 28,000 and 30,000. Further, PDE IV is a dimer while

PDE II is most probably a tetramer. A precursor-product relationship between the two forms; however, becomes evident if it is assumed that protease treatment removes a part of the subunit of PDE II which has binding domains for dimer-dimer interaction in an isologous tetramer (Monod et al,1965). This would result not only in reducing the monomer molecular weight, but also change the nature of the quaternary structure of the derived protein. It is very likely that the two apparently different subunits in PDE IV are derived from the native and proteolytically modified forms of PDE II (Fig. 5), respectively. A relationship between the 28,000 and 30,000-dalton subunits of PDE IV is implied (but not proven) from our finding that only one NH_2 terminus, aspartate, is found in the dimer. However, since the end group of Mr = 94,000 subunit has not been determined, it is still possible that one polypeptide has a masked end group. Moreover the peptide maps of both the 28,000 and 30,000-dalton subunits are also quite similar, indicating their origin from related forms of PDE II.

Apart from the relationship between phosphodiesterase forms, the question of great interest is whether the conversion of PDE II to the various forms has any regulatory significance. Since PDE I and PDE IV become prominent in vitro by manipulation treatment of crude extracts (e.g. PDE I appears on aging and PDE IV when

extracts are supplemented with calcium), it seems reasonable to assume that they are of not much significance in vivo or are perhaps even artifacts associated with cell homogenization. Production of PDE III, however, seems to have regulatory significance as shown in an earlier publication (Ball et al, 1980) and discussed in Chapter 4).

With the findings reported here and elsewhere (Chapter 3 and 4) about the control of the activity of phosphodiesterase, it would seem that cAMP phosphodiesterases in different tissues are regulated in two ways. The low affinity phosphodiesterase (Km for cAMP above 20 μM) found in brain and several other tissues is activated by calmodulin (Strada and Thompson, 1978). The high affinity phosphodiesterases (Km for cAMP, 2-5 μM), such as the one described here and from rat liver plasma membranes (Marchmont et al. 1981) are probably activated by phosphorylation (Ball et al,1980, Marchmont et al,1981 and Chapter 3 of this thesis) and some by proteolytic modification (Chapter 4 of this thesis, Ball et al,1980, Strewler et al,1978).

Apart from the regulatory properties of the various phosphodiesterases, a comparison of the enzyme forms described here with phosphodiesterases from the other sources should be made. Helfman et al (1981) have recently summarized data on the subunit structure and other properties of various phosphodiesterases purified

so far from various tissues. Amongst the calmodulin-independent enzymes, the only other low Km phosphodiesterase, apart from the one described here, is the enzyme from dog kidney (Thompson et al,1979). This enzyme however has a molecular weight of 60,000 and is apparently a monomer, in contrast to PDE II, which has a subunit molecular weight of about 94,000 and is a tetramer. The amino acid composition of the rat muscle and dog kidney enzyme is also completely different (Helfman et al,1981, Thompson et al,1979). Thus, there does not seem to be any obvious relationship of the enzyme described here with enzymes from other sources.

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CHAPTER 3. REGULATION OF CYCLIC ADENOSINE 3':5'-MONOPHOSPHATE PHOSPHODIESTERASES. EVIDENCE FOR A PHOSPHORYLATION-DEPHOSPHORYLATION MECHANISM OF CONTROL IN RAT SKELETAL MYOBLASTS AND ADULT MUSCLE

3.1 Introduction

cAMP, or the compounds which augment the levels of cAMP in cells, are known to increase the activity of low Km cAMP phosphodiesterase (Wells and Hardman, 1977; Loten et al., 1978; Alvarez et al., 1981).

Ball (1979) had demonstrated that when L6 myoblasts were exposed to Bt_2cAMP and MIX, a decline in the snake venom activation of phosphodiesterase took place in less than 30 min. This phenomenon could also be duplicated in cell free extracts by treating them with cAMP and ATP (Ball, 1979). In continuation of this preliminary work, I could demonstrate the activation of phosphodiesterase by Bt_2cAMP and MIX in vivo and cAMP and ATP in vitro. The in vitro activation did not occur in the presence of protein kinase inhibitor. These and related observations led us to suggest (Ball et al., 1980) that the activation of phosphodiesterase involves phosphorylation of the enzyme by a cAMP-dependent protein kinase. Proof of this suggestion has not been forthcoming however because of the problem of existence of multiple forms of phosphodiesterases in myoblasts (See Chapter 2). These forms have been designated as PDE I, PDE II, PDE III and PDE IV depending upon the order of their elution in gel

exclusion chromatography on Bio-Gel A-1.5 (See Chapter 2). PDE II can be distinguished from other forms not only by its molecular weight (about 400,000) but also by the fact that it is the only form which is activated 2-5 fold by various proteases, including the proteases in snake venom. Various lines of evidence (Chapter 2) suggest that PDE II is the primary form of the enzyme in myoblasts with other forms resulting from secondary modification. PDE II can be shown to be readily converted to PDE I by aggregation and to PDE IV and probably PDE III by proteolysis. This knowledge coupled with the availability of methods for the purification of PDE I and PDE IV (Chapter 2) to homogeneity have led us to investigate whether the activity of PDE II is regulated by phosphorylation-dephosphorylation mechanism. The results are described in the following sections.

3.2 Materials and Methods

3.2.1 Chemicals

ATP-γ-S and AMP-PNP were purchased from Boehringer-Mannheim. [γ-^{32}P] ATP (sp. act. 3000 Ci/mmole) and ^{32}P-orthophosphoric acid were purchased from New England Nuclear. All other chemicals, media constituents and enzymes were from various commercial sources which have been listed in Materials and Methods section of Chapter 2.

3.2.2 Cell Culture

A clone of rat skeletal myoblast cell line, L6 was used. When large number of cells were required for the purpose of purifying PDE II, they were grown in roller bottles (surface area, 850 cm^2) in Dulbeccos modified Eagles medium containing 10% horse serum and 5 µg/ml of gentamycin. After reaching confluency cells were harvested by using 1 mM EGTA in phosphate buffered saline. The cells were collected by centrifugation and suspended in a suitable volume of buffer (Buffer A), containing, 100 mM sodium phosphate, pH 7.5, 20 mM NaF, 1 mM EGTA, 1 mM TSF, 1 mM imidazole, 0.1% 2-mercaptoethanol and 30% ethylene glycol. For other experiments, cells were grown at an initial density of 3500/cm^2 in dishes of appropriate size. After 4 days of growth the cells were harvested by scraping in buffer B consisting of 50 mM Hepes, pH 7.5, 1 mM EGTA, 1 mM TSF and 0.1% 2-mercaptoethanol. Cell-free extracts were prepared by homogenizing the cells for 15 sec at a setting of 3 in a Polytron homogenizer (Brinkman Instruments, Rexdale, Canada). The extracts were centrifuged for 15 min at 4°C at 18,000 xg.

3.2.3 Activation of Phosphodiesterase in Cell Free Extracts

Supernatants of the cell free extracts made in Buffer B were diluted 1:2 into a mixture containing

the necessary amounts of ATP, cAMP, NaF or other factors. Protein concentration was usually between 0.5 to 1 mg/ml. When present, Mg-ATP concentration was 0.5 mM, cAMP, 0.1 mM, and NaF, 10 mM. The mixture was incubated at 37°C for 10 min. Reactions were stopped by cooling the mixture on ice. Removal of nucleotides was done either by dialysis (against 2 x 100 volumes of Buffer B) or desalting, using Sephadex G-25 columns.

3.2.4 Activation of PDE II by Protein Kinase

Incubation mixtures for activation of PDE II contained, suitable amounts of the enzyme, 50 mM sodium phosphate, pH 7.5, 0.5 mM Mg-ATP, 10 mM NaF, 0.5 mM EGTA, 0.5 mM TSF, 0.5 mM imidazole, 0.05% 2-mercaptoethanol and 15% ethylene glycol. When required cAMP (0.1 mM) and suitable amounts of protein kinase were also included. Incubations were done at 30°C for 10 min. Rest of the procedure is the same as described above in section 3.2.3.

3.2.5 Purification of Phosphodiesterases and Other Enzymes

Homogeneous preparations of PDE I and PDE IV were obtained from rat skeletal muscle as described in Chapter 2. PDE II was partially purified from both rat skeletal muscle and L6 myoblasts through 0-35% ammonium sulfate precipitation, polyethylene glycol precipitation,

batchwise DEAE-cellulose absorption and elution, and chromatography on Bio-Gel A-1.5 M columns exactly as described in Chapter 2. Peak fractions eluting from the Bio-Gel column at a ve/vo ratio of 1.3 were used as a source of PDE II.

Cyclic AMP-dependent protein kinase was purified from rat skeletal muscle according to the procedure of Beavo et al. (1974). Peak 1 from DEAE-cellulose column was used to purify the holoenzyme.

3.2.6 Deactivation of Phosphodiesterase

Incubation mixtures contained, 25 mM Hepes, pH 7.5, 0.5 mM EGTA, 0.5 mM TSF, 0.05% 2-mercaptoethanol, 5 mM $MgCl_2$ and, suitable amounts of potato acid phosphatase. Incubation were done at 30°C for the required lengths of time and reactions were stopped by adding 20 mM sodium phosphate (pH 7.5) and 20 mM NaF.

3.2.7 Phosphorylation of Phosphodiesterases

Incubation mixtures for phosphorylation experiments contained, unless otherwise indicated, 50 mM sodium phosphate, pH 7.5, 0.5 mM $Mg^{+}[\gamma\text{-}^{32}P]$ ATP (sp. act. 100-750 cpm/pmole), 10 mM NaF, 0.5 mM EGTA, 0.5 mM TSF, 0.5 mM imidazole, 0.05% 2-mercaptoethanol and 15% ethylene glycol. When required, cAMP (0.1 mM), suitable amounts of protein kinase and phosphodiesterase

(either as cell free extracts, semipurified PDE II, pure PDE I or pure PDE IV) were also included. The mixture was incubated at 30°C for 10 min, unless otherwise indicated. Reactions were stopped by cooling the mixture on ice.

3.2.8 Dephosphorylation of Phosphodiesterase

Dephosphorylation was done exactly the way as deactivation, as described in section 3.2.6.

3.2.9 Polyacrylamide Gel Electrophoresis and Autoradiography

Polyacrylamide gel electrophoresis under non-denaturing conditions was performed as described earlier in Chapter 2 except that the buffer used for elution of enzyme from gel slices was Tris-phosphate, pH 7.5, containing 5 mM EGTA, 1 mM TSF, 1 mM imidazole, 30% ethylene glycol, 0.1% 2-mercaptoethanol and 300 µg/ml bovine serum albumin. SDS slab gel electrophoresis was done according to the procedure of Laemmeli (1970) using 10% acrylamide. The gels were stained for 30 min in a solution containing 0.1% coomassie brilliant blue R250 in 50% methanol and 10% acetic acid. Gels were destained in 5% methanol and 7.5% acetic acid. Molecular weight standards used were phosphorylase b

(94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100) and α-lactalbumin (14,400). The proteins were radioiodinated by chloramine T procedure (Bolton, 1977). For autoradiography, gels were dried and exposed to Kodak XAR-5 films for 1-5 days at -20°C in a Kodak X-Omatic cassette with the aid of Cronex Lightning Plus intensifying screens.

3.2.10 Other Procedures

See Materials and Methods section of Chapter 2.

3.3 Results

3.3.1 Short Term Activation of Phosphodiesterase in vivo

Ball (1979) had shown earlier that when myoblast cells were treated with Bt_2cAMP and MIX and phosphodiesterase activity assayed at various time intervals, a time dependent loss of venom activatibility of phosphodiesterase took place. However, this loss of venom activatibility was hard to interpret as it could occur due to several reasons, viz., increase in the basal activity of the enzyme, decrease in the activity of the enzyme obtained in the presence of venom, or both. In order to find out which one of these mechanisms was responsible for the loss of venom activatibility,

the experiment was repeated. Four-days-old cells were treated with Bt_2cAMP and MIX and phosphodiesterase activity was assayed at various time intervals. The results (Fig. 1) show that there was about 2-fold increase in the basal activity of the enzyme in about 30 min. However activity obtained in the presence of snake venom remained unchanged. This resulted in the decrease in the ratio of venom activatibility from about 2.0 to about 1.0.

The presence of actinomycin D or cycloheximide had no effect on the increase in the activity or decrease in ratio suggesting that there was no requirement of de novo synthesis of transcriptional or translational products.

To show that the activation of phosphodiesterase was due to cAMP, compounds which augment the formation of cAMP in the cells were also tested for activation. It is clear from Table 1 and Fig. 2, that exposure to these compounds leads to an activation of the enzyme. PGE_1 activates phosphodiesterase about 2 fold. Half-maximal activation was reached in about 2-3 min. The amounts of isoproterenol and PGE_1 required were enough to initiate precocious cell fusion (Curtis and Zalin, 1981).

FIGURE 1

THE RATIO OF THE ACTIVITY OF

PHOSPHODIESTERASE IN THE PRESENCE

AND ABSENCE OF SNAKE VENOM

To 4-day-old myoblast, 0.7 mM Bt_2cAMP and 0.1 mM methylisobutyl xanthine was added at zero time and the cells were harvested at the time indicated. Activity of phosphodiesterase was measured in the absence (o) and presence (●) of snake venom as described in the text and ratio (▲) calculated. Controls did not receive methylisobutyl xanthine and Bt_2cAMP (Δ). Some plates were also exposed to 1 μg/ml of actinomycin D (■) or 10 μg/ml of cycloheximide (□) in the presence of Bt_2cAMP and methylisobutyl xanthine. Activity ratios were measured in these cells only after 60 min. The specific activity figures shown should be multiplied by 100 to obtain values of picomoles of product formed per min per mg of protein.

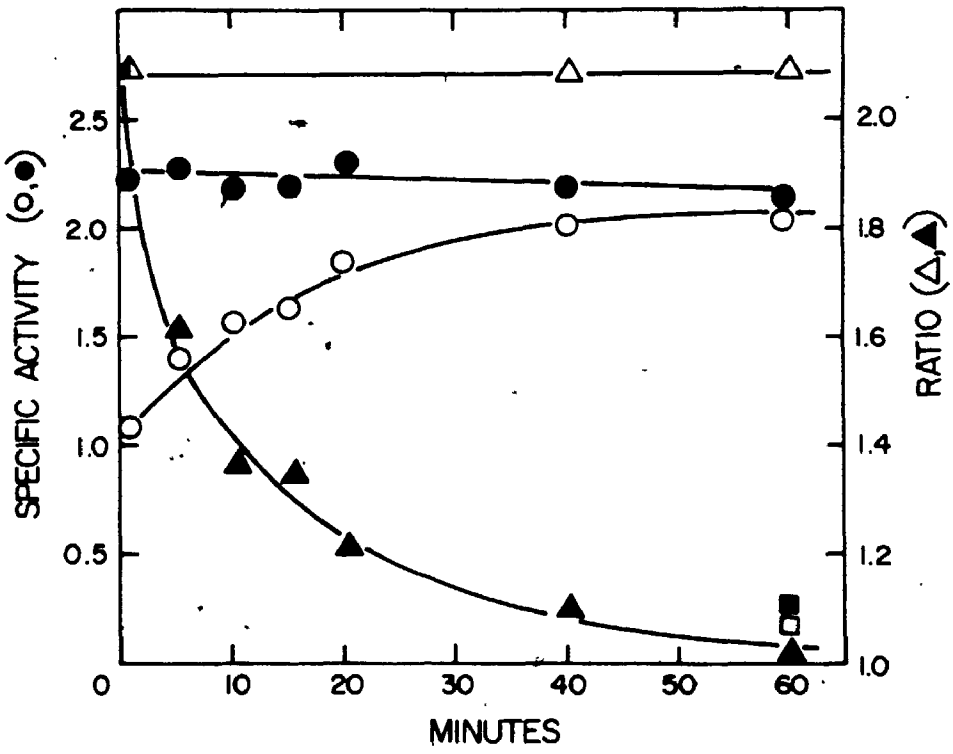

SPECIFIC ACTIVITY (○,●)
RATIO (△,▲)
MINUTES
0
10
20
30
40
50
60
0.5
1.0
1.5
2.0
2.5
1.0
1.2
1.4
1.6
1.8
2.0

TABLE 1

PHOSPHODIESTERASE ACTIVATION IN MYOBLASTS

The listed compounds were added to 4-day-old cells 1 hr before the measurement of enzyme activity at the following concentrations: Bt_2cAMP, 1 mM; isoproterenol, 0.1 mM; methylisobutyl xanthine, 0.1 mM; cycloheximide, 10 µg/ml. Activity of phosphodiesterase was measured in the presence and absence of snake venom as described in the text.

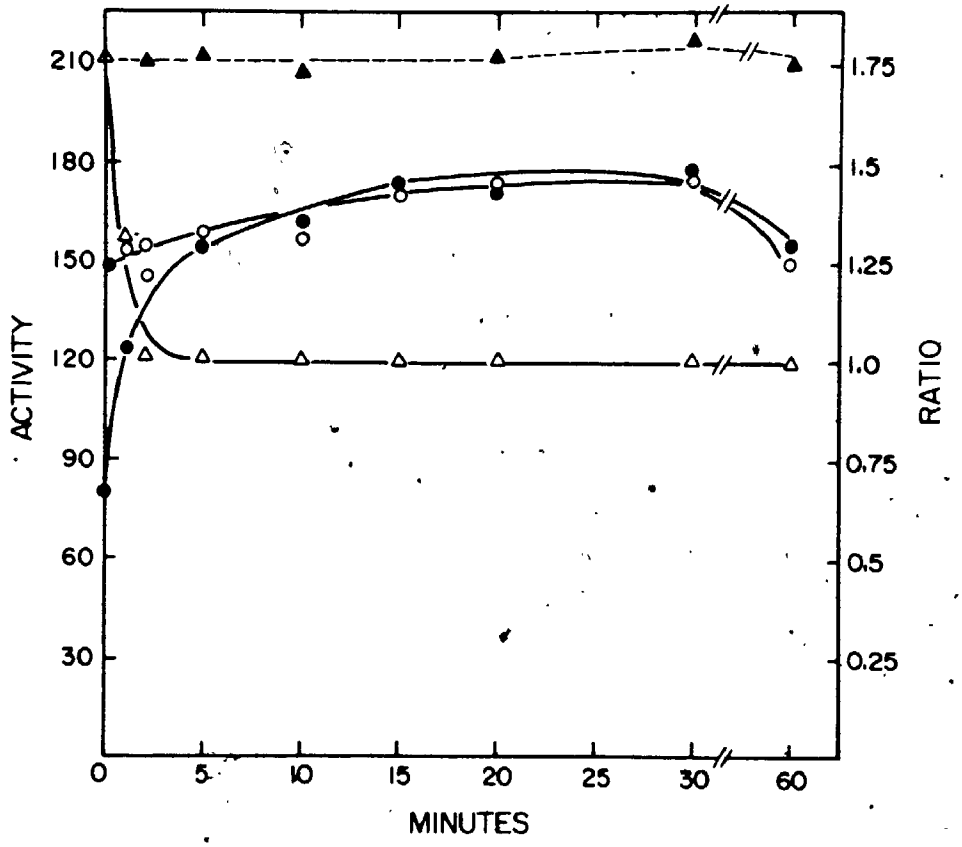
Additions	Basal Activity[a]	Activity[a] in the presence of venom	Ratio
None	75	180	2.4
Isoproterenol	129	177	1.4
Methylisobutyl xanthine	138	186	1.4
Bt_2cAMP	133	178	1.3
Methylisobutyl xanthine, Bt_2cAMP	159	182	1.1
Methylisobutyl xanthine, Bt_2cAMP, cycloheximide	169	190	1.1

[a]Activity is given as picomoles product formed per min per mg of protein.

FIGURE 2

EFFECT OF PGE_1 ON PHOSPHODIESTERASE ACTIVITY

To 4-day-old myoblasts, 5 µg/ml of PGE_1 was added at zero time and the cells were harvested at the time indicated. Activity of phosphodiesterase was measured in the absence (●) and presence (o) of snake venom and ratios (Δ) calculated. Controls did not receive PGE_1. Phosphodiesterase activity was also assayed in the absence and presence of snake venom in the control cells and the ratios (▲) calculated.


210
180
150
120
90
60
30
ACTIVITY
1.75
1.5
1.25
1.0
0.75
0.5
0.25
RATIO
0
5
10
15
20
25
30
60
MINUTES

3.3.2 Activation of Phosphodiesterase *in vitro*

Ball (1979) had earlier reported the loss of venom activatibility when broken cell preparations of myoblasts were treated with cAMP and ATP, together. In order to test whether this was also due to an increase in the total activity of the enzyme, an *in vitro* activation experiment was repeated. For this purpose cells were harvested after 4 days of growth in a buffer containing 50 mM Hepes, pH 7.5 instead of Tris-HCl (see Materials and Methods). Cell-free extracts were made in the usual manner and diluted 1:2 in a buffer containing 25 mM Hepes, pH 7.5. After incubation of the extract with various additions for 10 min at 37°C, the mixture (0.4 ml) was cooled on ice and either dialyzed for 24 h at 4°C against two changes of 25 mM Hepes buffer, pH 7.5, or applied to a Sephadex G-25 column to remove excess cAMP prior to enzyme assays. Results obtained with either method were comparable with minimum losses in enzymatic activity (less than 10%). Loss of venom activatibility of PDE II occurred in the *in vitro* system only in the presence of ATP and cAMP, as shown in Table 2. As expected, the absence of venom activatibility was accompanied by an increase in the basal activity of the enzyme. While addition of ATP and NaF to the extracts did not result in a significant decrease in the venom

TABLE 2

ACTIVATION OF PHOSPHODIESTERASE IN VITRO

Myoblast extracts were incubated as described in the text with various chemicals listed below. After dialysis the activity of the phosphodiesterases was measured in the presence and absence of the snake venom and the ratios calculated. When present, NaF concentration was 10 mM; cAMP, 0.1 mM; and Mg-ATP, 0.5 mM. A total of 200 µg of protein kinase inhibitor (PKI) having 36 phosphorylation inhibitory units was used when required, Details of the procedures are described in the text.

Additions	Basal Activity[a]	Activity[a] in the presence of venom	Ratio
NaF	120	257	2.14
ATP, NaF	128	249	1.95
cAMP, NaF	136	294	2.16
cAMP, NaF, ATP	230	251	1.09
PKI, NaF	127	262	2.06
cAMP, NaF, ATP, PKI	123	262	2.13

[a]Activity is given as picomoles of product formed per min per mg of protein.

activatable to non activatable ratio, both ATP and cAMP were necessary to produce non activatable phosphodiesterase activity (i.e. more active enzyme). Addition of the protein inhibitor of protein kinase to a mixture containing ATP, NaF and cAMP inhibited the activation (Table 2). Gel filtration on Bio-Gel A 1.5-M of the extracts incubated in vitro with ATP and cAMP for 10 min gave a single activity peak of PDE II. However PDE II was now more active and no longer activatable by snake venom (Fig. 3).

The concentration of ATP necessary to cause modification of PDE II is shown in Fig. 4. The decrease in the ratio was maximum at about 0.1 to 1 mM. Notably Ball (1979) had already shown that the concentration of cAMP to elicit the half maximal effect was about 5×10^{-7} M. Concentration of both nucleotides which are effective in modification are within physiological ranges.

