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PARTIAL CHARACTERIZATION OF CYCLIC AMP-DEPENDENT PROTEIN KINASES IN GUINEA-PIG LUNG EMPLOYING THE SYNTHETIC HEPTAPEPTIDE SUBSTRATE, KEMPTIDE

IN VITRO SENSITIVITY OF THE SOLUBLE ENZYME TO ISOPRENALINE, FORSKOLIN, METHACHOLINE AND LEUKOTRIENE D_4

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Abstract—This paper describes the partial characterization of soluble cyclic AMP-dependent protein kinase (A-kinase) in guinea-pig lung using Kemptide, a synthetic serine-containing heptapeptide, and examines the sensitivity of this enzyme to drugs which are reported to increase and to decrease the intracellular concentration of cyclic AMP. Differential centrifugation of lung homogenates revealed that 78% of A-kinase was present in the 31,000 g_{max} × 15 min supernatant fraction. Both basal and cyclic AMP-stimulated phosphotransferase activity of this 'soluble' enzyme were abolished by the heat-stable inhibitor of A-kinase. Soluble A-kinase was Mg^{2+}-dependent (apparent K_m and K_{act} 8.6 and 2.6 mM, respectively) and was stimulated nine-fold by saturating concentrations of both cyclic AMP (K_{act}: 131 nM) and cyclic GMP (K_{act}: 28.7 μM) at a protein (enzyme) concentration of 1.3 μg. Kinetic analysis of the effect of Kemptide and ATP revealed linear, Hanes plots with Michealis constants of *ca.* 12 and 13 μM, respectively. Chromatography of the soluble enzyme over DEAE-cellulose resolved three peaks of catalytic activity when fractions were assayed in the presence of cyclic AMP (10 μM): (i) free catalytic subunits (5%), (ii) Type I isoenzyme (5%) and (iii) Type II isoenzyme (90%). The A-kinase activity ratio was markedly increased in lung pre-treated with the smooth muscle relaxants isoprenaline and forskolin. This biochemical effect was both time- and concentration-dependent and was temporally associated with the ability of these drugs to reduce lung parenchymal tone. In contrast, the contractile agonists, methacholine (Mch) and leukotriene (LT) D_4 exerted opposite effects on A-kinase activity. Thus, Mch significantly reduced cyclic AMP levels and lowered basal A-kinase activity whilst the converse was true for LTD_4. For both drugs this biochemical effect accompanied contraction of the lung. Pre-treatment of lung tissue with flurbiprofen, an irreversible inhibitor of cyclo-oxygenase *in vitro*, abolished the ability of LTD_4 to increase the A-kinase activity ratio suggesting that this biochemical response was mediated indirectly through the stimulated biosynthesis and release of a prostanoid(s) able to activate adenylyl cyclase; the increase in tension induced by LTD_4, however, was not significantly affected by flurbiprofen pre-treatment. Collectively, these data support the concept that soluble A-kinase activity in guinea-pig lung can be regulated by changes in intracellular cyclic AMP and that activation and/or inhibition of this biochemical cascade may influence alterations in lung contractility. The ability of Mch to reduce A-kinase activity suggests that protein dephosphorylation may represent a novel mechanism by which agonists that interact with muscarinic cholinoceptors control or regulate cell function.

β_2-Adrenoceptor agonists currently provide the mainstay in the treatment of asthma [1, 2]. In man these drugs are effective bronchodilators, decrease the reactivity of asthmatic airways to constrictor stimuli and may also possess efficacy at reversing inflammatory mediator-induced microvenular permeability changes [3–6]. The intracellular second messenger system responsible for ultimately eliciting these beneficial effects on respiratory function probably involves increased adenylyl cyclase (ATP: pyrophosphate lyase [cyclising]; EC 4.6.1.1) activity with a consequent increase in the tissue cyclic AMP content [7–12]. Although the precise sequence of biochemical events linking agonism of the β_2-adrenoceptor with a reduction in airway calibre is poorly understood, activation of cytosolic A-kinase (ATP: protein phosphotransferase EC 2.7.1.37) is thought to be an essential pre-requisite [10, 13–16].

In contrast to the stimulatory effect that β-adrenoceptor agonists exert on adenylyl cyclase, certain putative mediators of allergic bronchoconstriction (e.g. the peptidoleukotrienes) and the neurotransmitter acetylcholine have been reported to produce a rapid and well sustained decrease in the intracellular concentration of cyclic AMP both in airway smooth muscle and in lung tissue [17–20]. In view of the apparent rapidity of these responses

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together with the fact that some of these agonists can inhibit tracheal smooth muscle adenylyl cyclase activity [21], it is possible that, in addition to agonist-induced inositol phospholipid hydrolysis, inhibition of cyclic AMP formation may be intimately involved with tension generation in airway smooth muscle [18–21].

In view of this possibility we decided to initiate a series of experiments designed to examine the effect of various agonists which influence cyclic AMP biosynthesis on A-kinase activity *in vitro* with a view to understanding further possible mechanisms of excitation–contraction coupling and uncoupling in airway smooth muscle. Our rationale for looking at A-kinase was based upon the assumption that changes in cellular cyclic AMP content are likely to be of physiological significance *only* if a corresponding alteration in the activity of this enzyme is also observed.

Although guinea-pig lung is routinely employed as a pharmacological screen for potential bronchodilator drugs, surprisingly little is known about the biochemical characteristics of A-kinase in this tissue, the enzyme through which many of the beneficial effects of current anti-asthma therapies are believed to be mediated. Moreover, whereas the effects of various hormonal interventions have been extensively studied on cyclic AMP levels in lung and many other tissues, few reports have described the effects of excitatory and inhibitory drugs on A-kinase activity. We have, therefore, made an attempt to fill this gap in knowledge. In this paper we first describe some of the more important characteristics of soluble A-kinase in guinea-pig lung employing Kemptide, a phosphate acceptor which is devoid of many of the disadvantages inherent in more traditional substrates [22]. In addition, we briefly report on the sensitivity of this enzyme to drugs which affect cyclic AMP levels in smooth muscle. Guinea-pig lung was chosen for these studies since it is a rich source of A-kinase and one of the few easily obtained tissues which exhibits a high density of specific binding sites for cholinomimetics, β-adrenoceptor agonists and leukotrienes (LTs) [12, 23–25]. Although lung parenchyma is cellularly heterogeneous [26], these receptor types are located predominantly on cells thought to play a major role in the allergic asthmatic response [27–30].

MATERIALS AND METHODS

Chemicals, drugs and analytical reagents. The following were purchased from the Sigma Chemical Co. (Poole, U.K.): ATP (free acid), atropine sulphate, bacitracin, benzamidine, bovine serum albumin (BSA; grade III), cyclic AMP, A-kinase (Type I, code P-4890, Lot 34F 9515), cyclic GMP, (*dl*) 1,4-dithiothreitol (DTT), EDTA (tetrasodium salt), glycerol, β-glycerol phosphate, human haemoglobin (fraction III), heparin, histone IIa, 3-isobutyl-1-methylxanthine (IBMX), (-)isoprenaline-D-bitartrate, Kemptide, magnesium acetate, magnesium chloride, methacholine chloride (Mch), phenylmethylsulphonylfluoride (PMSF), crude protein kinase inhibitor (PKI; code P-5015, lots 93F 9580 and 105F 9705), sodium acetate, sodium deoxycholate, sodium fluoride, soybean trypsin inhibitor and Triton X-100. All other chemicals, drugs and analytical reagents were obtained from the following sources: [^{32}P]ATP (Amersham, Bucks, U.K.), DEAE-cellulose (DE-52) and P81 phosphocellulose ion exchange paper (Whatman), flurbiprofen—sodium salt (Boots, Nottingham, U.K.), forskolin (Cal-Biochem, Frankfurt, F.R.G.), ICI 118551 (Imperial Chemical Industries, Macclesfield, U.K.), leukotriene D_4 (LTD_4; Merck Frosst, Quebec, Canada), activated charcoal-Norit A, phosphoric and trichloroacetic acids and salts for buffers and constituents of the assay cocktail (BDH, Poole, U.K.).

