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Protein Phosphorylation in Human Peripheral Blood Lymphocytes

SUBCELLULAR DISTRIBUTION AND PARTIAL CHARACTERIZATION OF ADENOSINE 3′:5′-CYCLIC MONOPHOSPHATE-DEPENDENT PROTEIN KINASE

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Cytoplasmic and membrane fractions prepared from human peripheral-blood lymphocytes both contained cyclic AMP-dependent protein kinase activity and endogenous protein kinase substrates. Protein kinase activity in the particulate fractions was not eluted with 0.25M-NaCl, suggesting that it was not derived from non-specifically adsorbed soluble cytoplasmic protein kinase. Nor was the particulate protein kinase activity eluted by treatment with cyclic AMP, suggesting that the catalytic subunit is membrane-bound and arguing against cyclic AMP-induced translocation of particulate activity. Cyclic AMP-dependent protein-phosphorylating activity in the cytoplasmic fraction was highly sensitive to inhibition by Mn^{2+}, and was co-eluted from DEAE-cellulose primarily with type-I rabbit skeletal-muscle kinase. Cyclic AMP-dependent phosphorylating activity in the plasma-membrane fractions was stimulated at low [Mn^{2+}] and inhibited only at high [Mn^{2+}]. When solubilized with Nonidet P-40, plasma-membrane protein kinase was co-eluted from DEAE-cellulose with type-II rabbit muscle kinase. These differences, together with the strong association of the particulate kinases with the particulate fraction, suggest the possibility of compartmentalized protein phosphorylation in intact lymphocytes.

Many agents that produce mitogenic activation of lymphocytes, including antigens and plant lectins, appear to express their activity at the plasma membrane, leading to speculation on the mechanism of propagation of the mitogenic signal within the cell. Alterations in the metabolism of cyclic nucleotides have been implicated in the intracellular modulation of lymphocyte activation (Smith *et al.*, 1971; Hadden *et al.*, 1972; Parker *et al.*, 1974; Wedner & Parker, 1976). Previous studies from this laboratory (Wedner & Parker, 1975) have demonstrated an early increase in the phosphorylation of non-nuclear proteins in intact human lymphocytes treated with mitogenic lectins. The lectin-induced increase in phosphorylation was partially mimicked by treatment with N^6-monobutyryl cyclic AMP. Subsequently, Byus *et al.* (1977) reported that a cyclic AMP-dependent protein kinase was activated 4h after stimulation of lymphocytes by mitogenic lectins.

Murray and co-workers recovered at least two protein kinase activities from lymphocyte homogenates (Murray *et al.*, 1972; Kemp *et al.*, 1975). These activities were resolved by ion-exchange chromatography and differed in responsiveness to cyclic AMP and univalent cations. These observations have been confirmed and additional protein kinases have been demonstrated in lymphocyte cytosol (Barbier & Colbert, 1975; Piras *et al.*, 1977).

Although it is apparent that soluble fractions of lymphocytes contain multiple cyclic AMP-dependent and -independent protein kinases, no studies of particulate lymphocyte protein kinases have been reported. Since lectin-sensitive adenylate cyclase in lymphocytes is detected only in the plasma-membrane fraction (Snider & Parker, 1977), and immunofluorescent detection of cyclic AMP indicates that mitogen-induced alterations in cellular cyclic AMP content are most pronounced in this location (Parker *et al.*, 1974), attempts to demonstrate protein kinase activities in lymphocyte plasma membranes are of particular interest. The present study investigates the subcellular distribution and regulation of protein-phosphorylating activities in human peripheral-blood lymphocytes and provides an initial characterization of the particulate and soluble protein kinases.

Materials and Methods

Materials

Reagents and their sources were as follows: [γ-^{32}P]ATP (>100Ci/mmol, ICN Pharmaceuticals,

Irvine, CA, U.S.A.), DEAE-cellulose DE-52 (Whatman, Clifton, NJ, U.S.A.), theophylline (Schwarz/Mann, Orangeburg, NY, U.S.A.), protamine, casein, histones, ATP, cyclic AMP, NADH, AMP, cyclic GMP, bovine serum albumin, heat-stable protein kinase inhibitor from rabbit skeletal muscle and cytochrome *c* (Sigma, St. Louis, MO, U.S.A.), and Nonidet P-40 (Particle Data Laboratories, Elmhurst, IL, U.S.A.).

Cell purification

Human peripheral-blood lymphocytes were prepared from heparinized blood by dextran sedimentation and isopycnic centrifugation on Ficoll/Hypaque density gradients as previously described (Eisen *et al.*, 1972). This preparation consisted of 93–98 lymphocytes per 100 nucleated cells and contained fewer than 30 platelets and five erythrocytes per 100 lymphocytes. In selected experiments, lymphocytes were further purified by passage over a nylon-wool column yielding a preparation of 99–100% lymphocytes (Eisen *et al.*, 1972). Some 5×10^8–8×10^8 lymphocytes could be obtained from a single donor by these techniques.

Preparation of subcellular fractions

Subcellular fractions were prepared by Dounce homogenization and discontinuous sucrose-density-gradient centrifugation by a modification of the method of Snider & Parker (1977). Preliminary experiments using a variety of buffers with pH values ranging from 6.2 to 7.5 indicated that optimal phosphorylation was obtained with 50mM-sodium acetate, pH7.3. The pH of the phosphorylation reaction mixture was shown not to change over the course of incubation. Purified cell preparations were suspended at 2×10^8 cells/ml in Mg/acetate buffer (2mM-magnesium acetate/50mM-sodium acetate, pH 7.3) and were allowed to swell for 30min at 4°C. Dounce homogenization, depletion of nuclei and subsequent fractionation were carried out at 4°C as previously described (Snider & Parker, 1977). Five subcellular fractions were recovered: 16000*g* supernatant; 20% (w/v) sucrose fraction; 20–30% (w/v) sucrose-gradient interface; 30–40% (w/v) sucrose-gradient interface; 40–55% (w/v) sucrose-gradient interface.