To determine whether ATP acts as an allosteric activator or as a phosphate donor, freshly prepared extracts from myoblasts were incubated with cAMP and various analogues of ATP. After 10 min incubation at 30°C, the mixture was cooled on ice and dialyzed for 24 h at 4°C against two changes of buffer B, to remove excess cAMP prior to enzyme assays. The phosphodiesterase activity was then measured in the

FIGURE 3

PHOSPHODIESTERASE PROFILES IN MYOBLAST EXTRACTS TREATED WITH ATP AND cAMP IN VITRO

An extract from 4-day-old myoblasts, containing 3.75 mg of protein was incubated without (control) or with 10 mM NaF, 0.1 mM cAMP, 0.5 mM Mg-ATP for 10 min. Following dialysis, as described in the text, the extracts were loaded on the Bio-Gel A-1.5 M columns (1.5 x 60 cm). Fractions of 1.7 ml were collected and assayed in the presence (o) or absence (o) of snake venom. Panel A represents control and Panel B the enzyme profile from treated extract. Activity units are pmoles product formed per min per ml of fraction using 0.05 µm cAMP.

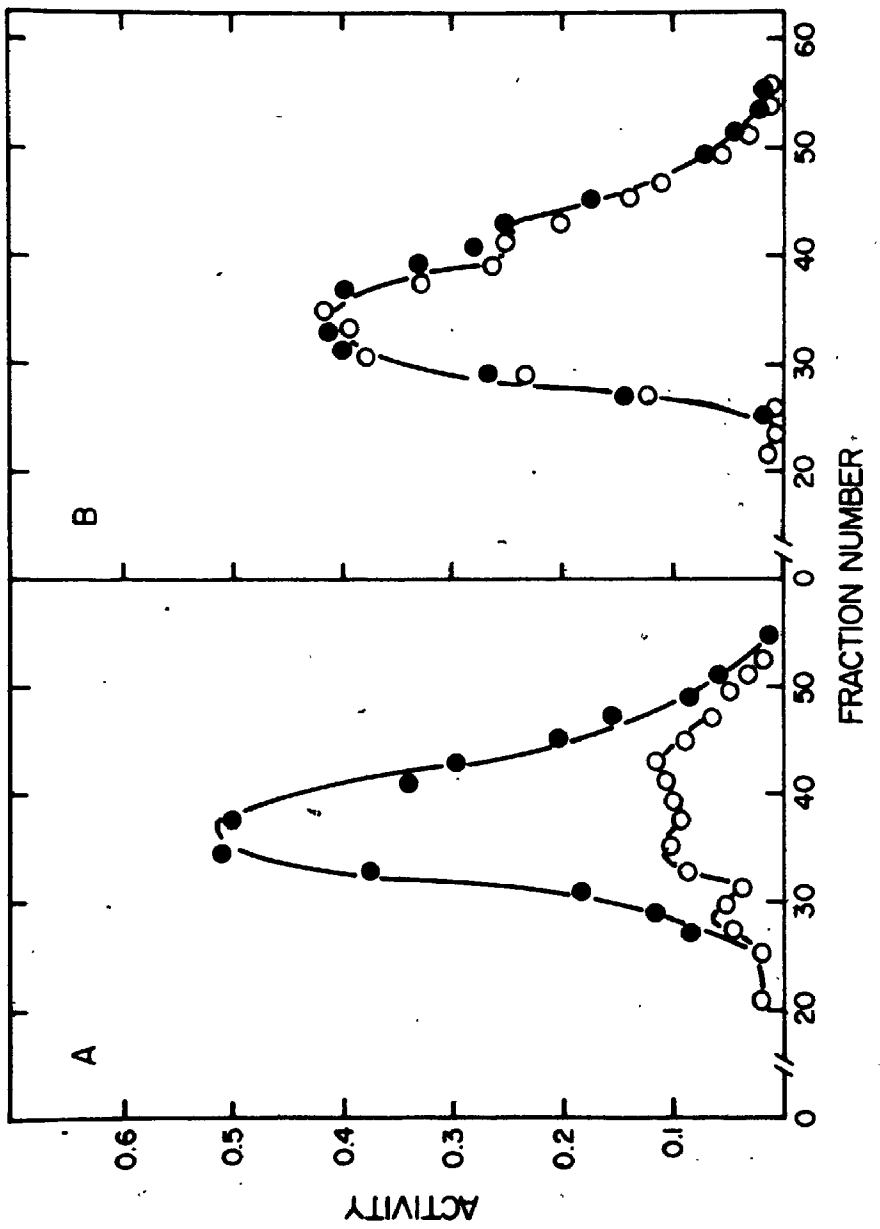
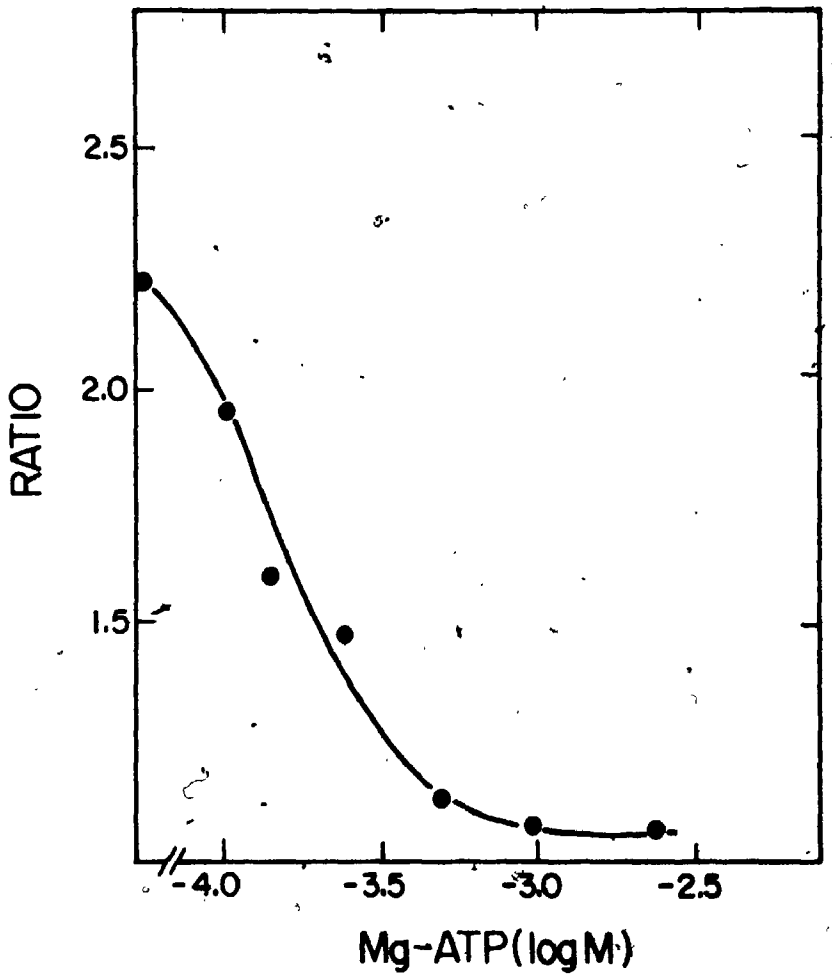

A
B
ACTIVITY
0.6
0.5
0.4
0.3
0.2
0.1
0
20
30
40
50
60
FRACTION NUMBER

FIGURE 4

ALTERATION OF THE VENOM-ACTIVATABLE PHOSPHODIESTERASE ACTIVITY IN VITRO

Extracts from 4-day-old myoblasts were treated, as described in the text, in the presence of 10 mM NaF and 0.5 mM methylisobutyl xanthine. To investigate dependence of the enzyme alteration on ATP (concentrations indicated), 1 mM cAMP was added to the incubation mixtures. The extract was dialyzed before use to remove endogeneous cAMP or ATP. Following dialysis of the treated extracts, the activity was measured in the presence and absence of snake venom and the ratios calculated.


2.5
2.0
1.5
RATIO
-4.0
-3.5
-3.0
-2.5
Mg-ATP(log M)

presence and absence of snake venom. As shown in the earlier experiments, the basal activity is increased about 2-fold when extracts are incubated with both cAMP and ATP or ATP-γ-S (Table 3). Simultaneously, venom activatibility is lost. ATP as well as ATP-γ-S are both known to be efficient donors of phosphate or thiophosphate groups, respectively in protein kinase mediated reactions such as activation of phosphorylase b (Gratecos and Fischer, 1974) and modification of myosin light chains (Sherry et al, 1978) and it is likely that they are able to modify the enzyme in myoblast extracts. The non-hydrolyzable analogue of ATP, AMP-PNP, however is not able to activate phosphodiesterases or decrease their activatibility by snake venom (Table 3). These observations suggest that during activation by cAMP and ATP a covalent modification, most likely phosphorylation of phosphodiesterase occurs.

To probe further into the requirement for a cAMP-dependent protein kinase in the activation of PDE II, the activity of partially purified PDE II was examined in the presence and absence of exogenous protein kinase. PDE II, purified more than 100-fold and free from PDE I and PDE IV (See Chapter 2), was incubated with pure cAMP-dependent protein kinase in the presence of various compounds of interest and the activity of the enzyme was measured. Table 4 summarizes the results

TABLE 3

PHOSPHODIESTERASE ACTIVATION IN MYOBLAST EXTRACTS

The listed compounds were incubated with crude extracts from 4-day-old myoblasts for 10 min. After dialysis the activity of the phosphodiesterases was measured in the presence and absence of snake venom and ratios calculated. The concentration of NaF and cAMP was 10 mM and 0.1 mM, respectively, Mg-ATP and ATP analogues were added at a concentration of 1 mM. Details of the procedures are described in the text.

Additions	Basal Activity[a]	Activity[a] in the presence of venom	Ratio
cAMP, NaF	125	264	2.11
cAMP, NaF, ATP	240	254	1.05
cAMP, NaF, ATP-γ-S	258	275	1.07
cAMP, NaF, AMP-PNP	138	249	1.80

[a]Activity is given as picomoles of product formed per min per mg of protein.

TABLE 4

ACTIVATION OF PARTIALLY PURIFIED PDE II BY cAMP-DEPENDENT PROTEIN KINASE

Partially purified PDE II was incubated with the listed substances in phosphate buffer at 30°C for 10 min. The mixtures were then cooled on ice and the nucleotide removed by passing them through Sephadex G-25. Activity of phosphodiesterase was measured in the absence and presence of snake venom and ratios were calculated. When present, the concentration of the reagents were: cAMP, 0.1 mM; Mg-ATP, 0.5 mM and protein kinase (PK), 100 µg/ml.

Additions	Basal Activity[a]	Activity[a] in the presence of venom	Ratio
PDE II	34.2	63.1	1.85
PDE II, PK	41.0	72.2	1.76
PDE II, PK, cAMP	36.4	65.6	1.80
PDE II, PK, Mg-ATP	51.1	66.4	1.30
PDE II, PK, Mg-ATP, cAMP	80.9	97.7	1.20

[a]Activity is expressed as picomoles of product formed/min/ml.

of this investigation. It should be noted that protein kinase, Mg-ATP and cAMP are all required to obtain a maximum activation of about 2 fold. Along with this activation the susceptibility of the enzyme to venom proteases is simultaneously lost, suggesting a causal relationship between the two events.

Active PDE II which was produced in the above mentioned experiment had a 2-fold increase in Vmax value as compared to the control PDE II preparations. However, there was no change in the Km of the enzyme when cAMP was used as the substrate (Table 5).

In passing, it may be also mentioned that purified PDE IV or PDE I could not be activated by cAMP dependent protein kinase.

3.3.3 Reversibility of Activation in vitro

If activation of phosphodiesterase is due to phosphorylation (Chapter 4), it should be possible to "deactivate" the enzyme by treatments which are known to dephosphorylate proteins. Accordingly, phosphodiesterases were activated in crude extracts of myoblasts by cAMP and ATP, as outlined earlier (Table 3). After dialysis, separate samples were supplemented at 30°C with either 5 mM magnesium chloride or with acid phosphatase. At various time intervals, aliquots were removed and phosphodiesterase activity was measured in

TABLE 5

KINETIC CONSTANTS OF PDE II

Semipurified PDE II was incubated with cAMP, ATP and protein kinase (as described in table 4) to produce active PDE II. Control extracts were incubated without ATP. After dialysis, kinetic studies were done by using cAMP as the variable substrate. K_m and V_{max} values were calculated from Lineweaver-Burk plots.

Enzyme Preparation	K_m µm	V_{max} units/mg protein
Control PDE II	2.5 (+sv)[a]	3182 (+sv)[a]
	2.2 (-sv)[b]	1208 (-sv)[b]
Active PDE II	2.3 (+sv)[a]	3408 (+sv)[a]
	2.1 (-sv)[b]	2948 (-sv)[b]

[a]Values obtained when the kinetic studies were done in the presence of snake venom (250 µg/ml).

[b]Values obtained when the kinetic studies were done in the absence of snake venom.

the presence and absence of snake venom. The results for acid phosphatase-treated extracts are presented in Fig. 5. Similar results were obtained with magnesium supplemented extracts. It can be seen from Fig. 5 that phosphodiesterase activity begins to decrease after the addition of phosphatase and the decrease levels off by 30 min. Simultaneously, venom protease activatibility reappears, as is evident from the ratios of phosphodiesterase activity obtained in the presence and absence of venom (Fig. 5) which changes from about 1.0 at the beginning of the experiment to almost 2.0 after 30 min. The 'deactivation' of phosphodiesterase is completely blocked by the addition of 20 mM phosphate and 20 mM NaF, compounds which inhibit the activity of phosphatase.

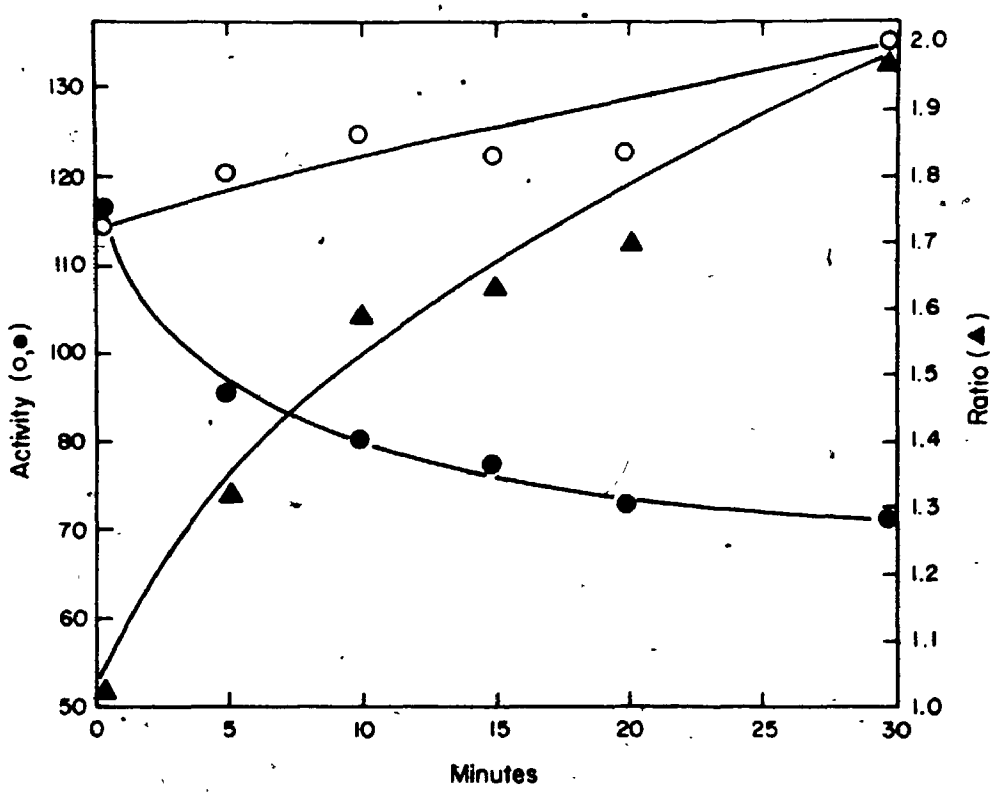
Although we have demonstrated earlier (Chapter 2) that out of several forms of phosphodiesterases in myoblasts, only PDE II is activated by venom proteases, and perhaps is the only form which is 'activated' and 'deactivated' in our experiments reported above, a possibility exists that changes in phosphodiesterase activity in crude extracts may be caused by the modification of some other proteins. In order to examine this possibility, the myoblast extracts were exposed to ATP and cAMP, and after dialysis, as described previously,

FIGURE 5

REVERSIBILITY OF PHOSPHODIESTERASE

ACTIVATION

A cell free extract from 4-day-old myoblasts was treated with 0.1 mM cAMP and 0.5 mM Mg-ATP to produce active phosphodiesterase. The extract was dialyzed against buffer B and incubated with 1 mg/ml of potato acid phosphatase. Aliquots of suitable volume were withdrawn at various time intervals indicated. The phosphatase action was terminated by the addition of 20 mM NaF and 20 mM phosphate pH 7.5, and cooling the mixture on ice. Phosphodiesterase activity was assayed in the absence (●) and presence (o) of snake venom and the ratios (▲) calculated. Activity of the enzyme is expressed as pmoles of product formed per mg of protein.


Activity (o,●)
Ratio (▲)
Minutes
130
120
110
100
80
70
60
50
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0
5
10
15
20
25
30

were divided into 2 portions, to one of which was added acid phosphatase and the other served as control. After 30 min, the extracts were passed through calibrated Bio-Gel A-1.5 M columns and fractions were analyzed for enzyme activity. The results presented in Fig. 6 show that phosphodiesterase activity emerges in two peaks with ve/vo ratios of approximately 1.3 and 1.6. These peaks, correspond to PDE II and PDE III, respectively. It is noteworthy that after treatment with ATP and cAMP (Fig. 6A) PDE II is not activatable by venom proteases, but after 'deactivation' by phosphatase (Fig. 6B) it becomes activatable. In addition, the total activity in PDE II peak drops significantly following deactivation. These experiments suggest that phosphorylation-dephosphorylation, if it occurs, affects the activity of PDE II.

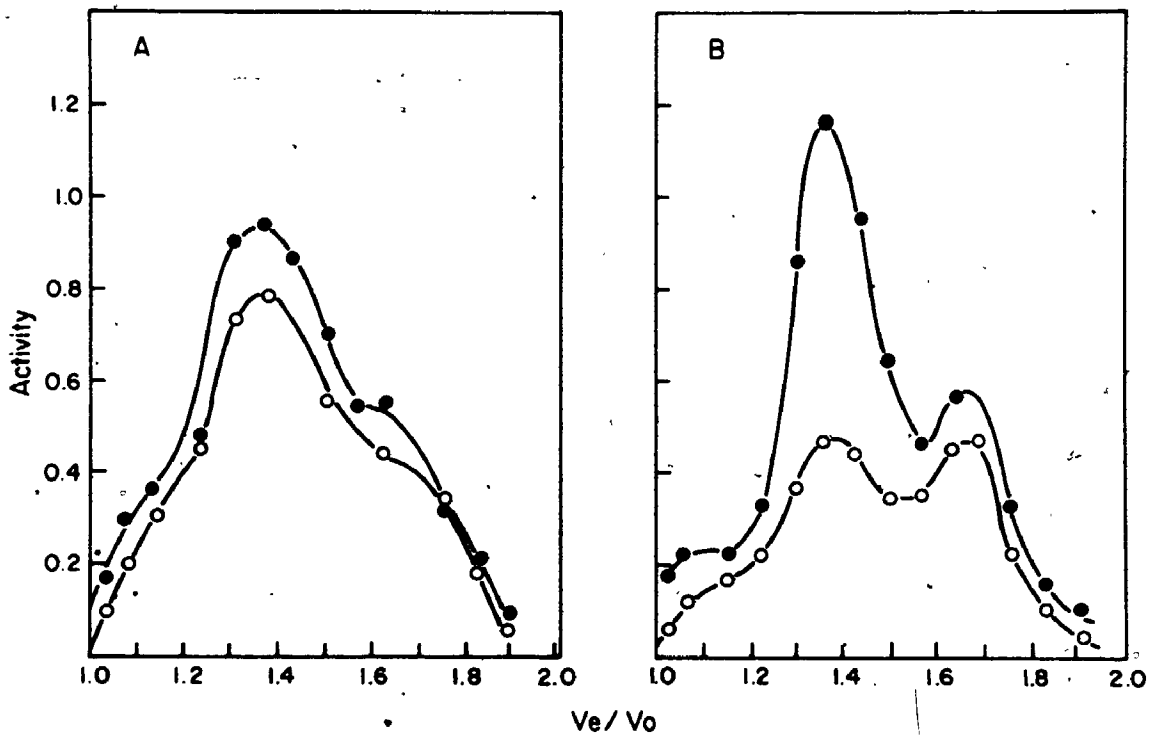
3.3.4 Purification of Phosphorylated PDE II From Rat Muscle

In order to prove unequivocally that phosphorylation of PDE II does occur, during the *in vitro* activation of phosphodesterase II described in the previous chapter, we decided to purify PDE II from muscle homogenate. Myoblast extracts were not used because of the difficulty in growing large number of cells required for the purification of the phosphorylated phosphodiesterase

FIGURE 6

BIO GEL A 1.5 M COLUMN PROFILES AFTER PHOSPHATASE TREATMENT OF MYOBLAST EXTRACTS

Myoblast phosphodiesterase was activated *in vitro* as described in legend to table 1. Dialyzed extract was treated with potato acid phosphatase, 1 mg/ml at 30°C for 30 min. Reaction was stopped by adding 20 mM NaF and 20 mM phosphate pH 7.5. The extract was immediately loaded on a Bio-gel A-1.5 M column (1.5 x 60 cm) and fractions of 1.0 ml were collected. Phosphodiesterase activity was assayed in the absence (o) and presence (●) of snake venom. Panel A represents the enzyme profile obtained from untreated extract and panel B represents the profiles from extract treated with phosphatase. Activity units are pmoles of product formed per ml per min, using 0.05 µm cAMP.


A
B
Activity
1.2
1.0
0.8
0.6
0.4
0.2
1.0
1.2
1.4
1.6
1.8
2.0
Ve/ Vo

to homogeneity. We ascertained that phosphodiesterase is 'activated' and 'deactivated' in adult muscle homogenates exactly as in myoblast extracts.

Cell free extracts from muscle were prepared as described in chapter 2 and were incubated with [γ-^{32}P]-ATP in the presence and absence of cAMP. After desalting the extracts on Sephadex G-25, PDE II was purified by DEAE-cellulose absorption and chromatography through Bio-Gel A-1.5 M column, as outlined in Chapter 2.

Active fractions from the Bio Gel column were pooled and chromatographed on a MIX-Sepharose column. The enzyme was eluted by 1 M KCl, as described earlier. Peak fractions were subjected to SDS slab gel electrophoresis, stained for protein, and exposed to X-ray film for 24 h. The 90-94 K band was subsequently cut out and counted to quantitate the amount of ^{32}P in the band.