Isolation, preparation and treatment of lung tissue. Male Camm–Hartley guinea-pigs of between 250 and 300 g were used. Each animal was heparinized (140 units/kg), left for 10 min and then killed by cervical dislocation and exsanguinated. The thorax was opened and the lungs were perfused *in situ* with heparinized Krebs–Henseleit Solution (KHS) through the right ventricle and pulmonary artery. The left atrium was removed from the heart to permit the outflow of the perfusion fluid. When the perfusate was free of blood the lungs were excised and placed in a petri dish containing oxygenated (95% O_2/5% CO_2) KHS. The lower lung lobes were isolated from the major airways and blood vessels and tissue showing significant haemorrhage was discarded. Subpleural parenchymal lung strips (*ca.* 3 mm × 3 mm × 15 mm) were then cut and mounted, under an initial stretching tension of 20 mN, in 10 mL tissue baths containing oxygenating KHS maintained at 37°. Each strip was left to equilibrate for 60 min and then removed from the tissue bath, blotted on dry absorbent paper and quick-frozen by submersion in liquid nitrogen. Lung tissue was then stored at −80° until A-kinase activity was determined. When used, drugs were added after the initial equilibration period for the indicated time and then processed as above. Drug-induced changes in tension were measured isometrically with Grass FT03.C force-displacement transducers and were displayed on a Grass 79D polygraph. The composition of the KHS was as follows (mM): NaCl 118, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.7, $CaCl_2 \cdot 6H_2O$ 2.5.

Preparation and assay of soluble A-kinase. Each frozen tissue was homogenized (Polytron PT 10 20 350D) for 1 × 15 sec burst at setting 8 in 20 vol. (w/v) of ice-cold buffer A [5 mM KH_2PO_4, 5 mM K_2HPO_4 (pH 6.8), 10 mM EDTA, 10 mm DTT, 500 μM IBMX; I = 87 mM]. When the effect of drug treatment on A-kinase activity was measured, tissues were homogenized in buffer A supplemented with 20 mM NaCl and 140 mM KCl (I = 247 mM). The resulting homogenate was centrifuged at 31,000 g_{max} for 15 min at 4° in a fixed arm rotor (r_{av} = 8.95 cm) to form soluble (supernatant) and particulate (pellet) fractions. The soluble enzyme was then diluted 16-fold in buffer A.

Soluble A-kinase activity was measured using a modification [31, 32] of the method originally described by Witt and Roskoski [33]. Assays were performed in triplicate at 30° and initiated by the addition of 25 μL (3–4 μg protein) of the soluble

extract to 65 μL of a reaction medium (pH 6.8) containing (final concentration): 10 mM KH_2PO_4, 10 mM K_2HPO_4, 10 mM $Mg\cdot(CH_3COO)_2$, 500 μM IBMX, 100 μM ATP supplemented with 100 cpm/pmol [^{32}P]ATP (sp. act. 20–40 Ci/mmol) and 64 μM Kemptide [34] (Leu-Arg-Arg-Ala-Ser-Leu-Gly) in the absence and presence of 10 μM cyclic AMP. The heat-stable PKI originally identified by Walsh *et al.* [35] was used to define A-kinase activity (see Results and Discussion). When particulate A-kinase was measured, 10 mM NaF was included in the assay cocktail to minimize ATPase and phosphatase activities that co-solubilize with A-kinase. In some experiments, indicated in the text and figure legends, 100 μg/mL histone IIa was used instead of Kemptide. In these cases higher concentrations (25–40 μg protein per tube) of soluble enzyme were required. The reaction was terminated after 8 min by pipetting 70-μL aliquots of the mixture onto 2 cm × 2 cm phosphocellulose paper squares (P 81) which were left for 30 sec and then immersed in 75 mM phosphoric acid. The paper squares were then extensively washed (4 × 5 min) in fresh phosphoric acid to remove any non-specifically bound labelled ATP and inorganic phosphate, immersed in ethanol (5 min) and diethylether (5 min) and then allowed to dry. The bound ratioactivity (representing phosphokemptide/phosphohistone) was quantified by liquid scintillation spectrometry (Packard TriCarb 460 CD) in ASC (Amersham). One unit of enzyme activity was defined as that amount of A-kinase which catalysed the incorporation of 1 nmol of phosphate from ATP into Kemptide/histone IIa in 1 min at 30°. In some experiments A-kinase activity is expressed as an activity ratio which is the ratio of the specific activity obtained in the absence of exogenous cyclic AMP divided by the specific activity of A-kinase obtained in the presence of a concentration of cyclic AMP required to maximally activate the enzyme.

Chromatographic separation of A-kinase isoenzymes. Unless otherwise stated all chromatographic procedures were performed at 4°. For the separation, identification and quantitation of A-kinase isoenzymes and free catalytic subunits approximately 100 mg of frozen tissue were homogenized in 20 vol. (w/v) of buffer B [2.5 mM KH_2PO_4, 2.5 mM K_2HPO_4 (pH 6.8), 1 mM EDTA, 10 mM DTT, 10 μM PMSF, 10 μg/mL soybean trypsin inhibitor, 17 μg/mL benzamidine and 100 μg/mL bacitracin; I = 12.3 mM], centrifuged to form soluble and particulate enzyme fractions as described above and then diluted in buffer B such that the conductivity of the enzyme preparation was ~1 mmho (at 4°). A-Kinase associated with the particulate material was detergent-solubilized by resuspension of the pellet in buffer B containing 0.2% (v/v) Triton X-100 followed by gentle stirring at 4° for 60 min. After recentrifugation the solubilized membrane-associated enzyme, now present in the supernatant, was diluted as for the soluble enzyme. The crude enzyme preparations were then applied to a column (1 × 10 cm) of DEAE-cellulose (DE-52) pre-equilibrated with buffer B which was then washed with a further 40 mL of the same buffer to remove unbound protein. The low conductivity of buffer B permitted the binding of free catalytic subunits to the DEAE-cellulose which would otherwise elute with the column wash. A-Kinase isoenzymes and free catalytic subunits were then eluted from the column with a linear NaCl gradient running from 0 to 350 mM in a total volume of 160 mL. The flow rate was adjusted to 180 μL/min and 40 × 4 mL fractions were collected. Aliquots of each fraction were then assayed for A-kinase activity as described above. Approximately 83% of the total activity applied to the column was recovered.

Purification of the heat-stable inhibitor of A-kinase. Preliminary studies revealed that the PKI purchased from Sigma contained impurities which resulted in incomplete inhibition of *bona fide* A-kinase activity (see Ref. 36). Moreover, major differences between the effectiveness of different lots of PKI (93F 9580 and 105F 9705 in these experiments) rendered this product unreliable when accurate estimates of A-kinase activity ratio were to be made (see below). The PKI was therefore further purified.

Approximately 100 mg of the crude PKI were dissolved in 20 mL of buffer C [5 mM sodium acetate, 20 μM EDTA (adjusted at room temperature to pH 5.3 with 0.5 M acetic acid), I = 3.7 mM] and applied to a column (0.7 × 12 cm) of DEAE-cellulose (DE-52) pre-equilibrated with the same buffer. The column was washed with 50 mL buffer C and subsequently developed with a linear sodium acetate gradient running from 5 mM to 300 mM at a flow rate of 540 μL/min; 30 × 3 mL fractions were collected. Aliquots (18 μL) from each fraction were then tested for their ability to inhibit the phosphorylation of histone IIa by the method described above using *bona fide* Type I A-kinase isolated from rabbit skeletal muscle as the enzyme source. Three peaks of PKI activity, corresponding to different charged species of PKI and denoted I_1, I_2 and I_3 by Walsh and his associates [37], were recovered from the column eluting at 85, 130 and 165 mM sodium acetate, respectively. The peak fractions comprising I_1 and I_2 (>97% of the total inhibitory activity) were pooled, neutralized with 1 M KOH and dialysed in Spectrapor 3 dialysis tubing (MWCO 3500) for 10 hr against three changes (2 L each) of buffer D (500 μM γ-glycerol phosphate, 20 μM EDTA, 2 mM DTT adjusted to pH 7 with 1 M KOH). The resulting non-diffusible material (*ca.* 21 mL) containing the PKI was lyophilized, reconstituted in 1 mL of buffer B containing 10% (v/v) glycerol, aliquoted and stored at −80°. When required PKI was diluted 1:15 in buffer B.

The ionic strength of buffers A, B and C was calculated using the standard equation: $I = \frac{1}{2}\Sigma c_i Z_i^2$ (c_i = ionic concentration; Z_i = the valency of the ionic species at the working pH) employing the known pK_a values of phosphate (7.2) and EDTA (6.27) at 25° and pH 6.8 and EDTA (4.76) at 25° and pH 5.3.

Miscellaneous measurements. The protein content of 31,000 g_{max} × 15 min supernatant fractions was measured according to the method of Lowry *et al.* [38] using BSA as standard. The contribution made to the soluble protein content by contaminating haemoglobin was determined spectrophotometrically at 411 nm and substracted away from the total measured protein.

Statistical analysis. Values in the text represent the

mean ± SE of N observations. Where statistical analysis was appropriate data were analysed non-parametrically using the statistical package 'Number Cruncher Statistical System' (written by Dr J. H. Hintz, 865 East 400 North Kaysville, Utah, U.S.A.). The specific tests used to compare data in these studies are indicated in the legends to figures and tables. Significance was accepted when $P < 0.05$.