Measurement of protein and enzyme activities

5′-Nucleotidase, NADH–ferricyanide oxidoreductase, cytochrome *c* oxidase and lactate dehydrogenase activities were determined as previously described (Snider & Parker, 1977). β-Glucuronidase activity was determined after 18h of incubation at 37°C with phenolphthalein glucuronide as substrate (Brittinger *et al.*, 1968). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Measurements of phosphorylation

Protein-phosphorylating activity was measured as the overall incorporation of ^{32}P from [γ-^{32}P]ATP into trichloroacetic acid-precipitable material. Conditions for the phosphorylation reaction were selected on the basis of preliminary experiments, in which the time of incubation and the concentrations of ATP, cyclic nucleotides and bivalent cations were varied. The standard assay conditions reflect those that yield maximal cyclic AMP responsiveness of phosphorylation. Phosphorylation was routinely carried out at 25°C in a final volume of 0.05ml containing 1mM-magnesium acetate, 3mM-theophylline, 50mM-sodium acetate, pH7.3, and 3–50μg of lymphocyte protein as indicated. Cyclic AMP (usually 2μM) and exogenous substrates (50μg) were present as indicated. Subcellular fractions were preincubated with the reaction constituents for 30s, then the phosphorylation reaction was initiated by the addition of 20μM-[γ-^{32}P]ATP (60–100μCi/ml). After an additional 30s of incubation, the reaction was terminated by the addition of 5μl of bovine serum albumin (100mg/ml) followed immediately by 3ml of cold 6% (w/v) trichloroacetic acid. Samples were maintained at 4°C for 30min, then centrifuged at 3500*g* for 20min. The supernatants were discarded and the pellets were washed three times by rapidly dissolving in 0.2ml of cold 0.2M-NaOH followed immediately by reprecipitation with 3ml of cold 6% trichloroacetic acid and centrifugation as above. Protein-bound ^{32}P was measured in a Searle Mark III liquid-scintillation counter.

Solubilization of protein kinase activity and ion-exchange chromatography

Subcellular fractions were obtained from the lymphocytes derived from 500ml of fresh blood. The 16000*g* supernatant fraction was dialysed for 1h at 4°C in a rapid dialyser (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.) against 200 times its volume of 10mM-potassium phosphate/1mM-EDTA, pH6.8 (potassium phosphate buffer). The dialysed sample was mixed with an equal volume of 1% (w/v) Nonidet P-40 in potassium phosphate buffer yielding a final Nonidet P-40 concentration of 0.5% (w/v). The plasma-membrane and microsomal/mitochondrial fractions were sedimented by centrifugation at 100000*g* for 60min at 4°C. The membrane pellets were each resuspended in 1.5ml of potassium phosphate buffer containing 0.5% Nonidet P-40 and were homogenized by five strokes in a tight-fitting Dounce homogenizer. The Nonidet P-40-treated preparations were separated into soluble and insoluble fractions by centrifugation at 100000*g* for 60min. In order to assay insoluble protein kinase activity, the 100000*g* pellets were again resuspended by homogenization in potassium phosphate buffer containing 0.5% Nonidet P-40.

The soluble Nonidet P-40 extracts were diluted to 0.25% Nonidet P-40 with an equal volume of potassium phosphate buffer and applied to small columns (1cm×2cm) of DEAE-cellulose (Whatman DE-52, equilibrated in potassium phosphate buffer containing 0.25% Nonidet P-40). The columns were washed with 4ml of the same buffer, then eluted with a 20ml linear gradient of 0–0.3M-NaCl in potassium phosphate buffer containing 0.25% Nonidet P-40. Fractions of volume 0.75ml were collected from the time of sample application.

Acid hydrolysis

Incorporation of ^{32}P into phosphoserine and phosphothreonine was measured by limited acid hydrolysis followed by high-voltage paper electrophoresis by the method of Allerton & Perlmann (1965).

Statistical significance was evaluated by Student's *t* test.

A note on terminology

Most of these experiments were performed with unpurified enzyme preparations. As such, the degree of phosphorylation observed reflects a dynamic equilibrium between the effects of protein kinases and phosphoprotein phosphatases. The use of the term 'phosphorylating activity' is intended to imply this equilibrium activity.

Results

Subcellular fractionation

Subcellular fractions, obtained by Dounce homogenization and discontinuous sucrose-density-gradient centrifugation of purified human peripheral blood lymphocytes, were characterized by their content of marker-enzyme activities (Table 1). The 16000*g* supernatant fraction contained 65–80% of the non-nuclear protein recoverable from the crude homogenate. This fraction was depleted of plasma-membrane (5′-nucleotidase; Song & Bodansky, 1967), microsomal (NADH–ferricyanide oxidoreductase; Landon & Norris, 1963), mitochondrial (cytochrome *c* oxidase; Beaufay *et al.*, 1959) and

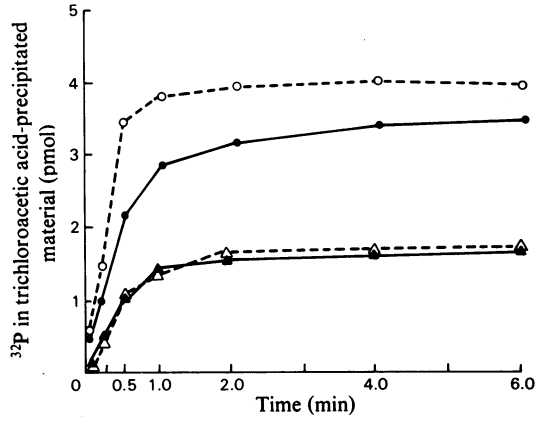

Fig. 1. *Time course of endogenous phosphorylation*
Subcellular fractions were incubated at 25°C with [γ-^{32}P]ATP in the absence of exogenous phosphate acceptor for the indicated times as described in the Materials and Methods section. ○, ●, 16000*g* supernatant (30μg of protein); △ ▲, 30–40% interface (10μg of protein). Phosphorylation was performed in the absence (closed symbols) or presence (open symbols) of 2μM-cyclic AMP. Values reported are means of triplicate determinations. Similar results were obtained in two other experiments.

Table 1. *Marker-enzyme distribution in lymphocyte subcellular fractions*
Subcellular fractions of purified human peripheral blood lymphocytes were prepared by isopycnic centrifugation in discontinuous sucrose gradients as described in the Materials and Methods sections. Marker-enzyme activities are expressed as specific activity relative to that in the crude homogenate. Determinations were performed in duplicate and reported values represent the means of five or more preparations.