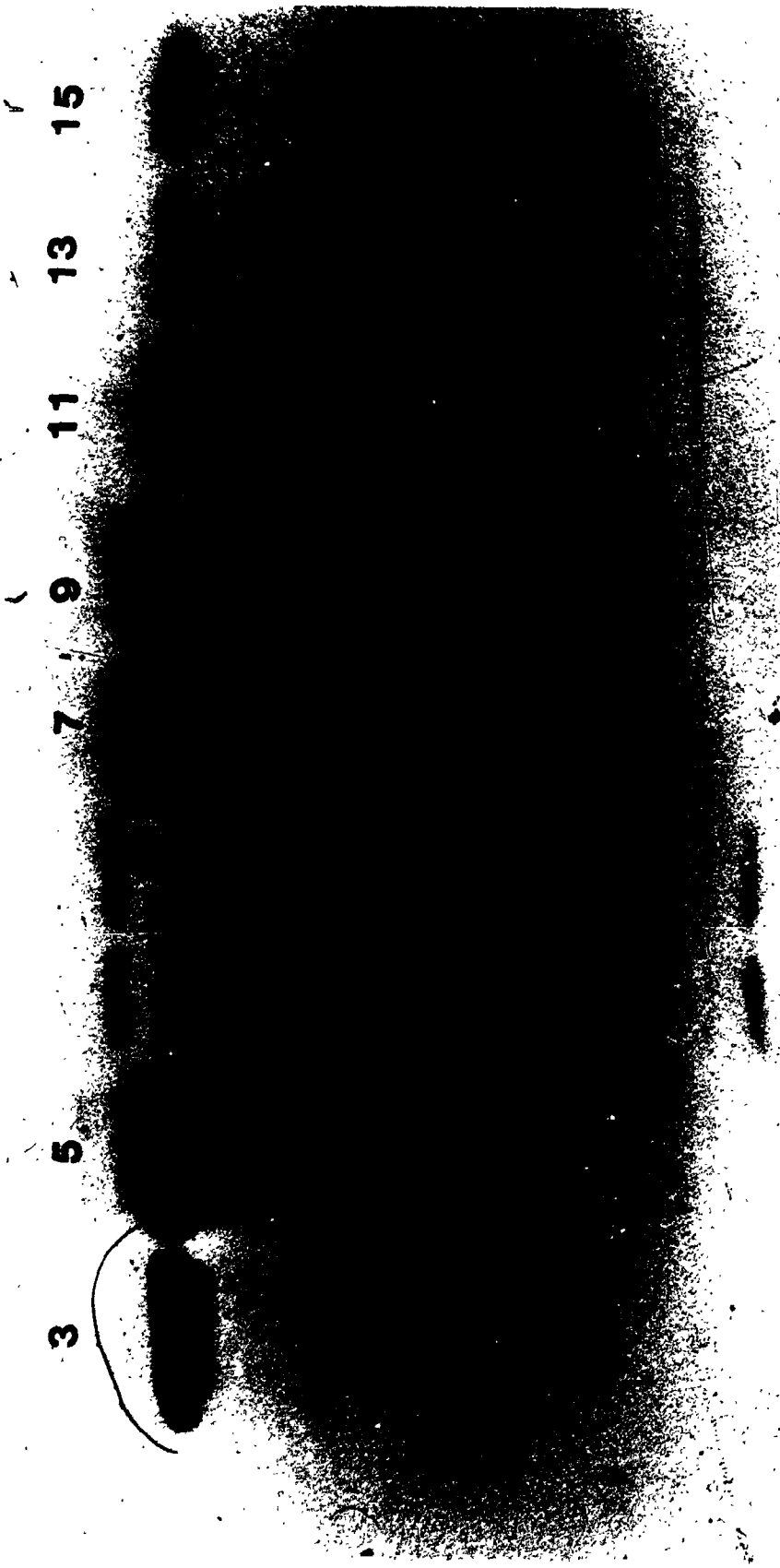
The results of this investigation are presented in Fig. 7. The autoradiograms show that peak fractions from MIX-Sepharose columns contain a single protein, whose subunit molecular weight is 90-94,000 daltons. As mentioned earlier, PDE II polymerizes to higher molecular weight aggregates (PDE I) when chromatographed through MIX-Sepharose column thus affording a suitable procedure for its purification (Chapter 2). PDE II has only one type of subunit with a molecular weight of

FIGURE 7

AUTORADIOGRAM OF THE PEAK FRACTIONS

FROM MIX-SEPHAROSE COLUMN

Freshly dissected rat leg muscle was homogenized in 2 times its volume of buffer A for 2 min. After centrifugation at 15,000 g for 30 min, the supernatant was collected. This crude extract was phosphorylated as described in Materials and Methods. Reaction mixture (2.0 ml) contained 1.5 ml of the extract. The specific activity of [γ-^{32}P] ATP was 750 cpm/pmole. After phosphorylation, the mixture was desalted on Sephadex G-25 and PDE II was semipurified through DEAE-cellulose absorption and Bio-Gel A-1.5 M column (1.5 x 60 cm) as described in the text. Active fractions of PDE II from Bio gel column were pooled and chromatographed on MIX-Sepharose column (0.8 x 4 cm). The column was washed successively with 100 ml of 0.1 M phosphate buffer (Buffer A) and then with 0.7 M phosphate buffer, pH 7.5 containing other components of Buffer A and 1 M KCl. 50 fractions of 0.5 ml each were collected and assayed for phosphodiesterase activity. Peak fractions (number 3 to 15) were subjected to SDS slab gel electrophoresis using 0.2 ml of the effluent. Autoradiography was done as described in the text. The numbers on top of the lanes are column fractions. Unnumbered lanes have radioiodinated molecular weight standards (94K, 67K, 43K, 30K, 20.1K and 14.4K).


15
13
11
9
7
5
3

90-94,000 daltons. Presumably then, the phosphorylated band obtained on SDS-gel electrophoresis is the PDE II subunit. When phosphorylation by [γ-^{32}P]-ATP in cell-free extracts was attempted in the absence of cAMP, the 90-94 K subunit was again found to be phosphorylated, but the amount of radioactivity incorporated was much less than in the presence of cAMP. In one typical experiment, for instance, when radioactive bands from gels after autoradiography were cut out and counted, radioactivity in the subunit obtained in the absence of cAMP was 553 cpm while in the subunit in the presence of cAMP it was 1195. This experiment suggests that phosphorylation of PDE II is brought about by a cAMP-dependent protein kinase present in crude cell-free extracts of muscle.

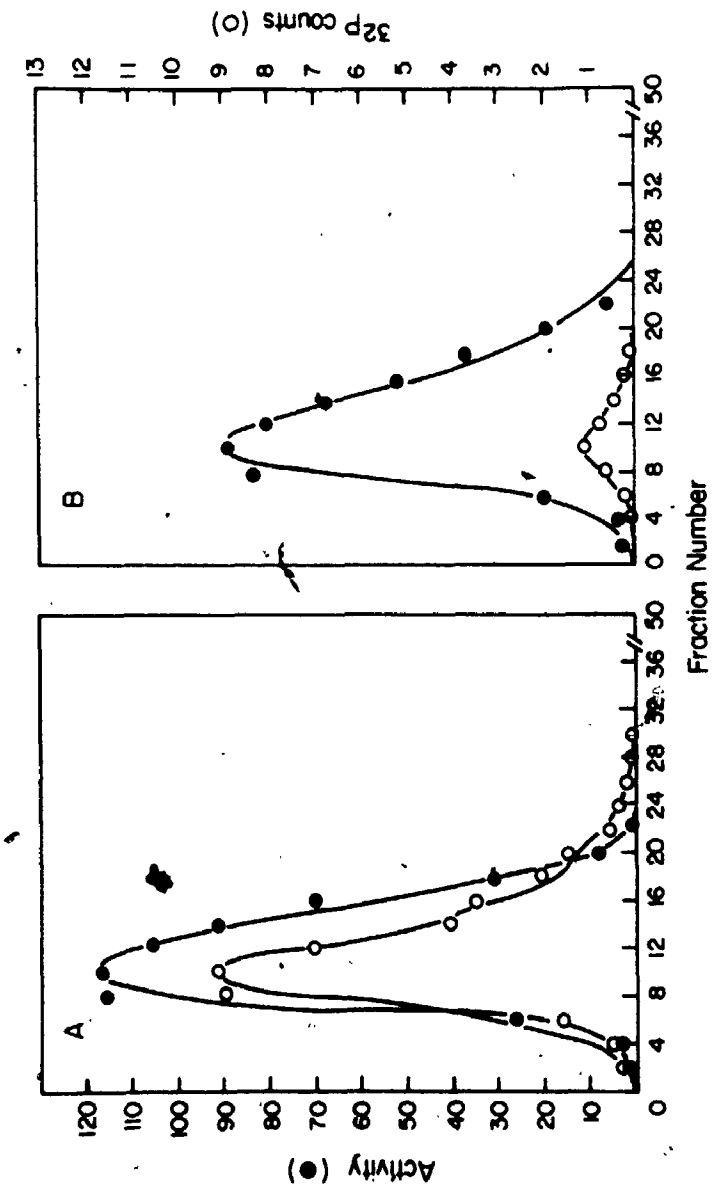
Since we had shown previously that partially purified PDE II can be also activated by adding exogenous cAMP-dependent protein kinase (Table 4), we attempted to show phosphorylation of PDE II during this activation. Semipurified PDE II was activated in the presence of [γ-^{32}P]-ATP and the other components as described in Materials and Methods. The extracts were passed through a MIX-Sepharose column and eluted with KCl in the usual manner. As shown in Fig. 8, there is a correspondence of the enzyme activity peak with the radioactive peak. It may be again pointed out

FIGURE 8

MIX-SEPHAROSE COLUMN

PROFILES OF PHOSPHODIESTERASE

About 100 fold purified PDE II (5 mg total protein) from rat skeletal muscle was incubated with cAMP dependent protein kinase (100 µgm) in the presence of [γ-^{32}P]-ATP (700 cpm/pmole) and other components required for phosphorylation in a total volume of 1.0 ml. After incubation at 30°C for 10 min, the reaction mixture was cooled on ice and passed through MIX-Sepharose column and PDE I was eluted as described in the legend to fig. 7. Phosphodiesterase activity was assayed in the usual manner (●). Aliquots of the fractions were counted for ^{32}P (o). Activity of the enzyme is expressed as dpm obtained per 10 µl per 10 min. Dpm should be multiplied by 1000 to get the actual counts. ^{32}P counts are per 20 µl and should be multiplied by 100 to get the actual count. Panel A, profile obtained in the presence of protein kinase. Panel B, profile obtained in the absence of protein kinase.


A
B
Activity (●)
32P counts (○)
Fraction Number

that on MIX-Sepharose only one type of protein (PDE I) is eluted by our procedures, which has a single phosphorylated subunit as shown in Fig. 9. However little phosphorylation was detected in the control without protein kinase.

Phosphorylation of muscle PDE II by exogeneous cAMP-dependent protein kinase was also shown by using another procedure. In this case, semipurified PDE II was activated by cAMP-dependent protein kinase in the presence of [γ-^{32}P]-ATP as described above. The extracts were run immediately on both SDS gel electrophoresis (Fig. 10) or electrophoresis under nondenaturing conditions (Fig. 11). In the latter case, PDE activity was eluted from the gels and subjected to SDS gel electrophoresis (Fig. 12). The results presented in Fig. 10 show the phosphorylation of semipurified PDE II band (90 K) (Fig. 10 D,F). However, no phosphorylated protein was visible when the incubation was done in the absence of protein kinase (Fig. 10 B,E). Notably when protein kinase alone was incubated, a phosphorylated band was seen. This band has been identified to be autophosphorylated protein kinase (Fig. 10 A,C).

Phosphorylated PDE II was further purified using nondenaturing gel electrophoresis (Fig. 11). The slices which gave phosphodiesterase activity also had 90 K phosphorylated band when subjected to SDS gel

FIGURE 9

AUTORADIOGRAM OF PHOSPHODIESTERASE

FROM MIX-SEPHAROSE COLUMN

Peak fractions (fraction number 8,10,12,15) from the MIX-Sepharose column (fig. 8) were subjected to SDS slab gel electrophoresis and autoradiography was done as described in Materials and Methods. The numbers above the channels indicate fraction numbers. Unnumbered lane has radioiodinated molecular weight standards (94K, 67K, 43K, 30K, 20.1K and 14.4K).

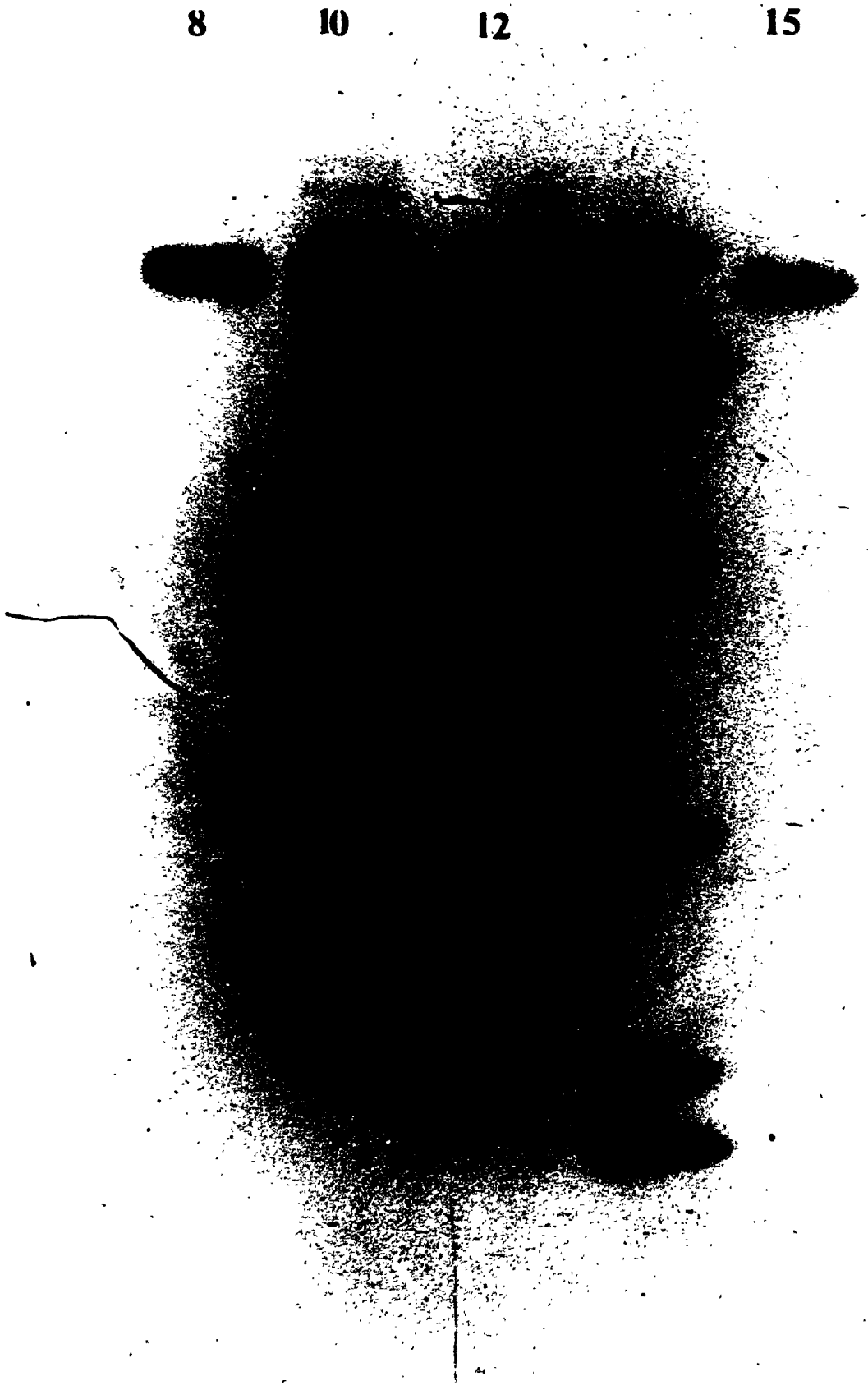

8
10
12
15

FIGURE 10

SDS-GEL ELECTROPHORESIS OF

PHOSPHORYLATED PDE II

About 100 fold purified PDE II from rat skeletal muscle was incubated with cAMP dependent protein kinase in the presence of [γ-^{32}P]-ATP as described in legend to fig. 7. A control without protein kinase and one without PDE II was also incubated under identical conditions. After the incubation at 37°C for 10 min, an aliquot from each sample was TCA precipitated and subjected to SDS-gel electrophoresis. The gel was stained, destained, dried and autoradiographed as usual. Lanes A and C show the phosphorylated extracts (control) which contained protein kinase alone (no PDE II). Lanes B and E show phosphorylated extracts (control) which contained PDE II alone (no protein kinase). Lanes D and F show phosphorylated extracts containing PDE II and protein kinase both. Unmarked lane shows radioiodinated protein standards (94K, 67K, 43K, 30K, 20.1K and 14.4K).

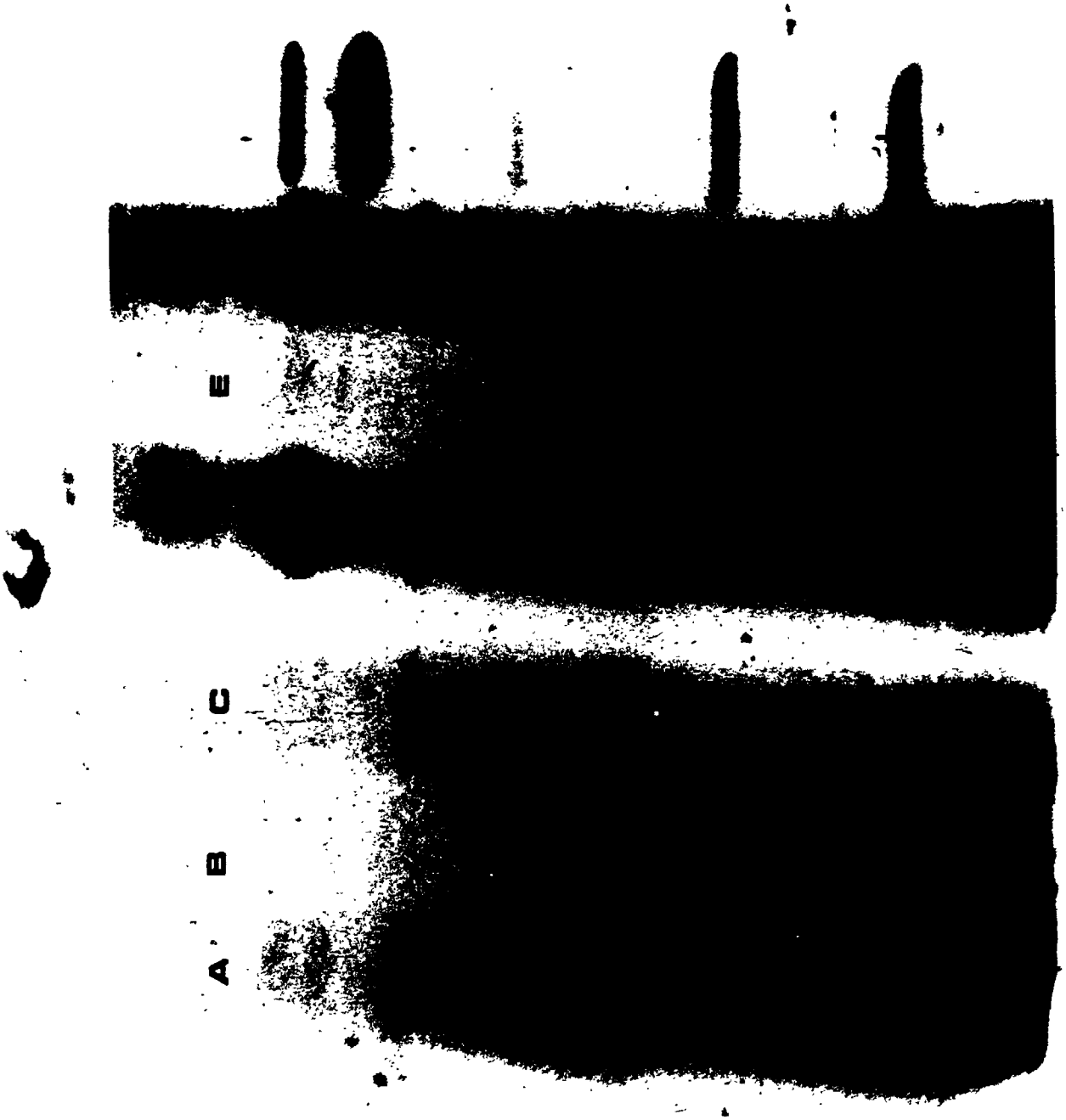

E
C
B
A

FIGURE 11

NONDENATURING GEL ELECTROPHORESIS

OF PHOSPHORYLATED PDE II

Phosphorylated PDE II produced in the previous experiment (Fig. 10) was subjected to electrophoresis under non denaturing conditions. Control which contained PDE II alone (i.e. no protein kinase) was also electrophoresed. Both the gels were sliced and phosphodiesterase activity eluted in buffer as described in Materials and Methods. Phosphodiesterase activity was assayed in the presence (o) and absence (●) of snake venom. Activity is expressed as pmoles of cAMP hydrolyzed per min per ml. Panel A shows the activity profile obtained from the control extract without protein kinase. Panel B shows the activity profile obtained from phosphorylated (active) extract.