RESULTS AND DISCUSSION

Preliminary studies revealed that A-kinase was located predominantly (>78%) in the supernatant fraction of guinea-pig lung homogenates. This 'soluble' enzyme effectively catalysed the transfer of phosphate from ATP to both histone IIa and Kemptide, the kinetics of which were linear with respect to time (up to 10 min) and protein concentration (histone IIa: up to 40 μg; Kemptide: up to 10 μg) in the absence and presence of cyclic AMP (10 μM).

Although the Triton X-100-solubilized particulate enzyme was apparently identical to soluble A-kinase, with respect to its elution profile from DEAE-cellulose (see below and Fig. 1B), this enzyme was resistant to stimulation by isoprenaline (10 μM; data not shown). It was thus concluded that soluble A-kinase may be the more important activity in guinea-pig lung with respect to β-adrenoceptor-mediated

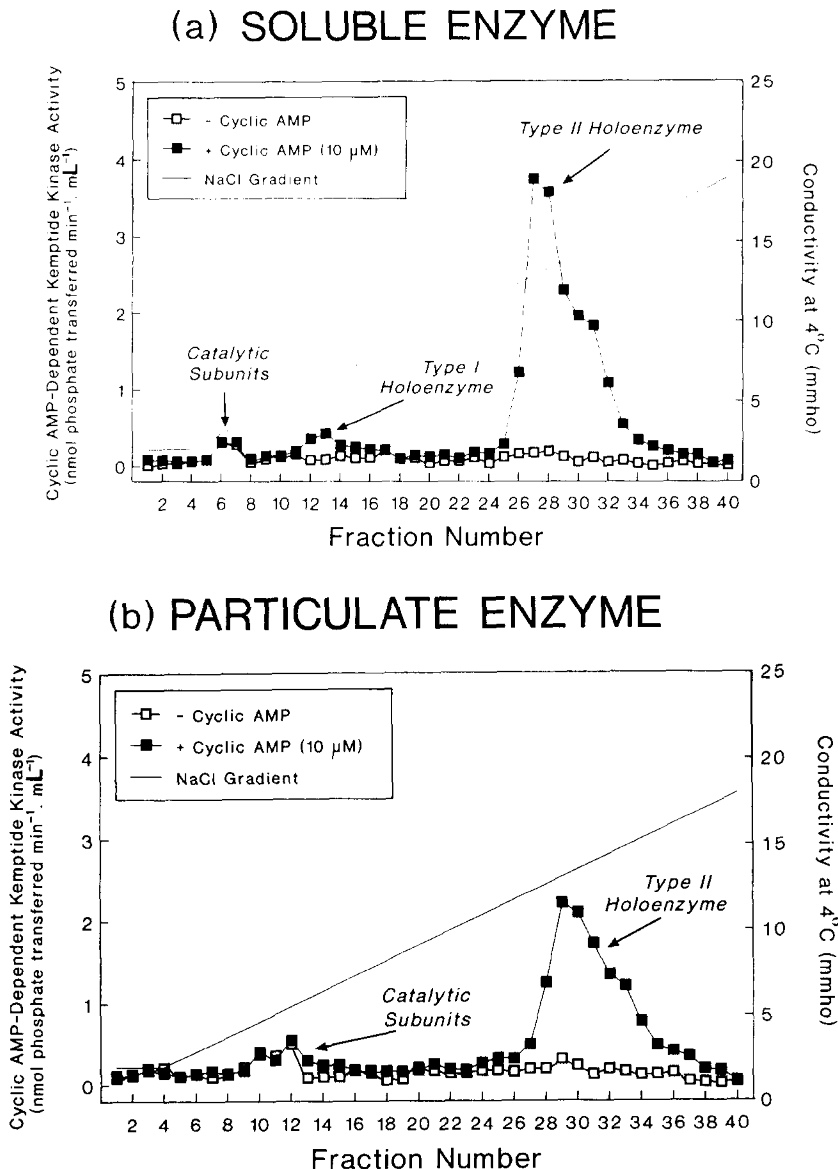


Fig. 1. Elution of soluble and particulate A-kinase after chromatography over DEAE-cellulose. Soluble (a) and Triton X-100-solubilized particulate (b) material obtained following centrifugation of guinea-pig lung homogenates at 31,000 g_{max} × 15 min was chromatographed over a column of DE-52 anion exchange resin as described in Materials and Methods. Fractions were then assayed for A-kinase activity in the absence and presence of cyclic AMP (10 μM). The data shown represent phosphotransferase activity that was inhibited by 20 μL post-DEAE-cellulose PKI.

phenomena. For this reason the particulate enzyme was not characterized further in this study.

Effect of the heat-stable inhibitor of A-kinase on basal and cyclic AMP-stimulated phosphotransferase activity

One of the main problems in measuring A-kinase activity by phosphocellulose paper adsorption or by trichloroacetic acid precipitation is that these similar methodologies permit other phosphotransferase activities to be expressed. This problem is especially pronounced when histones are used as the substrate since they are readily phosphorylated by many different protein kinases. It is essential, therefore, to establish what proportion of the total phosphotransferase activity is attributable to A-kinase if accurate estimates of the endogenous activation state of this enzyme are to be made. Torphy *et al.* [14] have described a simple and elegant *in vitro* method to accurately quantify A-kinase-induced protein phosphorylation based on the use of PKI, an endogenous eicosapeptide reported to specifically inhibit A-kinase activity [35, 39].

Figure 2a shows the effect of post-DEAE-cellulose PKI on soluble phosphotransferase activity assayed in the absence and presence of a saturating concentration of cyclic AMP (10 μM) using histone IIa as the substrate. PKI produced a concentration-dependent inhibition of basal and cyclic AMP-stimulated phosphotransferase activity with a maximum effect seen at 3–4 μL per tube. When protein kinase activity was assayed in the absence of cyclic AMP, PKI (20 μL per tube) inhibited only 60% of the total amount of phosphate incorporated into histone (Fig. 2b). This fraction of the resting activity (A) represents free catalytic subunits and reflects the endogenous basal activation state of A-kinase. In the presence of cyclic AMP (10 μM), protein kinase activity increase *ca.* five-fold (B + C). Inclusion of PKI (20 μL per tube) into the assay cocktail, however, did not completely prevent cyclic AMP-stimulated phosphohistone formation (i.e. cyclic AMP effected a small (8%), but significant, increase in histone phosphorylation (C) independent of A-kinase activation; Fig. 2b). Collectively, these data corroborate the findings of Torphy *et al.* [14] and Miller *et al.* [40] who respectively observed similar PKI-resistant phosphotransferase activities in canine trachealis and lower oesophageal sphincter smooth muscle from the opossum, man and dog. Although not established in this study (see below), cyclic nucleotide-*independent* protein kinase(s) and cyclic GMP-dependent protein kinase (G-kinase) are the likely candidates responsible for the PKI-resistant