Fraction	Total protein (μg)	5′-Nucleotidase	NADH–ferricyanide oxidoreductase	Cyto-chrome oxidase	Lactate dehydrogenase	β-Glucuronidase
Crude homogenate	14300	1	1	1	1	1
16000*g* supernatant	7700	0.12	0.30	0	1.04	0.60
20% sucrose	580	0.53	0.79	0	0.43	1.01
20–30% interface	145	3.86	0.65	0	0.35	1.56
30–40% interface	245	17.00	0.78	0	0.35	2.29
40–55% interface	1360	2.12	7.37	3.42	0.10	3.01

lysosomal (β-glucuronidase; de Duve *et al.*, 1955) marker enzyme activities. Because of its relative enrichmeent in cytosol marker-enzyme activity (lactate dehydrogenase; Keck & Choules, 1962) and failure to sediment at 16000*g*, this fraction is designated a soluble cytoplasmic fraction. The 20% sucrose fraction contains 5–6% of the recoverable protein and is depleted of all marker-enzyme activities except β-glucuronidase. It does not correspond to an identifiable subcellular organelle. The 20–30% and 30–40% sucrose-gradient-interface fractions contain 1.4 and 2.4% respectively of the recoverable protein. They are enriched 4- and 17-fold respectively in plasma-membrane-marker-enzyme activity, with substantially less enrichment in lysosomal-marker-enzyme activity. The 40–55% sucrose-gradient-interface fraction comprises 12–15% of the recoverable protein and is enriched primarily in microsomal- and mitochondrial-marker-enzyme activities. Approx. 10–20% of the protein from the crude homogenate is lost in a pellet at the bottom of the sucrose gradient. The particulate fractions are variably contaminated with lysosomal-marker-enzyme activity.

Endogenous phosphorylation

All of the subcellular fractions displayed self-phosphorylation in the absence of exogenous substrates, indicating the presence of both protein kinase and protein kinase substrates. Examination of the time course of endogenous phosphorylation (Fig. 1) indicated that, under the assay conditions used, maximal phosphorylation was achieved within 1 min in both the cytoplasmic and plasma-membrane fractions. Where stimulation of phosphorylation by added cyclic AMP was observed, it was maximal at 30s. Therefore for subsequent experiments a reaction time of 30s was routinely used. Initial Mg^{2+} dose–response curves indicated that maximal cyclic AMP-induced augmentation of phosphorylation was observed at added 1mM-Mg^{2+} (results not shown). This concentration of Mg^{2+} was routinely used in subsequent experiments.

^{32}P incorporation into endogenous trichloroacetic acid-precipitable material reflects the amount of both protein kinase substrate and protein-phosphorylating activity. For this reason, measurements of endogenous phosphorylation may not accurately reflect the

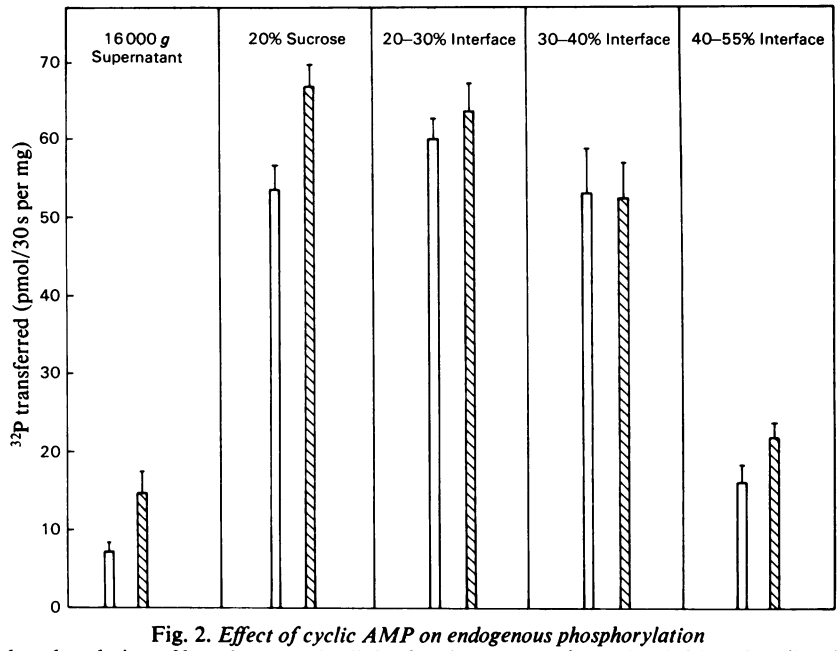

Fig. 2. *Effect of cyclic AMP on endogenous phosphorylation*

Endogenous phosphorylation of lymphocyte subcellular fractions was performed at 25°C as described in the Materials and Methods section. Phosphorylation reaction mixtures contained 23–35μg of cytoplasmic protein, 17–22μg of protein from the 20% sucrose fraction, 5–8μg of protein from the 20–30% interface, 11–15μg of protein from the 30–40% interface or 19–26μg of protein from the 40–55% interface. Data are expressed as pmol of ^{32}P transferred from [γ-^{32}P]ATP to trichloroacetic acid-precipitable material/mg of lymphocyte protein in 30s. Endogenous phosphorylation was measured in the absence (□) or presence (■) of 2μM-cyclic AMP. All determinations were performed in triplicate and the values reported are means ± 2 S.E.M. for six experiments.

quantity of protein kinase activity in a fraction. Nonetheless, they are useful in studying differences between subcellular fractions in terms of the sensitivity of phosphorylation to modulation by pharmacological agents.

Cyclic nucleotide effects on endogenous phosphorylation in the various fractions are shown in Fig. 2. Cyclic AMP and cyclic GMP were tested over a 0.01–10 μM range. Phosphorylation in the 16000*g* supernatant was augmented 80–120% by the addition of 2 μM-cyclic AMP ($P<0.01$). The response in the 20% sucrose fraction was also stimulated by added cyclic AMP, but to a lesser degree (25–40%; $P<0.02$). Phosphorylation in the plasma-membrane fractions appeared to be unresponsive to added cyclic AMP, although, as shown below and in the following paper (Chaplin *et al.*, 1979), several exogenous substrates and some individual endogenous protein substrates did show increased phosphorylation in the presence of added cyclic AMP. The response in the microsomal/mitochondrial fraction was modestly, but significantly, stimulated by added cyclic AMP (10–20%; $P<0.05$). Fractions sensitive to added cyclic AMP all showed half-maximal stimulation of phosphorylation at 0.1–0.5 μM-cyclic AMP (results not shown). Cyclic GMP produced no detectable effects on endogenous phosphorylation.

Partial acid hydrolysis of ^{32}P-labelled samples followed by high-voltage paper electrophoresis revealed that more than 70% of the trichloroacetic acid-precipitable ^{32}P radioactivity in each of the fractions was present as phosphoserine or phosphothreonine. More than 95% of the ^{32}P was rendered trichloroacetic acid-soluble by incubation of the samples in 1 M-NaOH for 2h at 56°C, whereas less than 5% was hydrolysed by treatment with 1 M-HCl under identical conditions. These data indicate that most of the macromolecule-associated ^{32}P measured in these experiments is present as protein-bound phosphate monoesters.