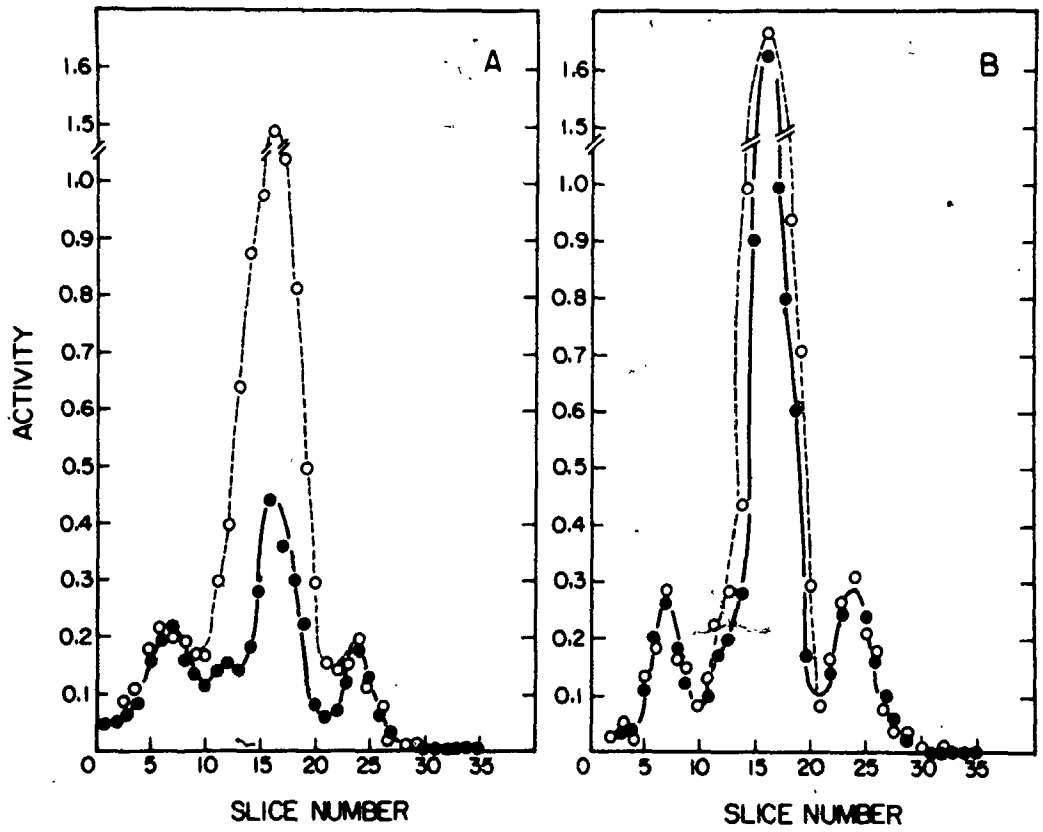
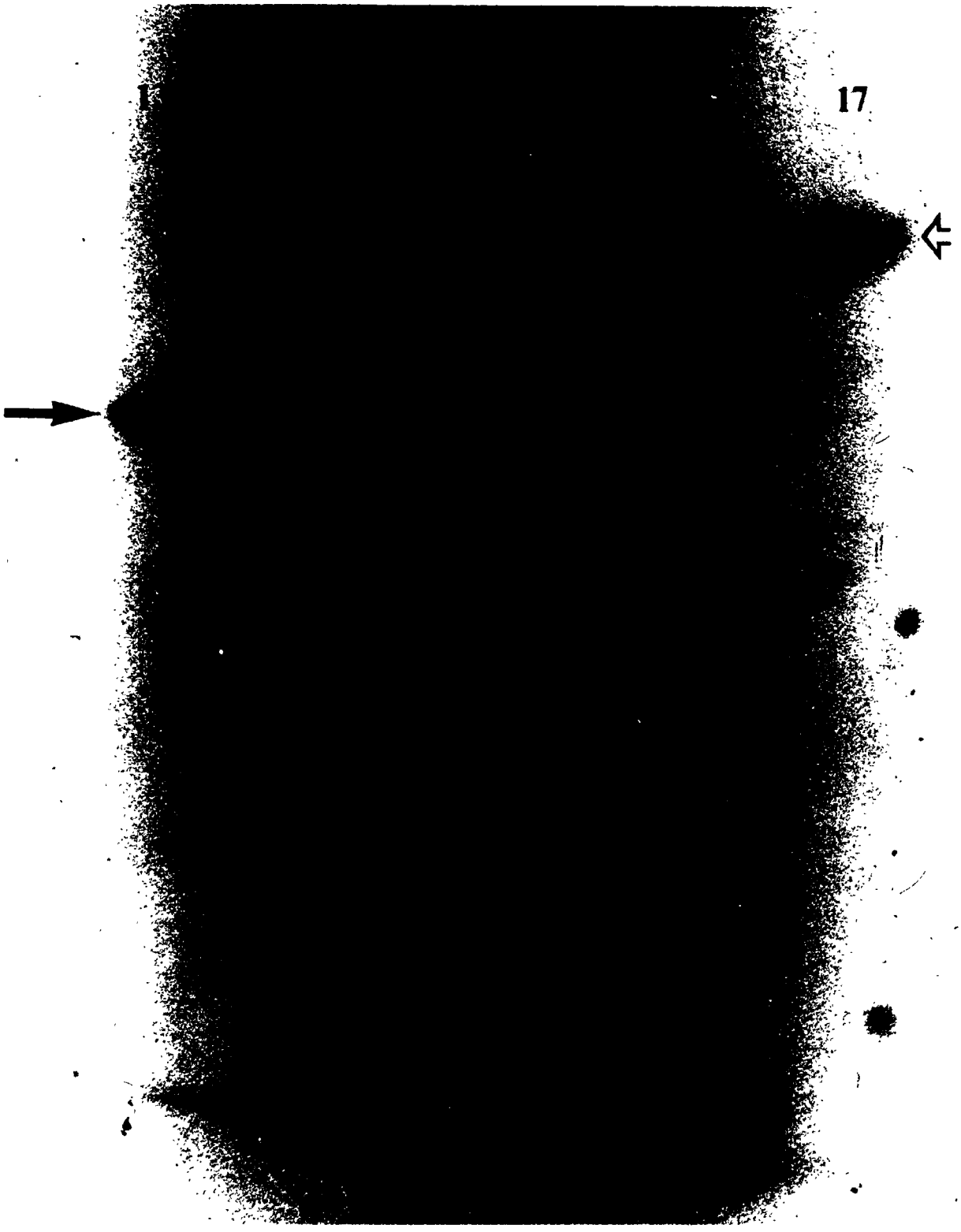

A
B
ACTIVITY
SLICE NUMBER
SLICE NUMBER
0
5
10
15
20
25
30
35
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.5
1.6

FIGURE 12

AUTORADIOGRAM OF PHOSPHORYLATED PDE II

Fractions (slices) containing PDE II activity in the previous experiment (Fig. 11B) were subjected to SDS-gel electrophoresis. After electrophoresis, the gels were stained, destained and autoradiographed as described in Materials and Methods. The numbers at the top of the lane represent the slice numbers of Fig. 11B. Unnumbered lane has radioiodinated molecular weight standards. Solid arrow indicates the position of protein kinase and hollow arrow marks the position of PDE II.


17

electrophoresis (Fig. 12). Noteworthy is the fact that gel electrophoresis under the nondenaturing conditions could also separate protein kinase band (50 K) from PDE II band (90 K) as shown in Fig. 12.

3.3.5 Phosphorylation of PDE II in Myoblast Extracts

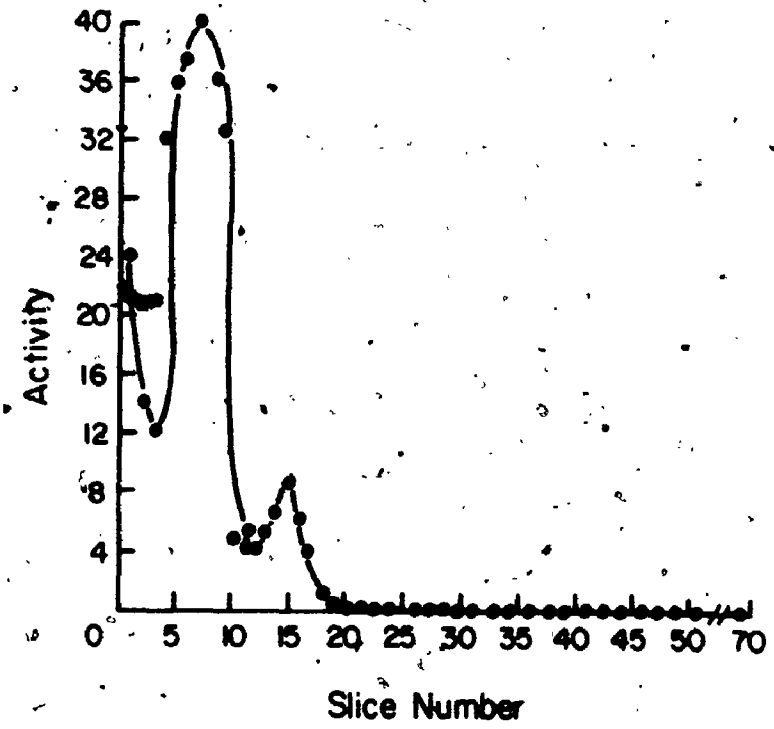
Since adult muscle consists of a mixture of several cell types and has been shown by others (Thompson and Appleman, 1971; Gain and Appleman, 1978) to contain multiple forms of phosphodiesterases which are absent in myoblasts, it is important to show phosphorylation of PDE II in myoblast extracts, where only one primary phosphodiesterase exists (Chapter 2). Owing to the paucity of cell material, PDE II from myoblast extracts was purified only through chromatography on Bio-Gel A-1.5 M columns. This approximately 10-15 fold purified preparation was incubated with protein kinase [γ-^{32}P]-ATP and cAMP as described earlier with PDE II preparations from adult rat skeletal muscle. The mixture was then chromatographed on a MIX-Sepharose column and enzyme eluted by KCl. Since the material applied to the affinity column was relatively impure, we ascertained that the peak of protein emerging from the column has enzyme activity binding at PDE I position in gel electrophoresis under nondenaturing conditions. This is shown in Fig. 13. Two bands of activity appear,

FIGURE 13

POLYACRYLAMIDE GEL ELECTROPHORESIS

UNDER NONDENATURING CONDITIONS

About 15 fold purified PDE II from myoblasts was phosphorylated by using cAMP dependent protein kinase in the presence of [γ-^{32}P]-ATP (specific activity 500 cpm/pmole). Amount of protein used was 3 mg in a final volume of 1.0 ml. Other components were added as described in Materials and Methods. After incubation at 30°C for 10 min, the reaction mixture was cooled on ice and passed through MIX-Sepharose column and PDE I fractions collected. PDE I peak activity fractions were subjected to polyacrylamide gel electrophoresis under non denaturing conditions and enzyme was eluted from the gel slices as described in Materials and Methods. Activity of the enzyme was assayed in the absence of snake venom (●). Activity is expressed as dpm per 20 µl per 1 h. The counts shown should be multiplied by 1000 to get the actual counts.


Activity
40
36
32
28
24
20
16
12
8
4
0
5
10
15
20
25
30
35
40
45
50
70
Slice Number

the major one being PDE I. When the affinity column effluent is electrophoresed on SDS-gels a distinct band at about 94 K can be visualized after autoradiography (Fig. 14). Several other minor radioactive bands are also present, however reflecting the rather impure state of the material applied to the affinity column.

3.3.6 Phosphorylation of PDE I and PDE IV

We have shown previously (Chapter 2) that PDE I and PDE IV probably arise from PDE II; the former by aggregation and the latter by proteolysis. The question thus arises as to whether phosphorylation sites are preserved in these derived forms, though it should be mentioned that there was no effect on the activity of either PDE I or PDE IV when they were separately treated with cAMP-dependent protein kinase in the presence of cAMP and ATP. To find whether these two forms of phosphodiesterases were phosphorylated or not, PDE I and PDE IV were purified to homogeneity from rat muscle by the procedures described elsewhere in this thesis (Chapter 2) and were incubated with protein kinase, cAMP and [γ-^{32}P]-ATP as was done with PDE II. The mixture containing PDE I was loaded on MIX-Sepharose to remove the protein kinase and nucleotides. PDE I was eluted by the usual procedure and was subjected

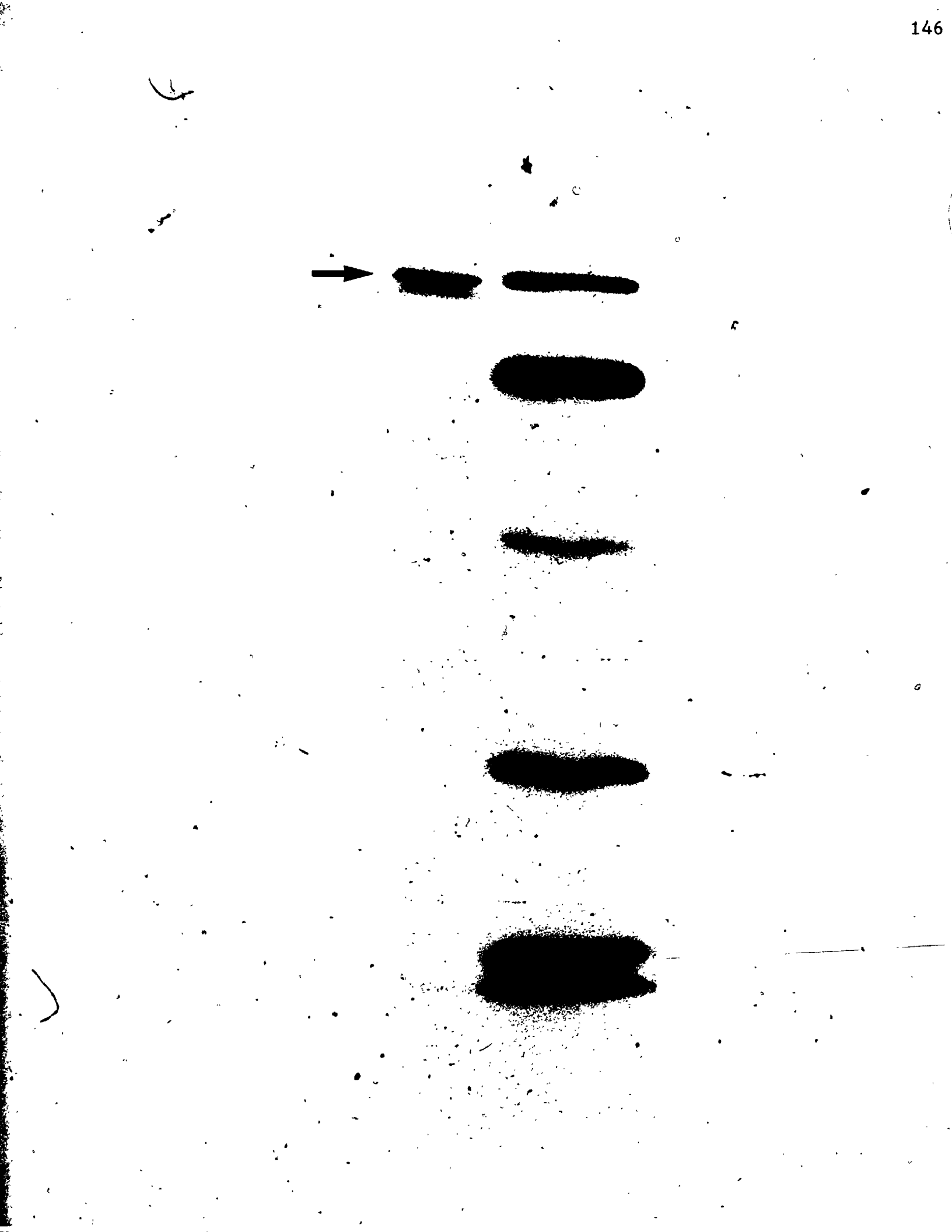
FIGURE 14

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

OF SAME FRACTIONS COLLECTED FROM

MIX-SEPHAROSE COLUMNS

PDE I which was obtained from MIX-Sepharose column as described in the legend to Fig. 13 was subjected to SDS slab gel electrophoresis and autoradiographed as described in Materials and Methods. The arrow indicates the position of 94K subunit of PDE I. Unmarked lane has radioiodinated molecular weight standards (94K, 67K, 43K, 30K, 20.1K and 14.4K).



to SDS-polyacrylamide gel electrophoresis and the radioactive bands were located on X-ray film. PDE I was phosphorylated by protein kinase as shown in Fig. 15 but stoichiometry of phosphorylation was not determined. PDE IV when incubated with protein kinse, cAMP and [γ-^{32}P]-ATP, however, failed to yield a radioactive product. This result is not surprising in view of the probable proteolytic origin of PDE IV. It is possible that amino acid sequences containing the phosphorylation sites are either excised during proteolysis, or these sites become inaccessible to protein kinase due to an altered conformation of the protein.

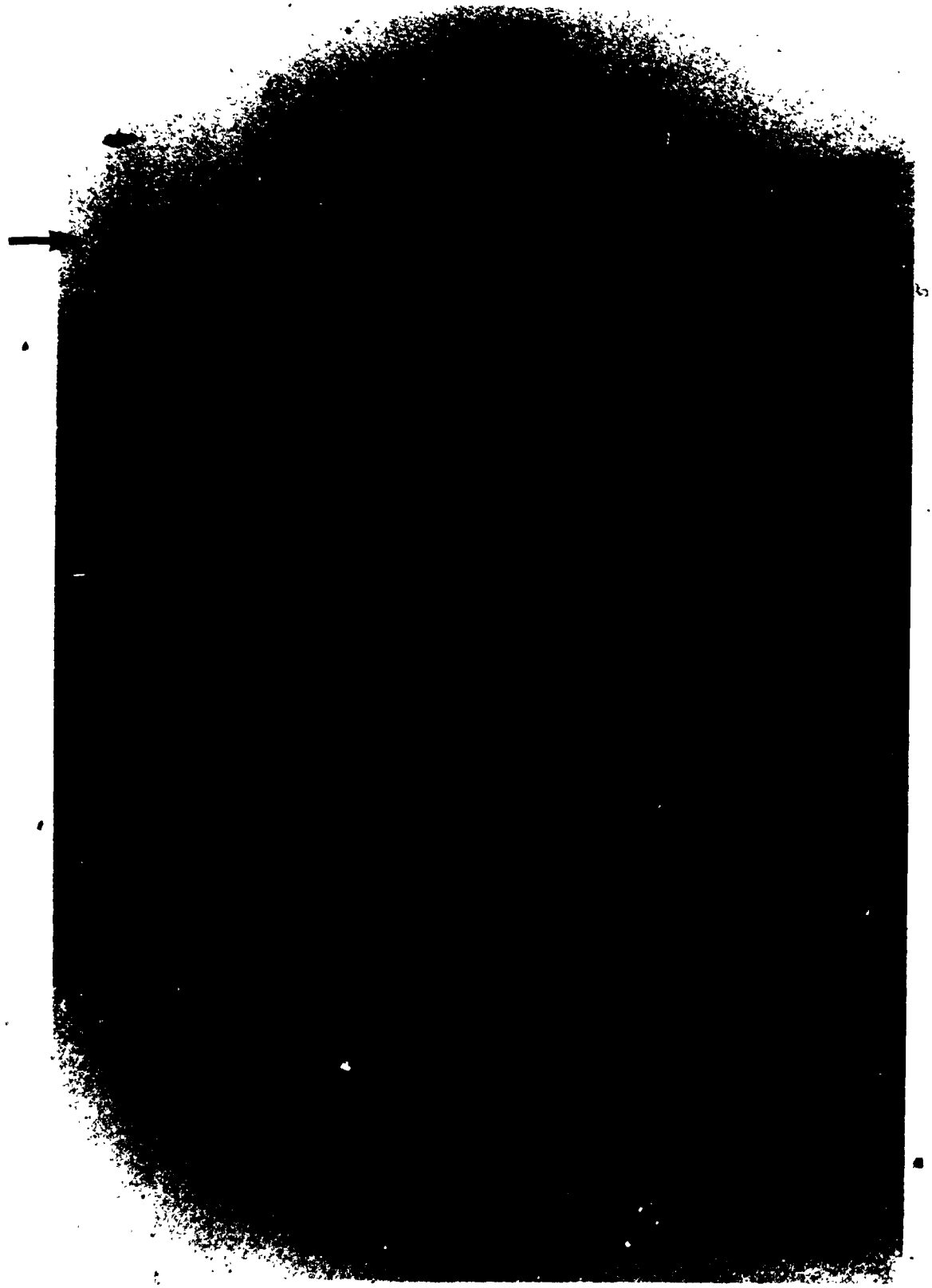
3.3.7 Dephosphorylation of Phosphorylated Phosphodiesterase

Experiments discussed above, indicate that *in vitro* activation of phosphodiesterase in rat myoblasts is probably due to the phosphorylation of PDE II. Since every modification of protein due to phosphorylation can be reversed by dephosphorylation, we made attempts to dephosphorylate the phosphodiesterase. In fact, we had earlier shown that activated phosphodiesterase can be deactivated *in vitro* by phosphatase (Fig. 5). Phosphodiesterase was activated in the crude extracts of myoblasts, in the presence of [γ-^{32}P]-ATP as described in 'Methods'. After 10 min incubation at 30°C, the

FIGURE 15

PHOSPHORYLATION OF PDE I IN VITRO

Pure preparations of PDE I (1 mg) was incubated with cAMP-dependent protein kinase and [γ-^{32}P]-ATP (500 cpm/pmole) and other components as described in Materials and Methods. Final volume of the incubation mixture was 1.0 ml. After incubation at 30°C for 10 min, the mixture was passed through MIX-Sepharose column. After washing the column with usual procedure, to remove protein kinase, PDE I was eluted with 1 M KCl in buffer A. Fractions containing the enzyme activity were subjected to SDS slab gel electrophoresis and autoradiographed as usual. The arrow marks the position of phosphodiesterase band. Radioiodinated molecular weight standards shown are 94K, 67K, 43K, 30K, 20.1K and 14.4K.



crude extract was loaded on Bio-Gel A-1.5 M to partially purify the phosphorylated PDE II. Fractions containing the enzyme activity were pooled, concentrated, and treated with acid phosphatase. Controls without phosphatase were also run. Aliquots were drawn at various time intervals and subjected to SDS-gel electrophoresis and autoradiographed. A time dependent dephosphorylation of 94 K band was seen. This dephosphorylation was almost complete (Fig. 16) in about 30 min. Interestingly, the pattern of dephosphorylation is quite similar to the deactivation of the phosphodiesterase as was seen earlier (Fig. 5), suggesting a causal relationship between deactivation and dephosphorylation.

3.3.8 In vivo Phosphorylation of Phosphodiesterase

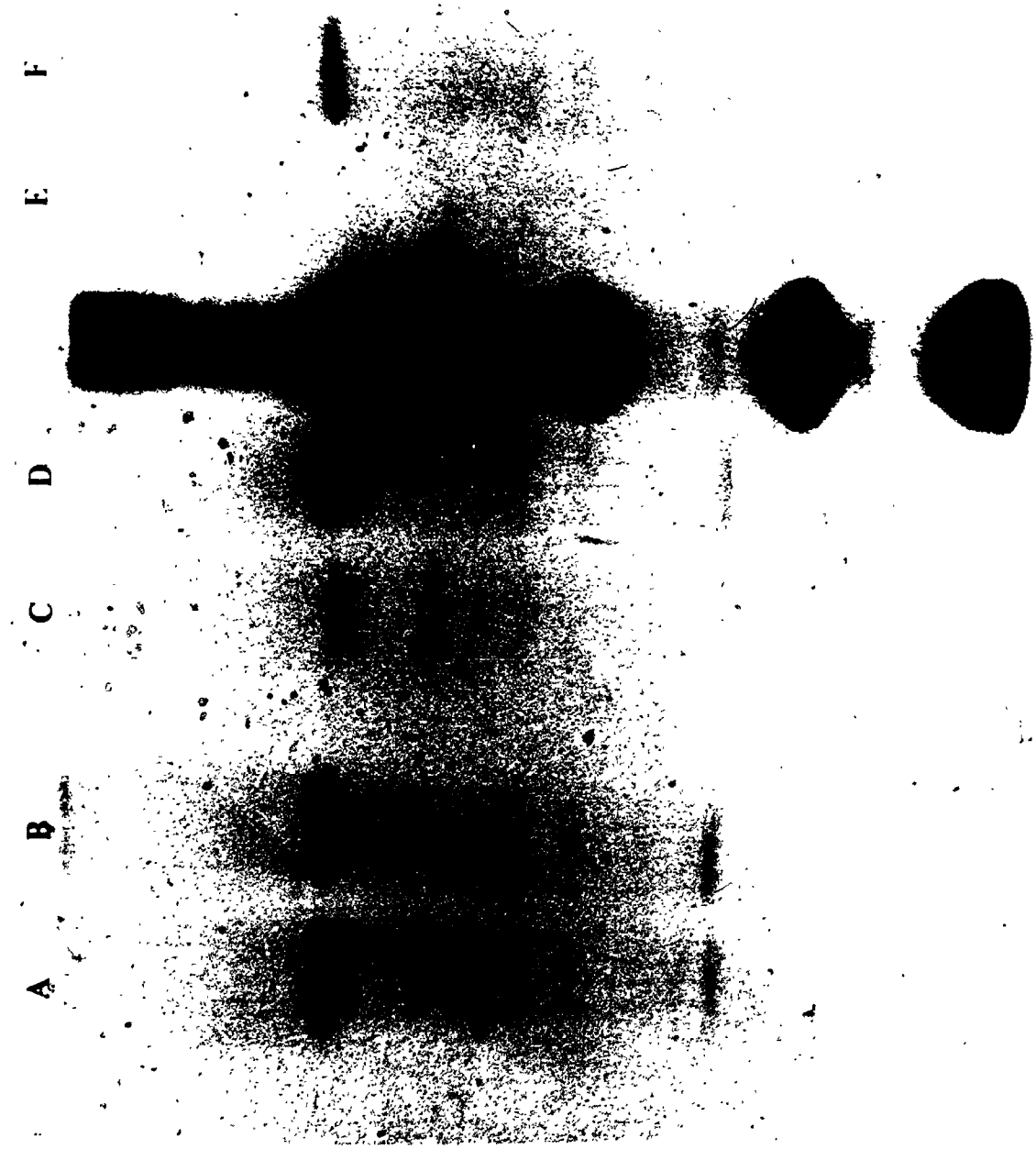
Preliminary studies have been done to show phosphorylation of PDE II in the intact cells. Myoblast cells were prelabelled with ^{32}Pi as described in 'Methods'. To labelled cells, Bt_2cAMP and MIX were added and incubations were done for 1 h. After incubation, cell free extracts were made and PDE II was purified through Bio-Gel A-1.5 M column. Fractions containing the enzyme activity were pooled and subjected to electrophoresis under non denaturing conditions and PDE II eluted in the usual manner. The

FIGURE 16

AUTORADIOGRAM OF PHOSPHORYLATED

MYOBLAST CRUDE EXTRACTS

A Cell free extract from 4-day-old myoblasts was treated with 0.1 mM cAMP and 0.5 mM [γ-^{32}P]-ATP (sp. act. 500 cpm/pmole) to produce active (phosphorylated) phosphodiesterase. After dialysis, the extract was incubated with 1 mg/ml of acid phosphatase. Aliquots of suitable volume were withdrawn at 0', 5' and 30'. The phosphatase action was terminated by the addition of 20 mM NaF and 20 mM phosphate pH 7.5 and subjected to SDS gel electrophoresis. Gels were stained, destained, dried and autoradiographed as described in Material and Methods. Lanes A,C,E represent phosphorylated proteins present in the extract after 0', 5' and 30' of phosphatase treatment respectively. Lanes B,D,F represent phosphorylated proteins present in the extract at 0', 5' and 30' in control extracts. Unmarked lane shows radioiodinated molecular weight standards (94K, 67K, 43K, 30K, 20.1K and 14.4K).