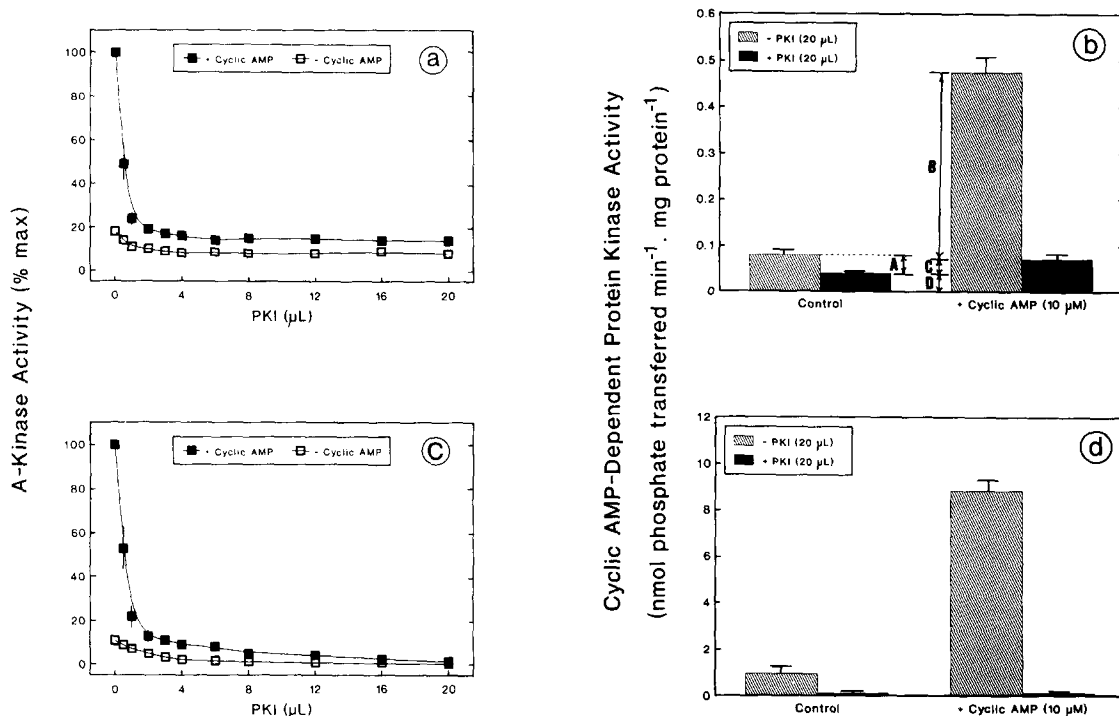


Fig. 2. Effect of partially purified protein kinase inhibitor on A-kinase-induced phosphorylation of histone IIa and Kemptide. Panels (a) and (c) illustrate the effect of PKI on basal (□) and cyclic AMP (10 μM)-stimulated (■) phosphotransferase activity in the 31,000 g_{max} × 15 min supernatant fraction of guinea-pig lung homogenates using histone IIa and Kemptide as the respective substrates. In (b) and (d) basal and cyclic AMP-dependent protein kinase activities were determined in the absence (hatched histograms) and presence (filled histograms) of a maximally effective concentration of PKI (20 μL per incubate) and were defined according to the method of Torphy *et al.* [14]. A-kinase, G-kinase and cyclic nucleotide-independent protein kinase activities are given by B, C and D respectively. In the absence of exogenous cyclic AMP the basal A-kinase activity, representing free catalytic subunits, is given by A. Values are the mean ± SE of six independent observations.

activity seen in the absence (D) and presence (C) of cyclic AMP [14, 40].

It is clear from the above discussion that failure to allow for contaminating phosphotransferase activities can result in artificially high A-kinase activity ratios. It is essential, therefore, to use post-DEAE-cellulose PKI routinely to precisely quantify what proportion of the total phosphotransferase activity is attributable to A-kinase, especially if histones are to be used as the phosphate acceptor.

Although PKI allows changes in A-kinase activity to be quantified more accurately, the methodology and subsequent data analysis is tedious. Ideally, a substrate that is phosphorylated only by A-kinase would effectively circumvent the need to use the PKI. In 1977 Kemp and colleagues [34] published a paper in which A-kinase activity was measured using a serine-containing heptapeptide (subsequently named Kemptide) as phosphate acceptor. Since this substrate corresponds very closely to the hexapeptide sequence present at the active site of porcine hepatic pyruvate kinase (an endogenous substrate for A-kinase [34]) we thought that this phosphate acceptor may be more resistant to phosphorylation by cyclic AMP-*independent* protein kinases. To test this hypothesis the ability of PKI to inhibit A-kinase-

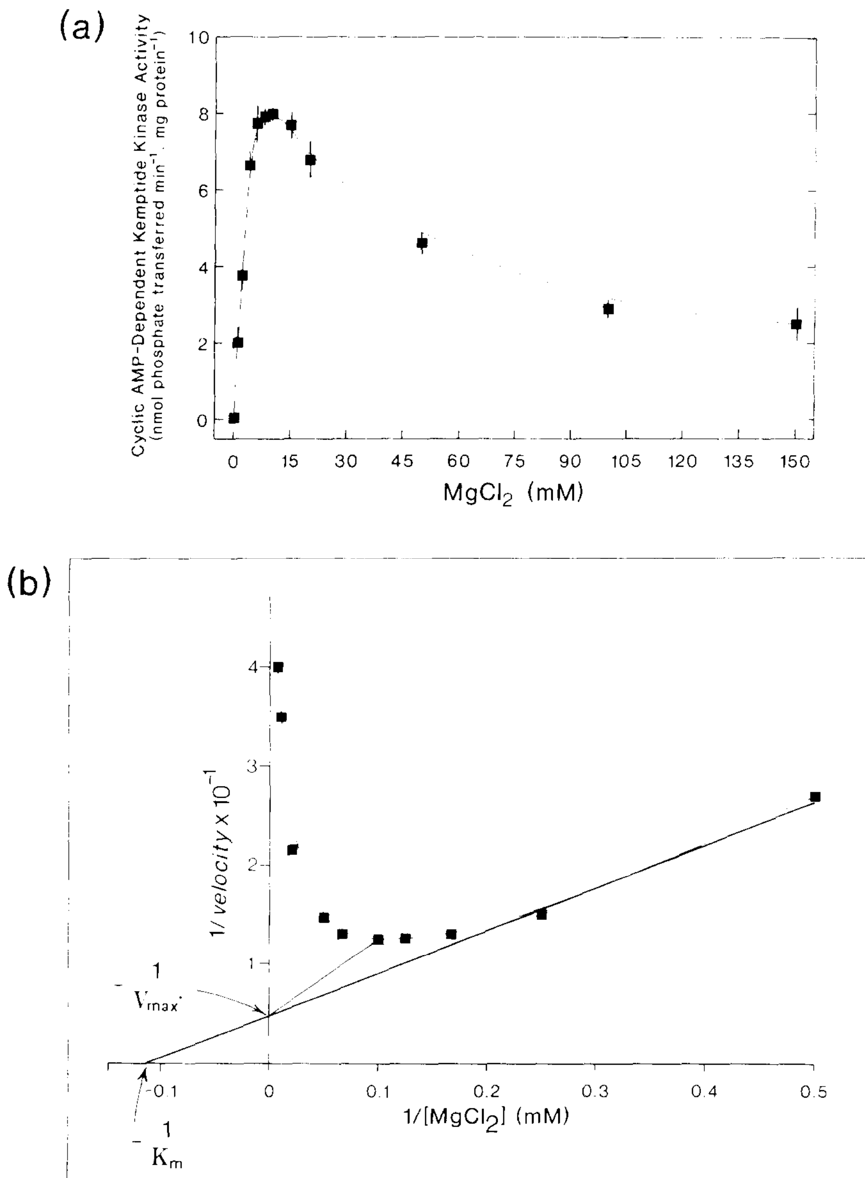


Fig. 3. Effect of the magnesium ion concentration on soluble A-kinase activity. In (a), cyclic AMP (10 μM)-stimulated A-kinase activity in the 31,000 g_{max} × 15 min supernatant fraction of guinea-pig lung homogenates is shown in the absence and in the presence of $MgCl_2$ (1–150 mM) using Kemptide as the phosphate acceptor. The kinetic constants, K_m and V^*_{max} (see text), of Mg^{2+} were determined from Lineweaver–Burk plots of these data (b) after correction for the inhibitory effect of Mg^{2+} seen at high concentrations [45]. Each point represents the mean ± SE of four independent observations.

stimulated phosphoKemptide formation was investigated using guinea-pig lung as the enzyme source. The results of these studies (Fig. 2c and d) show that both basal and cyclic AMP-stimulated phosphotransferase activities were abolished by PKI (20 μL per tube) indicating that the phosphorylation of Kemptide was catalysed exclusively by soluble A-kinase. Under these conditions, therefore, application of the PKI methodology described by Torphy *et al.* [14] is no longer required.

In addition to being specifically phosphorylated by A-kinase, Kemptide was also a much better substrate (*ca.* 15-fold) than histone IIa. The specific activities of cyclic AMP (10 μM)-stimulated, PKI-sensitive phosphotransferase activity utilizing histone IIa and Kemptide as substrate were 0.54 ± 0.03 (N = 25) and 8.29 ± 2.4 (N = 19) nmol/min/mg protein respectively.

Magnesium-dependency of A-kinase-induced protein phosphorylation

Illustrated in Fig. 3a is the effect of Mg^{2+} on soluble A-kinase activity using Kemptide as the substrate. In the absence of Mg^{2+} no detectable phosphotransferase activity was observed. Inclusion of millimolar quantities of this cation in the assay cocktail, however, markedly stimulated A-kinase in a concentration-dependent manner with an apparent K_{act} of 2.6 ± 0.2 mM (N = 6). In the presence of 10 mM EDTA the maximum rate (7.9 ± 0.2 nmol/min/mg protein; N = 4) of Kemptide phosphorylation occurred at 10 mM $MgCl_2$ in good agreement with the Mg^{2+} sensitivity of A-kinase in other tissues [14, 41–44]. Concentrations of Mg^{2+} greater than 10 mM inhibited cyclic AMP-stimulated A-kinase activity in concentration-dependent manner (Fig. 3a). Lineweaver–Burk plots of these data (Fig. 3b), after correction (see Ref. 45 for details of kinetic analysis) for the inhibition of A-kinase activity that occurred at high Mg^{2+} concentrations, yielded an apparent K_m for Mg^{2+} of 7.9 ± 0.5 mM (N = 6) and a theoretical maximum velocity, V^*_{max} (assuming no inhibition by Mg^{2+}), of 21.7 ± 4.3 nmol/min/mg protein. An identical K_m (7.2 ± 0.9 mM; N = 6), K_{act} (2.0 ± 0.1 mM) and Mg^{2+} optimum (10 mM) was obtained when histone IIa was employed as the phosphate acceptor (data not shown).