The presence of exogenous phosphate-acceptor proteins altered the cyclic AMP responsiveness of certain subcellular fractions (Fig. 3). Protamine, casein and histones H1 and H2B all served as phosphate acceptors. Phosphorylation of casein was cyclic AMP-responsive in the 16000*g* supernatant fraction ($P<0.02$), weakly but significantly ($P<0.05$) responsive in the 20% sucrose fraction, and unresponsive in the particulate fractions. Phosphorylation of protamine was not affected by the addition of cyclic

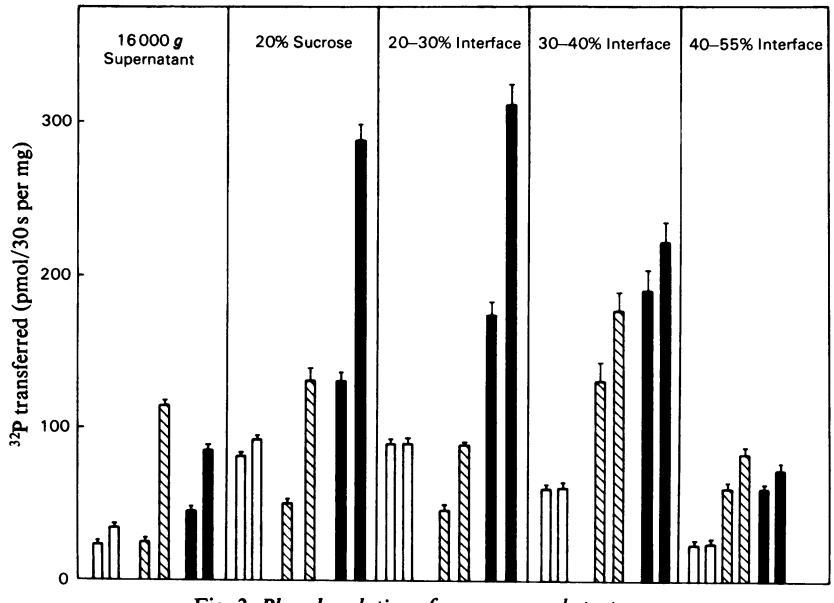

Fig. 3. *Phosphorylation of exogenous substrates*

Subcellular fractions in the quantities listed in Fig. 2 were incubated for 30s at 25°C with exogenous phosphate acceptors (1 mg/ml) and [γ-^{32}P]ATP as described in the Materials and Methods section. Exogenous phosphate acceptors were partially hydrolysed casein (□), histone H1 (▨) and histone H2B (■). Cyclic AMP (2 μM) was present for the second bar in each case. Data are expressed as pmol of ^{32}P transferred from [γ-^{32}P]ATP to trichloroacetic acid-precipitable material/mg of lymphocyte protein in 30s. All determinations were performed in triplicate. Values reported are the means ± 2 S.E.M. for five experiments.

AMP in any of the subcellular fractions. In all subcellular fractions, phosphorylation of histone H1 was stimulated by cyclic AMP ($P<0.01$), as was phosphorylation of the histone H2B (for the 16000*g* supernatant, 20% sucrose and 20–30% interface, $P<0.01$; for the 30–40% and 40–55% interfaces, $P<0.05$). Cyclic GMP (0.01–10 μM) had no detectable effect on the phosphorylation of any of the exogenous substrates.

When 1 mg of exogenous protein kinase substrate/ml was present in the assay, ^{32}P incorporation into trichloroacetic acid-precipitable material reflected primarily the phosphorylating activity of a fraction, not the endogenous substrate content. When calculated on the basis of phosphorylation of histone H2B, more than 70% of the total recoverable phosphorylating activity (assayed in the presence of cyclic AMP) was found in the 16000*g* supernatant. The 20% sucrose, plasma-membrane and microsomal/mitochondrial fractions contained 15, 4–5 and 7–10% of the phosphorylating activity respectively.

Effect of Mn^{2+} on phosphorylation

In the presence of 1 mM-Mg^{2+}, Mn^{2+} altered the phosphorylation of type-IIA mixed histones (Fig. 4). In the absence of added cyclic AMP, Mn^{2+} had no effect on phosphorylation in the 16000*g* supernatant and a very mildly inhibitory effect in the 20% sucrose fraction. In contrast, phosphorylation in the absence of cyclic AMP was stimulated by Mn^{2+}, modestly in the 30–40% interface and markedly in the 40–55% interface fractions. Cyclic AMP-dependent phosphorylation in the cytoplasmic and microsomal/mitochondrial fractions was strongly inhibited by Mn^{2+} (50% at 0.1 mM-Mn^{2+}). In the plasma-membrane fraction, cyclic AMP-dependent phosphorylation was stimulated at 0.1 mM-Mn^{2+}, with inhibition being seen only at higher concentrations. Comparable results were obtained when the effect on Mn^{2+} on endogenous phosphorylation was examined (results not shown).

Nature of the particulate protein kinase activity

Under conditions of low ionic strength, catalytic subunits of protein kinase have been reported to adsorb non-specifically to particulate fractions of rabbit heart (Corbin *et al.*, 1977). Experiments were performed to evaluate the possibility of such a non-specific association in lymphocyte homogenates. Crude homogenates were adjusted to 0.25 M-NaCl, after which subcellular fractions were isolated in the usual way. When expressed as specific activity, a slight but statistically insignificant increase in phosphorylating activity was observed in the 16000*g* supernatant fraction of homogenates treated with 0.25 M-NaCl (Fig. 5). Salt treatment resulted in a 40–50% decrease in phosphorylating activity in the 20% sucrose fraction, and a 20–25% decrease in the microsomal/mitochondrial fraction. On the other hand, in the two plasma-membrane fractions, the