F
E
D
C
B
A

activity profiles are shown in Fig. 17. The gel slices containing the phosphodiesterase activity were subjected to SDS slab gel electrophoresis and autoradiographed. The results are shown in Fig. 18. Activity peak obtained in non denaturing gels gave a major 94 K band, though there were also a few minor bands, copurified with our PDE. Moreover, 94 K bands obtained from cAMP treated cells was more intense than from control cells.

3.3.9 Activation of Phosphodiesterase by Insulin

It is very probable from the above results that in our system cAMP can activate phosphodiesterase. It was interesting to know if insulin, a hormone which is known to depress cAMP levels, and activate phosphodiesterase in other systems (see Chapter 1 of the thesis) also can activate the enzyme in myoblasts. Exposure of cells to 5 µg/ml insulin resulted in about a 2-fold increase in the activity of the enzyme. The enzyme remained activatable by snake venom just like the enzyme from control cells (Fig. 19).

3.4 Discussion

Phosphodiesterase activity is increased by exposing myoblasts to Bt_2cAMP and MIX for brief periods of time. Activation is also brought about by isoproterenol and

FIGURE 17

POLYACRYLAMIDE GEL ELECTROPHORESIS

UNDER NONDENATURING CONDITIONS

4-day-old myoblasts were labelled with ^{32}P-orthophosphoric acid (100 µci/ml) for 30 min at which time 0.7 mM Bt_2cAMP and 0.1 mM MIX were added for another one h. Control plates received buffer alone. Cells were homogenized and phosphodiesterase partially purified as described in the text. The electrophoresis was done under nondenaturing condition and phosphodiesterase activity eluted in buffer. The enzyme was assayed in the presence (o) and absence (●) of snake venom. Activity is expressed as pmoles of cAMP hydrolyzed per min per ml. Panel A, activity profile obtained from control cells. Panel B, activity profile obtained from cAMP treated cells.

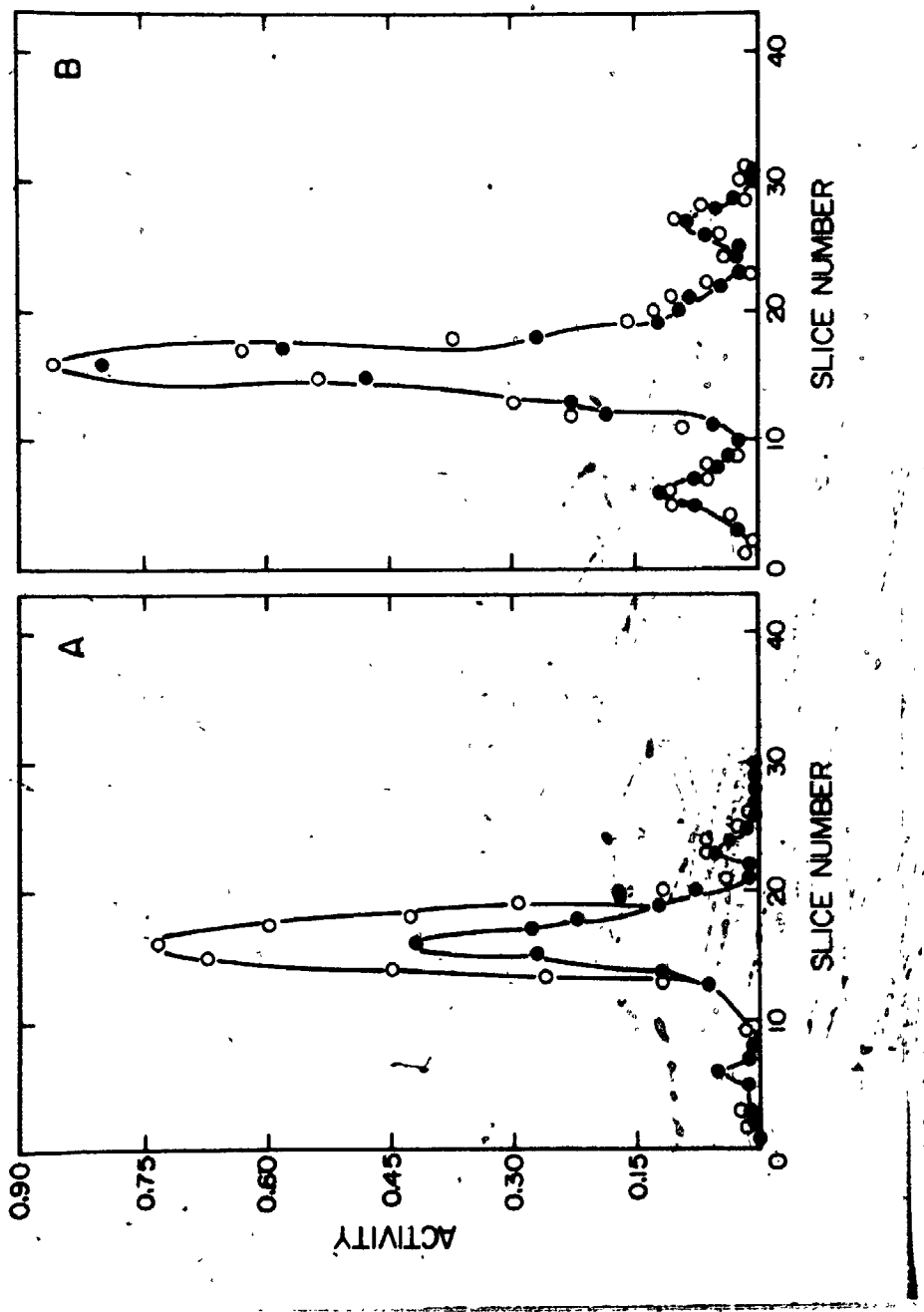

A
B
ACTIVITY
0.90
0.75
0.60
0.45
0.30
0.15
0
10
20
30
40
SLICE NUMBER
SLICE NUMBER

FIGURE 18

AUTORADIOGRAM OF IN VIVO

PHOSPHORYLATED PDE II

Active fractions from the previous experiment (Fig. 17) were subjected to SDS gel electrophoresis and autoradiographed by the usual procedure. Panel A, shows the autoradiogram of control PDE II partially purified fractions. Panel B, shows the autoradiogram of PDE II prepared (partially purified) from cAMP treated cells. Numbers on top of the lanes represent the slice number corresponding to Fig. 17. The arrow indicates the position of PDE II.

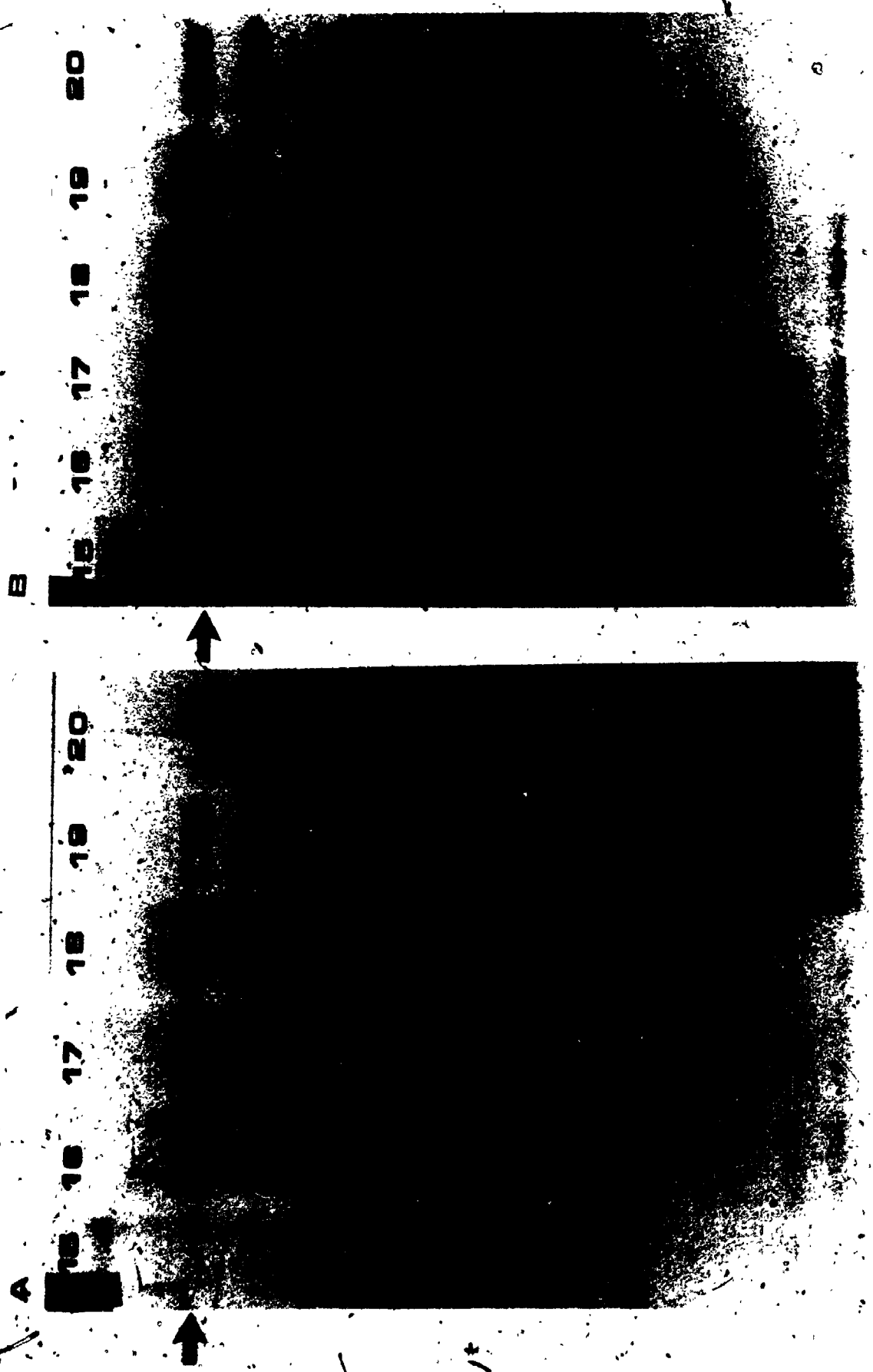
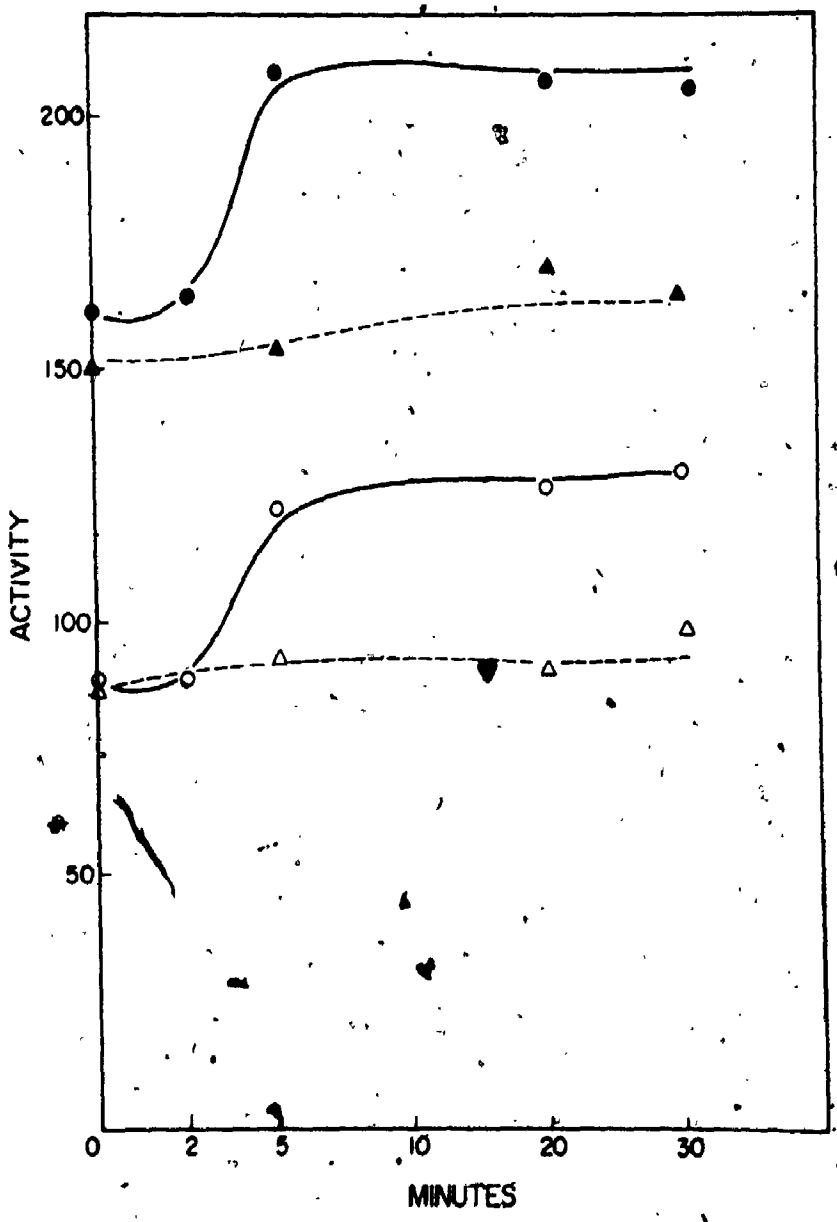

B
A

FIGURE 19

EFFECT OF INSULIN ON

PHOSPHODIESTERASE ACTIVITY

To 4-day-old myoblasts, 5 µg/ml of insulin was added at zero time and the cells were harvested at the time indicated. Activity of phosphodiesterase was measured in the absence (o) and presence (●) of snake venom. Controls did not receive insulin. Activity of phosphodiesterase was also measured in controls, in the absence (Δ) and presence (▲) of snake venom.


200
150
100
50
ACTIVITY
0
2
5
10
20
30
MINUTES

PGE_1, compounds which are known to augment the concentrations of cAMP in the cells (Curtis and Zalin, 1981). The process of activation did not require synthesis of new protein indicating that modification of preexisting phosphodiesterase occurs. Phosphodiesterase which is normally activated 2 to 5-fold by proteases was not activated when produced by cells in the presence of cAMP. These observations along with the general belief that all the effects of cAMP are mediated via a cAMP-dependent protein kinase (Kuo and Greengard, 1969) prompted us to investigate the possibility of regulation of phosphodiesterase by a phosphorylation-dephosphorylation mechanism.

cAMP and ATP when added together to the cell free extracts of myoblasts could also activate phosphodiesterase in vitro, exactly as in vivo. The ability of protein kinase inhibitor to inhibit this activation showed the involvement of activity of protein kinase in this process. Similarly the inability of AMP-PNP (an analog of ATP, γ-phosphate of which is not available for any phosphotransferase reaction) to activate PDE. also suggested that ATP is hydrolyzed during this reaction. Involvement of cAMP-dependent protein kinase in the activation was confirmed by the demonstration that semipurified PDE II could be activated by purified cAMP dependent protein kinase. All these experiments

are suggestive of the involvement of a protein phosphorylation step in the process of activation of phosphodiesterase.

Active PDE produced after *in vitro* activation could be deactivated by the addition of phosphatase. This treatment restored the protease activatibility of the enzyme which was lost during the activation process. The results suggest the involvement of a dephosphorylation step during the deactivation of the active form of phosphodiesterase.

Further evidences presented argue for a phosphorylation-dephosphorylation mechanism in the control of activity of phosphodiesterase in both myoblasts as well as in adult muscle. Our earlier studies (Chapter 2) have indicated that PDE II is probably the primary form of phosphodiesterase in myoblasts and as we have shown in the present work, this is the only form which is activated by phosphorylation *in vitro*. PDE I, the aggregated form of PDE II, is phosphorylated but does not show any augmentation in activity after modification. PDE IV, the lowest molecular weight form of PDE, and probably derived from PDE II by proteolysis (Chapter 2), can not be phosphorylated under conditions which lead to phosphorylation of PDE II. We have been unable to obtain any similar data on the fourth phosphodiesterase PDE III, because it is very unstable and is rapidly

converted to a form resembling PDE IV. It is thus very difficult to purify in order for in vitro experiments to be undertaken. As discussed in Chapter 4, PDE III is probably derived from PDE II in vivo by proteolysis triggered by cAMP through unknown mechanisms.

PDE II of L6 myoblasts as well as that of adult muscle is very probably regulated by cAMP-dependent phosphorylation. This phosphorylation seems to have physiological significance since several criteria proposed by Krebs and Beavo (1979) to establish phosphorylation-dephosphorylation as regulatory devices for a given protein are fulfilled in the case of PDE II. Thus in cell free extracts of L6 myoblasts which have a cAMP-dependent protein kinase and a phosphoprotein phosphatase (Huang and Tao, 1980), we can show that the regulatory properties of PDE II change in the appropriate manner, under conditions which either lead to phosphorylation or dephosphorylation. In other words, conditions favoring phosphorylation lead to activation of PDE II and conditions favoring dephosphorylation lead to a loss of activation. These changes are in appropriate direction because for a degradative reaction, such as the one catalyzed by phosphodiesterase, phosphorylation would be expected to activate the reaction (Cohen, 1980). Like the enzyme in the cell

free extracts, partially purified PDE II can be also shown to require cAMP-dependent protein kinase for phosphorylation and consequent activation.

Attempts were also made to show the in vivo phosphorylation of PDE II after ^{32}P labelling of the cells. However due to lack of enough myoblast material, it was very difficult to purify the phosphorylated form of PDE II to homogeneity, though partially purified PDE II had a phosphorylated protein band (Fig. 18). Clearly, direct in vivo phosphorylation of PDE II will have to be demonstrated before phosphorylation can be unequivocally considered as a control mechanism.

It may perhaps also be pointed out that while we have adduced evidence from several lines of experimentation that it is actually PDE II which is phosphorylated, we have not unequivocally proven that this is the case. The possibility, however slight, still remains that a contaminant, which copurifies with PDE II, may be the actual protein which undergoes phosphorylation. This uncertainty could be resolved easily if we had a specific antibody against PDE II. This antibody could be used to purify phosphorylated PDE II after labelling in vitro and in vivo. However, despite our repeated efforts we have been unable so far

to obtain an antibody preparation against any of the forms of myoblast phosphodiesterases.

It may be mentioned at this juncture that phosphorylation (and dephosphorylation) is not the only means by which PDE is regulated in myoblasts. When myoblasts are incubated with cAMP for long term (more than 10 hr), the amount of PDE II decreases and the level of PDE III increases considerably (Chapter 4 and Ball et al., 1980). The latter is not activated like PDE II, by proteases. We can show (Chapter 4) that certain non-differentiating mutants of myoblasts are unable to produce PDE III in the presence of cAMP, although PDE II still appears to undergo phosphorylation. A possibility thus exists that a unique protease is either produced de novo or activated in cells by cAMP which is responsible for the conversion of PDE II to PDE III (See Chapter 4). Phosphorylation of PDE II may then serve not only to enhance its activity in the short term, but also make it susceptible to proteolysis (to PDE III) to cope with the demand for a high Vmax enzyme in the chronic presence of cAMP. Indeed it is known that phosphorylation of some enzymes, such as liver pyruvate kinase, makes them more sensitive to proteolytic modification (Bergstrom et al 1975). Until proven, these points are only conjectural, but are presented for their heuristic

Apart from the phosphorylation of phosphodiesterase as demonstrated here for myoblasts and adult muscle, reports of phosphorylation have also appeared with enzymes from other systems. In crude extracts of Mucor rouxii phosphodiesterase undergoes activation in the presence of cAMP and ATP, presumably by phosphorylation. (Moreno et al,1982, Galvagno et al,1979). In rat liver plasma membranes, a high affinity phosphodiesterase has been shown to undergo phosphorylation and activation in the presence of cAMP, ATP and notably insulin (Marchmont and Houslay, 1980; Marchmont and Houslay 1981). All three reagents are required for phosphorylation and activation to occur. The enzyme in liver membrane, however, appears to be different than myoblasts or muscle PDE. The monomeric or subunit molecular weight of the liver enzyme is 52,000 (Marchmont et al,1981), compared to 90-94,000 in muscle. The requirement for insulin in the phosphorylation of PDE is interesting but the molecular mechanism of insulin action is still obscure.