Determination of the Michaelis constant for Kemptide and ATP

In the presence of Kemptide (100 μM), ATP activated A-kinase in a concentration-dependent manner with K_m (derived from Hanes analysis of the data) of 14.1 ± 1.0 μM (N = 4). The Michaelis constant for Kemptide obtained at a saturating concentration (200 μM) of ATP was 11.7 ± 1.3 μM (N = 4).

Determination of the activation constants of cyclic AMP and cyclic GMP

Cyclic AMP produced a concentration-dependent stimulation of soluble A-kinase which resulted in a

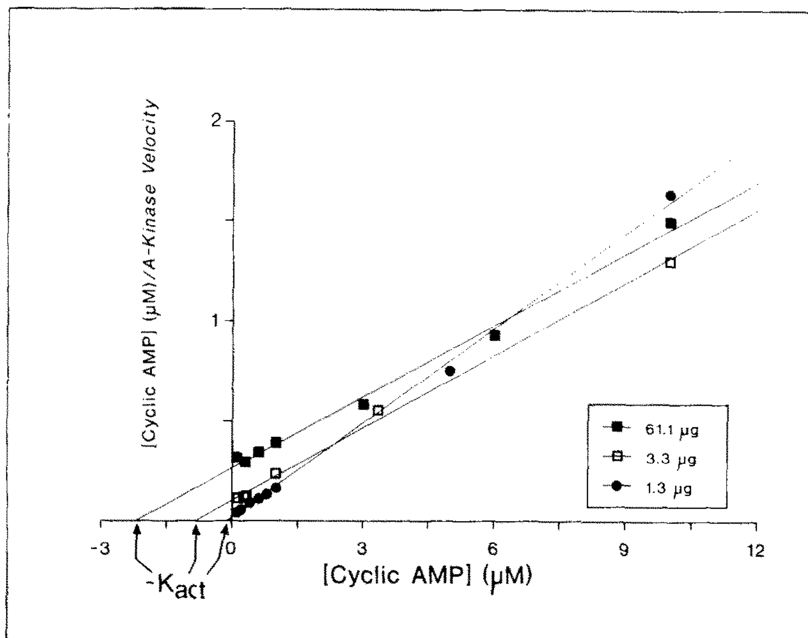
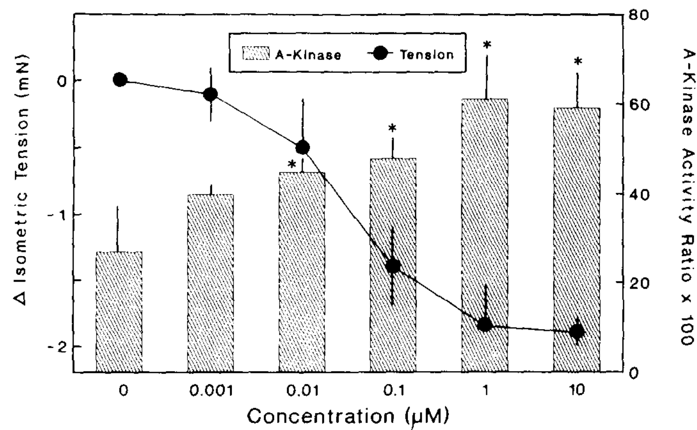


Fig. 4. Hanes analysis of the effect of cyclic AMP on soluble A-kinase activity at different enzyme concentrations. A-kinase activity in the 31,000 g_{max} × 15 min supernatant fraction of guinea-pig lung homogenates was measured using Kemptide as the phosphate acceptor. The assay was performed using 1.3 (●), 3.3 (□) and 61.1 (■) μg protein per tube in the presence of cyclic AMP (0.1–10 μM). When a high enzyme concentration was used (61.1 μg) the incubation time of the assay was abbreviated from 8 min to 60 sec. Data are plotted according to Hanes where the velocity of Kemptide phosphorylation is expressed as that observed *above* basal activity (i.e. stimulated activity − basal activity). Each point on the graph represents the results obtained from a representative enzyme preparation (see Table 1).



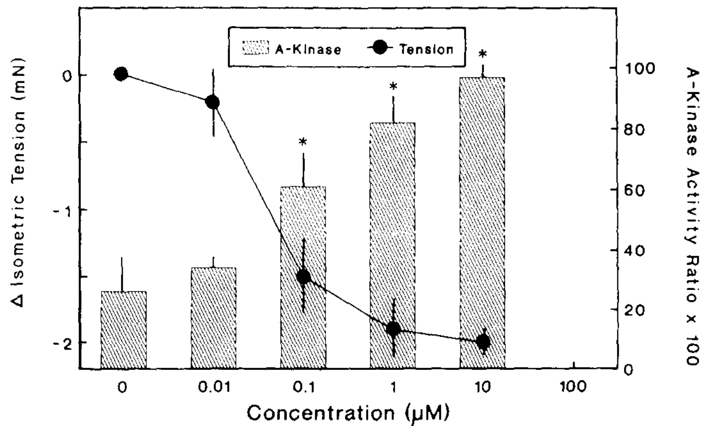
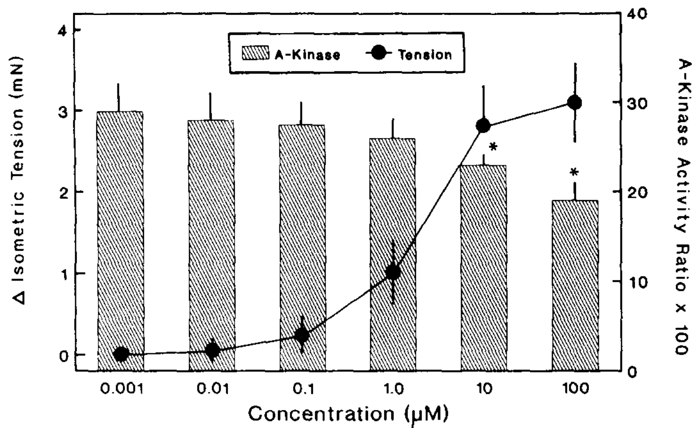


Fig. 5. Effect of isoprenaline, forskolin, methacholine and leukotriene D_4 on tension and on the A-kinase activity ratio in guinea-pig lung parenchyma. Strips of lung parenchyma were set up for isometric tension recording as described in Materials and Methods. After the equilibration period (60 min) tissue were challenged cumulatively with increasing concentrations of either (a) isoprenaline (1 nM–10 μM), (b) forskolin (10 nM–10 μM), (c) Mch (1 nM–100 μM) or (d) LTD_4 (0.1 nM–1 μM). In the case of the isoprenaline and forskolin studies, lung was initially pre-contracted with KCl (30 mM). When the mechanical response (●——●) had plateaued lung strips were removed from the bath and rapidly frozen in liquid nitrogen for the subsequent determination of the A-kinase activity ratio (histograms). Values represented by the histograms and data points refer to the mean ± SE of six independent observations for each drug at each concentration. *$P < 0.05$ with respect to pre-drug values, Kruskal–Wallace ANOVA with multiple comparisons.

nine-fold increase in PKI-sensitive enzyme activity when Kemptide was used as a substrate. At low concentrations of A-kinase (1.3 μg protein per tube) Hanes analysis yielded a K_{act} for cyclic AMP of 131 nM (Fig. 4). Although this value is consistent with others reported in the literature, where histones have generally been used as substrate [14, 40, 42, 46–49], it nevertheless presents an anomaly in that it is almost certainly lower than the resting cellular cyclic AMP concentration which varies from 0.15 to 1.5 μM depending upon the cell type. If 131 nM is truly representative of the *in vivo* K_{act} of cyclic AMP, then A-kinase would be expected to be more than 50% activated under basal conditions. The data in Figure 5 and in Table 1 however, show a basal activity ratio (*ca.* 25–30%) considerably less than is suggested by this K_{act}. The reason for this apparent discrepancy became clear when it was found that the K_{act} of cyclic AMP was markedly dependent upon the concentration of enzyme used (Fig. 4; Table 2). Thus, increasing the effective enzyme concentration from 1.3 to 61.1 μg protein per tube effected a parallel rightwards shift in the concentration–activation curve for A-kinase such that the K_{act} of cyclic AMP