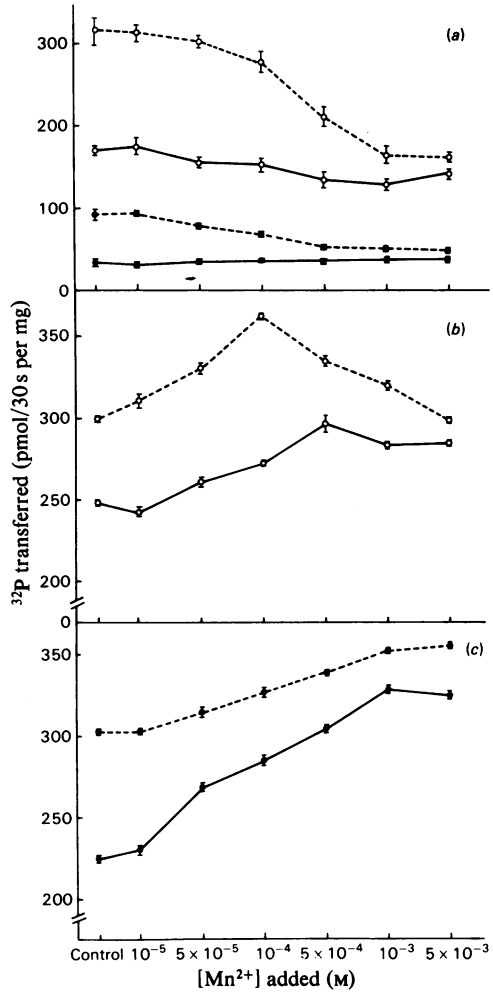

Fig. 4. *Effect of Mn^{2+} on phosphorylation of mixed histones by subcellular fractions*

Phosphorylation was carried out at 25°C in the presence of 1 mM-Mg^{2+} and various concentrations of $MnCl_2$ as indicated. Broken lines indicate phosphorylation in the presence of 2 μM-cyclic AMP. (*a*) ●, 16000*g* supernatant (20 μg of protein); ○, 20% sucrose fraction (17 μg of protein). (*b*) 30–40% interface fraction (plasma membrane) (10 μg of protein). (*c*) 40–55% interface fraction (microsomal/mitochondrial) (20 μg of protein). Data are expressed as pmol of ^{32}P transferred from [γ-^{32}P]ATP to mixed histones (1 mg/ml)/mg of lymphocyte protein in 30s. Values reported are means ± 2 S.D. for triplicate determinations. Similar results were obtained in two additional experiments.

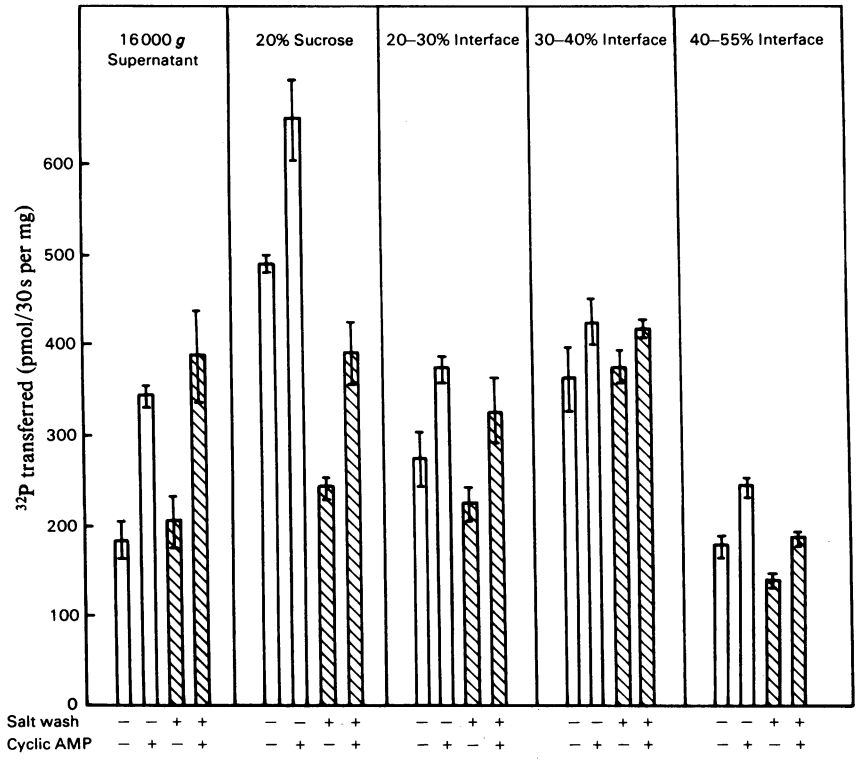

Fig. 5. *Subcellular fractionation in the presence of* 0.25 M-*NaCl*

Lymphocytes were homogenized at 2×10^8 cells/ml in Mg/acetate buffer. Nuclei were removed by low-speed centrifugation. One-half of the preparation was untreated and one-half was brought to 0.25 M-NaCl. Subcellular fractions were then prepared in the usual way. Phosphorylating activity in the untreated (□) and NaCl-treated (■) subcellular fractions was measured in the presence or absence of 2 μM-cyclic AMP with mixed histones as substrate. Data are expressed as pmol of ^{32}P transferred in 30s from [γ-^{32}P]ATP to histones/mg of lymphocyte protein. Quantities of lymphocyte protein in the phosphorylation reactions are as listed in Fig. 3. Values reported are means ± 2 S.D. for triplicate determinations. Similar results were obtained in two other experiments.

specific activity of phosphorylating activity was unaffected by NaCl treatment. Treatment with 0.25 M-NaCl did not alter the cyclic AMP-dependence of phosphorylation in any of the fractions.

The effect on the particulate phosphorylating activity of exposure to cyclic AMP during fractionation was studied in crude particulate fractions: 16000*g* pellets obtained in the usual way were resuspended by Dounce homogenization in Mg/acetate buffer, exposed to 2 μM-cyclic AMP in the presence and absence of 0.25 M-NaCl, and recentrifuged at 16000*g*. Supernatants and pellets were studied for phosphorylating activity with mixed histone as substrate (Fig. 6).

In the untreated fraction, approx. one-quarter of the histone-phosphorylating activity failed to sediment upon recentrifugation. Both supernatant and pellet activities were stimulated by added cyclic AMP. As expected from the experiments with isolated sucrose-gradient fractions, NaCl treatment increased the amount of phosphorylating activity in the supernatant to approx. one-third of the total However, cyclic AMP did not increase the phosphorylating activity released into the supernatant, even at concentrations of NaCl reported to be high enough to dissociate non-specifically adsorbed catalytic activity from membranes (Corbin *et al.*, 1977). Thus the lymphocyte particulate phosphorylating activity remains tightly associated with the membrane, even in the presence of both high salt concentrations and cyclic AMP, arguing that it represents a true membrane constituent rather than a non-specifically adsorbed contaminant.