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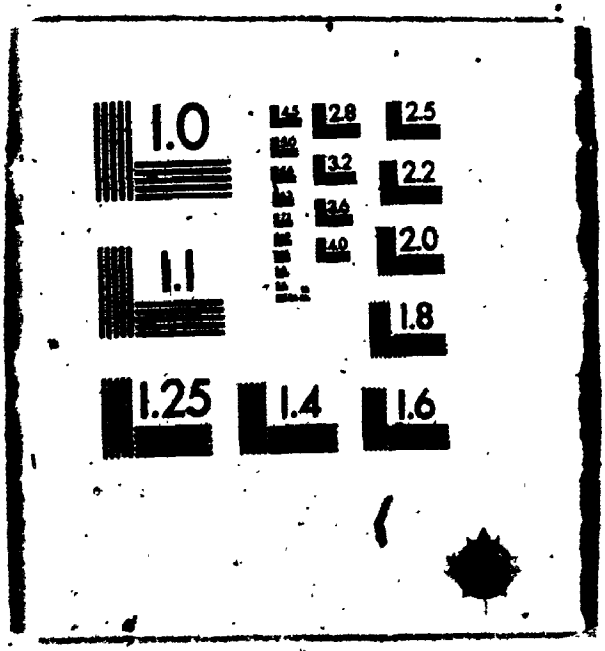
3 3

OF / DE



CHAPTER 4. REGULATION OF CYCLIC ADENOSINE 3':5' MONOPHOSPHATE PHOSPHODIESTERASES. ALTERED PATTERN IN TRANSFORMED MYOBLASTS

4.1 Introduction

Rat skeletal myoblasts contain 4 forms of cAMP phosphodiesterases termed PDE I, PDE II, PDE III and PDE IV (Chapter 2). They are named according to the order of their elution from a Bio-Gel A-1.5 M column with PDE I emerging first and PDE IV last. We have shown earlier that PDE I is derived from PDE II by aggregation and PDE IV by proteolysis (Chapter 2). The relationship between PDE II and PDE III is not clear, but we believe that the latter form also arises from PDE II by a proteolytic process. The distinguishing characteristics of PDE II is that it is activatable 2 to 4 fold by treatment with very low concentrations of various proteases. None of the other forms is similarly activated. When actively growing myoblasts are exposed for brief periods (60 min or less) to exogenous Bt_2cAMP PDE II gets converted to a form which, while retaining its elution characteristics during gel filtration, is no longer susceptible to protease activation. This modification of PDE II can be shown to occur in cell-free extracts in the presence of cAMP-dependent protein kinase, ATP and cAMP (See Chapter 3) and most probably involves phosphorylation of the enzyme. The modified PDE II exhibits about 2-fold increase in V_{max} compared to the unmodified

enzyme without any change in its K_m for cAMP. Modification of PDE II is probably a transient regulatory device used by myoblasts for control of the levels of cAMP *in vivo* and has been termed 'short-term activation' (Chapter 3). When myoblasts are exposed chronically to cAMP (10 hours or more), the activity of PDE II decreases, but the activity of PDE III increases correspondingly. Unlike short-term activation, the increase in enzyme activity is prevented by cycloheximide. The increase in PDE III activity has been termed 'long-term induction' (Ball *et al*,1980). Since PDE II activity decreases during long-term induction, the possibility arises that it serves as a precursor for PDE III. It has been hypothesized that during chronic exposure to cAMP, a protease is either activated or induced in cells which is then responsible for the conversion of PDE II to PDE III. However, since myoblasts contain a large variety of different types of proteases (Kaur and Sanwal, 1981), attempts have not been made to try to purify the putative inducible protease. Rather, indirect approaches have been sought to prove the existence of a cAMP-inducible or activatable protease in myoblasts. Since cAMP levels probably regulate the growth rate of cells (Pastan *et al*,1975), it occurred to us that fast growing variants of myoblasts, because of the low concentration of cAMP they are expected to contain

(Pastan et al,1975), may have alterations in the long-term induction of PDE III, which may signify the absence of the putative protease in these variants. One of the easy ways of obtaining variants altered in their growth pattern is to select fusion-defective variants (Kaufman and Parks, 1977).

In the following report we show that spontaneously or virally transformed, fusion-defective, variants of myoblast lines show an altered pattern of long-term regulation of phosphodiesterases. Although these results do not shed any new light on the mechanism of regulation, they do suggest that phosphodiesterases may have a considerable role to play in the control of cAMP levels in transformed cells.

4.2 Materials and Methods

4.2.1 Cell Cultures

Two rat skeletal myoblast lines L6 and L8 first obtained by Yaffe (1968) were used. The cells were grown in Dulbecco's modified Eagle's medium containing 10% horse serum and 5 µg/ml gentamycin. Fu-1 variant of L8 line which was unable to fuse was obtained from S. Kaufman, University of Illinois, Urbana. Cells were plated at a density of 6700/cm^2 in 75 cm^2 petri dishes.

4.2.2 Isolation of Transformed Myoblasts

Spontaneously transformed cells were obtained by the method of MacPherson and Montagnier (1964) as described by Kaufman and Parks (1977). A clone of L8 myoblasts, E63, was grown up and the cells were plated over a layer of 0.5% agar made up in growth medium with 10% horse serum. The cells were overlayed with 0.3% agar. Large colonies developing after 10 days were picked from the plates and cloned. Two independent isolates, JRu2 and JRu5, were used in the present investigation.

To obtain virally transformed myoblasts, a population of L6 cells was infected with Schmidt-Ruppin (D) strain of Rous Sarcoma virus and colonies were picked after growth of the cells in soft agar. A clone, L6 (RSV), was used in the present investigation. As judged by immunoprecipitation assays it had $p60^{src}$ antigen (Brugge and Erikson, 1977).

4.2.3 Hybridization Procedure

From a azaguanine-resistant L6 cell line (Az-2), described earlier (Dufresne et al,1976), an α-aminitin, double mutant was selected. This mutant (Az-2, ama^R) was unable to fuse unlike its Az-2 parent. The ama^R marker was used because it is dominant (Pearson, 1981). The double mutant was used in crosses with the transformed lines. Hybridization was accomplished by

using polyethylene glycol as described earlier (Rogers et al,1978), and hybrids were selected in HAT medium (Dufresne et al,1976), containing 3 µg/ml α-aminitin. One hybrid line from each of the two crosses, (Az-2, ama^R) x (JRu2) and (Az-2, ama^R) x (JRu5), was kept for further analysis. The former is referred to as Hy-1 and the latter Hy-2. The hybrids and parental lines were karyotyped according to the procedure outlined earlier (Dufresne et al,1976).

4.2.4 Chromatography and Enzyme Assays

Cells were harvested after the required number of days in culture (generally 4 days) by scraping in Buffer A (25 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM imidazole, and 10 mM 2-mercaptoethanol) containing 0.1 mM EGTA. Cell-free extracts were prepared by homogenizing cells in Buffer A for 15 s at a setting of 3 in a Polytron homogenizer (Brinkman Instruments, Rexdale, Canada). The extract was centrifuged for 15 min at 4°C at 18,000 xg.

Phosphodiesterases were chromatographed on Bio-Gel A-1.5-M in Buffer A containing 0.1 mM EGTA and eluted with the same buffer as described earlier in Chapter 2. Recoveries of phosphodiesterase activity in different experiments generally ranged from 75 to 95%. Phosphodiesterase activity was estimated using the two-step assay of d'Armiento et al (1972), as

described earlier in Chapter 2. If not otherwise specified, cAMP was initially present in assays at a concentration of 0.05 μM. The reaction was linear up to at least 60 min or until 25% of the substrate was hydrolyzed. Blanks were constructed using buffer or boiled enzyme (which gave identical results). Protein was estimated by the method of Lowry et al. (1951). Molecular weights of the enzymes were determined by gel exclusion chromatography on Bio-Gel A-1.5-M columns, as described earlier in Chapter 2.

4.2.5 Other Procedures

See Materials and Methods of Chapter 2 and Chapter 3.

4.3 Results

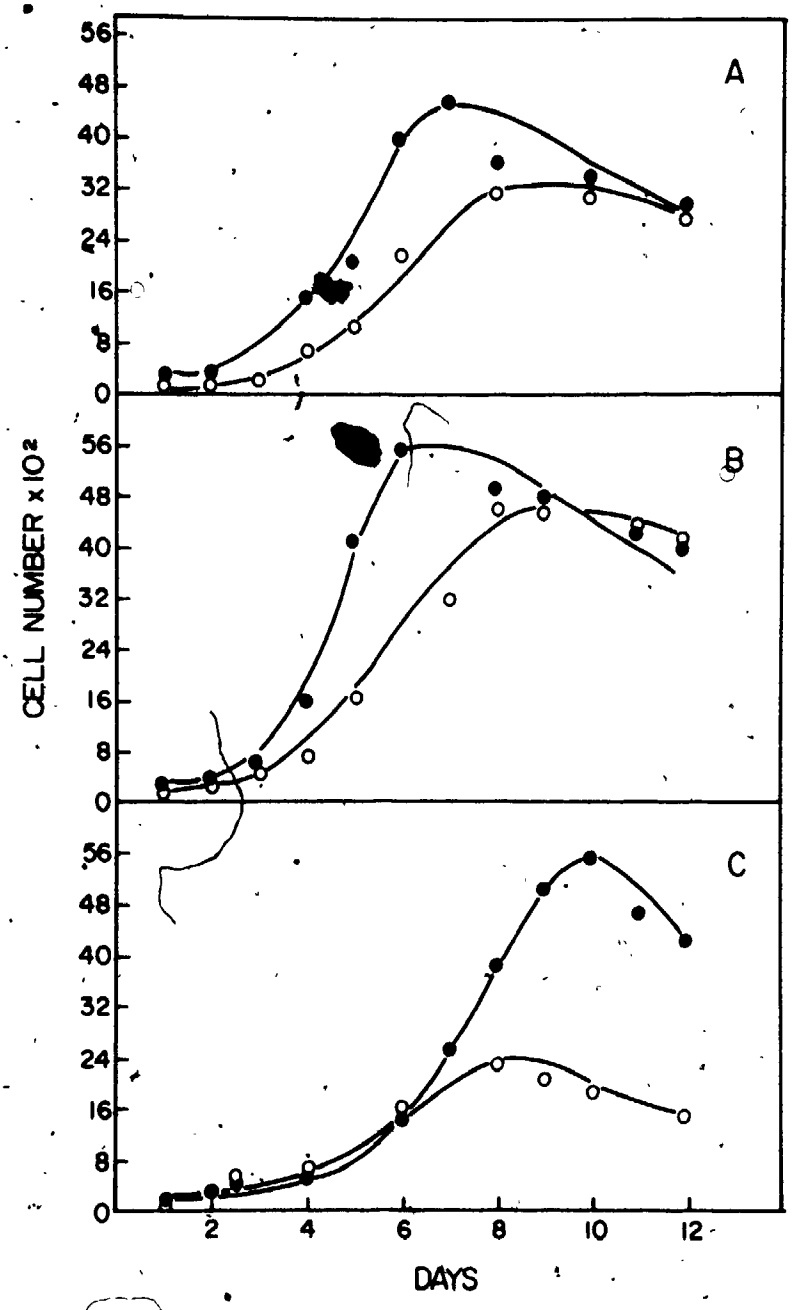
4.3.1 Growth Characteristics of Various Cell Lines

Apart from their growth on soft agar, the various transformed lines can be distinguished from normal myoblasts by their growth behaviour in medium containing 1% horse serum. Normal myoblasts grow slowly in 1% horse serum compared to 10% serum and the final cell densities achieved are also much lower (Fig. 1). The transformed cells (both spontaneous and virally transformed) also grow slowly in 1% serum but the final cell densities achieved in both low and high concentration (10%) of serum are comparable (Fig. 1).

FIGURE 1

GROWTH CURVES OF MYOBLASTS

Myoblasts were plated at a density of 5 x 10^3 cells/well in 12 well tray in MEM medium. Cells were trypsinized from each well on every second day and accounted using a coulter counter. (●) represents the growth curve in 10% horse serum; (○) represents the growth curve using 1% horse serum. Panel A, JRu2; Panel B, JRu5; Panel C, L8.


A
B
C
CELL NUMBER x 10²
DAYS
0
8
16
24
32
40
48
56
2
4
6
8
10
12

The morphology of the transformed myoblasts is also different from the normal cells. The cells transformed by RSV are quite rounded, while the normal myoblasts are spindle shaped. Spontaneously transformed cells are not as altered as the virally transformed ones, but do have a distinguishably different shape compared to normal cells (Fig. 2).

4.3.2 Characteristics of Hybrid Cells

Hy-1 and Hy-2 were found to have chromosome numbers of 109 and 108, respectively. The non-transformed parental line (Az-2, ama^R) had 65 chromosomes while the JRu2 and JRu5 had 53 and 59 chromosomes respectively. Both Hy-1 and Hy-2 behaved like the transformed cells with regard to growth and morphology. They were also found to be capable of growth on soft agar. The transformed trait was thus dominant.

4.3.3 Activation of Phosphodiesterases in Various Cell Lines

To investigate whether activation of phosphodiesterase by cAMP occurred in the various cell lines, the cells were exposed to cAMP and MIX for 1 h or 16 h and the total phosphodiesterase activity was measured in cell free extracts. The results of this investigation are presented in Table 1. All cell lines,

FIGURE 2

MORPHOLOGY OF TRANSFORMED MYOBLASTS

Cells were grown in MEM with 10% horse serum. Two-day-old cells were fixed with methanol for 1 min, air dried and stained with 6% giemsa solution. Magnification x 400. Panel A, L6 cells; Panel B, L6 (RSV); Panel C, JRu5 and Panel D, hy-2.

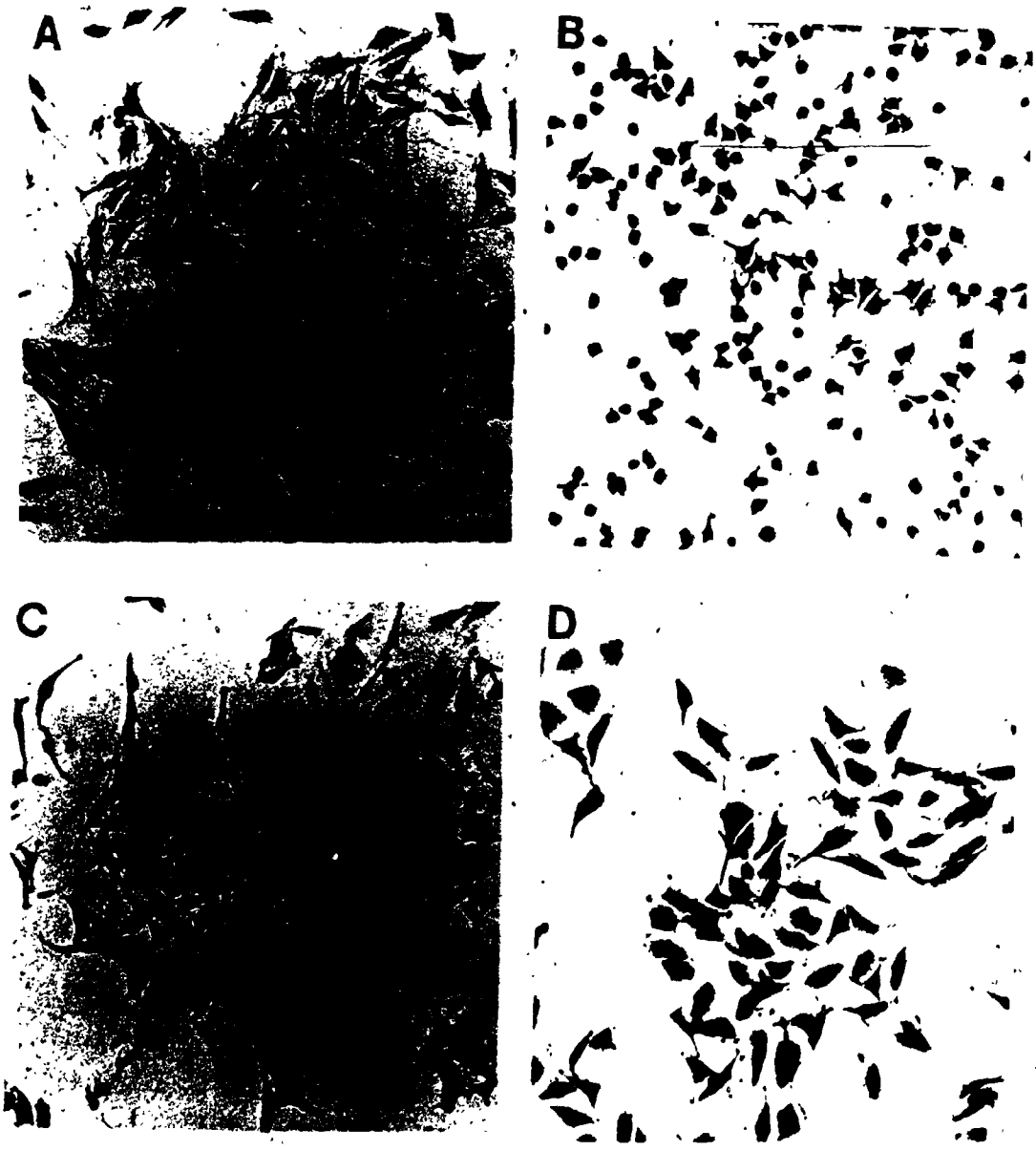

A
B
C
D

TABLE 1

ACTIVATION OF PHOSPHODIESTERASES IN MYOBLASTS

Four-day-old myoblast monolayers were exposed to 0.7 mM Bt_2cAMP and 0.1 mM MIX for either 1 or 16 hours. At the end of the incubation period, phosphodiesterase activity was assayed as described in the text. All experiments were done in duplicate and the values were averaged. Enzyme activity is expressed as percentage of control (no additions).

Cell Line	Percent Activation	
	1 h exposure	16 h exposure
L6	256	220
L8	246	200
Fu-1	227	235
JRu2	184	209
JRu5	202	200
L6(RSV)	266	180
Hy-1	160	163
Hy-2	247	246

including the transformed cells and hybrids, showed activation of 1.6 to 2.7 fold. In this regard the variants resembled the wild-type cells.

To find whether activation of the enzyme in transformed cells was specific to cAMP, as we had shown earlier for L6 (Ball et al,1980), spontaneously transformed JRu5 cells were grown in the presence of various compounds for 16 hr and phosphodiesterase activity was measured. As shown in Table 2, Bt_2cAMP by itself caused about 1.8-fold increase in the activity of the enzyme. MIX had little effect on the activity by itself, but when added together with Bt_2cAMP gave about 2.4 fold increase in enzyme activity. Isoproterenol which causes augmentation of cAMP levels in vivo in muscle cells (Curtis and Zalin 1981), had no effect on activation. This is not peculiar to the transformed myoblasts because we had shown earlier that isoproterenol also does not affect phosphodiesterase in L6 cell lines (Ball et al,1980). More importantly, Bt_2cGMP was incapable of activating the enzyme. The increase in phosphodiesterase activity by cAMP was blocked by cycloheximide and actinomycin D suggesting that the increase in activity probably involves de novo synthesis of the enzyme.

TABLE 2

SPECIFICITY OF INDUCTION IN SPONTANEOUSLY TRANSFORMED MYOBLASTS

The listed chemicals were added to cells (JRu5) 16 hours before measurement of the enzyme activity at the following concentrations: Bt_2cAMP, 1 mM; MIX, 0.1 mM; Bt_2cGMP, 1 mM; Isoproterenol 0.1 mM; sodium butyrate, 1 mM; cycloheximide, 10 µg/ml; actinomycin D 1 µg/ml. Measurements were made in duplicate and the values averaged. Enzyme activity is expressed as percentage of the controls (no addition).

Addition	Percent Induction
Bt_2cAMP	180
MIX	124
Bt_2cAMP, MIX	240
Bt_2cGMP	124
Isoproterenol	98
Sodium butyrate	107
Bt_2cAMP, MIX, cycloheximide	92
Bt_2cAMP, MIX, actinomycin D	117

4.3.4 The Regulation of Phosphodiesterases in Various Cell Lines

We had shown earlier (Ball et al, 1980), that freshly prepared extracts of L6 myoblasts when passed through Bio-Gel A-1.5 M columns, show the presence of two phosphodiesterases, one appearing at a Ve/Vo ratio of 1.3 (PDE II) and another at 1.6 (PDE III). Only the former was activatable about 3-fold by snake venom proteases. When cells were grown for 16 h in the presence of cAMP, PDE II activity decreased, but PDE III activity increased about 2 to 4 fold. An entirely similar situation exists in L8 line (Fig. 3). Just like L6 myoblasts, after exposure to cAMP, PDE II becomes non-activatable by snake venom and PDE III increases considerably. However, when the spontaneously transformed derivative of L8, JRu5, was exposed to Bt_2cAMP and MIX for 16 hr and cell-free extracts were chromatographed, the results were entirely, and surprisingly, different (Fig. 4). PDE II activity increased, but PDE III activity remained almost at the basal level, as in the untreated cells. PDE II which was activatable by proteases in untreated JRu5 cells (Fig. 4) also became refractory to protease activation in cells exposed to cAMP. Both PDE II and PDE III emerged at the expected location from the columns suggesting that gross molecular weight alterations had not occurred in these enzyme forms in JRu5 cells.

FIGURE 3

GEL EXCLUSION CHROMATOGRAPHY

OF NORMAL MYOBLAST EXTRACTS

Four-day-old myoblasts (L8) were treated for 16 hours either with buffer alone (controls) or with 0.7 mM Bt_2cAMP and 0.1 mM MIX. Extract containing 3.5 mg of protein from both the control (A) and treated cultures (B) was applied to Bio-Gel A-1.5 M column (1.6 x 50 cm). The flow rate was 15 ml/h. Fractions of 1.0 ml were collected. They were assayed in the presence (o) and absence (●) of snake venom. Units of activity are picomoles of product formed per min per ml of fraction. Void volume (Vo) of column was determined using blue dextran. Ve is the elution volume of the particular fraction. Ve/Vo was calculated for each fraction of the column.