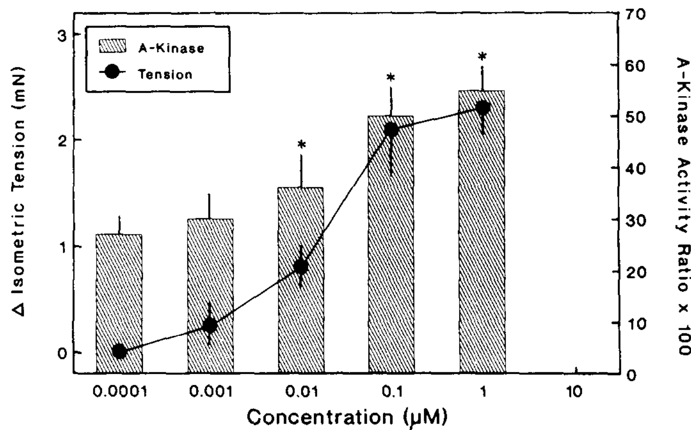


Fig. 5. Cont.

for this catalysis was increased *ca.* seven-fold (see Fig. 4 for Hanes transformation of these data); the V_{max} was not significantly affected by protein concentration as indicated by the parallel slopes of the Hanes plot at each enzyme concentration (Fig. 4). Collectively, therefore, these data indicate that dilution of A-kinase markedly underestimates the 'real' K_{act} of cyclic AMP and underscores the necessity to employ assay conditions which approximate, as closely as possible, to those found *in vivo* if an accurate estimate of this kinetic parameter is to be made. Cyclic GMP also promoted Kemptide phosphorylation in a concentration-dependent manner (data not shown). The concentration–stimulation curve for this cyclic nucleotide was abolished by the inclusion of PKI (20 μL per tube) in the assay cocktail indicating that cyclic GMP was stimulating A-kinase. At a protein concentration of 1.3 μg per tube the K_{act} of cyclic GMP determined by Hanes analysis of the data was 32.8 ± 3.3 μM (N = 4), approximately 150-fold higher than the K_{act} of cyclic AMP; the same V_{max} (9.1 ± 1.4 nmol/min/mg protein; N = 4) was, however, attained.

Separation of soluble and particulare A-kinase isoenzymes by DEAE-cellulose chromatography

There is a mass of evidence from chromatographic, electrophoretic and photoaffinity labelling studies to support the idea that at least two charged species of A-kinase exist (see Refs 50 and 51 for reviews). These species or isoenzymes have been named Type I and Type II based upon the order from which they elute from anion exchange columns. It is important to identify and quantify the relative proportions of each species within a tissue since the isoenzymes differ not only in their net charge, but in a number of potentially functional ways including their susceptibility to autophosphorylation, response to alterations in Mg^{2+} concentration, dissociation–reassociation kinetics and sensitivity to activation by cyclic AMP [51].

The A-kinase present in the 31,000 g_{max} × 15 min

Table 1. Spontaneous reassociation of the regulatory and catalytic subunits of A-kinase in low ionic strength buffer

Treatment	Activity ratio × 100*	
	I = 87 mM	I = 247 mM
Control	9.3 ± 1.3 (10)	27.9 ± 0.8 (40)
Isoprenaline (10 μM)†	27.9 ± 4.9 (6)	53.8 ± 2.2 (28)
Leukotriene D_4 (200 nM)‡	21.9 ± 2.9 (5)	53.7 ± 2.3 (7)
Isoprenaline (10 μM) + forskolin (10 μM)†	43.0 ± 5.2 (6)	98.2 ± 4.1 (4)

Soluble A-kinase was isolated from control and drug-treated guinea-pig lung in low (I = 87 mM) and high (I = 247 mM) ionic strength buffer; activity ratios were then calculated as described in Materials and Methods using Kemptide as the substrate. Values in parentheses represent the number of individual determinations.

* The ability of A-kinase to phosphorylate Kemptide was determined 10 min post-homogenization.

† Ten minute contact time.

‡ Five minute contact time.

supernatant fraction of guinea-pig lung homogenates eluted from DE-52 columns in three main peaks (Fig. 1a). The activity present in the first peak eluted at 25 mM NaCl and was seen irrespective of whether the fractions were assayed in the absence or presence of exogeneous cyclic AMP (10 μM). Since the activity in this peak was abolished by PKI (data not shown) it was concluded that this phosphotransferase activity was attributable to free catalytic subunits. The second and third peaks eluted from the column at 68 and 198 mM NaCl, respectively. These peaks of activity, however, were only seen when fractions were assayed in the presence of exogenous cyclic AMP (10 μM; Fig. 1a) suggesting that they represented the inactive holoenzymes of Type I and Type II A-kinase, respectively. In four independent experiments the free catalytic subunits, Type I holoenzyme and Type II holoenzyme represented 5.1 ± 1.2%, 4.8 ± 1.7% and 90.1 ± 2.1% of the total cyclic AMP-stimulated A-kinase activity, respectively.

The elution profile of Triton X-100-solubilized A-kinase associated with the 31,000 g_{max} × 15 min pellet is shown in Fig. 1b. Only two peaks of PKI-sensitive activity were observed eluting at 28 and 193 mM NaCl, respectively. The first peak represented free catalytic subunits and amounted to 9.5 ± 2.4% (N = 4) of the total A-kinase activity. The second peak, comprising the majority of the enzyme (90.5 ± 2.7%; N = 4), was only seen in the presence of cyclic AMP and represented the Type II holoenzyme. Comparison of soluble and the particulate elution profiles showed that the catalytic subunits in each fraction and the Type II isoenzymes eluted at the same ionic strengths.

Effect of isoprenaline, forskolin, leukotriene D_4 and methacholine on A-kinase activity

We and others have previously reported that isoprenaline, forskolin and LTD_4 each promote the accumulation of cyclic AMP in guinea-pig lung and trachea whilst Mch lowers the levels of this cyclic nucleotide [18, 20, 52, 53]. Since it is generally assumed that any alteration (increase or decrease) in the intracellular level of cyclic AMP is of physiological significance only if a corresponding change in the activation state of A-kinase is also observed, it was important to establish if these changes in intracellular cyclic AMP content were accompanied by similar alterations in A-kinase activity.

Table 2. Apparent activation constants of cyclic AMP for soluble A-kinase at different enzyme concentrations

Enzyme concentration (μg/protein tube)	K_{act} (μM)	V_{max}* (nmol/min/mg protein)
1.3 ± 0.2 (6)	0.131 ± 0.011	7.81 ± 0.04
3.3 ± 0.1 (8)	0.731 ± 0.036†	6.66 ± 0.39
61.1 ± 2.8 (8)	2.15 ± 0.11†‡	7.17 ± 0.38

The 31,000 g_{max} × 15 min supernatant fraction of guinea-pig lung homogenates was assayed for A-kinase activity in the presence of increasing concentrations of cyclic AMP using Kemptide as the substrate. Apparent activation constants (K_{act}) were estimated from the Hanes plots shown in Fig. 4 at three different protein concentrations. Values in parentheses represent the number of individual determinations.

* V_{max} represents the change in Kemptide phosphorylation with respect to basal activity.

† $P < 0.002$ with respect to 0.131 μM.

‡ $P < 0.0001$ with respect to 0.731 μM.

Data analysed by Kruskal–Wallace ANOVA with multiple comparisons.

To gain a reasonably accurate estimate of the activation state of A-kinase in intact tissues spontaneous reassociation of free catalytic and regulatory subunits and non-enzymatic dissociation of the inactive holoenzymes must be prevented during the preparation and assay of this enzyme [42, 54]. The optimal conditions required to preserve the *in vivo* activation state of A-kinase depend upon the relative proportion of the constituent isoenzymes [42, 54]. Thus, low ionic strength conditions must be used to extract A-kinase from tissues which contain predominantly the Type I isoenzyme since this prevents the spontaneous, non-enzymatic dissociation (A-kinase activation) of the catalytic and regulatory subunits [54]. In contrast, for guinea-pig lung and other tissues which contain predominantly the Type II species (see above), use of homogenization buffers of high ionic strength has been emphasized [54] since this condition prevents the reassociation (A-kinase activation) of the catalytic and regulatory subunits [54].

Tissues were, therefore, homogenized in buffer A supplemented with 20 mM NaCl and 140 mM KCl (I = 247 mM). To ensure that the salt-supplemented buffer was of sufficient ionic strength to prevent subunit reassociation, the activity ratio in control and isoprenaline-treated lung was determined at various times (10 to 100 min) after tissue homogenization. As shown in Fig. 6, the A-kinase activity ratios were unchanged during the 100 min incubation period. Specific activities were similarly unaffected (data not shown). These data were in stark contrast to the significant subunit reassociation that occurred in control and isoprenaline-treated lung when this tissue was homogenized in a low ionic strength (I = 87 mM) buffer (Table 1).

Having established that conditions of high ionic strength were appropriate, the effect of isoprenaline, forskolin, Mch and LTD_4 were examined on A-kinase activity together with the effects of these agents on lung parenchymal tone.