Solubilization of protein kinase activity with Nonidet P-40

When particulate subcellular fractions were treated with 0.5% (w/v) Nonidet P-40 and centrifuged at

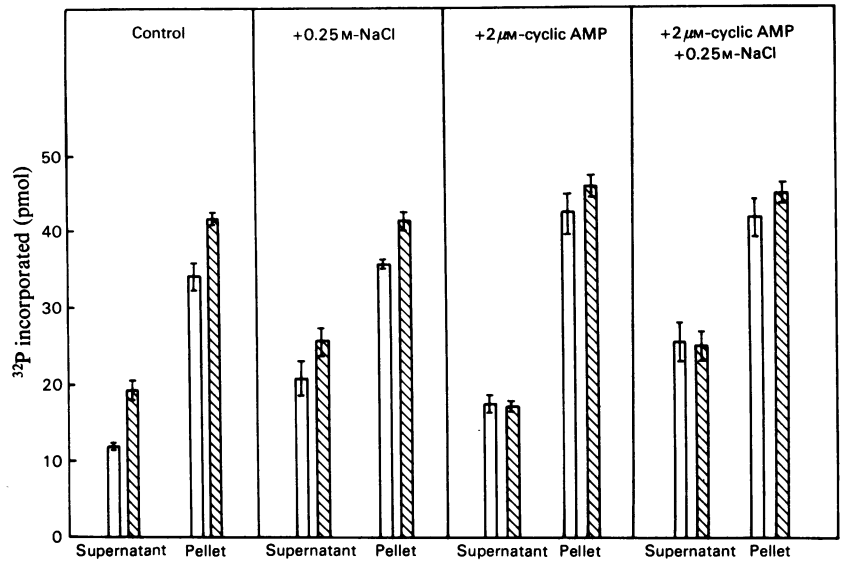

Fig. 6. *Treatment of the particulate fraction with cyclic AMP before sedimentation*
Lymphocytes were homogenized, depleted of nuclei, and a crude particulate fraction was prepared as usual by centrifugation at 16000*g*. The crude particulate fraction was resuspended in Mg/acetate buffer at 2×10^8 cell equivalents/ml by homogenization and was treated with 2 μM-cyclic AMP in the presence or absence of 0.25 M-NaCl. Samples were then recentrifuged at 16000*g* for 15 min at 4°C. Supernatants were harvested and pellets were resuspended by homogenization in a volume equal to that of the supernatants. Phosphorylating activity in the supernatants and pellets was measured in the presence (■) or absence (□) of added 2 μM-cyclic AMP with mixed histones as phosphate-acceptor protein. Data are expressed as pmol of ^{32}P transferred from [γ-^{32}P]ATP to histones by 25 μl of lymphocyte fraction in 30s. Values reported are means ± 2 S.D. for triplicate determinations. Similar results were obtained in two additional experiments.

Table 2. *Nonidet P-40 solubilization of protein kinase activity, assayed with mixed histones*
Subcellular fractions from human lymphocytes were solubilized in potassium phosphate buffer containing 0.5% (w/v) Nonidet P-40. The crude extracts were centrifuged at 100000*g* for 60 min, yielding soluble and insoluble fractions. The insoluble material was resuspended in potassium phosphate buffer containing 0.5% (w/v) Nonidet P-40 by homogenization in a volume equal to that of the crude extract. Protein kinase activity was measured with mixed histones as the substrate and in the presence and absence of the heat-stable protein kinase inhibitor from rabbit skeletal muscle (0.5 mg/ml) and in the presence or absence of 2 μM-cyclic AMP. Data are expressed as pmol of ^{32}P transferred from [γ-^{32}P]ATP to histones/min by the entire sample and represent means ± S.D. for triplicate determinations.

Fraction	Presence of cyclic AMP	Protein kinase activity (pmol of ^{32}P incorporated/min) −Inhibitor	+Inhibitor
30–40% Interface			
containing 0.5% Nonidet P-40	−	33 ± 2.2	17 ± 1.1
	+	61 ± 3.1	17 ± 0.8
100000*g* supernatant	−	30 ± 1.8	17 ± 1.3
	+	61 ± 2.1	18 ± 1.0
100000*g* pellet	−	34 ± 3.2	20 ± 0.2
	+	44 ± 4.2	20 ± 1.4
40–55% Interface			
containing 0.5% Nonidet P-40	−	53 ± 2.1	47 ± 1.4
	+	87 ± 0.9	80 ± 1.1
100000*g* supernatant	−	37 ± 1.7	25 ± 0.9
	+	90 ± 2.1	33 ± 1.3
100000*g* pellet	−	31 ± 2.0	29 ± 2.3
	+	41 ± 4.2	39 ± 2.6

100000*g* for 60min, protein kinase activity was recovered in both the supernatants and the pellets (Table 2). Treatment with 0.5% Nonidet P-40 apparently solubilized 100% of the original protein kinase activity from the plasma-membrane fraction, but rehomogenization of the insoluble material in Nonidet P-40-containing potassium phosphate buffer revealed substantial additional activity. Similarly, apparently 100% of the protein kinase activity in the microsomal/mitochondrial fraction was solubilized by Nonidet P-40 extraction, but substantial additional activity could be recovered by rehomogenization of the insoluble material. The heat-stable protein kinase inhibitor from rabbit muscle inhibited soluble and insoluble kinase activities in the plasma-membrane fraction by 70 and 55% respectively. This inhibitor had almost no effect on kinase activity in the crude Nonidet P-40 extract of the microsomal/mitochondrial fraction, but did inhibit the solubilized activity by 65%. The rehomogenized insoluble material was again negligibly inhibited by the inhibitor.

DEAE-cellulose column chromatography of Nonidet P-40-solubilized protein kinases

Protein kinase activity solubilized by Nonidet P-40 from lymphocyte subcellular fractions was chromatographed on small DEAE-cellulose columns in the presence of 0.25% Nonidet P-40. Eluted kinase activity was assayed with mixed histones as substrate. Under the conditions of chromatography, partially purified type-I and type-II protein kinases from rabbit skeletal muscle were well resolved. Protein kinase activity derived from the cytoplasmic fraction (Fig. 7*a*) was composed of one major peak, which was co-eluted from DEAE-cellulose with type-I rabbit muscle kinase, and two minor peaks, which corresponded to neither type-I nor type-II rabbit muscle kinase. All three peaks of activity were stimulated by addition of 2μM-cyclic AMP. The kinase activity of each peak could be inhibited by more than 85% by the inclusion in the assay of 0.5mg of inhibitor/ml (results not shown). In three separate experiments, 94, 93 and 90% of the applied protein kinase activities (assayed in the presence of cyclic AMP) were recovered from