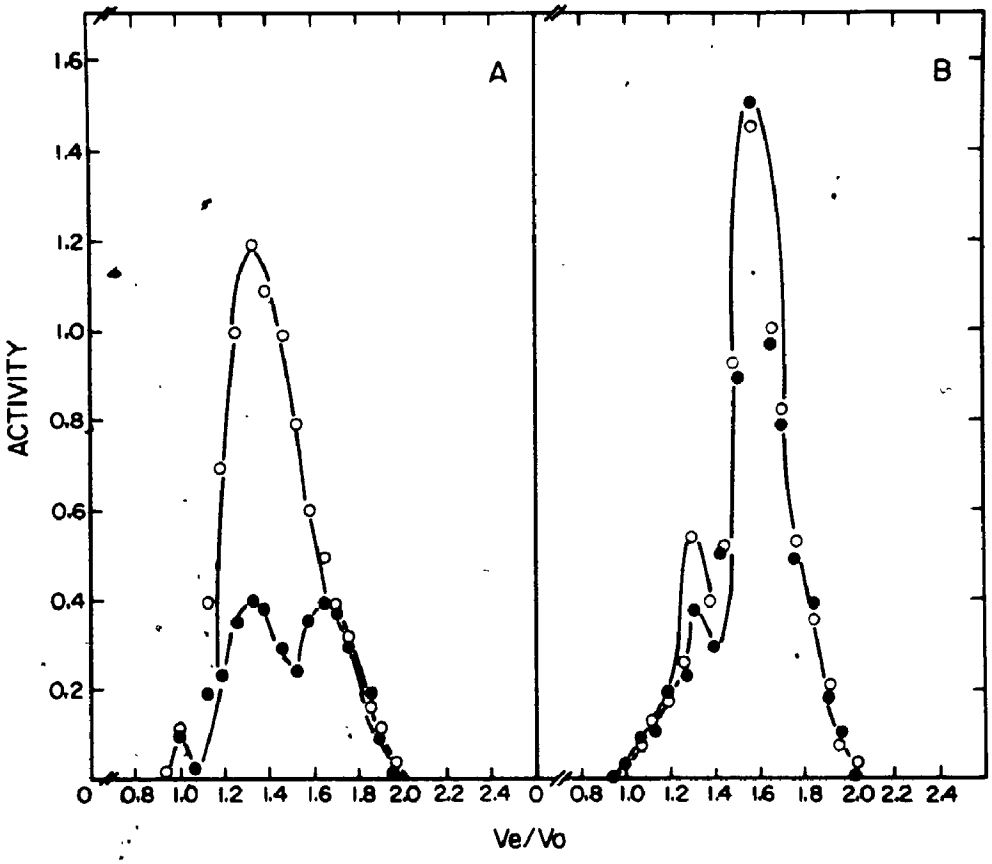
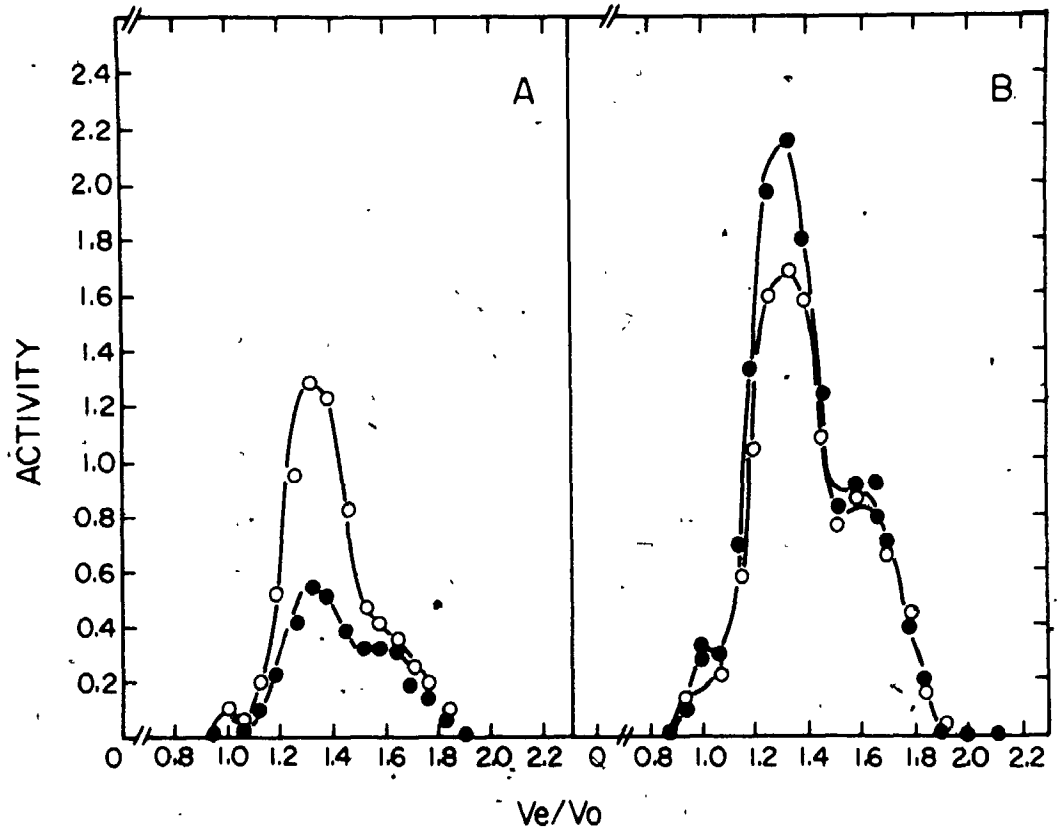

A
B
ACTIVITY
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
Ve/Vo

FIGURE 4

GEL EXCLUSION CHROMATOGRAPHY OF SPONTANEOUSLY TRANSFORMED MYOBLAST EXTRACTS

Four-day-old myoblasts (JRu5) were treated for 16 hours either with buffer alone (controls) or with 0.7 mM Bt2cAMP and 0.1 mM MIX. The extract containing 4.0 mg of protein from both the control (A) and treated cultures (B) was applied to Bio-Gel A-1.5 M column as described in legend to Fig. 3. The rest of the details are exactly as described in legend to Fig. 3.


A
B
ACTIVITY
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
Ve/Vo

The other spontaneously transformed cells, fu-1 and JRu2, also behaved exactly as JRu5, with regard to induction of PDE III by cAMP. The RSV transformed cell line, L6 (RSV), also was similar to the spontaneously transformed lines, i.e., in the presence of Bt_2cAMP, PDE III was not induced (Fig. 5); only PDE II activity increased and became resistant to activation by proteases.

4.3.5 Dominance of the Transformed Phenotype in the Regulation of Phosphodiesterases

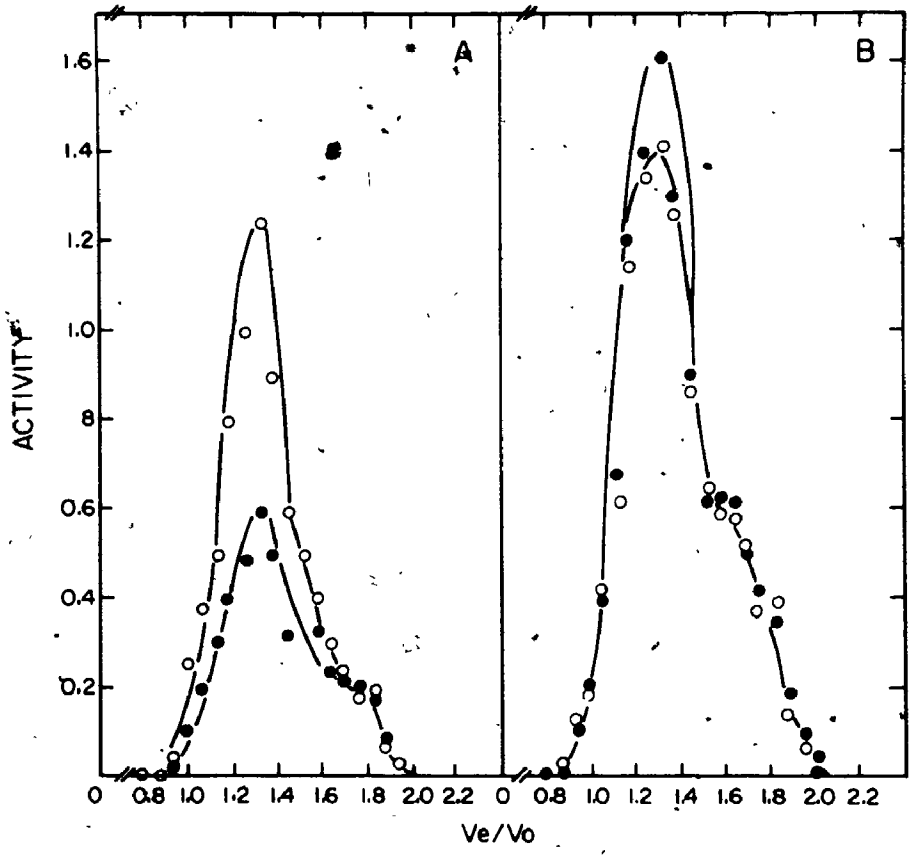
We have proposed elsewhere that in normal myoblasts long term induction of PDE III in response to exposure to cAMP may possibly involve the activity of a protease (Ball et al, 1980). This putative protease was postulated to convert PDE II to PDE III and was considered to be either produced or activated (if pre-existent) in the presence of cAMP. If this hypothesis were correct, the absence of PDE III induction in transformed cells would then simply be due to an absence or malfunctioning of the postulated protease. To test whether a protease is involved in cAMP induction of PDE III, we grew L8 cells in the presence of cAMP and MIX with added leupeptin, a potent inhibitor of protease activity in myoblasts (Kaur and Sanwal, 1981). The extracts were then chromatographed as outlined earlier.

FIGURE 5

GEL EXCLUSION CHROMATOGRAPHY OF

VIRALLY TRANSFORMED MYOBLAST EXTRACTS

Four-day-old myoblasts (L6-RSV) were treated for 16 hours either with buffer alone (controls) or with 0.7 mM Bt$_2$cAMP and 0.1 mM MIX. Extract containing 5 mg of protein from both the control (A) and treated cultures (B) was applied to Bio-Gel A-1.5 M column as described in legend to Fig. 3. Rest of the details are exactly as described in legend to Fig. 3.


A
B
ACTIVITY
1.6
1.4
1.2
1.0
8
0.6
0.4
0.2
0
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
Ve/Vo

In Fig. 6 are presented the results of this investigation. As expected, cells grown without any additions gave two peaks, one (PDE II) eluting at a Ve/Vo ratio of 1.3 and another (PDE III) at 1.6 (Fig. 6A). Cells treated with cAMP produced a major peak of PDE III (Fig. 6B). However, when leupeptin was also included along with cAMP, the formation of PDE III was significantly reduced (Fig. 6C) and PDE II itself remained at an elevated level. Addition of cycloheximide did not show any induction (Fig. 6D). These results indicate possible involvement of a cAMP-activated or induced protease in the long term induction of PDE III. It may be mentioned in the passing that leupeptin by itself did not affect protein synthesis or the total phosphodiesterase activity of the cells.

The question now arose whether the altered regulation of PDE seen in transformed myoblasts is due to an absence or alteration of the putative protease (inhibited by leupeptin). If this were so, somatic hybrids between L8 (wild type) and transformed cells would be expected to behave exactly like the normal parent as far as PDE III induction in the presence of cAMP is concerned. However, when the hybrids Hy-1 and Hy-2 were exposed to cAMP and MIX for 16 hours and the extracts were chromatographed (Fig. 7), results

FIGURE 6

GEL EXCLUSION CHROMATOGRAPHY

OF MYOBLAST EXTRACTS

Four-day-old myoblasts (L6) were treated for 16 hours either with buffer alone; with 0.7 mM Bt_2cAMP and 0.1 mM MIX; with 0.7 mM Bt_2cAMP, 0.1 mM MIX and 20 µg/ml leupeptin or with 0.7 mM Bt_2cAMP, 0.1 mM MIX and 10 µg/ml cycloheximide. Extracts containing 2.5 mg protein from all the cultures were applied to Bio-Gel A-1.5 M column (1.6 x 50 cm). The flow rate was 15 m/h. Fractions of 1.0 ml were collected. Phosphodiesterase activity was assayed in the presence (o) and absence (●) of snake venom. Units of activity are picomoles of product formed per min per ml of fraction. Panel A, control extract; Panel B, cultures treated with Bt_2cAMP and MIX; Panel C, cultures treated with Bt_2cAMP, MIX and leupeptin; Panel D, cultures treated with Bt_2cAMP, MIX and cycloheximide.

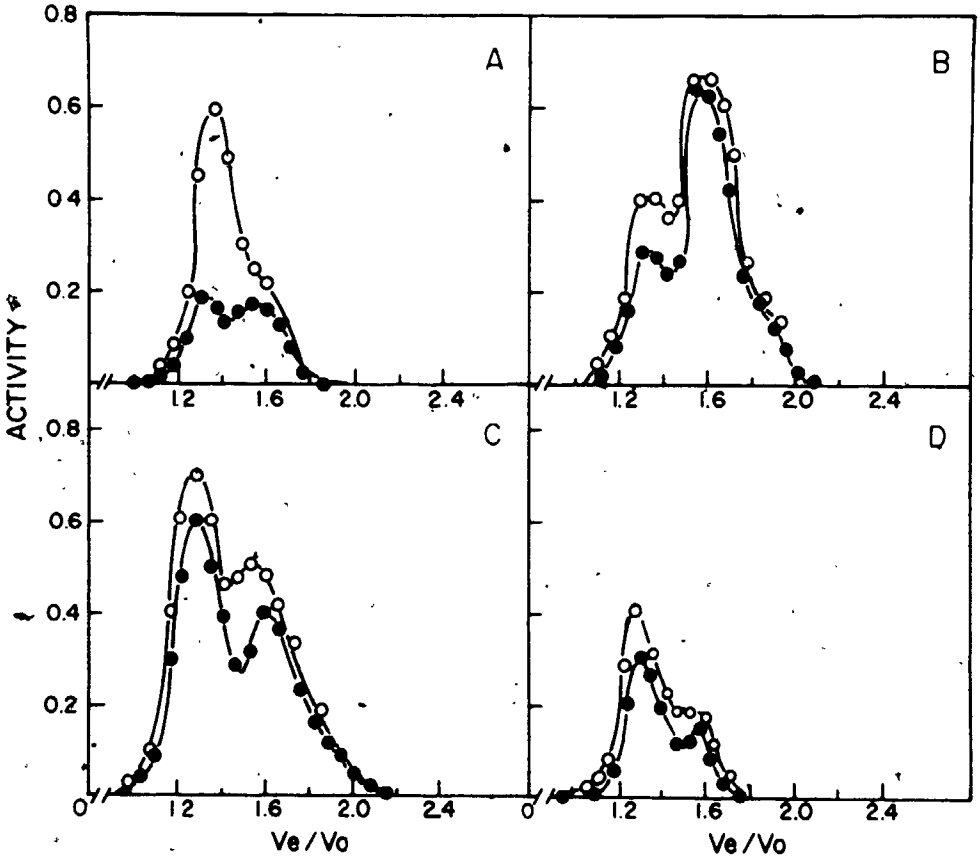
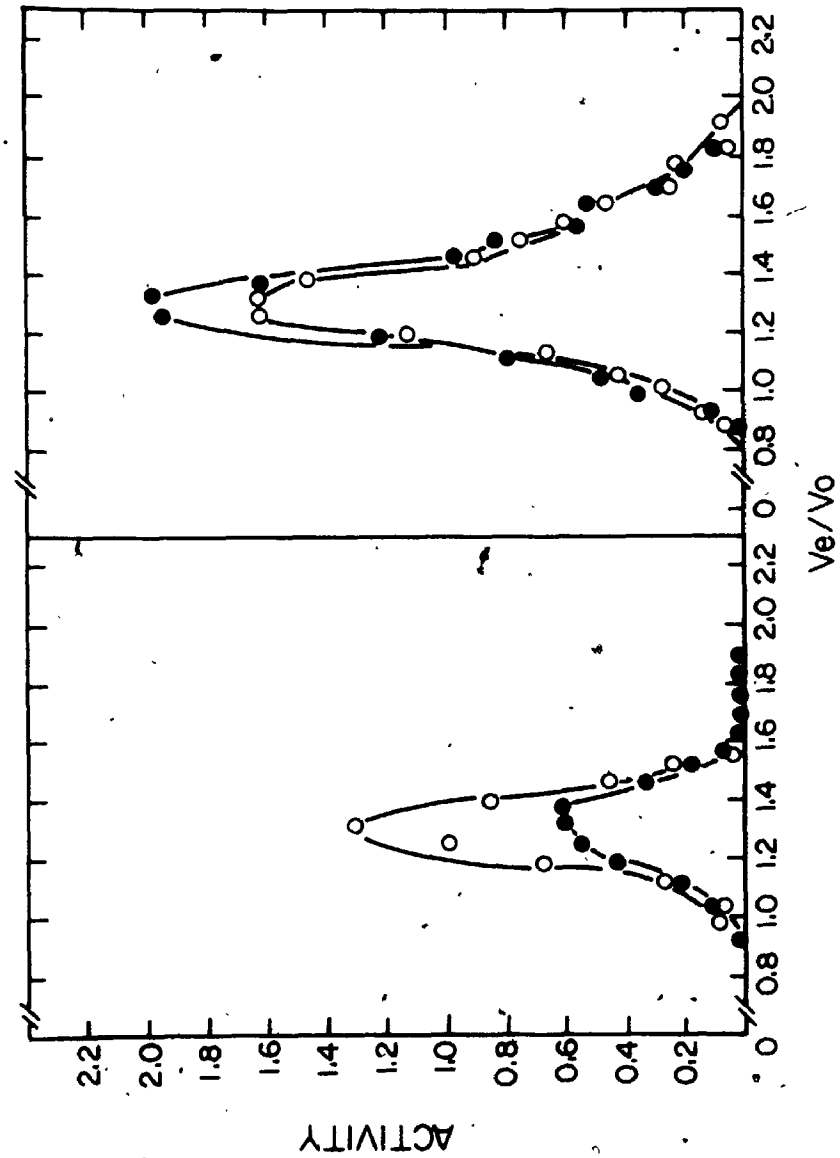

A
B
C
D
ACTIVITY
0.8
0.6
0.4
0.2
0
1.2
1.6
2.0
2.4
Ve / Vo

FIGURE 7

GEL EXCLUSION CHROMATOGRAPHY

OF HYBRID EXTRACTS

Four-day-old Hy-2 cells were treated for 16 hours either with buffer alone (controls) or with 0.7 mM Bt_2cAMP and 0.1 mM MIX. Extract containing 4.5 mg protein from both the control (A) and treated cultures (B) was applied on Bio-Gel A-1.5 M column. Rest of the details are exactly as described in legend to Fig. 3.


ACTIVITY
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
0
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
Ve/Vo

comparable to those with transformed cells were obtained, i.e., PDE III activity did not increase. It is thus clear that altered regulation in transformed cells is not due to a loss of function, but rather due to an 'acquisition' of some new dominant property by these cells.

4.3.6 Nature of PDE II in Transformed Cells

We have shown earlier that in transformed cells, activity of PDE II increases after exposure to cAMP. In addition, the enzyme is not activatable by snake venom proteases, as it is in cells grown in the absence of cAMP. The question, therefore, arose whether the enzyme produced in the presence of cAMP is different than that produced in its absence. To probe into this question we partially purified PDE II from extracts of cells grown in the absence or presence of cAMP by chromatography on Bio Gel A 1.5 M columns as outlined earlier (Chapter 2). An approximately 4-fold purification is obtained by this procedure. The effect of a number of reagents, known to influence PDE II activity (Chapter 2), were tested on the activity of the two preparations. In Table 3 some of the characteristics of these preparations including the kinetic properties are listed. Noteworthy is the fact that sodium thiocyanate activates the control enzyme

TABLE 3

SOME CHARACTERISTICS OF INDUCED PHOSPHODIESTERASE IN TRANSFORMED MYOBLASTS

Transformed myoblasts (JRu5) were treated with 0.7 mM Bt_2cAMP and 0.1 mM MIX for 16 hours. PDE II was partially purified from both these cultures using Bio-Gel A-1.5 M column as described in the text. Phosphodiesterase activities were assayed using 50 µg/ml of protein under various conditions.

Property	Control[a] enzyme	Induced[b] enzyme
Molecular weight[c]	450,000	450,000
Effect of proteases[d]	Activatable (2-3 fold)	Non activatable
Activation by NaSCN[e]	About 4-fold	About 2-fold
K_m[f]	2.5 µM (+sv)[g] 2.0 µM (-sv)[h]	2.5 µM (+sv)[g] 2.5 µM (-sv)[h]
V_{max}[f]	2220 (+sv)[g] 952 (-sv)[h]	2857 (+sv)[g] 3076 (-sv)[h]

[a] PDE II prepared from untreated cultures.

[b] PDE II prepared from cAMP treated cultures.

[c] Determined by gel exclusion chromatography on Bio Gel A-1.5 M. The column was calibrated with the following proteins: Ferritin (Mr = 800,000), lactate dehydrogenase (Mr = 140,000), pyruvate kinase (Mr = 237,000), creatine phosphokinase (Mr = 81,000), hemoglobin (Mr = 65,000).

[d]Enzyme preparation was assayed in the continuous presence of either 250 µg/ml of snake venom or 5 µg/ml of chymotrypsin (results were identical).

[e]Using 200 mM of NaSCN in the assay.

[f]Kinetic studies were done by using cAMP as variable substrate. K_m and V_{max} values were calculated from Lineweaver-Burk plots.

[g]Values obtained when the kinetic studies were done in the presence of snake venom (250 µg/ml).

[h]Values obtained when the kinetic studies were done in the absence of snake venom.

4-fold while only 2-fold activation is obtained for the 'induced' enzyme. The K_m of the enzyme from both sources is similar but there is a 2-fold increase in the V_{max} of the 'induced' enzyme. In addition, the latter enzyme is not activated by snake venom protease, while the 'control' enzyme is activated about 2-fold.

When the control enzyme was treated with proteases other than snake venom proteases, like chymotrypsin and trypsin, as expected, activation occurred. As an example when chymotrypsin was used, maximum activation (3-fold) was achieved with 1 µg of the protease. Concentrations higher than this inhibit the activity of the enzyme (Fig. 8) probably due to proteolytic degradation. 'Induced' PDE, on the other hand was not activated even slightly by the same concentration of chymotrypsin (Fig. 8). Results obtained with trypsin were entirely similar to those obtained with chymotrypsin.

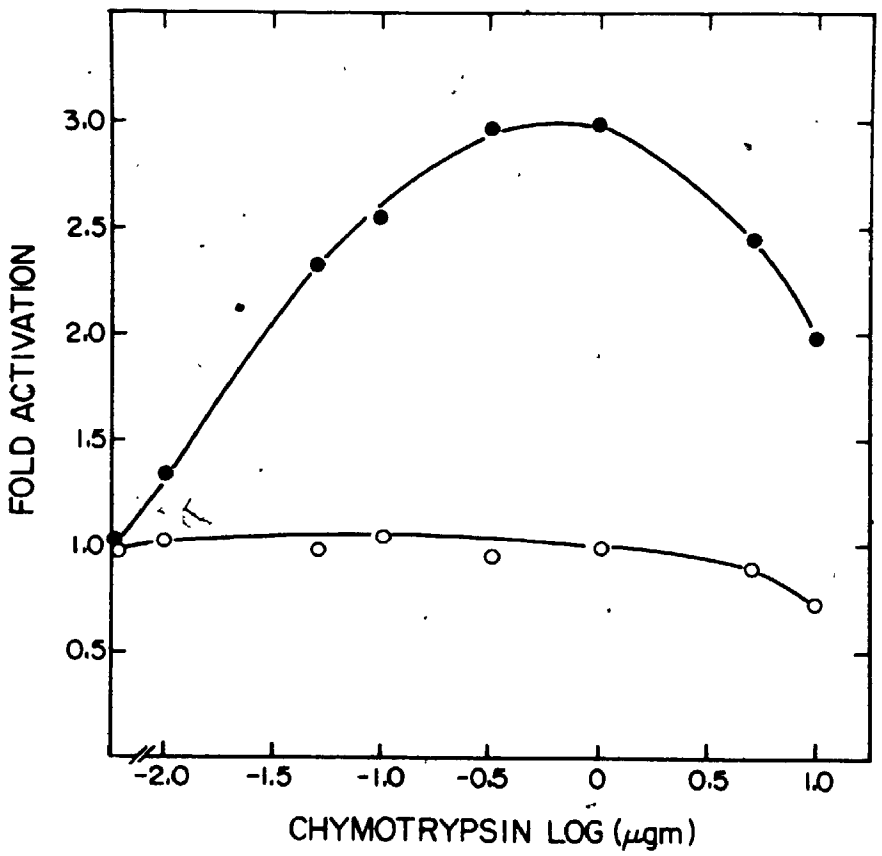
4.3.7 Conversion of Induced PDE II to Other PDE Forms

Earlier (Chapter 2) we had demonstrated that partially purified PDE II or that present in crude extracts of normal myoblasts (L6), could be converted to active PDE III and PDE IV *in vitro* by either a calcium-activated protease in the cell extracts or by

FIGURE 8

ACTIVATION OF PDE II BY CHYMOTRYPSIN

Four-day-old spontaneously transformed myoblasts (JRu5) were treated with either buffer alone or with 0.7 mM Bt_2cAMP and 0.1 mM MIX. PDE II was partially purified from both these cultures as described in the text. PDE II from both treated and control cultures was assayed at 30° in the presence of various concentrations of chymotrypsin and ratio (fold activation) calculated. Protein concentration was kept equal in all samples. (●), activation of PDE II prepared from control culture; (o), activation of PDE II prepared from treated cultures.