Isoprenaline and forskolin. Cumulative addition of both isoprenaline and forskolin to KCl (30 mM; EC_{40})-pre-contracted lung produced concentration-dependent relaxations that were associated in all cases with concentration-related increases in the A-kinase activity ratio (Fig. 5a and b). The concentration of forskolin (10 μM) that produced the maximum reduction in lung parenchymal tone produced almost complete activation of A-kinase whilst the maximally effective concentration of isoprenaline (1 μM) increased the activity ratio to *ca.* 60%. Indeed, a supramaximal concentration of isoprenaline (100 μM) did not further increase this ratio (data not shown). The respective IC_{50} values of isoprenaline and forskolin were 61.3 ± 3.3 nM (N = 6) and 39.3 ± 4.8 nM (N = 6) for tension generation and 569 ± 56.4 nM (N = 6) and 82.1 ± 10.2 nM (N = 6) for A-kinase activation. Figure 7a and b show the effect of isoprenaline (1 μM) and forskolin (10 μM) on the time course of relaxation and on A-kinase activation. In both cases the A-kinase activity ratio increased rapidly following addition of either drug that paralleled the expression of the mechanical response. In the case of isoprenaline the $t_{1/2}$ for A-kinase activation was less than 20 sec significantly less than the $t_{1/2}$ for complete relaxation (50 sec). Maximum activation of A-kinase (~60%) was reached within 5 min of exposure to isoprenaline at a time when maximum relaxation was observed. This level of activity remained sustained for the duration of the experiment (15 min) despite there occurring a rebound increase in tone in some tissues (Fig. 7a). Forskolin also produced a well sustained activation of A-kinase. In this case, however, the activity ratio continued to increase even after complete relaxation of the lung was observed such that 15 min after forskolin challenge, A-kinase was maximally activated. Given that cyclic AMP is able to elicit relaxation of smooth muscle this latter observation suggests that only a fraction of the phosphorylating capacity of A-kinase is necessary to inhibit lung

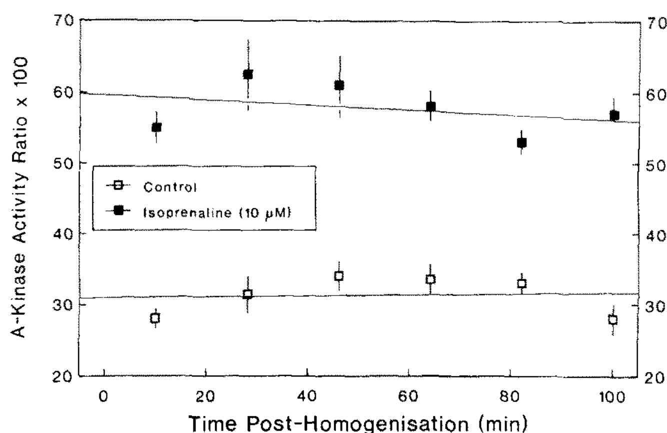


Fig. 6. Stability of basal and isoprenaline-stimulated A-kinase activity in the soluble fraction of guinea-pig lung homogenates. Control (□) and isoprenaline (■; 10 μM)-treated guinea-pig lung was homogenized, transferred to 1.5 mL capacity polypropylene assay tubes and centrifuged at 12,000 g_{max} × 3 min in a Gilson Microfuge. Supernatants were kept on ice for the times indicated above and then assayed for A-kinase activity as described in Materials and Methods. Each point represents the mean ± SE of 12 observations.

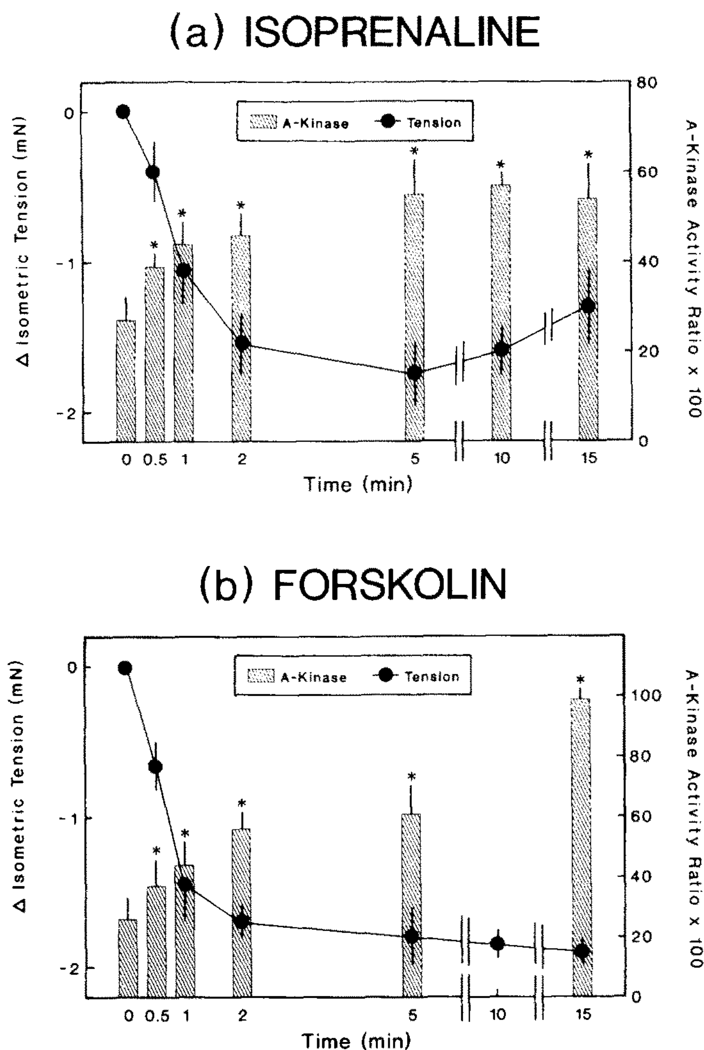
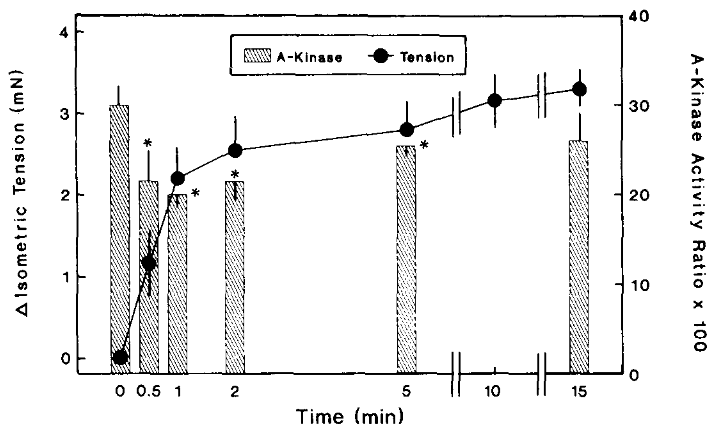


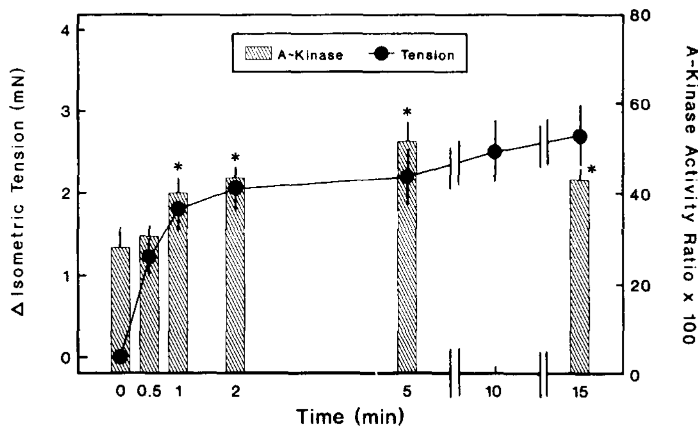
Fig. 7. Time course of isoprenaline-, forskolin-, methacholine- and leukotriene D_4-induced changes in lung parenchymal tone and in the activation state of soluble A-kinase. The effect of a single submaximal concentration of (a) isoprenaline (1 μM), (b) forskolin (10 μM), Mch (50 μM) and LTD_4 (200 nM) on tension (●——●) and on the A-kinase activity ratio (histograms) in guinea-pig lung was assessed at pre-determined time points after drug challenge as described in Materials and Methods. Values denoted by each data point and histogram represent the mean ± SE of six independent observations. *$P < 0.05$ with respect to pre-drug values, Kruskal–Wallace ANOVA with multiple comparisons.

parenchymal tone. The $t_{1/2}$ for forskolin-stimulated Kemptide phosphorylation was *ca.* 1.5 min.