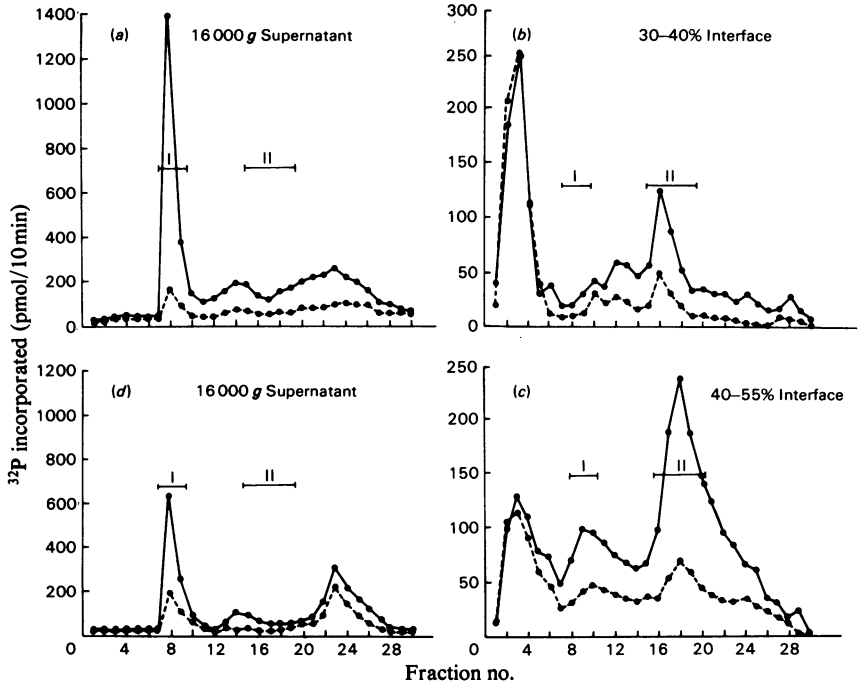

Fig. 7. *DEAE-cellulose chromatography of Nonidet P-40-solubilized lymphocyte protein kinases*
Protein kinase activity was solubilized from lymphocyte subcellular fractions with potassium phosphate buffer containing 0.5% Nonidet P-40 as detailed in the Materials and Methods section. Solubilized samples were diluted to 0.25% Nonidet P-40 with an equal volume of potassium phosphate buffer and chromatographed on small columns of Whatman DEAE-cellulose DE-52 as described in the Materials and Methods section. Column fractions were assayed for protein kinase activity with mixed histones (*a*, *b* and *c*) and casein (*d*) as phosphate-acceptor proteins. Fractions were assayed with (——) or without (----) 2μM-cyclic AMP. Horizontal bars indicate the elution position of rabbit skeletal-muscle protein kinase types I and II. Similar elution profiles were obtained in three separate experiments.

the column. Protein kinase activity solubilized from the plasma membranes was composed of two major peaks (Fig. 7*b*), the largest of which was not retained by the column and was unresponsive to added cyclic AMP. This fraction was inhibited by 83% by the protein kinase inhibitor (results not shown) and probably represented free catalytic subunits. The second peak of activity was co-eluted from DEAE-cellulose with type-II protein kinase from rabbit muscle and was stimulated 2.7-fold by the addition of cyclic AMP. In three separate experiments, 75, 72 and 71% of the applied protein kinase activities were recovered after chromatography. Protein kinase activity solubilized from the microsomal/mitochondrial fraction was eluted as three peaks (Fig. 7*c*), again the first not being retained by the column, exhibiting no stimulation with added cyclic AMP, being fully inhibited by the protein kinase inhibitor, and therefore probably representing free catalytic subunits of protein kinase. The second peak was coeluted with type-I rabbit muscle kinase and was stimulated 2-fold by added cyclic AMP. The third and largest peak was co-eluted from DEAE-cellulose with type-II rabbit muscle kinase. Its activity was augmented 4-fold by added cyclic AMP. Both cyclic AMP-dependent peaks were completely inhibited by the addition of 0.5 mg of inhibitor/ml (results not shown). In three separate experiments, 86, 85 and 80% of the applied protein kinase activities were recovered after chromatography.

DEAE-cellulose chromatography has been reported to resolve casein kinase activity in human lymphocyte homogenates from histone kinase activity (Kemp *et al.*, 1975; Piras *et al.*, 1977). In contrast with these results, when DEAE-cellulose column fractions derived from the cytoplasmic fraction were assayed for kinase activity with casein as substrate, casein kinase activities were co-eluted with the histone kinase activities (Fig. 7*d*). Casein kinase activity was also inhibited by protein kinase inhibitor from rabbit muscle.

Discussion

In the present study, cyclic AMP-dependent protein kinase activity assayed with exogenous phosphate-acceptor molecules was detected in particulate fractions as well as in the soluble cytoplasmic fraction derived from lymphocyte homogenates. Cyclic GMP-dependent protein kinase activity was not detected under any conditions. The particulate protein kinases could be distinguished from the soluble cytoplasmic protein kinases on the basis of fractionation on DEAE-cellulose, differing responses to treatment with $MnCl_2$, phosphorylation pattern with exogenous and endogenous substrates, and washing experiments designed to minimize non-specific adsorption.

(1) *DEAE-cellulose fractionation*

Most of the cyclic AMP-dependent protein kinase activity from the cytoplasmic fraction was co-eluted from DEAE-cellulose with type-I protein kinase from rabbit skeletal muscle. Two other minor peaks of activity were eluted at salt concentrations characteristic of neither type-I nor type-II protein kinase. These may be discrete molecular forms or may simply represent proteolytic degradation products of the type-I kinase. The predominant cyclic AMP-dependent protein kinase activities solubilized from the plasma membrane and microsomal/mitochondrial fractions were each co-eluted with type-II protein kinase.

(2) *Effect of* Mn^{2+}

The presence of Mn^{2+} altered protein phosphorylation in the presence and absence of cyclic AMP in different ways. Protein-phosphorylating activity in the absence of added cyclic AMP was essentially unaffected in the soluble fractions, but was stimulated in the plasma-membrane and microsomal/mitochondrial fractions (Fig. 4). Cyclic AMP-dependent protein phosphorylation in the plasma-membrane fraction was stimulated by low concentrations of Mn^{2+} (10–100 μM), then inhibited at higher concentrations. Cyclic AMP-dependent phosphorylation in all the other fractions was inhibited by Mn^{2+} at all concentrations higher than 10 μM. In complex tissue fractions such as these, the differential effects of Mn^{2+} on cyclic AMP-dependent and -independent phosphorylation could be expressed at many levels, including protein kinase, phosphoprotein phosphatase and endogenous modulatory molecules.