FOLD ACTIVATION
3.0
2.5
2.0
1.5
1.0
0.5
-2.0
-1.5
-1.0
-0.5
0
0.5
1.0
CHYMOTRYPSIN LOG (μgm)

several other proteases such as trypsin or chymotrypsin. It appeared plausible that the PDE II produced in transformed cells exposed to cAMP was modified structurally in such a way that it could no longer be degraded to smaller molecular weight form PDE III (and PDE IV). To test if induced PDE II was resistant to proteolytic degradation, the enzyme partially purified by column chromatography was incubated with high concentration (500 µg/ml) of snake venom at 30°C for 15 min and the mixture was again chromatographed on Bio-Gel A-1.5 M columns. All of the enzyme activity appeared now at a Ve/Vo ratio of 1.6, exactly at the place PDE III emerges from the column (Fig. 9). No trace of a PDE II peak remained. Thus, it is clear that the induced PDE II is still capable of being converted to smaller molecular weight form (PDE III) *in vitro* although it is incapable of this conversion *in vivo*.

4.3.8 Reversibility of Modification of PDE II in Transformed Cells

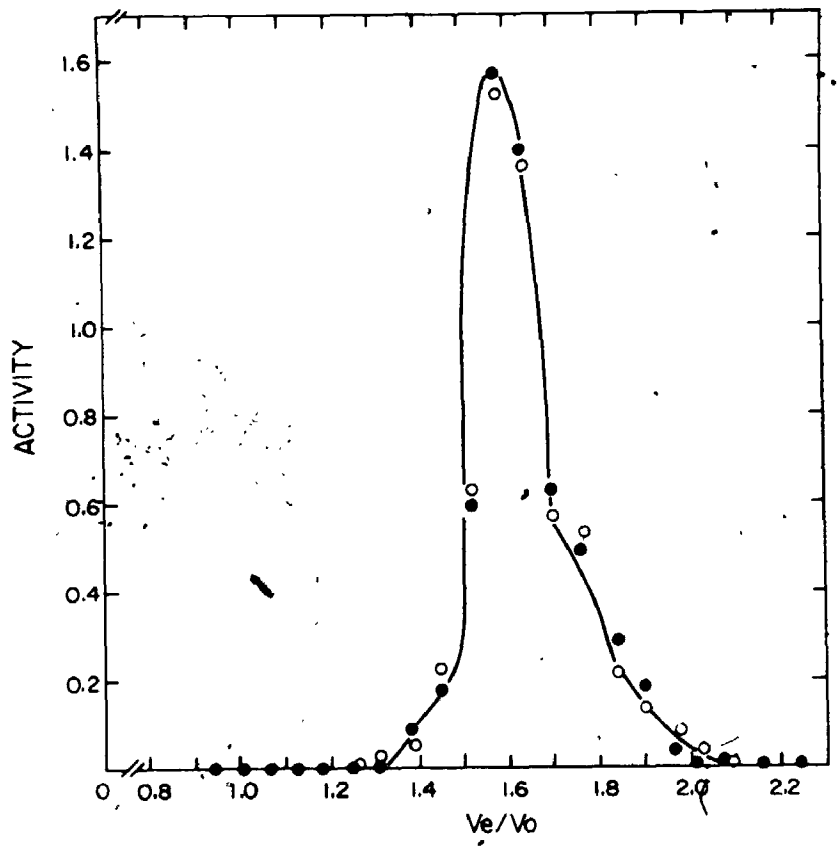
To obtain clues as to the nature of the modification of PDE II in transformed cells grown in the presence of cAMP, it was important to know if the modification *in vivo* was at all reversible. To test this, cells were exposed to Bt_2cAMP, and MIX for 16 h,

FIGURE 9

GEL EXCLUSION CHROMATOGRAPHY OF

PHOSPHODIESTERASE TREATED WITH SNAKE VENOM

PDE II was partially purified from spontaneously transformed myoblasts (JRu5) pretreated with 0.7 mM Bt_2cAMP and 0.1 mM MIX for 16 hours. PDE II was incubated with snake venom (20 µg/ml) at 30°C for 30 min and rechromatographed using Bio-Gel A-1.5 M column (1.6 x 50 cm). Phosphodiesterase activity was assayed both in the presence (●) and absence (o) of snake venom. Other details have been described in the legend to Fig. 3.


ACTIVITY
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
Ve/Vo

and after removal of these compounds, aliquots were incubated in the presence or absence of cycloheximide and actinomycin D. At various time intervals phosphodiesterase activity was assayed in the absence and presence of snake venom and ratio of the activities was calculated. The results are presented in Fig. 10. After the removal of cAMP and MIX, the enzyme regained its protease activatability in less than 2 hrs as the basal activity of the enzyme itself declined. However, when reversibility was tested in the presence of either cycloheximide or actinomycin D, there was no increase in the ratio of activity in the presence and absence of snake venom. At the concentration of the inhibitors used, the cells remained viable as tested by trypan blue exclusion test (Ball et al,1980). Thus, although the modification of phosphodiesterase in transformed cells is reversible, the process probably needs de novo synthesis of proteins.

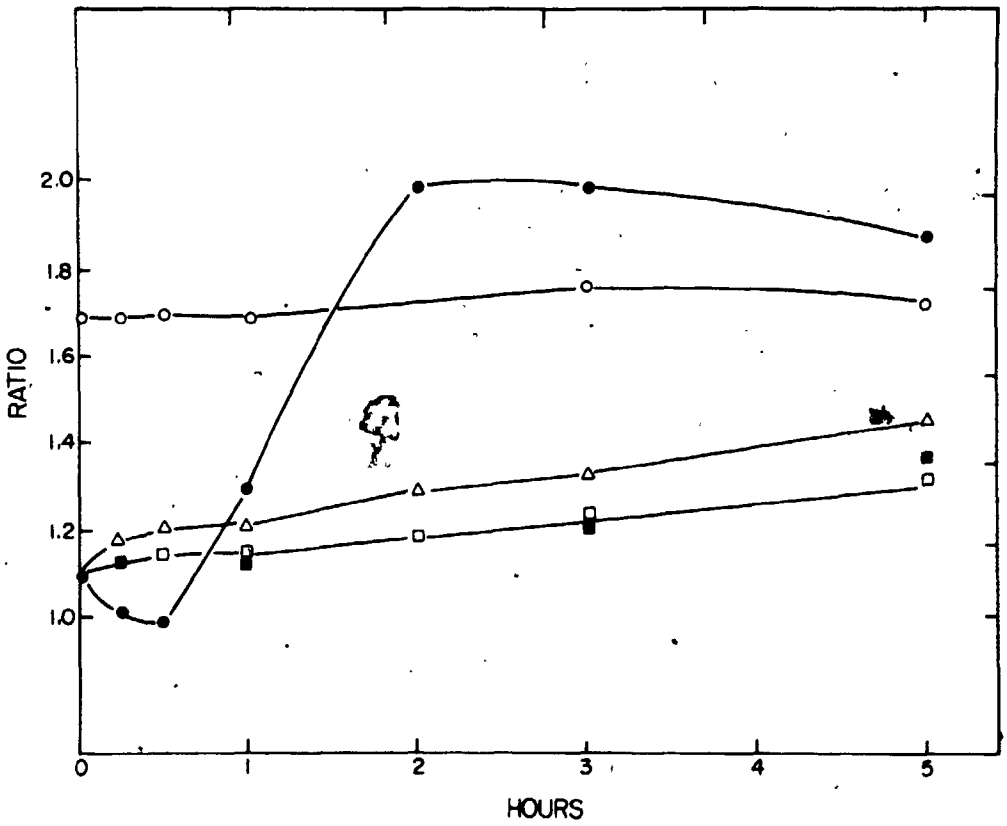
4.4 Discussion

There are two aspects of the work presented above which deserve comment. First is the altered regulation of cAMP phosphodiesterase in both spontaneous and virally transformed myoblasts. Myoblasts are ideal experimental material to study regulation of cAMP degrading enzymes because, unlike other cell types

FIGURE 10

REVERSIBILITY OF PHOSPHODIESTERASE INDUCTION IN TRANSFORMED MYOBLASTS

Cells were pretreated with 0.7 mM Bt_2cAMP and 0.1 mM MIX for 16 hours before the medium was changed at zero hour. Activity in the extracts made at indicated time intervals was assayed in the presence and absence of snake venom. Symbols used are: (o) control; (□) cells in the presence of inducers throughout; (●) cells changed to fresh medium; (Δ) cells changed to fresh medium with 1 µg/ml actinomycin D; (■) cells changed to fresh medium with 10 µg/ml of cycloheximide.


RATIO
2.0
1.8
1.6
1.4
1.2
1.0
0
1
2
3
4
5
HOURS

(Ball et al,1979), they seem to have only one variety of cAMP phosphodiesterase, viz., the low K_m form (Chapter 2). This form, referred to as PDE II, can easily be converted in vitro into a polymerised form called PDE I and a highly degraded, but still active form called PDE IV. These forms seem artifactual and probably have no regulatory significance. However, evidence gathered so far (Ball et al,1980) indicates that PDE II (Mr = 400-450,000) can be converted in vivo to PDE III (Mr = 120,000), when myoblasts are exposed to cAMP for periods longer than 8-10 hours. Since, under controlled proteolysis, purified PDE II can also be converted to PDE III in vitro (Chapter 2), we have proposed the hypothesis that cAMP, by activation or induction of a protease, brings about the conversion of PDE II to PDE III in vivo. What physiological advantages accrue to the cells by such conversion is not known, especially since PDE III has the same K_m for cAMP as PDE II (about 2 μM). However, the important finding made here is that in direct contrast to normal myoblasts PDE III is not induced in transformed cells; the form that accumulates in such cells during long-term exposure to cAMP is what appears to be the phosphorylated form of PDE II (Ball et al,1980), as judged by kinetic and other criteria. The physiological reasons for the necessity of altered

regulation is not entirely clear, however, unless they are concerned with the controls of the basal levels of cAMP *in vivo*. Transformed cells normally have low cAMP levels (Pastan *et al.*, 1975; d'Armeiento *et al.*, 1972).

At a first glance, it is puzzling that both the spontaneous and virally transformed myoblasts show an identical pattern of regulation of PDE. However, it appears from the work of Kaufman & associates (Kaufman and Parks, 1977; Kaufman *et al.*, 1980), that myoblasts isolated by the criteria of growth in soft agar show the presence of endogenous C type particles, and also possess a reverse transcriptase (characteristic of retroviruses) which is Mn^{2+} dependent. It is very likely, therefore, that like RSV and several other retroviruses, the induced endogenous virus of myoblasts has its own *src* gene which determines the structure of a protein kinase specific for tyrosine residues in protein substrates (Hunter *et al.*, 1981). The RSV and spontaneously transformed myoblasts would thus be physiologically equivalent as far as possession of a v-*src* kinase (obviously different in each case), is concerned. This relationship between the two types of transformed myoblasts have a bearing, as described later, on the nature of the mechanism which is postulated to be responsible for the regulation of PDE.

What then is the mechanism of the altered regulation of phosphodiesterases in transformed cells? We had postulated earlier (Ball et al, 1980), that a cAMP-induced or activated protease is involved in the conversion of PDE II to PDE III. We have shown in the present work that leupeptin inhibits formation of PDE III in normal myoblasts which supports the hypothesis suggested earlier. Activation or induction of a protease by cAMP in myoblasts, if it exists at all, would not be an isolated case of its kind. Rodeman & Goldberg (1982) have shown, for instance, that protein degradation in skeletal and cardiac muscle increases 20-40% in the presence of prostaglandin E2, possibly through the mediation of cAMP. Lysosomal proteases have also been shown to be induced by cAMP in cultured mouse myeloid leukemia cells (Honna et al., 1978). Similarly, plasminogen activator increases in the presence of cAMP in hepatoma cells (Barouski-Miller et al, 1982).

Despite the indirect evidence we have gathered, the increase in the activity of a particular protease by cAMP in myoblasts remains to be demonstrated. However, absence of PDE III 'induction' in transformed cells is obviously not due to the absence of the putative protease, as demonstrated by the fact that hybrids between transformed and normal cells behave like

transformed cells, i.e., do not induce PDE III in response to the presence of cAMP. Dominance of the transformed phenotype is a common finding, also in other systems (Jha et al,1980; Howell and Sager,1979).

If the hypothetical protease is present in transformed cells, as judged by dominance relationships in somatic hybrids, it is possible that it may not act on PDE II if it is altered in some way in transformed cells. If it is assumed that PDE II serves as a substrate for the pp60^{src} kinase in RSV transformed myoblasts, or the v-src kinase produced by endogenous virus in spontaneously transformed myoblasts, it may be sufficiently modified (by phosphorylation of a tyrosine residue on PDE II) not to be recognized as a good substrate by the protease which converts PDE II to PDE III. There is evidence, in the case of pp60^{src} that not only the structural proteins (Hunter, 1980) of the transformed cells, but also a metabolic enzyme (Rubsamen et al,1982) is phosphorylated by the viral kinase. Thus precedent exists for modification of enzymes by pp60^{src}. This hypothesis for the mechanism of altered regulation of PDE II in transformed cells, fits with all the experimental facts. It explains the absence of increase of PDE III in the presence of cAMP and the dominance of altered regulation in somatic hybrids. The only seemingly contradictory

observation made in the present work is the easy conversion *in vitro* of the PDE II produced in transformed cells in the presence of cAMP to PDE III by some proteases. However, this can be easily explained by the fact that in partially purified preparations of PDE II there is liable to be an alkaline phosphatase or other phosphatases which remove the phosphate from the tyrosine residue. In this form PDE II would become susceptible to the protease added *in vitro*. Indeed, it has been demonstrated by Swarup *et al* (1981) that alkaline phosphatase can act as an active protein phosphatase for proteins phosphorylated at tyrosine residues.

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CHAPTER 5. SUMMARY

5.1 Multiple Forms of Phosphodiesterases

Evidence presented in this thesis indicates that rat skeletal myoblasts and adult muscle contain a calmodulin independent form of cAMP phosphodiesterase. This enzyme exists in four forms termed PDE I, II, III and IV of which PDE II is the genetically determined form. Rest of the forms viz., PDE I, PDE III and PDE IV are all derived from PDE II. When partially purified PDE II is treated with ammonium sulphate, α-tocopheryl phosphate, or stored at 4°C or chromatographed on a methylisobutyl xanthine-Sepharose column, it is converted into PDE I, thus suggesting that PDE I is an aggregated form of PDE II. Partially purified PDE II and PDE III, when treated with various proteases, yield a form identical with PDE IV, indicating that PDE IV arises from PDE II by proteolytic cleavage. Since PDE II and PDE III both give rise to PDE IV upon proteolysis, it is possible that PDE III also arises from PDE II. All the four forms of phosphodiesterases have low K_m for cAMP ($\sim$ 2 μm), although they all can be distinguished by their molecular size (the approximate molecular weights of PDE I, II, III and IV are 1.5×10^6, 400,000, 120,000 and 60,000 respectively) and change. Furthermore, they all differ

in their sensitivity to activation by proteases and some other compounds. Only PDE II is activated by proteases, chaotropic agents and α-tocopheryl phosphate.

To further investigate the relationship of the various forms at a molecular level we have purified PDE I, PDE III and PDE IV to homogeneity. Due to instability of PDE II, we have not been able to purify it completely. PDE I consists of only one type of subunit with a molecular weight of 90-94,000; PDE III has a single subunit of molecular weight of about 60,000 and PDE IV has two subunits with molecular weights of 28,000 and 30,000. PDE I and PDE IV have aspartate as the NH_2-terminal amino acid.

5.2 Regulation of Phosphodiesterases by cAMP

In rat skeletal myoblasts, cAMP regulates the activity of cAMP phosphodiesterases in two ways. One is the 'short term activation' of the enzyme, a process which does not require de novo synthesis of protein and has been suggested to be a form of 'covalent regulation'. The second mechanism is the long term induction of phosphodiesterase, a process which requires de novo synthesis of protein, and therefore is termed 'genetic regulation'. Both these mechanisms have been described in a schematic form in Fig. 1.

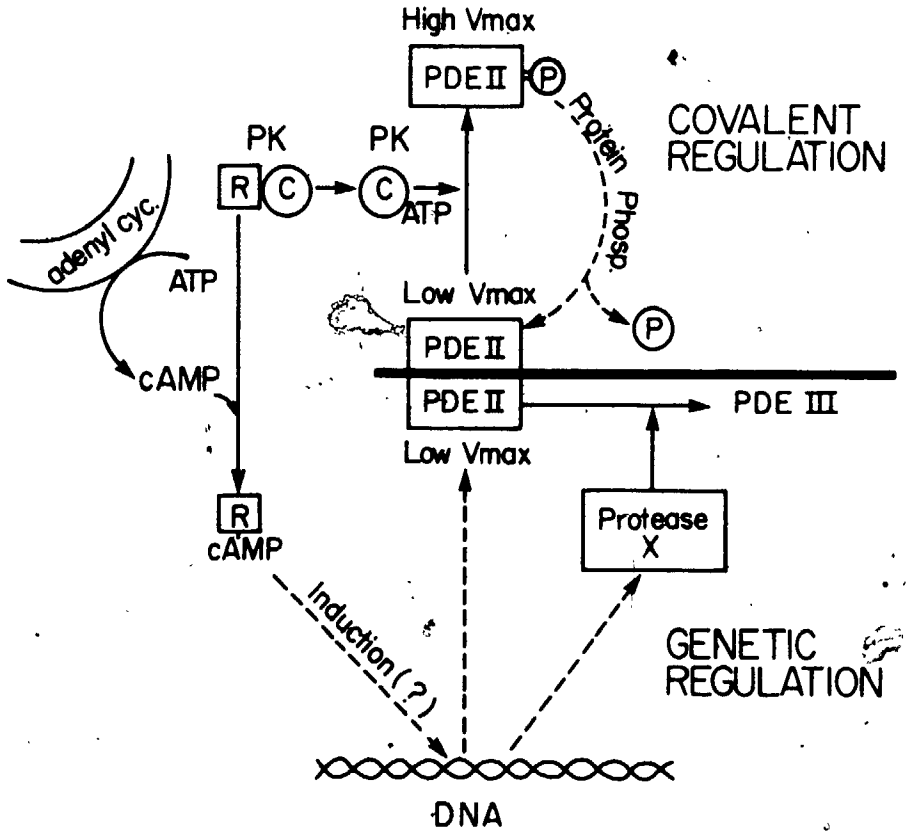
FIGURE 1

PROPOSED MODEL FOR THE

REGULATION OF PHOSPHODIESTERASES

BY cAMP

cAMP produced by the activation of adenylate cyclase (Adenyl cyc.), binds with protein kinase (PK) holoenzyme and dissociates its regulatory subunits (R) and catalytic subunits (C). Catalytic subunit participates in the activation of PDE II (low V_{max}) and produces phosphorylated PDE II (PDE II = P_f high V_{max} form). After cAMP levels are reduced, phosphorylated PDE II is dephosphorylated by phosphoprotein phosphatase (protein phosp.). The process of reversibility requires de novo synthesis of protein. Long term presence of cAMP induces the production of PDE III. A protease X has been proposed to participate in the in vivo conversion of PDE II to PDE III. Dotted lines (----) are genetic mechanisms.


High Vmax
PDE II
P
Protein Phosp
COVALENT REGULATION
PK
PK
R
C
C
ATP
adenyl cyc.
ATP
Low Vmax
PDE II
P
cAMP
PDE II
PDE III
Low Vmax
R
cAMP
Protease X
Induction (?)
GENETIC REGULATION
DNA

Covalent Regulation

We found that, addition of Bt_2cAMP (or compounds which augment the levels of cAMP *in vivo*), cause a 2-fold increase in the basal activity of phosphodiesterase. The process of activation takes less than 30 min, and results in the loss of snake venom activatibility of the enzyme. We have proposed that activation of phosphodiesterase is due to a cAMP-dependent phosphorylation of PDE II (low V_{max} form) to more active PDE II (high V_{max} form). In the absence of cAMP, the latter is converted back to low V_{max} form, probably as a result of the activity of phosphoprotein phosphatase. The major evidences for the involvement of a phosphorylation step in the control of phosphodiesterase activity are following:

(1) Phosphodiesterase can be activated *in vitro* when cell free extracts are treated with cAMP and ATP, under conditions which generally favour phosphorylation of proteins. Such activation does not occur when an inhibitor of protein kinase is also included in the reaction mixture.

(2) *In vitro* activation described above can be also shown using a biologically hydrolyzable analogue

of ATP, ATP-γ-S but not a nonhydrolyzable analogue such as AMP-PNP.

(3) No detectable change is observed in the gross molecular weight of PDE II produced after activation in vitro. This is an expected result as the addition of a few phosphate residues to PDE II would not change its molecular weight significantly.

(4) Active phosphodiesterase produced in the crude extracts can be deactivated and protease activatibility of the enzyme restored, by the addition of acid phosphate, suggesting the involvement of a dephosphorylation step in the deactivation process.

(5) Partially purified PDE II can also be activated in the presence of cAMP, ATP and protein kinase.

(6) A single phosphoprotein is purified, after phosphorylation of (a) the crude extracts, using endogeneous protein kinase or (b) semipurified PDE II using exogeneously added cAMP dependent protein kinase. In all cases, the purified

phosphoprotein behaves exactly like homogenous preparation of PDE I, phosphorylated *in vitro* by protein kinase.

From the above discussion it is clear that majority of criteria for the involvement of protein phosphorylation (Krebs and Beavo, 1979) have been fulfilled for the regulation of PDE and such a step is very probably involved in the short term activation of PDE II. Although we have considerable amount of evidence that PDE II itself is phosphorylated, we have not been able to prove it unequivocally.

Genetic Regulation

cAMP phosphodiesterase activity present in rat skeletal myoblasts is also increased (induced) when the cells are exposed to Bt_2cAMP for periods longer than 12 hours. The enzyme form produced during this process is PDE III as shown by gel filtration experiments. (Ball *et al.*, 1980). Formation of PDE III is inhibited when a protease inhibitor, leupeptin (Kaur and Sanwal, 1981) is also added to the medium along with Bt_2cAMP. It is therefore suggested that a protease activity is involved in the process of long term induction of phosphodiesterase (Fig. 1). Interestingly, both spontaneous and virally transformed cells, seem to lack the mechanism which generates

PDE III when the cells are chronically exposed to Bt_2cAMP. Instead these cells produce, snake venom nonactivatable PDE II. This altered mode of regulation of phosphodiesterase is dominant in hybrids between normal and transformed myoblasts, suggesting that altered regulation of phosphodiesterase is due to an 'acquisition' of some new property by transformed cells. It is suggested that in both spontaneous and Rous-Sarcoma virus transformed myoblasts, the src gene product probably phosphorylates PDE II in such a way that it cannot be converted in vivo to PDE III.

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