That the effect of isoprenaline was mediated through agonism of β-adrenoceptors was confirmed by the ability of the selective β_2-adrenoceptor blocking drug, ICI 118551 (400 nM), to antagonize isoprenaline-stimulated A-kinase activation (data not shown); the increase in the A-kinase activity ratio induced by forskolin was unaffected by this concentration of ICI 118551.

Methacholine. One piece of evidence which would support the hypothesis that Mch-induced reduction in cyclic AMP content is causally linked to the expression of some cellular response would be the demonstration that A-kinase activity is similarly inhibited in lung tissue treated with muscarinic cholinoceptor agonists. As illustrated in Fig. 5c challenge of guinea-pig lung with Mch resulted in concentration-dependent contractions (EC_{50}; 1.7 ± 0.1 μM, N = 6) that were associated by concentration-related reductions in the A-kinase activity ratio (IC_{50}; ~6 μM). Both the mechanical and biochemical effect of Mch were antagonized by atropine pretreatment (1 μM for 60 min) confirming that the effects of Mch were mediated through agonism of muscarinic cholinoceptors (data not shown). Temporally, the inhibitory effect of Mch was rapid (Fig. 7c) accompanying the development of tone but gradually returned towards the control activity ratio over the duration of the experiment (15 min). These A-kinase data, therefore, are consistent with the ability of muscarinic cholinoceptor agonist to reduce





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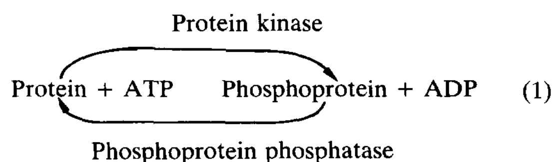
the intracellular cyclic AMP content in airway smooth muscle and lung tissue and illustrate that *basal* A-kinase activity can be inhibited as well as stimulated by drug intervention. Although the mechanism by which Mch lowers the intracellular cyclic AMP content in guinea-pig lung was not determined in these studies, inhibition of adenylyl cyclase activity and/or augmentation of cyclic nucleotide-dependent phosphodiesterase activity can equally well account for the inactivation of A-kinase.

If the above observations with Mch are confirmed in other systems with different agonists then, collectively, such data would represent relatively novel findings directly implicating protein dephosphorylation as a receptor-mediated mechanism controlling or modulating specific cell functions. That a reduction in A-kinase activity is, in some way, linked to the generation of smooth muscle tone is a difficult concept to rationalize given the overwhelming evidence that supports the idea that agonist-induced contraction of airway smooth muscle is initiated and, most probably maintained, by second messengers derived from the receptor-mediated hydrolysis of phosphatidylinositol (4, 5) bisphosphate [55, 56]. However, in the context of inositol phospholipid hydrolysis it is conceivable that a reduction in cyclic AMP content and, therefore, an attenuation of A-kinase-induced protein phosphorylation, rather than promoting tension generation *per se*, could assist the contractile process indirectly by reducing the threshold for the initiation of this response. Theoretically, one consequence of A-kinase inactivation would be to swing the equilibrium in Eqn 1 to the left thus favouring the dephosphorylation of certain phosphoproteins.



(1)

Possible candidates for dephosphorylation are myosin light chain kinase which, even under basal conditions, is 50% phosphorylated in some smooth muscles [57] and, based upon the recent observations of Supattopone *et al.* [58], the inositol, (1, 4, 5) trisphosphate receptor which exists in a low affinity state when phosphorylated by A-kinase.

It is important to include a caveat to follow the above discussion since many agonists that contract smooth muscle do not lower the intracellular cyclic AMP content (see Ref. 59 for references). Whether or not the decreases in cyclic AMP levels seen in the present experiments following Mch treatment are causally related to the cholinomimetic-induced contraction remains to be established.

Leukotriene D_4. LTD_4 produced concentration-dependent contractions of guinea-pig lung (EC_{50}: 21.6 ± 3.2 nM, N = 6) that were associated with concentration-related increases in the A-kinase activity ratio (EC_{50}: 32.6 ± 6.4 nM, N = 6; Fig. 5d). Although this latter observation was consistent with the ability of LTD_4 to increase the intracellular cyclic AMP content [52] this result was difficult to interpret given that increases in cyclic AMP and activation of A-kinase are generally associated with promoting smooth muscle relaxation [59]. Since tension began to develop before any measurable increase in the A-kinase activity ratio was observed (Fig. 7d) we hypothesized that the cyclic AMP-generating action of LTD_4 may be mediated indirectly. Given that LTD_4 effectively promotes the biosynthesis and release of prostaglandins and thromboxanes [60, 61] it seemed possible that a cyclo-oxygenase product may be the causative mediator of the observed increase in A-kinase activity. This was confirmed in a subsequent set of experiments where pre-treatment of lung tissue with the cyclo-oxygenase inhibitor, flurbiprofen (8 μM for 60 min), significantly lowered the basal A-kinase activity ratio (from 27–15%) and abolished the ability of LTD_4 to increase A-kinase activity (Fig. 8). As reported previously [60], flurbiprofen abolished spontaneous tone, that is characteristic of guinea-pig lung, but did not significantly affect the magnitude of the tension response induced by subsequent LTD_4 challenge.

The activation of A-kinase by LTD_4 was not as rapid as that seen with with isoprenaline ($t_{1/2}$ *ca.* 45 sec) consistent with the fact that the secondary release of a cyclo-oxygenase product(s) is/are responsible for activating this enzyme. Temporally, the increase in the activity ratio was transient peaking after 5 min drug contact thereafter decaying despite the continued presence of LTD_4 (Fig. 7d).

It is important to note that the activation of A-kinase by isoprenaline, forskolin and LTD_4 was attributable solely to an increase in the concentration of free catalytic subunits since total PKI-sensitive phosphotransferase activity was unchanged (data not shown).

CONCLUSION

The results of this study demonstrate the existence of a rich source of soluble A-kinase in guinea-pig lung that is sensitive to drugs that both increase and decrease the cellular cyclic AMP content. These data, therefore, support the concept that alterations in A-kinase-induced protein phosphorylation are specifically directed as a consequence of changes in intracellular cyclic AMP. The ability of Mch to inactivate A-kinase is a potentially important observation and suggests a role for protein dephospho-

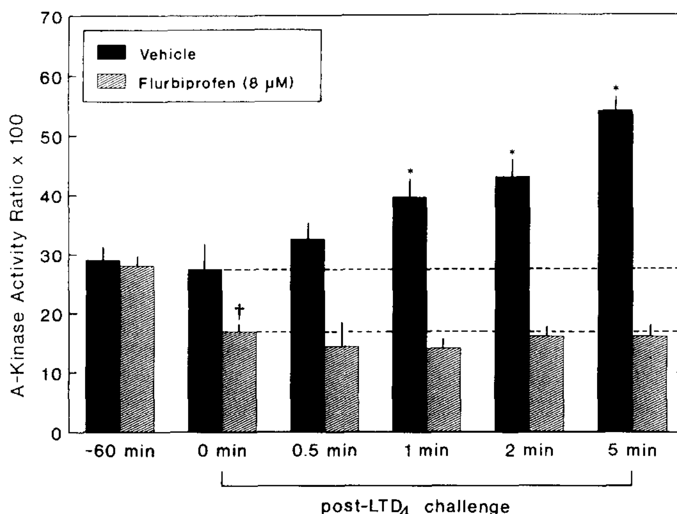


Fig. 8. Effect of the cyclo-oxygenase inhibitor, flurbiprofen, on LTD_4-induced activation of soluble-A-kinase. The effect of LTD_4 (200 nM) on soluble A-kinase activity was determined 0, 0.5, 1, 2 and 5 min after pre-treatment (60 min) of lung strips with either vehicle (KHS; filled histograms) or with the cyclo-oxygenase inhibitor, flurbiprofen (8 μM; hatched histograms). Values denoted by the histograms represent the mean ± SE of six determinations at each time point. *$P < 0.05$, significant increase in A-kinase activity ratio with respect to vehicle-pre-treated 0 min controls. †$P < 0.05$, significant decrease in A-kinase activity ratio with respect to −60 min flurbiprofen-pre-treated tissue. Data analysed by Kruskal–Wallace ANOVA with multiple comparisons.

rylation in the control of muscarinic cholinoceptor-mediated phenomena. In spite of previous evidence to the contrary in some systems [59], the possibility that some agonists are able to assist and/or promote tension generation by lowering the intracellular cyclic AMP concentration clearly warrants further investigation. Methodologically, employing Kemptide as an exogeneous substrate in A-kinase assays is far superior than using histones both in the specificity and extent to which this heptapeptide is phosphorylated by soluble A-kinase. These properties of Kemptide undoubtedly make it a substrate of choice when assaying A-kinase in tissue extracts which also contain high levels of other contaminating phosphotransferase activities.

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