(3) *Phosphorylation patterns with exogenous substrates*

Differences between the soluble and particulate protein kinases were also seen in their ability to catalyse cyclic AMP-dependent phosphorylation of exogenous substrates. Phosphorylation of histones was stimulated by cyclic AMP in both soluble and particulate fractions. However, phosphorylation of casein was stimulated by cyclic AMP only in the soluble fractions, not in the particulate fractions.

(4) *Attempts to diminish adventitious contamination*

Membranes were prepared in the presence of 0.25 M-NaCl in order to elute catalytic subunits of protein kinase non-specifically adsorbed during homogenization in hypo-osmotic media (Corbin *et al.*, 1977). Recovery of plasma-membrane protein-phosphorylating activity was unaltered by fractionation in buffers containing 0.25 M-NaCl; 15–25% of the total protein-phosphorylating activity in the microsomal/mitochondrial fraction was solubilized by fractionation in 0.25 M-NaCl; however, the cyclic AMP-dependence of the response was unaltered.

Thus, by a variety of criteria, the cyclic AMP-dependent protein-phosphorylating activity in the membrane fractions appears to be a true endogenous membrane component and not a contaminant from the soluble cytoplasmic fraction of the cell. Attempts to determine whether the particulate protein kinase is an integral or a peripheral membrane protein have failed because the conditions used to make this distinction inactivate the enzyme.

Quantification of the total amount of protein kinase activity in particulate subcellular fractions has been hampered by our inability to evaluate the accessibility of membranous protein kinases to exogenous phosphate-acceptor proteins. When protein-phosphorylating activity was quantified by using histones as substrate, less than 20% of the total non-nuclear protein kinase was found to reside in the particulate fractions of the cell. However, this was a minimal estimate, as seen in Table 2. When particulate subcellular fractions were extracted only once with the non-ionic detergent Nonidet P-40, all of the activity observed in the original fraction was recovered in soluble form. When the non-solubilized material was homogenized a second time with Nonidet P-40, the overall recovery of activity was increased by 50–80%, indicating the existence of considerable latent activity in intact membranes. Whether any of this latent activity is expressed in intact cells is unknown.

It has been proposed that activation of plasma-membrane protein kinases might release catalytic subunits, allowing translocation to other compartments of the cell (Jungmann *et al.*, 1975). However, in the present experiments, protein kinase catalytic activity was not released from membranes by treatment with cyclic AMP at concentrations sufficient to activate the enzyme fully (Fig. 6), suggesting that, in lymphocytes, particulate protein kinases do not interact with the membrane solely through their regulatory subunit as described in rabbit heart (Corbin *et al.*, 1977) and that cyclic AMP-induced translocation of plasma-membrane protein kinase probably does not occur.

Interestingly, although all of the subcellular fractions could catalyse cyclic AMP-dependent phosphorylation of histones, only the soluble fractions showed significant cyclic AMP-dependent stimulation of phosphorylation of endogenous substrates. Cyclic AMP marginally stimulated net ^{32}P incorporation into endogenous proteins in the microsomal/mitochondrial fraction and produced no response in the plasma-membrane fractions. Since net incorporation of ^{32}P into all trichloroacetic acid-precipitable substrates was being measured, cyclic AMP-dependent phosphorylation of a small number of endogenous substrates might be difficult to detect against a large background of cyclic AMP-independent phosphorylation. Indeed, in the following paper (Chaplin *et al.*, 1979), we will present evidence that a small number of plasma-membrane proteins do undergo cyclic AMP-dependent phosphorylation *in vitro*, but that the detection and quantification of these responses requires analysis by two-dimensional polyacrylamide-gel electrophoresis. In addition, the accessibility of membrane substrates to the cyclic AMP-dependent protein kinases may be importantly affected by non-cyclic AMP-dependent mechanisms, such as changes in membrane fluidity, by the availability of modulator proteins, or by other mechanisms that require integrity of the cell for their proper expression.

In all of the subcellular fractions, considerable protein kinase activity was present in the absence of added cyclic AMP, regardless of whether the phosphate acceptors were histones or endogenous proteins. When histones were the substrate, between 30 and 80% of the total activity was cyclic AMP-independent, depending on the subcellular fraction examined (Fig. 3), and when endogenous proteins were the substrate, 50–100% of the total activity was cyclic AMP-independent (Fig. 2). Whether this activity reflects the presence of truly cyclic AMP-independent protein kinases or reflects cyclic AMP-dependent protein kinases partially activated by endogenous cyclic AMP or by dissociation of the enzyme after interaction with phosphate-acceptor proteins (Miyamoto *et al.*, 1971) remains to be fully elucidated. The presence of cyclic AMP-independent protein kinase in the subcellular fractions is suggested by the residual activity found in the presence of the heat-stable protein kinase inhibitor (Table 2). However, when solubilized fractions were subjected to DEAE-cellulose chromatography, all of the peaks of protein kinase activity could be inhibited by more than 90% by the addition of the protein kinase inhibitor. Moreover, when casein, which is normally a good substrate for cyclic AMP-independent protein kinases, was used as the phosphate-acceptor protein for kinase fractions purified on DEAE-cellulose, no new cyclic AMP-independent protein kinases were observed. In addition, the protein kinase activity obtained with casein as substrate could be fully inhibited by the inhibitor (results not shown). Although other interpretations clearly are possible and further DEAE-cellulose chromatography studies under different conditions (Kemp *et al.*, 1975; Piras *et al.*, 1977) are desirable, we suspect that most or all of the protein kinase activity that we have measured in lymphocyte non-nuclear fractions is derived from either the holoenzyme or free catalytic subunit of cyclic AMP-dependent protein kinase. This implies that the residual activity seen in the presence of the inhibitor is an artifact caused by a blocking factor or proteolytic inactivation of the kinase inhibitor. Supporting this view is the finding that the presence of Nonidet P-40-insoluble material in the microsomal/

mitochondrial fraction potently blocked inhibition of protein kinase by the inhibitor (Table 2).

The data presented here indicate that protein kinase activities and phosphate-acceptor proteins are present in particulate as well as in soluble lymphocyte fractions. The differential substrate specificity and regulation of membrane and cytoplasmic phosphorylating systems together with the apparently permanent association of the particulate kinases with the particulate fractions suggest the possibility of compartmentalization of protein phosphorylation in intact cells.

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